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RESEARCH TOPICS

# TOWARDS TRANSLATING RESEARCH TO CLINICAL PRACTICE: NOVEL STRATEGIES FOR DISCOVERY AND VALIDATION OF BIOMARKERS FOR BRAIN INJURY

Topic Editors
Stefania Mondello, Ronald L. Hayes, András Büki, Frank C. Tortella and Kevin K. Wang

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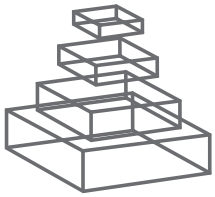

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# TOWARDS TRANSLATING RESEARCH TO CLINICAL PRACTICE: NOVEL STRATEGIES FOR DISCOVERY AND VALIDATION OF BIOMARKERS FOR BRAIN INJURY

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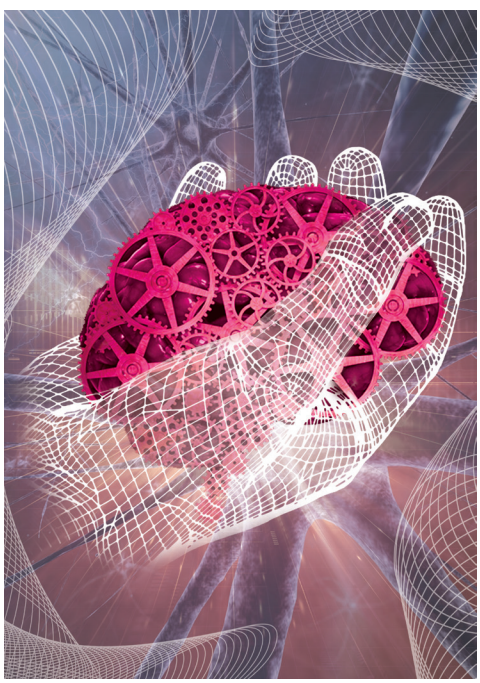

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Traumatic brain injury (TBI) is a major cause of death and disability and one of the greatest unmet needs in medicine and public health. TBI not only has devastating effects on patients and their relatives but results in huge direct and indirect costs to society. Although guidelines for the management of patients have been developed and more than 200 clinical trials have been conducted, they have resulted in few improvements in clinical outcomes and no effective therapies approved for TBI.

It is now apparent that the heterogeneity of clinical TBI is underlain by molecular phenotypes more complex and interactive than initially conceived and current approaches to the characterization, management and outcome prediction of TBI are antiquated, unidimensional and inadequate to capture the interindividual pathophysiological heterogeneity. Recent advances in proteomics and biomarker development provide unparalleled opportunities for unraveling substantial injury-specific and patient-specific variability and refining disease characterization. The identification of novel, sensitive, objective tools, referred

to as biomarkers, can revolutionize pathophysiological insights, enable targeted therapies and personalized approaches to clinical management.

In this Research Topic, we present novel approaches that provide an infrastructure for discovery and validation of new biomarkers of acute brain injury. 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.

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. Thus, this Research Topic includes reviews of 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 and fluid percussion injury (FPI). Consideration is also given to the design and results from human clinical trials validating biomarkers of mild, moderate and severe traumatic brain injury (TBI). Human studies encompass detailed analyses of the potential utility of brain damage markers as diagnostic, prognostic, and therapeutic adjuncts in settings of specific interest including pediatric brain injury, sports concussions and military injuries. Relationships between levels of biomarkers and long term complications are also outlined.

Finally, suggestions are provided for the way forward, with an emphasis on need for a multidimensional approach that integrate a panel of pathobiologically diverse biomarkers with clinical variables and imaging-based assessments to improve diagnosis and classification of TBI and to develop best clinical practice guidelines.

# Table of Contents


**06** ***Brain Injury Markers: Where are We?***
Stefania Mondello and Frank C.Tortella

**08** ***Prefactory Comments: Promise and Enigma of Biomarkers for Brain Injury***
Andrew I. R. Maas

**09** ***Integration of Proteomics, Bioinformatics, and Systems Biology in Traumatic Brain Injury Biomarker Discovery***
J. D. Guingab-Cagmat, E. B. Cagmat, R. L. Hayes and J. Anagli

**21** ***The Role of Markers of Inflammation in Traumatic Brain Injury***
Thomas Woodcock and Maria Cristina Morganti-Kossmann

**39** ***Microglia Activation as a Biomarker for Traumatic Brain Injury***
Diana G. Hernandez-Ontiveros, Naoki Tajiri, Sandra Acosta, Brian Giunta, Jun Tan and Cesar V. Borlongan

**49** ***Application of Blood-Based Biomarkers in Human Mild Traumatic Brain Injury***
Alex P. Di Battista, Shawn G. Rhind and Andrew J. Baker

**55** ***The Potential for Bio-Mediators and Biomarkers in Pediatric Traumatic Brain Injury and Neurocritical Care***
Patrick M. Kochanek, Rachel P. Berger, Ericka L. Fink, Alicia K. Au, Hülya Bayir, Michael J. Bell, C. Edward Dixon and Robert S. B. Clark

**64** ***Amyloid-β Peptides and Tau Protein as Biomarkers in Cerebrospinal and Interstitial Fluid Following Traumatic Brain Injury: A Review of Experimental and Clinical Studies***
Parmenion P. Tsitsopoulos and Niklas Marklund

**81** ***Assessing Neuro-Systemic & Behavioral Components in the Pathophysiology of Blast-Related Brain Injury***
Firas Kobeissy, Stefania Mondello, Nihal Tümer, Hale Z. Toklu, Melissa A. Whidden, Nataliya Kirichenko, Zhiqun Zhang, Victor Prima, Walid Yassin, John Anagli, Namas Chandra, Stan Svetlov and Kevin K. W. Wang

**100** ***Repetitive Traumatic Brain Injury and Development of Chronic Traumatic Encephalopathy: A Potential Role for Biomarkers in Diagnosis, Prognosis, and Treatment?***
Ryan C. Turner, Brandon P. Lucke-Wold, Matthew J. Robson, Bennet I. Omalu, Anthony L. Petraglia and Julian E. Bailes

**111** ***The Diagnosis of Traumatic Brain Injury on the Battlefield***
Kara E. Schmid and Frank C. Tortella

**116** ***A Military-Centered Approach to Neuroprotection for Traumatic Brain Injury***
Deborah A. Shear and Frank C. Tortella

**122** ***Biomarkers of Hypoxic-Ischemic Encephalopathy in Newborns***
Martha Douglas-Escobar and Michael D. Weiss

**127** ***Biomarkers of Brain Injury in the Premature Infant***
Martha Douglas-Escobar and Michael D. Weiss

**134** ***Can S100B Predict Cerebral Vasospasms in Patients Suffering From Subarachnoid Hemorrhage?***
Moshgan Amiri, Ramona Astrand and Bertil Romner

**139** ***Increased Seizure Susceptibility in Mice 30 Days After Fluid Percussion Injury***
Sanjib Mukherjee, Suzanne Zeitouni, Clarissa Fantin Cavarsan and Lee A. Shapiro

**150** ***Controlled Cortical Impact and Craniotomy Induce Strikingly Similar Profiles of Inflammatory Gene Expression, but With Distinct Kinetics***
Mouna Lagraoui, Joseph R. Latoche, Natalia G. Cartwright, Gauthaman Sukumar, Clifton L. Dalgard and Brian C. Schaefer

**164** ***Cerebrospinal Fluid Biomarker Candidates for Parkinsonian Disorders***
Radu Constantinescu and Stefania Mondello

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# Brain injury markers: where are we?

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Traumatic brain injury (TBI), a growing public health problem, appears to result not only from major andprimary injury but also from a complex interplay among inflammatory, biochemical, and neurohormonal changes, as well as genetic components acting on brain tissue. As a result, characterization and classification of TBI requires multidimensional approaches that are able to encompass the diverse and highly complex clinical picture of TBI across the continuum of severities and broad spectrum of pathobiological processes. Emerging evidence suggests that an increasing number of biologic substances, commonly referred to in today's vernacular as biomarkers, can provide unprecedented opportunities for detecting and classifying injury, and identifying pathophysiologic mechanisms potentially leading to more effective targeted therapies.

In this Research Topic, we include comprehensive reviews of the current literature on this topic ranging from proteomics techniques applied for the first time to central nervous system (CNS) biomarker discovery (1) to potential clinical applications of existing biomarkers of brain injury in specific settings such as ICU, pediatric TBI (2), and the military-relevant battlefield casualty (3). In particular, to address the unique circumstances and consequences of sustaining a TBI in combat and the demand for specific practices of management and care of soldiers, presentations (3, 4) have been included from outstanding researchers of the Combat Casualty Care Research Program (CCCRP) for Brain Trauma and Neuroprotection, a program specifically focused on developing neuroprotective and neurorestorative strategies for military-relevant TBI. We have also added a chapter on blast TBI to emphasize the potential problem of TBI following exposure to blast (5). Finally, we expanded discussions to explore the potential of brain damage biomarkers as tools for predicting long-term consequences of TBI (6) and to outline their roles in other CNS diseases such as neurodegeneration (Parkinson's disease) (7), subarachnoid hemorrhage (8), and hypoxic ischemic encephalopathy (9, 10).

We have strived to assemble a multidisciplinary group of internationally recognized researchers and clinicians highly relevant to this research domain (11–13). As the translation of brain damage biomarkers has already transformed from research tools to being aids in clinical decision-making, this Research Topic will be evolutionary reading for neurotrauma scientists and clinicians interested in the potential of a simple biofluid-based diagnostic test to refine the clinical characterization of TBI offering more accurate disease phenotyping. Such improved molecular characterization integrated with traditional approaches, including clinical examination and structural and functional neuroimaging, will allow the field to develop improved clinical practice guidelines and tailor therapeutic interventions to the patient's individual pathophysiology, thereby leading to effective management and improved patient outcome.

This Research Topic would not have been possible without the support and help of many people. First, we thank the chapter authors for devoting their time and effort to produce valuable contributions that provide comprehensive frameworks and critical insights. We also thank the members of the editorial board for their dedicated assistance and for providing informed perspectives on the chapters. Last, and most important, we thank all patients with TBI and their families for their invaluable contributions. To improve their outcome and quality of life represents our ultimate goal and our greatest source of inspiration to foster knowledge in this critical research area.

## AUTHOR NOTE

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# Prefactory comments: promise and enigma of biomarkers for brain injury

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Traumatic brain injury (TBI) represents one of the greatest unmet needs in medicine and public health (Maas et al., 2008). It is a major cause of death and disability and leads to great personal suffering to victims and relatives, as well as huge direct and indirect costs to society (Finkelstein et al., 2006; Faul et al., 2007; Gustavsson et al., 2011). TBI is considered "the most complex disease in our most complex organ." We now recognize that TBI is not just an acute event but can trigger a chronic process, with progressive injury over hours, days, weeks, months, and even years. The challenges posed by TBI are huge.

Notwithstanding improved understanding of disease mechanisms, appropriate characterization of TBI is complex, and even establishing a reliable diagnosis in subjects with mild injuries, can be challenging. It remains difficult to "look into the brain" and to track disease processes on a continuous basis *in vivo*. Despite the current availability of robust prognostic models, estimates of outcome in individual patients often have a large confidence interval. The emerging field of biomarkers has great potential for improving characterization of TBI: in the acute and subacute phases, biomarkers can aid in the diagnosis of TBI, in tracking disease processes and for establishing more confident prognostic estimates. In the more chronic phases, they may indicate ongoing progressive damage with neuronal and glial cell loss.

This special issue of Frontiers in Neurology on biomarkers in brain injury provides a comprehensive summary of our current knowledge in the emerging field of biomarkers across the TBI spectrum from initial injury to long term outcome. Despite the extremely promising results presented, this issue also illustrates some of the gaps in our knowledge, thus stimulating further research and development. Why, for example, is it so much more difficult to find reliable serum biomarkers for brain injury than for, for example, myocardial disease (e.g., troponin)? Could this be perhaps that we still have insufficient knowledge of how degradation products from brain tissue are removed into the venous blood? Is this directly into the venous system, or indirectly by flow of the extracellular fluid draining via the cerebrospinal fluid into the sagittal sinus? Basic understanding of such mechanisms would be of great relevance toward optimal biomarker sampling. Whilst many studies on the prognostic value of biomarkers show clear prognostic effects, it should be realized that numbers in these studies in general are small and that the added value of biomarkers as prognostic indicators over and above other predictors has not yet been adequately shown in multivariable analyses. The concept of being able to differentiate between neuronal and glial injury based upon biomarkers is exciting. This topical issue will also address relations between laboratory markers and other biomarkers such as imaging modalities. By definition characterization and classification of brain injury is multidimensional. Better characterization with the aid of biomarkers can be expected to facilitate Precision Medicine, a concept recently advocated by the US National Academy of Science (2011). Precision Medicine aims for appropriate targeting of management and individualizing treatment approaches based upon more precise characterization of the disease process. Achieving these goals and establishing the role of biomarkers herein will require confirmation of promising results from proof of concept studies in larger numbers. This can best be accomplished in multidisciplinary, international collaborations, collecting high quality prospective data in observational studies in parallel to continued basic science research.

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# Integration of proteomics, bioinformatics, and systems biology in traumatic brain injury biomarker discovery

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Traumatic brain injury (TBI) is a major medical crisis without any FDA-approved pharmacological therapies that have been demonstrated to improve functional outcomes. It has been argued that discovery of disease-relevant biomarkers might help to guide successful clinical trials for TBI. Major advances in mass spectrometry (MS) have revolutionized the field of proteomic biomarker discovery and facilitated the identification of several candidate markers that are being further evaluated for their efficacy as TBI biomarkers. However, several hurdles have to be overcome even during the discovery phase which is only the first step in the long process of biomarker development. The high-throughput nature of MS-based proteomic experiments generates a massive amount of mass spectral data presenting great challenges in downstream interpretation. Currently, different bioinformatics platforms are available for functional analysis and data mining of MS-generated proteomic data. These tools provide a way to convert data sets to biologically interpretable results and functional outcomes. A strategy that has promise in advancing biomarker development involves the triad of proteomics, bioinformatics, and systems biology. In this review, a brief overview of how bioinformatics and systems biology tools analyze, transform, and interpret complex MS datasets into biologically relevant results is discussed. In addition, challenges and limitations of proteomics, bioinformatics, and systems biology in TBI biomarker discovery are presented. A brief survey of researches that utilized these three overlapping disciplines in TBI biomarker discovery is also presented. Finally, examples of TBI biomarkers and their applications are discussed.

**Keywords: proteomics, biomarkers, traumatic brain injury, bioinformatics, systems biology**

## INTRODUCTION

Tremendous efforts have been put into the discovery of biomarkers that can diagnose disease or injury. A quick search for scholarly articles that include the word biomarker can yield more than half a million hits. However, the overall status of biomarker development and clinical validation is very disappointing. There are only a handful of novel biomarkers that are of clinical relevance, and the rate at which a biomarker is introduced to the market is dismal. One estimate shows that since 1998, new protein biomarkers that were approved by the US Food and Drug Administration fell to one per year (Rifai et al., 2006). The reasons for this trend are numerous and one strategy to reverse the fall is a better understanding of the whole process itself. One key strategy in hunting for that robust biomarker is the combination of scientific disciplines. In traumatic brain injury (TBI), an interdisciplinary approach is employed by combining the methods and tools from three fields, namely, proteomics, bioinformatics, and systems biology.

Proteomics is the study of protein populations or proteomes. The term "proteome" was coined in 1995 (Wasinger et al., 1995) to describe the protein complement of a genome. This came after realizing that at least half of the proteins encoded by the human genome have no known functions. The move to study the message (mRNA or cDNA) and focus on the product of the message (proteins) gave birth to proteomics. Proteomics assesses the expression level of proteins, post translational modifications and interactions of proteins within a tissue, cell, subcellular compartment, or biofluid. The goal is to obtain a large scale and a global view of physiological conditions and disease processes. However, studying the global systems of proteins has produced a large amount of data, and making sense of the complex data generated became a problem. In the beginning, it was clear that processing a vast amount of data requires the aid of computers. Like genomics a decade ago, proteomics tackled the problem by enlisting the help of bioinformatics and later on, systems biology.

Bioinformatics combines mathematics and computer technology to deal with the analyses of large numbers of proteins while systems biology unveils the global network of physiological environments. Bioinformatics has become an integral part of proteomics, strategically mining data for sensible results. Systems biology on the other hand tries to look at the big picture by mapping interactions of isolated proteins, akin to looking at the ecosystem of the whole forest, rather than just the individual trees.

The triad of proteomics, bioinformatics, and systems biology has been applied to study protein behaviors in myriad disease pathologies. It was no different with neurological conditions such as TBI, Alzheimer's disease, and stroke. Neuroproteomics (Choudhary and Grant, 2004), a field under the proteomics umbrella, has

zeroed in these disorders, extracting insights into the dynamics and interactions of proteins in these disease states (Ottens et al., 2006, 2010; Bayes and Grant, 2009; Alzate, 2010; Shoemaker et al., 2012).

One of the neurological conditions that received a fair amount of media attention lately is TBI. Although TBI is known as the "silent epidemic," the public is beginning to be aware of the injury as war veterans come home from war-zone blasts. Even the military has acknowledged that TBI is the "signature injury of the conflicts in Iraq and Afghanistan" (Risdall and Menon, 2011).

Increasing media coverage to concussive injury has increased lately. This is partly due to the increase in suicides of football players. High profile cases of professional football players have captivated the public, highlighted by the suicide of Dave Duerson. Mr. Duerson, a professional football player, was suspected to have suffered TBI during his playing years. He was found dead with a gunshot to his chest, not in his head, to preserve his brain for science.

Statistically, TBI is one of the leading causes of disability in the United States. It is considered one of the major health problems annually claiming 5% of the lives of the two million victims. Around 25% are hospitalized and approximately half are treated and released after emergency care (Smith et al., 2003; Johnson et al., 2004). It is estimated that by 2030, the public health impact of TBI will increase (Mathers and Loncar, 2006). This should alarm us as road traffic accidents will be the most common cause of blunt trauma, making TBI the fourth leading cause of disability.

The disturbing reality for victims and their families is that currently, there are no FDA-approved treatments or therapy (except for pain relievers) that can alleviate the effects of TBI. One of the most pressing needs however, is the accurate diagnosis and monitoring of patients. Physicians should be guided if patients respond to the treatment and improve. But to this day, clinicians are limited only by parameters such as brain pH, $pO_2$, intracranial pressure (ICP), and temperature. Brain imaging techniques such as Computer Tomography (CAT) and Magnetic Resonance Imaging (MRI) scans have provided information of damaged regions non-invasively, but only looking at the injury in a short time. The limitations of traditional diagnosis have hindered the overall progress in understanding the condition, highlighting the need for more accurate diagnostic tools. The goal is that a robust biomarker or panels of biomarkers will complement existing diagnosis, and eventually replace the more traditional ones.

In this paper, advances and limitations of proteomics, bioinformatics, and systems biology will be discussed. We shall then try to integrate the three fields in relation to biomarker discovery, and limiting the discussion only to protein biomarkers in TBI. This article is structured as follows. In Section "Biomarkers, TBI Models, Proteomics, Bioinformatics, and Systems Biology, Their Definition," we define biomarkers, TBI animal models, proteomics, bioinformatics, and then systems biology. In Section "Protein Profiling," we shall review the methods, challenges, and technical difficulties inherent in identifying proteins. Section "Biomarker Applications" deals with the present panels of proteins that can be used as a biomarker for TBI.

## BIOMARKERS, TBI MODELS, PROTEOMICS, BIOINFORMATICS, AND SYSTEMS BIOLOGY, THEIR DEFINITION

Biomarkers are indicators of normal biological processes or disease states. A biomarker can also be a gage of pharmacological response in therapeutic interventions (Lesko and Atkinson, 2001).

The idea in biomarker discovery is that organs secrete specific molecules that can indicate a physiological malfunction. In general, these are any biomolecules that can serve as a fingerprint showing up from samples of affected tissue or peripheral fluids of the affected area. In the context of TBI and proteomics, ideal biomarkers are proteins that are only present in the brain, leaked out from the blood brain barrier and into the person's blood or cerebrospinal fluid (CSF) during or after brain injury. These molecular signatures should be proportional to the impact and the extent of damage in the brain, and should reflect differences between age groups and sex.

Numerous animal models of TBI have been developed to understand the heterogeneous nature of brain injury (recently reviewed by Chopp et al.) (Xiong et al., 2013). Due to their low cost and the presence of more standardized outcome measurements, rodent models are particularly used to study TBI although bigger animals are closer to human physiology. Controlled cortical impact (CCI) uses a controlled degree of impact by a pneumatic or electromagnetic impact device (Lighthall, 1988; Dixon et al., 1991). Penetrating ballistic-like brain injury (PBBI) model mimics severe to moderate TBI such as gunshot wounds. PBBI is induced by transmission of high energy projectiles and a leading shockwave producing a temporary cavity in the brain that is many times the size of the projectile itself (Williams et al., 2005). Another widely used model is the fluid percussion injury (FPI) where a contusion force is incurred by the movement of a fluid in a chamber. In the drop-weight impact acceleration injury, the skull (with or without craniotomy) is exposed to a weight that is dropped from a certain height and injury severity can be altered by adjusting the mass of the weight and the height from which it falls. The more recent TBI models are the blast models that mimic TBI induced by explosive devices. Blast-induced brain injuries have been predominant among military personnel who have been exposed to a blast but do not have external injuries (Warden, 2006; Benzinger et al., 2009). Different variations of blast TBI animal models have been developed to elucidate the effects of primary blast waves on the brain (Wang et al., 2005; Cheng et al., 2010; Svetlov et al., 2010; Risling et al., 2011). Elucidation of the mechanisms of blast injury, identification of biomarkers and, eventually, the development of strategies for mitigating blast-induced brain injury will benefit from further design optimization, characterization, and standardization experimental parameters of blast TBI models.

While TBI can occur as a result of auto accidents, violence, or sports injuries it has left the shadows with the war in Iraq and Afghanistan. Twenty-first century warfare exposes military personnel to blast injuries resulting from high order explosives. The Kevlar helmet, although an excellent protection against penetrating brain injury, offers little protection from blast injuries (Lew, 2005; Okie, 2005). Accurate statistics are not currently available, but it is estimated that more than 50% of all casualties from the

Afghanistan and Iraq theaters have sustained head injuries (Warden, 2006) compared to 15–25% from twentieth century conflicts (Carey, 1996). Of the 1.4 million TBIs that occur annually, the vast majority, between 75 and 90% are mild or moderate (mTBI) (Jager et al., 2000; Gerberding, 2003). Mild and moderate TBI, also called concussion, occurs when an impact or forceful motion of the head results in a brief alteration of mental status, such as confusion, disorientation, brief memory loss, or brief loss of consciousness. Because they produce a number of imprecise perceptual symptoms without diagnosable objective structural brain alterations, mTBIs are challenging to diagnose (Lyeth et al., 1990; Hamm et al., 1993; Kibby and Long, 1996; Margulies, 2000). Furthermore, many sufferers fail to recognize the potential severity and seriousness of their injury thus do not seek medical attention (Alexander, 1995; Kushner, 1998). TBI is thus under-diagnosed and under-represented in medical statistics. However, even brief alterations in mental status can inflict profound and persistent impairment of physical, cognitive, and psychosocial functioning (Binder, 1997; Ruff and Jurica, 1999). Furthermore, TBI is an epigenetic risk factor for Alzheimer's and Parkinson's diseases (Smith et al., 2003; Szczygielski et al., 2005). Although TBI is a major focus of casualty care in combat areas and the principal cause of mortality and morbidity due to improvised explosive devices (IEDs) and other hazards, there are no FDA-approved pharmacologic therapies that have been demonstrated to improve functional outcomes.

Proteomics is defined by many as the study of the protein complement of the genome, the proteome (Blackstock and Weir, 1999; Stults and Arnott, 2005). The proteome is the set proteins from the whole organism or specific organ at specific physiological conditions.

Several definitions of bioinformatics can be found in the literature today. What suits us is the idea that bioinformatics is a tool to mine vast amounts of data using computer technology and mathematics (Hagen, 2000; Kumar and Mann, 2009).

Systems biology came into picture as soon as proteins were identified from proteomics experiments. For example, low concentration proteins can now be identified in an injured brain; however, a list of individual proteins may not make sense. To understand the connections of isolated proteins, systems biology came in.

The science of systems biology is still considered to be in its infancy and a consensus on its definition has not been fully reached (Ideker et al., 2001; Kitano, 2002a,b; Chuang et al., 2010). For us, it is an approach to study the complex interactions of biological systems. It examines, assembles, and maps the properties and regulations of tightly interconnected biological systems.

## PROTEIN PROFILING

Identification of proteins is one of the main goals of biomarker discovery. The conventional method of identifying proteins as a marker for disease is by measuring a specific compound known to be part of the pathophysiology. In TBI for example, the presence of glial fibrillary acidic protein (GFAP) in the blood can mean damage to the glia. Also, tau and spectrin protein breakdown products in the blood indicate damage to the axons. One can also examine the unregulated breakdown products of necrotic cell death. Breakdown products of calpain mediated proteolysis can be used as biomarkers of TBI. This is the same for products of apoptotic cell death, from the activation of caspase (Büki et al., 1999; Farkas et al., 2005; Svetlov et al., 2009; Risdall and Menon, 2011).

A novel method, which is the subject of this review, is the data driven and high-throughput approach of discovery. In this strategy, the samples from normal and TBI patients are compared, screening for differences between the two. This approach consistently uses mass spectrometry (MS) and most of the time it is *discovery driven* instead of being *hypothesis driven* (Stults and Arnott, 2005). In discovery driven types of experiments, information is collected and then patterns are sought. Unlike hypothesis driven research that disproves or proves a defined hypothesis, discovery driven research collects a huge amount of information first then extracts questions and answers from lots of data. It may sound like a "blind shot" to find answers, but our current technology enables us to do this. If history is a good indicator, it worked with genomics and metabolomics, so performing discovery driven experiments with an entire proteome is logical.

### HISTORICAL BACKGROUND

It was almost 40 years ago when two-dimensional electrophoresis was invented and described in a paper (O'Farrell, 1975), giving way to the separation of more complex mixtures. A few years after, in the early 1980s, the first profiling of human CSF (Merril et al., 1983) and mammalian brain (Klose and Feller, 1981) were reported. These started the systematic classification of proteins from the brain. By the mid-80s, the first proteomic database SWISS-SPROT was established (Bairoch and Boeckmann, 1991; Bairoch and Apweiler, 1997; Peitsch et al., 1997). In the end of that decade, two ionization techniques for MS analysis were introduced, making large protein analyses possible (Karas and Hillenkamp, 1988; Fenn et al., 1989). High-throughput and gel free proteomics came into being when liquid chromatography (LC) was integrated with MS around 1996 (Appella et al., 1995).

Ten years later, the profile of a mouse's brain was created, identifying 7,792 proteins (Wang et al., 2006), ranging in abundance from tens of copies to hundreds of thousands of copies.

### MS-BASED NEUROPROTEOMICS WORKFLOW

In a typical neuroproteomics experiment, proteins from the brain or spinal cord tissue are extracted as a mixture of proteins. Depending on the experiment, obtaining the proteins can be done with tissue homogenization, cellular fractionation, or affinity fractionation. Then the complex mixture is further separated to reduce its complexity.

Three of the common separation tools for protein separations are two-dimensional electrophoresis (2DE), one-dimensional electrophoresis (1DE), and a two-dimensional LC. In the more common bottom-up proteomics, after subjecting a sample to one of the three methods mentioned above, the proteins are then digested by an enzyme prior to analysis by MS. After protein separation and digestion, the resulting peptide mixture is further resolved by a nanoflow liquid chromatography (nanoLC) based on the peptides hydrophobicity prior to introduction into the mass spectrometer by nano-electrospray ionization. Many TBI proteomic biomarker studies have relied on the bottom-up approach. Putative protein biomarker candidates were identified

in rat CCI model using 1D-SDS-PAGE prior to bottom-up proteomic analysis (Will Haskins). An improved two-dimensional platform employing a protein pre-fractionation step by cation-anion exchange and ID-SDS-PAGE prior to bottom-up proteomic analysis was used in subsequent TBI biomarker studies from our group (Kobeissy et al., 2006; Ottens et al., 2007). Kochanek's group was the first to use 2D-PAGE in TBI biomarker study (Jenkins et al., 2012). Siman et al. (2004) performed MALDI-MS following 2D-PAGE of proteins released from TBI cell culture model to identify acute TBI protein biomarkers. 2D-PAGE and mass spectrometric analysis have been implemented in oxidative stress TBI biomarker studies (Opii et al., 2007). An alternative to the above approach is shotgun proteomics (Wolters et al., 2001; McDonald and Yates, 2002, 2003; Wu and MacCoss, 2002). The complex mixture in shotgun analyses is directly digested without prior separation or fractionation. Variations of this method exist but all shotgun proteomics begins with a mixture of proteins. For example, a complete protein digest without prior separation can be separated by LC and then analyzed by MS in real time. A shotgun proteomic approach based on nanoLC in conjunction with matrix-assisted laser desorption/ionization time of flight tandem MS (MALDI-TOF MS/MS) was utilized to quantitatively analyze the protein content of consecutive ventricular CSF samples of severe TBI patients (Hanrieder et al., 2009). Recently, our group has applied shotgun proteomics to profile the neuronal-glial biomarkers released into conditioned media collected from MTX-, NMDA-, and STS-treated cell cultures (Guingab-Cagmat et al., 2012).

One application of MS is in the identification of intact proteins (i.e., without enzyme digestion) referred to as top-down approach. In the context of proteomics, top-down is an emerging technology but more difficult to implement compared to the more widely used bottom-up approach. For proteomics, top-down has the advantage of preserving the forms of proteins present *in vivo* by measuring them intact, rather than measuring peptides produced from them by proteolysis. This approach is particularly useful in characterization of post translational modifications which may be challenging to analyze with enzymatic digestion. But in order to perform this kind of analysis, an expensive instrumentation is a requirement. Most of the laboratories however don't have the luxury of having a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS) (Marshall et al., 1998; Shi et al., 1998), or the relatively less expensive Orbitrap mass spectrometer (Thermo's Orbitrap Elite) or access to these kinds of instruments. The very advantage of these kinds of instruments is that they are highly sensitive and capable of ultra-high resolution. The downside however is that maintenance of FT-ICR-MS is very expensive since it requires cooling a very strong magnet, on top of an expensive machine. The Orbitrap mass analyzer traps ions using an electrostatic field, instead of a magnet. The cost and maintenance are now relatively lower, but still an expensive machine. Possibly due to these reasons, top-down proteomics is yet to be accepted and widely implemented to TBI studies.

Presently, technologies that focus on identifying less abundant proteins are gaining traction. These methods are usually based on MS, and the requirement is that a step prior to injection into the mass spectrometer is added. Broadly, the steps prior to MS can be categorized into chemical modifications and direct enrichments. An example of chemical modification is affinity tagging. A popular tagging method is ICAT or isotope coded affinity tags. It is used to quantify and identify plasma biomarkers of TBI. These kinds of experiments can identify several candidate proteins, from tens to hundreds. Protein biomarkers in serum of pediatric patients with severe TBI were identified by ICAT-LC-MS/MS (Haqqani et al., 2007a,b). Another approach is isobaric tagging for relative and absolute quantification (ITRAQ). An example is the study by Crawford et al. (2012) on the identification of protein markers of TBI outcome. Here, CCI mouse model was used to identify plasma biomarkers specific to mild or severe TBI at 24 h, 1 month, or 3 months post-injury. In addition, they used apolipoprotein E 3 and 4 transgenic mice, which demonstrate relatively favorable and unfavorable outcomes respectively, following TBI to identify proteins that are significantly modulated in response to the TBI*APOE genotype interaction representing potential prognostic biomarkers. ITRAQ has also been applied in the identification of serum biomarkers and demonstrating their potential for predicting elevated intercranial pressure in TBI patients (Hergenroeder et al., 2008).

Direct enrichment entails some separation prior to MS analysis. These separation strategies usually apply chromatography, SDS-PAGE, or antibody. For example, cation-exchange chromatography, SDS-PAGE, and then LC, were performed on a rat CCI model to identify putative protein biomarkers post 48 h TBI (Kobeissy et al., 2006; Ottens et al., 2007). The results included 59 differential protein components of which 21 decreased and 38 increased in abundance after TBI. Proteins with decreased abundance included collapsing response mediator protein 2 (CRMP-2), glyceraldehyde-3-phosphate dehydrogenase, microtubule-associated proteins MAP2A/2B, and hexokinase. Conversely C-reactive protein, transferrin, and breakdown products of CRMP-2, synaptotagmin, and alphaII-spectrin were found to be elevated after TBI.

## BIOINFORMATICS IN IDENTIFYING PROTEINS

Proteomics experiments to identify proteins are tedious. It is akin to breaking a huge and complicated puzzle and then putting the pieces together again. With our puzzle analogy, manual integration of the pieces (smaller peptides) is impossible to complete without the help of computers.

The need for algorithms to identify proteins married bioinformatics to proteomics. Once the developers of algorithms were on board, they needed to know some of the rules of protein science. One of these is for example in sample digestion. If trypsin was used in the digestion, this enzyme is known to *only* cleave proteins after both lysine and arginine, *as long* as the next amino acid sequence is not proline (Fraser and Powell, 1950). This and many other rules have to be grasped by software developers.

Other rules that computer scientists should fundamentally understand are mass spectra. Historically speaking, MS of digested proteins was performed predominantly by matrix-assisted laser desorption ionization time of flight or MALDI-TOF. MALDI (Karas and Hillenkamp, 1988) is a kind of ionization that is regarded as "soft," enabling large biomolecules to be ionized and carried to the mass analyzer. The ionization requires two things:

the energy from the laser and the matrix. Although the mechanism of MALDI is still in question, it is believed that the ionization of the analyte happens after the matrix absorbs the energy from the laser, the matrix imparting the energy to the analyte, thereby ionizing the sample.

Once the calculated experimental spectrum or mass lists are produced, these are matched against a protein database. Another set of spectrum, a theoretical one, is also matched to the database. Theoretical and experimental results are compared and computed, to have confidence in the identified proteins (Maggio and Ramnarayan, 2001; Colinge and Bennett, 2007; Matthiesen, 2007; Webb-Robertson and Cannon, 2007).

The above method, performed in MALDI-TOF, is commonly referred to as peptide mass fingerprinting (PMF). PMF's requirement is that a single spectrum should contain the peptides of the protein. The introduction of LC and ESI however removed the single spectrum requirement. With LC experiments, identification of peptides became more challenging.

In a typical LC-MS/MS analysis, one can predefine the number of the most intense peaks to be selected for dissociation. For example, in our laboratory, we subject the 10 most intense peptide signals to tandem MS (MS/MS) fragmentation (data dependent scanning). Every second, the MS analyzes the sample and produces a full MS Scan of ∼20,000 intact peptides. Based on the initial full MS scan, the mass spectrometer, following the user's settings, selects again and fragments up to 10 distinct peptides, producing another set of MS/MS spectra.

During the selection of peptide however, the same peptide can be selected more than once. To avoid this problem, a dynamic exclusion strategy is usually implemented. For example, if a peptide was selected three times already over the span of 18 s, that peptide is placed in the exclusion list for the next 25 s. The cycle of subjecting the 10 most intense peptides to MS/MS and the production of s full MS scan is repeated until the chromatography is done.

Tandem MS (MS/MS) provides an additional degree of information in identifying proteins. One can see that in a single analysis, a large number of MS/MS spectra are produced. Assigning the peptide sequences responsible for the generation of the observed fragments is challenging. Since the fragmentation process in MS/MS follows some rules, rules that software developers exploit, it is now possible to identify proteins that are subjected to tandem MS.

Collision of an inert gas with large proteins (such as collision induced dissociation or CID) fragments the proteins apart into smaller peptides. This happens inside the trapping cell of the MS. The breaking of proteins follows a certain type of fragmentation pattern (most researchers follow the nomenclature introduced by Roepstorff and Fohlman, 1984). It is widely known that proteins in the gas phase can break into set of ions ("*b*, *y*, and *a*" type ions) (Bencsath and Field, 1988; Polfer et al., 2005; Liu and Schey, 2008; Chen et al., 2009; Paizs and Mann, 2012).

Even though the rules of producing specific ions are clear, problems still exist. Some compounding factors happen when there can be some additional peaks resulting from neutral losses (*b*-$H_2O$, *y*-$H_2O$), ammonia loss (*b*-$NH_3$), from contaminating peptides, small molecules, or even missing peaks. Some peaks can be shifted due to amino acid modifications. And as in any other analytical signal, the presence of noise even complicates the spectrum interpretation. These hinder the peptide sequence assignment to each spectrum.

## SOFTWARE FOR PEPTIDE AND PROTEIN IDENTIFICATION

The process of protein identification benefited from the maturation of two technologies, the computer hardware and database software. Protein database search has become a powerful approach to address the challenge of protein ID. Currently, numerous bioinformatics software for computational peptide identification from MS/MS data are available in the market (Xu and Ma, 2006).

The first computer program to use a database search was Sequest. Acquired by Thermo Scientific and commercially available through Proteome Discoverer (Thermo, San Jose, CA, USA. www.thermo.com), the development of this software can be traced back to Yates et al. (1995) in the early 1990s at the University of Washington. The scoring function in this package is heuristic in nature, and it was considered to be the first really useful bioinformatics technique in the field of proteomics. The software integrates correlational analysis between data dependent mass spectral scans and a FASTA protein database. Sequest searches and identifies peptides and the corresponding modifications that the user specifically queries. Using these peptide identifications, one can make inferences about the proteins in the sample.

In Sequest, the first process is the extraction of tandem mass spectra from the raw file. Theoretical candidate sequences from the digested proteins in the database are listed. Within a defined tolerance set by the end user, the algorithm determines which one matches the experimental peptides' molecular weight. A comparison of the candidate's *b* and *y* ions to the experimental spectra are made and scored as primary score (Sp). The primary score sorts the candidate sequences in descending order. Sequest uses two scoring functions, so after the initial candidate sequence is determined; the top peptides are taken off the list. A second function rescores the hits by computing a cross correlation, taking into account their height and mass position. The new candidates are resorted in descending order. After taking into account the possible random matches, the final list after resorting is the final SEQUEST scores (Xcorr). These top hits are reported back and stored into the search files (.msf). In addition to Sequest's Xcorr, users can export several other parameters such as Sp or DeltaCn. DeltaCn measures how good the XCorr is relative to the next best match. Overall, Xcorr is a robust measure of how accurate the match was between theoretical and experimental peaks.

Several algorithms came after Sequest. MASCOT (Perkins et al., 1999) and X! Tandem also became popular search engines. Owned by Matrix science, MASCOT is commercially available, although the scoring has never been patented or published. With MASCOT, the accuracy score is probability based. This is measured by Ion score, and a *P*-value gives a relative score. On the other hand, X! Tandem is an open source tool. These search engines approach the problems differently and uses different algorithms. With X! Tandem, hyper score and *E*-value are two of the parameters calculated.

In some instances however, there are situations that a protein database is not yet available. This can happen in the analysis of an organism where its genome sequence is incomplete or unavailable.

In addition, if one is only interested in identifying novel isoforms of the protein, often, the database is unavailable. A popular approach to tackle the problem is to perform a *de novo* (Shevchenko et al., 2001) sequencing. Spectrum identification in *de novo* analysis uses a database of candidate peptides consisting of all possible linear amino acid sequences (Xu and Ma, 2006). This method can be used also for searching peptide homologs and modifications. In the early days of the development of algorithms in *de novo* sequencing, researchers in this field attempted to reconstruct peptide sequences by making all of the amino acid combinations. This was not applicable though due to generic problems. However, the market has seen software for *de novo* sequencing. Algorithms in *de novo* sequencing usually filter the experimental mass list to remove noisy peaks. PEAKS (Ma et al., 2003) and PepNovo (Frank and Pevzner, 2005) are some of the software that facilitate fast *de novo* peptide identification. A hybrid between the *de novo* sequencing and protein database searching is known as tag-based approach. Sequence-tagging uses the *de novo* analysis to identify subpeptides or sequence tags hypothesized to occur in the sequence. In these kind of experiments, information is usually extracted from database that contains the tags (Mann and Wilm, 1994).

Since the sequencing results of *de novo* shows a close resemblance compared to the output of known protein database, *de novo* is usually used in validating the accuracy of database-derived protein identifications (Shadforth et al., 2005). Validation of the accuracy of one's result is one of the issues that are tackled by end users and software developers. Reviewers of top proteomic journals have pushed to address this issue. This will be discussed next.

### FALSE DETECTION RATE

False detection rate (FDR) measures the false positive proteins identified. FDR provides a statistically meaningful estimate of the uncertainty in protein identification. It is usually a good validation, for example in large data sets of brain proteins. Most proteomic journals require FDR to be reported. In measuring FDR, a decoy database is usually used. Decoy database for FDR calculations were pioneered by Gygi and co-workers, in which decoys consist of a randomized or scrambled sequence database (Elias and Gygi, 2010). The parameters used in regular search are applied to the decoy database search. Matches using decoy database search is not expected to be significant, and the number of matches found in a decoy search is a good estimate of the real FDR in the regular forward sequence database search.

Although there are two ways to implement a search in a decoy database, users preferentially use one from the other. The most preferred method is the concatenated approach. In this method, the decoy and the non-decoy databases are linked together.

The other method is a more conservative approach. The search of MS/MS data is separate from non-decoy to decoy databases and the number of matches for each database is counted.

### SYSTEMS BIOLOGY

After a database search and identification of proteins, usually a huge library of information is generated. The next step is to know the protein's functions and the connections of these identified proteins. Rather than focusing on individual molecular components, systems biology seeks to understand the dynamics that govern protein networks, the functional set of proteins that regulate cellular decisions related to TBI. From the perspectives of drug discovery and diagnostics, systems biology gives important and practical clues concerning the pathways relevant to brain injury and the effects that drugs might have on them. Therefore, it enhances the entire biomarker and therapeutic drug discovery, development, and commercialization process (cite Systems bio approach/Theranostics). Recently, protein biomarkers of TBI, induced by penetrating ballistic-like injury model (PBBI), were identified by the proteomics followed by systems biology analysis (Boutté et al., 2012). These proteins are ubiquitin carboxyl-terminal isozyme 1, tyrosine hydroxylase, and syntaxin-6. Using semi-quantitative western blotting analysis, the said proteins were found to be elevated after 72-h post-injury compared to control. It should not be a surprise that Ubiquitin carboxy-terminal hydrolase L1 protein (UCHL1) is already in clinical trial as a biomarker.

The connections or network of connections are pictured using nodes and links. The nodes can be a biomolecule, such as proteins or DNA. The link or the connections between these nodes represent the biochemical interactions or the connections can highlight relationships between nodes, such as the strength of predicted binding or physical interactions. Theories in the science of systems study and statistical mechanics, in conjunction with graph theory, can be applied to glean insights about the network. Mapping the connection of these proteins is the driving force of pathway-based biomarker discovery and diagnosis. Particularly in TBI, upregulated proteins after the injury are hunted and identified as possible diagnostic biomarkers. Numerous scientific publications containing networks of cellular pathways are scattered throughout archives and available data are growing fast. Historically, most of the repositories of large scale sequencing projects were mostly nucleic acid and amino acids. But this gave way to other biomolecules such as proteins. Lately, databases that store proteins have been steadily increasing. For example, the Database of Interacting Proteins can be queried for known protein-protein interactions or PPI (Xenarios et al., 2001).

The nuts and bolts of these bioinformatics software, which systems biology has integrated, are geared toward people with a strong background in computer science and statistics. Since we are the end users of this technology, we will focus on software that we are familiar with and have been using. Readers are directed to other sources of in-depth reviews with regards to systems biology. Three commercially available pathway analysis software include Pathway Studio (Ariadne Genomics, Rockville, MD, USA), Metacore (Thompson Reuters, New York City, NY, USA), and Ingenuity (Ingenuity Systems, Redwood City, CA, USA). These tools enable the identification of the relationship among proteins, small molecules, cell processes, and diseases. Pathway analysis provides information on what is known to interact with the proteins that are identified in the sample as well as association of these proteins to cellular processes.

## BIOMARKER APPLICATIONS

Clinically validated biomarkers are needed for the accurate diagnosis of mild TBI. This type of TBI is particularly hard to accurately measure and the situation is, made more challenging by patients

who sometimes hide their symptoms. There is no gold standard yet for diagnosing mild TBI (Shenton et al., 2012), not even by conventional assessment through neuroimaging techniques (Niogi and Mukherjee, 2010). The lack of a consensus definition of mild TBI further complicates the matter (Ruff and Jurica, 1999; Arciniegas and Silver, 2001) and the challenge lies in accurate diagnosis in managing post-injury. The Veteran's Administration Clinical Practice Guideline released a working document on criteria to diagnose mild TBI. These diagnostic criteria include an initial Glascow Coma Scale (GCS) of 13–15; less than a 30-min loss of consciousness; post traumatic amnesia up to 24 h after the injury and alteration of consciousness (Management of Concussion/mTBI Working Group, 2009). Other factors may compound this guideline. In addition to patients trying to hide the true injury, proper diagnosis is compounded by alcohol ingestion, polytrauma, sedatives, pain killers, and drugs of abuse.

A biomarker that is measurable in the blood would be useful in these kinds of situations, where a polytrauma exists. It was suggested that instead of using one biomarker, a panel of biomarkers could be helpful. Mondello et al. (2012a) have explored the ratio of GFAP and UCHL1 concentrations to assess patients with severe TBI.

Another type of injury that needs to be addressed by biomarkers from the blood is in diffuse axonal injury (Inglese et al., 2005). The microstructural axonal damage in this kind of injury is believed to be a challenge to detect by neuroimaging techniques such as computed tomography and conventional MRI.

Drug discovery is one of the fields that will greatly benefit from a signature marker for TBI. New therapeutic development traditionally has an extremely high triage rate because more than 90% of drugs that advance to Phase I clinical trials fail. Some argue such extreme loss can be overcome by guiding all new therapeutic development and clinical trials with a disease-relevant diagnostic test. Discovery of translational biomarker (from animal studies to clinical trials) might help to finally deliver the long sought after clinical trial success. "*Theranostics* represents the convergence between *Thera*peutics and diag*nostics* (Bissonnette and Bergeron, 2006; Hooper, 2006)." It has been viewed as the parallel use of new therapy and diagnostic tests for a human disease or disorder so as to facilitate drug development and clinical trials and to achieve optimal clinical outcomes in a population of patients. Importantly, in recognizing the emerging role of the theranostic approach, the FDA has recently drafted a Drug-Diagnostic Co-Development Concept Paper (Hinman et al., 2006) with the goal of setting guidelines for prospective co-development of a drug or biological therapy (drugs) and a device test in a scientifically robust and efficient way.

One example of a theranostic approach to drug development is a novel biomarker-guided approach in our laboratory that combines calpain-generated acute brain injury-tracking biomarkers with potent and selective calpain inhibitor drug candidates to fast-track and improve the chances of successful drug development for CNS injury. During brain injury, neural proteins or their breakdown products generated by calpains (μ-calpain and m-calpain) are released into the extracellular environment and eventually reach the CSF in relatively high concentration (Wang et al., 2005). In due time the proteins reach the blood stream either via the compromised blood brain barrier (BBB or via filtration of the CSF). Clearance and half-life of the biomarkers contribute to the final concentration that can be measured in the blood. The CSF volume of an adult human (CSF 125–150 mL) is about 30- to 40-fold less than the blood volume (4.5–5 L) which explains why the brain biomarker concentration is significantly higher in the CSF samples versus blood samples and makes the former valuable for drug development. Enabled by recent technological advances in proteomics, novel brain injury biomarkers that have elevated levels in biofluid such as CSF or blood after TBI have been discovered.

## POSSIBLE BIOMARKERS FOR TRAUMATIC BRAIN INJURY

We now know that despite the efforts in brain injury research to discover and develop disease tracking markers, currently there are no clinically validated biomarkers to diagnose TBI. Even though the search continues, several candidate biomarkers of TBI biomarkers are in the clinical validation pipeline. Extensive studies are being pursued to move these protein biomarkers to clinical validation. The aforementioned techniques in proteomic have been employed in the discovery for candidate biomarkers of TBI. Kobeissy et al. identified 59 differentially proteins 48 h post TBI using a CCI rat model. Proteins that were decreased in abundance included CRMP-2, glyceraldehyde-3-phosphate dehydrogenase, microtubule-associated proteins MAP2A/2B, and hexokinase (Kobeissy et al., 2006). Upregulated proteins included C-reactive proteins, transferrin, and breakdown products of CRMP-2, synaptotagmin, and alphaII-spectrin. Western blotting analysis confirmed the differential changes in the mentioned proteins. This study provided insight into the mechanism of TBI and generated candidate biomarkers that can aid in the evaluation of the severity and progression of injury as well as in the development of possible therapies. The need for strengthening the role of systems biology and its application to the field of neuroproteomics due to its integral role in establishing a comprehensive understanding of specific brain disorder and brain function in general was highlighted in a review by Kobeissy et al. (2008). The use of a systems biology-based approach to drug discovery and development for TBI based on the advances in genomics, proteomics, bioinformatic tools, and systems biology software has been shown (Zhang et al., 2010). Recently, Boutté et al. (2012) conducted a proteomic analysis and brain-specific systems biology in a rodent model of PBBI. In their study, a combination of two-dimensional gel electrophoresis and MS was used to screen for biomarkers in a rat model of PBBI. Brain-specific systems biology analysis of brain tissue identified 321 upregulated and 65 downregulated proteins 24 h post PBBI compared to sham controls. In their gene ontology analysis, the majority of upregulated proteins were cytoskeletal (10.5%), nucleic acid binding (9.3%), or kinases (8.9%). Most proteins were involved in protein metabolism (22.7%), signal transduction (20.4%), and development (9.6%). Pathway analysis indicated that these proteins were involved in neurite outgrowth and cell differentiation. Further confirmation of these proteins was conducted using semi-quantitative Western blotting. Among these proteins that indicated consistent increase in the brain tissue and CSF at several time points post PPBI were UCHL1, tyrosine hydroxylase, and syntaxin-6. Antibody-based platforms, antibody microarrays

(AbMA), and reverse capture protein microarrays (RCPM) complementing the classical methods based on 2D gel electrophoresis and mass spectrometry (2DGE/MS) has been proposed for discovery of potential biomarkers for blast neurotrauma (Agoston et al., 2009). Kwon et al. (2011) combined behavioral, proteomics, and histological studies to investigate stress and blast-induced TBI. In this study, exposure to repeated stress alone showed a transient increase in anxiety but no significant memory impairment or cellular and molecular changes. In contrast, repeated stress and blast resulted in lasting behavioral, molecular, and cellular abnormalities characterized by memory impairment, neuronal and glial cell loss, inflammation, and gliosis.

Listed below are examples of the most studied candidate protein biomarkers for TBI. These represent potential biomarkers of TBI that have shown high sensitivity and specificity in independent studies. UCHL1, SBDPs, and neuron-specific enolase (NSE) are presented as examples of neuronal and axonal protein biomarkers. For glial-specific markers, GFAP and S100beta are discussed below.

#### UBIQUITIN CARBOXY-TERMINAL HYDROLASE L1 PROTEIN

Ubiquitin carboxy-terminal hydrolase L1 protein is a cysteine protease that is predominantly expressed in neurons, although it is also expressed in small amounts in neuroendocrine cells. This enzyme is relatively small, around 25 kDa and comprises ∼2% of the total soluble protein in the brain. The other name for this protein is neuronal-specific protein gene product 9.5. Known function of UCHL1 is that it hydrolyzes the C-terminal bond of ubiquitin or unfolded polypeptides (Setsuie and Wada, 2007).

Several publications have indicated that UCHL1 can be a biomarker for TBI. Recently, the biokinetic parameters of UCHL1 were measured from a cohort of severely injured TBI patients (Brophy et al., 2011). A more recent study (Mondello et al., 2012b) demonstrated that UCHL1 can be used as a biomarker for severely injured TBI patients. Compared to control, the serum UCHL1 levels of TBI patients were significantly elevated measured after the acute phase and then over a week.

#### SPECTRIN BREAKDOWN PRODUCTS

AlphaII-spectrin is primarily found in neurons and is concentrated in axons and presynaptic terminals (Riederer et al., 1986). Upon activation in TBI, calpain cleaves the protein to breakdown products (SBDPs) of molecular weights 150 kDa (SBDP150) and 145 kDa (SBDP145) and casapse-3 cleaves it to a 120-kDa product (SBDP120). 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;). SBDPs concurrently indicate calpain and caspase-3 proteolysis of alphaII-spectrin, providing crucial information on the underlying cell death mechanisms. In CSF, distinct temporal release patterns of SBDP145 and SBDP120 were observed to reflect different temporal characteristics of protease activation (Mondello et al., 2010). Elevated levels of SBDPs in CSF from adults with severe TBI were reported 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. The temporal profile of SBDPs in non-survivors was also found to be different those of survivors (Mondello et al., 2010). Taken together, these findings 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.

#### NEURON-SPECIFIC ENOLASE

Neuron-specific enolase is a glycolytic pathway enzyme and highly expressed in neuronal cytoplasm. NSE has been shown to have the sensitivity and specificity to detect neuronal cell death (Selakovic et al., 2005). 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 (Ross et al., 1996; Herrmann et al., 2000; Selakovic et al., 2005).

#### GLIAL FIBRILLARY ACIDIC PROTEIN

Of the numerous candidate biomarkers for TBI, this protein holds the most promise. One of the strengths of GFAP as an ideal biomarker for TBI is that this protein is not found outside the central nervous system (Galea et al., 1995). First reported in 1971 (Eng et al., 1971), GFAP is found only in astroglial cytoskeleton. GFAP is an intermediate filament protein that forms networks that support the astroglial cells. Damage to the astroglial cells (astrogliosis) shows subsequent upregulation of GFAP. During injury, astroglial cells react by producing more GFAP. Evidence shows that serum GFAP is elevated with several types of brain damage, including TBI (Pelinka et al., 2004a,b; Nylén et al., 2006).

What makes GFAP specific to brain trauma is that even if the body is subjected to multiple forms of trauma, GFAP doesn't spike up without brain injury (Pelinka et al., 2004b; Vos et al., 2004). Thus, GFAP as a biomarker is a specific indicator of injury to the glia. There's also a high likelihood that GFAP can predict death or unfavorable outcomes (Vos et al., 2010; Zurek and Fedora, 2012). According to the proceedings of the military mild TBI diagnostic workshop (2010), validation studies in humans are already on-going (Marion et al., 2011).

#### S100ß

S100ß is mainly found in astroglia and Schwann cells (Donato, 1986; Donato et al., 1986a,b), and is one of the most well-known biomarkers of brain damage. The concentration of S100ß is known to increase in the CSF and serum after injury making this protein a potential biomarker for TBI (Townend et al., 2006). This protein is not influenced by hemolysis and has a biological half-life of 2 h. S100ß belongs to a family of low molecular weight (9–13 kDa) calcium-binding S100 proteins and is involved in signal transduction (Heizmann et al., 2002). Several studies have examined the value of this marker, demonstrating correlation with injury and outcome (Pelinka et al., 2003a; Berger et al., 2005; Kleindienst et al., 2007; Egea-Guerrero et al., 2012). However, several limitations have been found. First, S100ß is not specific to the brain, showing up in non-nervous cells such as adipocytes, epidermal, chondrocytes, melanoma cells, and Langerhans cells (Zimmer et al., 1995). The presence of this protein outside the central nervous system is compounded by the problem that general trauma without

brain injury can increase the said protein (Rothoerl and Woertgen, 2001). Second, S100ß spikes up after hemorrhagic shock, correlating the concentration to shock severity (Pelinka et al., 2003a,b,c). Because of this, it seems that S100ß cannot be used as a single biomarker for TBI. A recent study has looked at the ratio of S100ß against GFAP (Pelinka et al., 2004a), instead of looking at S100ß alone. In the study, the ratio of GFAP against S100ß was used to determine brain damage and prognosis. In another study, S100ß seemed to be a useful indicator of patients with intracranial lesion (Egea-Guerrero et al., 2012).

Another limitation in using S100ß as a biomarker for TBI is the relatively short serum half-life (Jackson et al., 2000). The obvious countermeasure to this problem is to measure the proteins right after injury; however, most mild TBI victims are not evaluated as soon as the injury occurs.

## CONCLUSION

Proteomics, with the advancement in MS along with the bioinformatics software, has opened opportunities to interrogate protein dynamics and provide insights into the biochemistry of TBI. Over the past years, proteomics has led to the discovery of many candidate biomarkers and is becoming the method-of-choice for preliminary candidate marker selection. However, identification of candidate biomarkers using this approach is proving to be only the initial step in the development of a useful biomarker. Systems biology coupled to data mining strategies has been applied to harness these large data sets into organized and interlinked databases that can be queried to identify non-redundant brain injury pathways. The pathways can be exploited to determine the utilities of these proteins as diagnostic biomarkers and/or therapeutic targets.

This review provides an overview of the integration of proteomics, bioinformatics, and systems biology in TBI biomarker discovery. At present, proteomic biomarker discovery experiments have generated a long list of TBI biomarker candidates. Clearly, the next step is translating a robust biomarker or panel of biomarkers to clinical use. Currently, sensitive and specific immunoassays are being developed to validate a number of TBI biomarkers in clinical samples. However, the high cost of assay development and availability of antibodies result in a bottleneck in the clinical validation pipeline of the long list of discovered potential biomarkers. Targeted proteomics is a growing trend among the proteomic community. Mass spectrometry-based measurements such as multiple reaction monitoring (MRM) is a promising technique that could revolutionize biomarker validation. The current technologies are still evolving to address fundamental problems in identifying low abundant protein biomarkers such as in the case of mild TBI. The trend of lower costs, highly sensitive instruments (Orbitrap), and better electronic hardware will most likely increase targeted proteomics experiments in the future.

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# The role of markers of inflammation in traumatic brain injury

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Within minutes of a traumatic impact, a robust inflammatory response is elicited in the injured brain. The complexity of this post-traumatic squeal involves a cellular component, comprising the activation of resident glial cells, microglia, and astrocytes, and the infiltration of blood leukocytes. The second component regards the secretion immune mediators, which can be divided into the following sub-groups: the archetypal pro-inflammatory cytokines (Interleukin-1, Tumor Necrosis Factor, Interleukin-6), the anti-inflammatory cytokines (IL-4, Interleukin-10, and TGF-beta), and the chemotactic cytokines or chemokines, which specifically drive the accumulation of parenchymal and peripheral immune cells in the injured brain region. Such mechanisms have been demonstrated in animal models, mostly in rodents, as well as in human brain. Whilst the humoral immune response is particularly pronounced in the acute phase following Traumatic brain injury (TBI), the activation of glial cells seems to be a rather prolonged effect lasting for several months. The complex interaction of cytokines and cell types installs a network of events, which subsequently intersect with adjacent pathological cascades including oxidative stress, excitotoxicity, or reparative events including angiogenesis, scarring, and neurogenesis. It is well accepted that neuroinflammation is responsible of beneficial and detrimental effects, contributing to secondary brain damage but also facilitating neurorepair. Although such mediators are clear markers of immune activation, to what extent cytokines can be defined as diagnostic factors reflecting brain injury or as predictors of long term outcome needs to be further substantiated. In clinical studies some groups reported a proportional cytokine production in either the cerebrospinal fluid or intraparenchymal tissue with initial brain damage, mortality, or poor outcome scores. However, the validity of cytokines as biomarkers is not broadly accepted. This review article will discuss the evidence from both clinical and laboratory studies exploring the validity of immune markers as a correlate to classification and outcome following TBI.

**Keywords: traumatic brain injury, biomarkers, inflammation, cytokines, chemokines**

## INTRODUCTION

Traumatic brain injury (TBI) has long been called a "silent epidemic" (Goldstein, 1990; Coburn, 1992). In the United States, it accounted for at least 1.5 million emergency room visits and hospitalizations annually between 1995 and 2001, a number that has certainly increased as a result of the wars in Iraq and Afghanistan (Langlois et al., 2006). Many survivors of TBI are left with long term disabilities, and even a mild TBI can leave people with cognitive impairments, difficulty in concentrating, fatigue, and headaches. It disproportionately affects young men, but is also increasingly common in the elderly population (Hukkelhoven et al., 2003). The financial burden to the United States has been estimated to exceed $56 billion annually (Finkelstein et al., 2006; Langlois et al., 2006; Rutland-Brown et al., 2006) while in 2008 alone it has been calculated to approximately AU$ 8.6 billion in Australia. Despite advances in prevention measures, surgical, and diagnostic techniques, there have been relatively few changes in the way TBI patients are managed, and so far no pharmacological treatment has been found to confer neuroprotection by targeting secondary injury mechanisms.

Currently, the main focus for TBI patient management is monitoring and maintenance of normal intracranial pressure (ICP), and cerebral perfusion pressure (CPP). One of the effects of elevated ICP is the reduction in CPP, which consequently leads to secondary ischemia. It is no surprise therefore that high ICP has been shown to be associated with mortality and poor outcome in TBI patients (Narayan et al., 1982; Treggiari et al., 2007). Although treatment of high ICP is certainly beneficial, being able to predict elevations in ICP and apply preventive or early interventions would be more ideal. At present, there is no method with which to predict changes in ICP, since neurological examinations and routinely administered computed tomography (CT) scans do not provide this information. To this end, the development of biomarkers that have predictive accuracy with regard to ICP would greatly improve patient management.

In addition to lowering elevated ICP, treatment and management of a TBI patient could depend on a wide range of factors. Human TBI is a very heterogeneous condition due to the intrinsic combination of focal and diffuse injuries and the individual response via secondary mechanisms of neurodegeneration. Understanding these processes would aid the development of an individual patient's tailored treatment plan. At present patients are categorized based on admission characteristics including age, pupil reaction, Glasgow coma scale scores (GCS), body temperature, blood glucose, non-cranial injuries (Hukkelhoven et al., 2005; Mushkudiani et al., 2008), and also observations from a CT scan (e.g., Marshall CT classification, primarily prognostically oriented Rotterdam score; Marshall et al., 1992; Maas et al., 2005). Magnetic resonance imaging (MRI) is routinely used in the clinic and is becoming very popular for the quantification of brain damage particularly in diffuse TBI. The more recently developed diffusion tension (DT)-MRI can detect with in great detail alterations in the microstructure of the white matter and shows considerable promise in the assessment of axonal damage (Salmond et al., 2006). However, these neuroimaging techniques reveal little or no information regarding secondary injury processes such as excitotoxicity, neuroinflammation, blood-brain barrier (BBB) breakdown, ischemic damage, and cell death. Biomarkers promise to arm clinicians with all this additional, patient-specific information. In the context of this review a biomarker is a molecule that can be measured in a patient, which reflects the pathology of TBI, how the pathology is likely to develop, and become predictive factors for long term neurological outcome. It would be of great benefit to the patient if an endogenous molecule could be identified to have an expression profile, which can be linked to the type or extent of secondary injury. With this information clinicians would be able to make more informed decisions regarding treatments most likely to lead to an optimal outcome. Furthermore, detection of early biomarker levels would be invaluable in identifying patients that are most likely to benefit from a specific experimental treatment. Indeed, the diverse nature of human TBI has been cited as a potential explanation for lack of a successful clinical trial (Statler et al., 2001). While it is now clear that there is no "silver bullet" with which all TBI patients can be treated, categorization of TBI patients using biomarkers in combination with traditional methods might allow for specific treatments to be given to those most likely to benefit.

Identifying secondary injury mechanisms may also be useful in determining the type of injury that a person has received and the degree of its progression in the long term. For example, the "War on Terror" has led to an increase in the incidence and awareness of blast injuries, which involve a rapid change in air pressure around the body. Blast injury is becoming increasingly common, yet symptoms often do not manifest until weeks or months after the incident (Zeitzer and Brooks, 2008). Even a mild TBI can leave survivors with long term cognitive deficits and behavioral problems, which impact on their daily lives (Corrigan et al., 2004; Pagulayan et al., 2006; Strandberg, 2009). Being able to specifically predict which patients are likely to develop these neurological symptoms would enable doctors to make referrals to specific rehabilitation programs, council patients and family members, and encourage vigilance in reporting such changes.

## THE DEVELOPMENT OF BIOMARKERS IN TBI

The most commonly used biomarkers include S100B, neuron-specific enolase (NSE), and myelin basic protein (MBP; Palfreyman et al., 1978; Thomas et al., 1978) reflecting the extent of tissue damage as well as having prognostic value for long term outcome (Baker et al., 2009; Svetlov et al., 2009; Kovesdi et al., 2010). There are several observational clinical TBI studies where S100B has been successfully correlated with initial brain injury severity (GCS), size of brain damage (on CT/MRI scans), and neurological outcome (Glasgow Outcome Scale/Extended; GOSE). However, it is only recently that biomarkers are being employed in the context of clinical trials with prospective collection of physiological data, outcomes, and the clinical assessment of efficacy of an intervention. The ultimate aim is to demonstrate the correlations between a drug's neuroprotection and the reduced concentration of biomarkers in TBI patients. Biomarkers are defined as sensitive early measure of outcome than the currently available neurological scores assessed only 6 months after TBI. In addition due to the common problem of TBI clinical trials being inadequate in patient numbers, the use of biomarkers could provide a more powerful tool to detect outcome differences in TBI patient populations.

At present, the most studied TBI biomarker is S100B, a low-molecular weight calcium binding protein secreted by astrocytes. The validity of S100B as a potential TBI biomarker relies on its constitutively low expression in serum and cerebrospinal fluid (CSF), which is rapidly released into the CSF and serum following brain injury. A very strong correlation of S100B levels and severity of injury has been reported (Savola et al., 2004), as well as a link between high S100B expression and unfavorable outcome (Herrmann et al., 2001; Townend et al., 2002; Vos et al., 2004; Rainey et al., 2009). However, S100B is not ideal as a TBI biomarker because it does not readily cross the BBB, its serum levels increase after peripheral trauma in the absence of brain injury (Anderson et al., 2001; Savola et al., 2004; Torabian and Kashani-Sabet, 2005), and it has not always been found to reliably predict outcome (Berger et al., 2007; Piazza et al., 2007). In the search for a reliable biomarker of TBI, other molecules have been assessed, including glial fibrillary acidic protein (GFAP), NSE, MBP, α-II-spectrin breakdown products (BDPs), ubiquitin C-terminal hydrolase-L1, and various cytokines (Dash et al., 2010; Schiff et al., 2012).

## THE POTENTIAL OF INFLAMMATORY CYTOKINES AS BIOMARKERS OF TBI

One of the integral features of TBI is the inflammatory reaction initiated and regulated by an array of pro- and anti-inflammatory cytokines. Cytokines are small, short-lived proteins produced by blood leukocytes and glial cells. They are quickly released in response to TBI and are rapidly sequestered. There are a large number of different cytokines, many with overlapping functions that form a complex network of inflammatory mediators. Cytokines that initiate or propagate an inflammatory response are said to be pro-inflammatory, while cytokines that inhibit the inflammatory response are called anti-inflammatory. The expression profile of each cytokine following brain injury has the potential to provide information about the extent of tissue damage, and can be easily and rapidly measured via immunological assays. However, cytokine concentrations vary depending on the tissue or fluid they

are measured in (brain tissue, CSF blood, serum, plasma, etc.), and the temporal profile of the cerebral immune response in rodent versus human data can present differences as well as commonality. Some of the most studied cytokines with respect to brain injury and their potential as biomarkers are discussed in this review. A summary of relevant literature is shown in **Table 1**.

## INTERLEUKIN-1

The Interleukin-1 (IL-1) family of cytokines are key mediators of the inflammatory response peripherally and centrally. The IL-1 related molecules are perhaps the best known in relation to acute TBI, having been widely studied in models of both focal and diffuse injury (Fan et al., 1995; Benveniste, 1998; Yatsiv et al., 2002; Brough et al., 2011). The IL-1 receptor type I (IL-1R) is thought to mediate many of the effects of the IL-1 cytokines, and is expressed on multiple cell types in the brain (Holmin et al., 1997; Csuka et al., 2000; Pinteaux et al., 2002; Lu et al., 2005a). However, there is also evidence to suggest that some of the effects of IL-1 cytokines are independent of the IL-1R (Touzani et al., 2002; Boutin et al., 2003; Loscher et al., 2003). Intranuclear actions of IL-1 to regulate gene transcription and RNA splicing may account for some of these actions (Luheshi et al., 2009).

The IL-1 family includes the closely related agonists IL-1α and IL-1β, the antagonist IL-1ra, and the other family member, the agonist IL-18 (Dinarello, 1994, 1998, 2009; Barksby et al., 2007). Of these, the IL-1β isoform is by far the most often reported in TBI. IL-1β is a pro-inflammatory cytokine and has been implicated in the release of phospholipase-2 (PLA2), prostaglandins, and the activation of cyclooxygenase-2 (COX-2; Chung and Benveniste, 1990; Aloisi et al., 1992; Molina-Holgado et al., 2000; Rothwell, 2003). Furthermore, the primary mechanism of action for IL-1β is believed to be the regulation of release of other cytokines. IL-1β has also been shown to play a role in apoptosis (Holmin and Mathiesen, 2000), adhesion of leukocytes to endothelial cells (Bevilacqua et al., 1985), BBB disruption (Quagliarello et al., 1991), and edema formation (Holmin and Mathiesen, 2000). The fundamental pro-inflammatory and neurotoxic function of IL-1β is demonstrated by studies that have aimed to attenuate IL-1 effects. For example, the antagonist IL-1ra has been found to reduce neuronal damage in rodent brain injury models (Relton and Rothwell, 1992; Yang et al., 1998). Improved cellular and behavioral outcomes from brain injury have been reported in rats treated with recombinant human IL-1ra (Toulmond and Rothwell, 1995), mice lacking the IL-1R (Basu et al., 2002), mice over-expressing IL-1ra (Tehranian et al., 2002), or by means of intraventricular administration of IL-1β or IL-1α antibodies to rats (Lu et al., 2005a,b). The intensive research into IL-1 with regard to TBI has led to it being considered as a biomarker of early neuroinflammation and consequent tissue damage.

### LABORATORY EVIDENCE

Studies in animal models of focal and diffuse TBI have consistently shown that basal levels of IL-1β are very low (O'Connor and Coogan, 1999; Krueger, 2008), and that an increase in IL-1β expression is detectable as early as 1 h after trauma (Fan et al., 1995; Kinoshita et al., 2002; Lu et al., 2005a,b; Kamm et al., 2006). In rodent brain homogenates peak mRNA and protein expression occurs between 12 and 24 h after injury (Fan et al., 1995; Ciallella et al., 2002; Ahn et al., 2004; Lu et al., 2005a,b, 2007; Kamm et al., 2006; Maegele et al., 2007; Semple et al., 2010a,b; Shojo et al., 2010); and levels of IL-1β mRNA within 24 h of injury do appear to be associated with injury severity (Kinoshita et al., 2002). Despite the lack of direct correlations with brain damage or outcome, we have shown that while IL-1β peaks at 2 h in the rat cortex following a diffuse axonal injury, when combined with post-traumatic hypoxia the expression of IL-1β is significantly enhanced and prolonged (Foda and Marmarou, 1994; Yan et al., 2011). The exacerbated production of IL-1β is therefore important to consider, especially when secondary injuries occur.

### CLINICAL EVIDENCE

IL-1β is barely detectable in the serum or CSF of healthy individuals, and has proved difficult to measure following human TBI (Kossmann et al., 1996, 1997; Hergenroeder et al., 2010). One recent study reported peak IL-1β in CSF of 1.4–25 pg/mL, and serum concentrations of 0.8–7.6 pg/mL (Singhal et al., 2002). More recently, measurements of IL-1β concentrations in post-mortem tissue from TBI patients have confirmed that a global upregulation occurs within a few minutes to hours of injury (Frugier et al., 2010). Similar small increases in IL-1β concentrations have been reported previously in stroke patients (Tarkowski et al., 1995). Although changes in IL-1β expression in CSF and serum following injury appear to be small, attempts have been made to correlate IL-1β levels with outcome. Serum levels of IL-1β taken within 6 h of TBI have been found correlate with GCS in a cohort of 48 patients (Tasci et al., 2003). In other studies in severe brain injury patients high CSF concentrations of IL-1β were associated with poor outcome and increased ICP (Hayakata et al., 2004; Shiozaki et al., 2005). In pediatric TBI, the CSF levels of IL-1β have been correlated with outcome assessed by the Glasgow outcome score (GOS; Chiaretti et al., 2005). Finally, in one study IL-1β and IL-1ra were measured in brain microdialyzates of 15 TBI patients, and better outcomes were reported in patients with a high IL-1ra/IL-1β ratio (Hutchinson et al., 2007). Despite these results, other groups have failed to correlate IL-1β to ICP or outcome (Winter et al., 2004; Stein et al., 2011).

## TUMOR NECROSIS FACTOR

Tumor Necrosis Factor (TNF; formerly TNFα) is a multifunctional cytokine most often referred to as a potent pro-inflammatory cytokine, produced by microglia and astrocytes. Early studies mostly in rat models of TBI, administration or inhibition of TNF suggested that increased expression of TNF is detrimental (Ramilo et al., 1990; Kim et al., 1992; Shohami et al., 1996; Knoblach et al., 1999; Trembovler et al., 1999). However, more recent work employing TNF and TNF receptor knockout mice have shown that mortality rates are increased and long term recovery impaired in these models of focal TBI (Scherbel et al., 1999; Sullivan et al., 1999; Stahel et al., 2000). These apparently conflicting data mirror findings from other inflammatory mediators and demonstrate the dual role of TNF as both a pro- and anti-inflammatory cytokine (Shohami et al., 1999; Lenzlinger et al., 2001; Morganti-Kossmann et al., 2002; Schmidt et al., 2005).

**Table 1 | Studies relevant to the development of cytokines as biomarkers of TBI.**

| Cytokine | Species | Injury/model | Tissue/fluid | Findings | Reference |
|---|---|---|---|---|---|
| IL-1β | Rat | LFP, weight-drop | Brain homogenates | Increase in mRNA expression occurs within 1 h and peak mRNA and protein expression is between 12 and 24 h after injury | Fan et al. (1995), Kinoshita et al. (2002), Lu et al. (2005a), Lu et al. (2005b), Kamm et al. (2006) |
| | Rat | Weight-drop | Plasma | No change in IL-1β expression following TBI | Kamm et al. (2006) |
| | Rat | LFP | Brain homogenates | mRNA expression of IL-1β is higher in severe versus moderate injury severity | Kinoshita et al. (2002) |
| | Rat | DAI-hypoxia | Brain homogenates | Increased and prolonged IL-1β expression when TBI is combined with post-traumatic hypoxia | Yan et al. (2011) |
| | Human | TBI | Post-mortem tissue | Increased mRNA expression within minutes of injury | Frugier et al. (2010) |
| | Human | TBI | CSF and serum | Peak expression of IL-1β in CSF and serum following TBI is very low | Kossmann et al. (1996), Kossmann et al. (1997), Singhal et al. (2002), Hergenroeder et al. (2010) |
| | Human | TBI | Serum | IL-1β levels within 6 h of injury correlate with GCS | Tasci et al. (2003) |
| | Human | Severe TBI and pediatric TBI | CSF | Elevated IL-1β expression associated with poor outcome and increased ICP | Hayakata et al. (2004), Chiaretti et al. (2005), Shiozaki et al. (2005) |
| | Human | TBI | Brain parenchyma, CSF, serum | No correlation of IL-1β expression with ICP or outcome | Winter et al. (2004), Stein et al. (2011) |
| IL-1β/IL-1ra | Human | Severe TBI | Brain parenchyma | High IL-1ra/IL-1β ratio is associated with better outcome | (Hutchinson et al. (2007) |
| TNF | Rat | TBI | Brain homogenates, brain slices | Increased mRNA and protein expression detectable at 1 h, and peak expression between 4 and 8 h post-TBI | Taupin et al. (1993), Shohami et al. (1994), Fan et al. (1996), Knoblach et al. (1999), Dalgard et al. (2012) |
| | Rat | LFP | Brain homogenates | TNF expression increases after severe TBI, but not mild TBI | Knoblach et al. (1999) |
| | Rat | DAI-hypoxia | Brain homogenates | DAI and post-traumatic hypoxia leads to increased expression of TNF versus DAI alone | Yan et al. (2011) |
| | Rat | CCI | CSF | Peak levels of TNF in CSF are not reached until 24 h after TBI | Stover et al. (2000) |
| | Human | TBI | CSF, serum, plasma | TNF is increased in CSF, serum, and plasma following TBI | Goodman et al. (1990), Ross et al. (1994), Morganti-Kossmann et al. (1997), Csuka et al. (1999) |
| | Human | TBI | Post-mortem tissue | TNF mRNA and protein can be detected in the brain within minutes of injury | Frugier et al. (2010) |
| | Human | Severe TBI | CSF | TNF protein concentrations peak in the CSF within 24 h | Hayakata et al. (2004) |
| | Human | Severe TBI | CSF, serum | Six hours after TBI, TNF expression is higher in CSF than in serum. TNF expression does not correlate with outcome | Shiozaki et al. (2005) |
| | Human | Severe TBI | CSF, serum | Increased serum TNF levels correlate with increased ICP and decreased CPP, but not outcome. TNF concentrations in CSF not linked to ICP, CPP, or outcome | Stein et al. (2011) |
| IL-10 | Rat | LFP | Brain homogenates | IL-10 expression increases rapidly, and remains elevated from 4 to at least 20 h after TBI | Knoblach and Faden (1998) |
| | Rat | CCI | Brain homogenates | IL-10 expression is reduced in the brains of TBI versus sham animals 1 day after surgery | Lee et al. (2012) |

*(Continued)*

**Table 1 | Continued**

| Cytokine | Species | Injury/model | Tissue/fluid | Findings | Reference |
|---|---|---|---|---|---|
| | Human | Severe TBI | CSF, serum, plasma | IL-10 expression in both CSF and serum increases rapidly following TBI, and is higher in CSF than in serum or plasma. There is no correlation of IL-10 with BBB integrity | Csuka et al. (1999), Maier et al. (2001), Woiciechowsky et al. (2002) |
| | Human | Severe TBI | CSF, serum | IL-10 expression is higher in serum than in CSF following TBI | Hayakata et al. (2004) |
| | Human | Pediatric TBI | CSF | Increased IL-10 levels in CSF are linked to mortality | Bell et al. (1997) |
| | Human | Severe TBI | CSF, serum | Increased IL-10 is linked to BBB dysfunction and mortality | Kirchhoff et al. (2008) |
| | Human | Severe TBI | CSF | IL-10 expression is higher in patients that had an unfavorable outcome | Shiozaki et al. (2005) |
| | Human | Severe TBI | CSF, serum | No link between IL-10 and outcome | Maier et al. (2001), Woiciechowsky et al. (2002), Lo et al. (2009, 2010), Stein et al. (2011) |
| IL-6 | Rat, mouse | LFP, PBBI, weight-drop | Brain homogenates, brain parenchyma | IL-6 expression is undetectable in normal brain, but increases rapidly, peaking at 2–8 h following TBI | Woodroofe et al. (1991), Taupin et al. (1993), Shohami et al. (1994), Maegele et al. (2007), Williams et al. (2007), Ziebell et al. (2011), Weckbach et al. (2012) |
| | Rat | CCI, DAI | CSF, serum | IL-6 expression is higher in CSF than in serum. IL-6 expression increases from 1 h and peaks at 2–5 h after injury | Woodroofe et al. (1991), Hans et al. (1999), Stover et al. (2000) |
| | Rat | CHI, poly-trauma | Serum | IL-6 expression in serum cannot discriminate between peripheral and CNS injuries | Maegele et al. (2007), Weckbach et al. (2012) |
| | Human | TBI | CSF, serum | Following TBI IL-6 expression increases to a greater extent in CSF than serum | Kossmann et al. (1996), Winter et al. (2004), Hillman et al. (2007), Chiaretti et al. (2008) |
| | Human | TBI | Plasma | IL-6 concentrations greater than 100 pg/mL are associated with severe TBI | Woiciechowsky et al. (2002) |
| | Human | TBI | Plasma | Increased IL-6 concentrations correlate with poor outcomes | Arand et al. (2001), Woiciechowsky et al. (2002) |
| | Human | Pediatric TBI | Serum | No correlation between IL-6 levels and outcome | Kalabalikis et al. (1999) |
| | Human | TBI | Serum | IL-6 levels within 17 h of injury can be used to predict elevated ICP | Hergenroeder et al. (2010) |
| | Human | TBI | Brain parenchyma | Higher parenchymal levels of IL-6 correlate with better outcomes | Winter et al. (2004) |
| | Human | TBI | Brain parenchyma | No relationship between IL-6 and ICP, brain oxygenation, or edema | Perez-Barcena et al. (2011) |
| IL-8 | Rat, mouse | CCI, weight-drop | Brain homogenates | IL-8 functional homologs CXCL-1 and CXCL-2 exhibit peak expression at 4–12 h after TBI | Otto et al. (2002), Dalgard et al. (2012) |
| | Human | TBI | CSF, serum | Following TBI, increased IL-8 expression can be measured in CSF and to a lesser extent in serum | Kossmann et al. (1997), Morganti-Kossman et al. (1997), Whalen et al. (2000), Kushi et al. (2003a) |
| | Human | Pediatric TBI | CSF | IL-8 levels following TBI correlate with mortality | Whalen et al. (2000) |
| | Human | Severe TBI | CSF, plasma | Lower IL-8 levels in plasma are associated with survival. CSF IL-8 levels do not vary between survivors and non-survivors | Gopcevic et al. (2007) |
| | Human | Severe TBI, pediatric TBI | Serum | Increases in IL-8 after TBI correlate with unfavorable outcome and are associated with mortality | Mussack et al. (2002), Kushi et al. (2003b); Lo et al. (2010) |

### LABORATORY EVIDENCE

In injured rat brain, increased TNF mRNA can be detected prior to the cytokine protein itself, and upregulation of TNF was shown to precede leukocyte infiltration to the site of injury (Riva-Depaty et al., 1994; Shohami et al., 1997). This suggests that TNF is produced early by resident brain cells in response to neuronal injury. Increases in TNF protein have been measured at 1 h, and peak levels were found between 4 and 8 h after injury (Taupin et al., 1993; Shohami et al., 1994; Fan et al., 1996; Knoblach et al., 1999; Dalgard et al., 2012). Early increases in TNF expression could prove to be useful in the clinical setting as a diagnostic/prognostic factor. In fact TNF was shown to reflect injury severity, since one study using the lateral fluid percussion (LFP) injury reported that while increases could be measured for severe injury, no change in TNF was recorded for a mild injury (Knoblach et al., 1999). Furthermore, in a recent study from our laboratory, we found that TNF is only increased in rats subjected to a combined diffuse brain injury and hypoxia, but not in diffuse brain injury alone (Yan et al., 2011). In contrast to previous published work, when using the closed brain injury model of focal TBI, we did not find increased expression of TNF in brain homogenates over 24 h (Bye et al., 2007; Semple et al., 2010a). Another interesting finding from animal studies is a difference in the timing of TNF peaks in the parenchyma versus CSF of rats. In brains of rats subjected to LFP injury TNF expression peaks at 4–6 h and is resolved by 24 h (Fan et al., 1996; Knoblach et al., 1999), yet in the controlled cortical impact (CCI) model TNF expression in the CSF did not peak until 24 h post-injury (Stover et al., 2000). Whether this represents a differential response to injury type, or a delay in movement of cytokine from parenchyma to CSF remains to be determined.

### CLINICAL EVIDENCE

Following TBI increases in TNF levels have been reported in the CSF and serum of patients (Goodman et al., 1990; Ross et al., 1994). In work from our group TNF concentrations were measured in the CSF and serum of TBI patients at 24 h intervals, and TNF was significantly elevated from controls (Morganti-Kossmann et al., 1997; Csuka et al., 1999). Given that TNF expression in animal models usually peaks and resolves within the first 24 h this is perhaps not surprising. The delayed and sustained increase in TNF measurements in human CSF (3 days to 3 weeks) as compared to rapid fluctuations observed in brain tissue may reflect different mechanisms of cytokine metabolism and degradation in these environments. Indeed, more recently we have shown that TNF mRNA and protein can be detected in post-mortem brain tissue from TBI patients that died within 17 min of injury (Frugier et al., 2010). In another study, Hayakata et al. (2004) examined CSF from 23 severe TBI patients and a peak in TNF of 20–30 pg/mL was recorded within 24 h. They then attempted to analyze the associations of TNF levels with raised ICP and poor outcome (GOS < 4 at 6 months), but no correlation was found. In a subsequent study the same group measured TNF in both the CSF and serum of 35 TBI patients with or without additional injury at exactly 6 h after injury (Shiozaki et al., 2005). They reported that TNF levels were higher in CSF (median 18 pg/mL) versus serum (median 5 pg/mL) regardless of the presence of additional injury. Again, there was no correlation between TNF and GCS, ICP, or neurological outcome. More recently, Stein et al. (2011) analyzed CSF and serum samples from 24 patients at 12 h intervals for 7 days after having sustained a severe TBI. In the same patients ICP and CPP were continually monitored so that associations between cytokine levels and subsequent changes in ICP or CPP could be investigated. They reported that increased serum, and not CSF, concentrations of TNF moderately correlate with subsequent increases in ICP or decreases in CPP. However, they did not find any relationship of any cytokine concentration [IL-1β, Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), or TNF] with outcome (GOS < 5).

## INTERLEUKIN-10

Interleukin-10 is regarded to be primarily an anti-inflammatory cytokine, having a potent inhibitory effect on production of several pro-inflammatory mediators including IL-1β and TNF, but also IL-1α, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6, IL-8, IL-12, and IL-18 (de Waal Malefyt et al., 1991, 1993; Fiorentino et al., 1991; D'Andrea et al., 1993; Gruber et al., 1994). The inhibition of IL-1β and TNF is its most important function, since these cytokines are known to play central roles in initiation and propagation of the inflammatory response. Indeed, rats subjected to LFP injury and treated with IL-10 have improved outcomes and reduced levels of IL-1β and TNF in brain tissues (Knoblach and Faden, 1998).

### LABORATORY EVIDENCE

Although the anti-inflammatory properties of IL-10 following TBI are well established, there is relatively little information regarding the expression profile of IL-10 following TBI in animals. Using the FPI model, one study found that in the brain IL-10 increased rapidly during the first 4 h following injury and remained elevated for at least 20 h thereafter (Knoblach and Faden, 1998). However, a more recent study reported a reduction in IL-10 expression in brains of rats 1 day after CCI (Lee et al., 2012). No changes instead were shown in diffuse brain injured rats over 4 days post-injury, whether with or without the addition of hypoxia (Yan et al., 2011). This suggests that such anti-inflammatory cytokine may play a role in a delayed phase after TBI. The discrepancy between the two studies indicates that there may be a differential expression profile of IL-10 based on the type of injury, since the weight-drop model produces a diffuse axonal injury in the absence of focal damage, whereas CCI is primarily a focal injury.

### CLINICAL EVIDENCE

We have shown that IL-10 is elevated in the CSF and serum of patients with isolated, severe TBI (Csuka et al., 1999). In the CSF IL-10 was elevated in 26 out of 28 patients (range: 1.3–41.7 pg/mL) versus controls, but in serum only 7 patients displayed elevated IL-10 (range: 5.4–23 pg/mL). The temporal profile was similar in both fluids, exhibiting a rapid early rise and peak followed by a slow decline. In addition to cytokine measurements, BBB function has been assessed in TBI patients using the CSF/serum albumin ratio, and no correlation between the two variables has been found (Csuka et al., 1999; Maier et al., 2001). The lack of association of IL-10 levels with BBB dysfunction, combined with the fact that

IL-10 CSF levels exceeded serum levels in most patients suggest an intrathecal origin for this cytokine. However, not all studies have corroborated this hypothesis, since serum levels have been reported to be significantly higher than CSF levels in some studies (Hayakata et al., 2004). The presence of additional injuries could easily account for this difference (Hensler et al., 2000; Dziurdzik et al., 2004; Shiozaki et al., 2005). Other studies have confirmed that in severe TBI IL-10 expression increases early, reaching a peak within 2–8 h of injury (Woiciechowsky et al., 2002). Higher levels of IL-10 have been linked to better outcome in some studies, but not in others. For instance, an early study reported a link between increased IL-10 levels in CSF and mortality in pediatric TBI (Bell et al., 1997), and more recent work in adult severe TBI made a similar link between increased IL-10 and mortality (Kirchhoff et al., 2008). The concentration of IL-10 in the CSF has also been shown to be higher in patients that have an unfavorable outcome (GOS < 4) assessed 6 months after injury (Shiozaki et al., 2005). However, other studies have failed to find any connection between IL-10 and outcome (Maier et al., 2001; Woiciechowsky et al., 2002). In pediatric TBI, Lo et al. measured serum IL-10 levels on day 1, and found they could not differentiate severe and non-severe injury or predict favorable outcome (Lo et al., 2009), even when paired with GCS (Lo et al., 2010). In a more recent study there was no correlation of IL-10 in serum or CSF with outcome assessed at 6 months using the GOSE scores (Stein et al., 2011). The IL-10 response to peripheral injuries as reported in multi-trauma patients could be part to blame for the difficulty in making associations between TBI variables and IL-10 levels (Shimonkevitz et al., 1999; Hensler et al., 2000; Dziurdzik et al., 2004; Shiozaki et al., 2005).

## INTERLEUKIN-6

Interleukin-6 has been extensively studied, and has been found to be involved in a large number of physiological and pathophysiological processes. IL-6 is known to regulate inflammation, immunity, bone metabolism, hematopoiesis, and neural development (Romano et al., 1997). In addition, a role for IL-6 has been implicated in aging, osteoporosis, autoimmune disease, Alzheimer's disease, and brain injury. Although IL-6 is not exclusively expressed in the CNS, it does exhibit a significant upregulation following brain injury (Morganti-Kossmann et al., 1992; Kossmann et al., 1995).

### LABORATORY STUDIES

Laboratory studies have shown that in the brain IL-6 is expressed by astrocytes (Benveniste et al., 1990; Van Wagoner and Benveniste, 1999), microglia (Woodroofe et al., 1991; Sebire et al., 1993), and neurons (Schobitz et al., 1993; Gadient and Otten, 1994; Ringheim et al., 1995; Sallmann et al., 2000). It inhibits the synthesis of TNF (Aderka et al., 1989), induces synthesis of nerve growth factor (NGF; Kossmann et al., 1996), inhibits *N*-methyl-D-aspartate (NMDA) mediated toxicity (Wang et al., 2009), and promotes neuronal differentiation and survival (Islam et al., 2009). Evidence suggests that expression of IL-6 is beneficial following neuronal injury (Penkowa et al., 2000, 2003). While IL-6 is often undetectable in normal brain, its acute release in response to injury is well documented (Woodroofe et al., 1991; Taupin et al., 1993; Shohami et al., 1994; Williams et al., 2007; Ziebell et al., 2011). In rodent models, experimental TBI induces an increase in IL-6 mRNA expression in brain tissue after 1 h (Williams et al., 2007), and peaks in protein expression have been reported between 2 and 8 h after injury (Taupin et al., 1993; Shohami et al., 1994; Hang et al., 2004; Ziebell et al., 2011). In CSF, increases in IL-6 protein can be detected within 1 h, with peak expression between 2 and 5 h after an experimental brain injury (Woodroofe et al., 1991; Hans et al., 1999; Stover et al., 2000). The rapid increase in IL-6 expression following injury, and its maximal levels detected within a few hours makes this cytokine a promising candidate biomarker. However, it may have limited utility in stratification of patients since a similar temporal profile of IL-6 production has been demonstrated in most studies irrespective of the injury model used (weight-drop, FPI, CCI, or stab wound). The concentration of IL-6 in serum is rarely reported, but lower magnitude increases can be detected following injury (Maegele et al., 2007; Weckbach et al., 2012). Since the primary source of IL-6 following TBI originates in the brain, a limited ability of IL-6 to cross the BBB could explain in part this discrepancy. Indeed, studies in rodents and ovines have indicated that IL-6 has a limited ability to cross the BBB following a peripheral injection (Banks et al., 1994), and that a specific and saturable transport mechanism is involved in movement of IL-6 across the BBB (Threlkeld et al., 2010). These findings suggest that measurement of IL-6 in serum is unlikely to be truly indicative of brain concentrations, but rather the integrity of the BBB. Furthermore, since peripheral injuries can lead to changes in circulating IL-6 levels, models of "poly-trauma" are being developed (Maegele et al., 2005; Weckbach et al., 2012). Using these models, the specificity of serum IL-6 as a biomarker for brain injury has been found to be poor. While serum IL-6 concentrations were significantly higher in poly-trauma versus LFP or tibia fracture alone at 6 and 24 h after injury, there was no difference between the latter two groups (Maegele et al., 2007). In another model involving combined blunt bilateral chest trauma, lower limb fracture, and closed head injury (CHI), serum IL-6 was measured at 2 and 4 h after injury and found to be significantly higher in three-hit poly-trauma (CHI, chest trauma, and limb fracture) versus other groups at 4 h (Weckbach et al., 2012). Again there were no differences between single-hit groups or even combined CHI and chest trauma versus chest trauma and limb fracture. Therefore, current data on IL-6 in animal models is controversial. The rapid increase in IL-6 expression observed after brain injury and its peak within hours make it suited as a biomarker. Unfortunately, its limited ability to cross the BBB and apparent lack of ability to discriminate injury types may limit its usefulness.

### CLINICAL EVIDENCE

Under physiological conditions in humans, IL-6 expression in plasma is accepted as being 0–42 pg/mL, whereas in CSF there are only a few studies which have detected IL-6 under physiologically normal conditions (Froon et al., 1996; Kossmann et al., 1996; Maier et al., 2005). The largest of these studies measured IL-6 in the CSF of 113 patients, and reported IL-6 concentrations of 1–23 pg/mL (Maier et al., 2005). Following injury, IL-6 concentrations in CSF can reach concentrations as high as 35,500 pg/mL, but there appears to be a lot of variability

in this response (Kossmann et al., 1996; Hillman et al., 2007). In addition, increases in serum IL-6 have been also been reported after TBI, with lower peak concentrations of 93–269 pg/mL (Winter et al., 2004; Chiaretti et al., 2005). Plasma levels of IL-6 greater than 100 pg/mL in the first 24 h following injury have been found to be associated with severe brain injury (Woiciechowsky et al., 2002). Data on the predictive ability of IL-6 in serum is limited and conflicting; in pediatric TBI serum IL-6 was reported to have no associations with neurological outcome (Kalabalikis et al., 1999), while others demonstrated that high IL-6 correlated with poor outcome (Arand et al., 2001; Woiciechowsky et al., 2002). More recently, measurements of serum IL-6 within 17 h of injury have been shown to identify patients at risk of developing elevated ICP (Hergenroeder et al., 2010). However, the authors also noted that lack of prognostic value of IL-6 for elevated ICP when patients also presented with extracranial injuries. Indeed, the presence of multiple injuries is common in TBI patients (Gennarelli et al., 1994; Meixensberger and Roosen, 1998) and should be considered when searching for or with the purpose of developing a biomarker. This is substantiated by evidence showing increased serum concentrations of IL-6 following orthopedic injury (Hergenroeder et al., 2010), burns (Agay et al., 2008), and exercise (Nybo et al., 2002; Febbraio and Pedersen, 2005). The poor predictive ability of IL-6 in the presence of multiple injuries is not surprising, and is corroborated by studies on experimental poly-trauma models described above. However, since the primary source of IL-6 in TBI are the cells of the CNS, including microglia, astrocytes, and neurons (Marz et al., 1998; Van Wagoner et al., 1999; Lau and Yu, 2001), more specific information on injury to the brain might be obtained from measurement of parenchymal cytokine production. This can be achieved using the technique of cerebral microdialysis, which can be adapted to recover cytokines and allow for continual sampling of brain parenchyma (Winter et al., 2002; Helmy et al., 2007). A wide range of cytokine concentrations can be measured in brain injured patients using this technique (Helmy et al., 2011). In a study involving 14 severe TBI patients, higher peak parenchymal levels of IL-6 were found to correlate with better GOS (Winter et al., 2004). Concentrations of NGF were also measured in this study, and while overall levels of NGF or IL-6 alone could not predict outcome, the ratio of NGF:IL-6 was significantly lower in survivors, and was correlated with GCS and GOS. In a more recent study including 16 patients with diffuse TBI, there was no relationship between parenchymal levels of IL-6 and ICP, brain tissue oxygenation, or the presence of brain swelling (Perez-Barcena et al., 2011). However, it must be noted that this study used averages of samples collected over 8-h periods, and could have missed some of he large-scale changes in cytokine concentrations known to occur in TBI. Furthermore, both of these studies suffered from a lack of statistical power, having relatively few patients. While further development of microdialysis techniques will undoubtedly provide us with some very useful information regarding the brain's response to injury, it is limited by its invasive nature, the expense of the probes, and the highly region-specific information obtained.

In summary, IL-6 is highly sensitive to brain injury and can be easily detected in serum, although current data on its ability to predict outcome and its correlation with ICP are limited and inconclusive. Its inability to discriminate between brain damage and peripheral injuries may limit its usefulness in poly-trauma patients, but further development of parenchymal microdialysis and its use in combination with other biomarkers may prove fruitful.

## INTERLEUKIN-8/CXCL8 AND MONOCYTE CHEMOATTRACTANT PROTEIN/CCL2

Interleukin-8 is a member of the CXC chemokine family (CXCL8), and is secreted by glial cells, macrophages, and endothelial cells (Aloisi et al., 1992; Nitta et al., 1992; Zhang et al., 2011). It is an important mediator for the activation and chemotaxis of neutrophils (Bickel, 1993). Early studies showed that IL-8 is released from astrocytes in response to other cytokines including IL-1β and TNF (Kasahara et al., 1991), both of which are expressed soon after brain injury (McClain et al., 1987; Woodroofe et al., 1991; Taupin et al., 1993). Increased IL-8 expression has been reported in many cancers (Xie, 2001), bacterial infections (Hirao et al., 2000), and is linked to cardiovascular disease (Apostolakis et al., 2009).

The monocyte chemoattractant protein-1 (MCP-1) or CCL2 is produced by astrocytes within hours after injury and its levels correlate with the amounts of recruited macrophages (Semple et al., 2010c). Since MCP-1 is regulated in an autocrine fashion, subsequent release of MCP-1 by infiltrated macrophages and microglia perpetuates cell migration in the injured brain. MCP-1 overexpression increased macrophage infiltration and neurological deficit in ischemia whereas its deletion attenuated infiltrates in brain injury, stroke, and multiple sclerosis models (Lu et al., 1998; Huang et al., 2001; Hughes et al., 2002; Chen et al., 2003).

### LABORATORY EVIDENCE

Rodents lack a direct homolog for IL-8, but chemokine (CXC motif) ligand-1 (CXCL-1), CXCL-2, CXCL-5, and CXCL-6 appear to be functional homologs, residing in the same gene cluster as IL-8 (human chromosome 4q13.3) and contributing to neutrophil recruitment in a number of animal models. In rat CCI, the expression of the chemokine CXCL-1 peaks at 4 h, and is reduced by 12 h, but remains elevated versus control for up to 7 days after injury (Dalgard et al., 2012). In our model of CHI, the synthesis of MIP-2 as well as other chemokines (MCP-1, MIP-1α, RANTES, and KC) in the cortex increased at 4–12 h preceding the infiltration of neutrophils and macrophages which peak at 24–48 h and 4–7 days, respectively. MIP-1α and MIP-2 concentration was reduced in the brain of TNF-KO mice after TBI, implying a role for TNF in regulating their expression (Otto et al., 2002). Amplified expression of chemokine receptors CXCR2 and CCR2 was localized on infiltrating neutrophils and macrophages, respectively at 1 and 4 days post-TBI (Otto et al., 2001; Semple et al., 2010b). While neutrophils depart by 1 week from the injured brain, the accumulation of macrophages persists over 4 weeks (Semple et al., 2010a). The prolonged presence of activated leukocytes within the pericontusional tissue is likely detrimental due to their ability to secrete neurotoxins leading to delayed neuronal death. The role of MCP-1 and IL-8/MIP-2 in secondary brain degeneration and neurological function was recently explored using MCP-1-KO and CXCR2-KO mice. The most striking data in MCP-1-KO mice showed a significant reduction in lesion volume, neuronal loss and macrophage

accumulation up to 46% over 4 weeks after TBI as opposed to wild-type mice (Semple et al., 2010a). Improved brain damage resulted in faster neurological recovery from 1 to 4 weeks. In CXCR2-KO mice, an 80% decline of neutrophil infiltration occurred at 12 h after TBI and coincided with reduced lesion and neuronal loss over wild-type controls (Semple et al., 2010b).

### CLINICAL EVIDENCE

In humans, IL-8 is detected at very low levels in the CSF and plasma of healthy individuals. Physiological plasma concentrations are 5–18 pg/mL, and in CSF are 5–72 pg/mL (Maier et al., 2005). Following TBI there is an increase in IL-8 concentration in serum and CSF (Kossmann et al., 1997; Morganti-Kossman et al., 1997). IL-8 appears to peak early following a TBI, with mean levels up to 29,000 pg/mL reported in CSF (Whalen et al., 2000; Kushi et al., 2003a). Increases in plasma levels of IL-8 following brain trauma have been reported, but are of lower magnitude and more variable. Peak concentrations of approximately 100 pg/mL are commonly demonstrated in severe TBI (Kossmann et al., 1997; Mussack et al., 2002; Kushi et al., 2003a). Increased IL-8 in CSF has been associated with mortality. In one study, CSF was obtained from 27 children who had sustained a severe TBI, 7 with meningitis, and 24 children with neither diagnosis. The increase in IL-8 levels in children with TBI was of similar magnitude to children with meningitis. Heightened IL-8 expression persisted for several days, and a significant correlation was found between IL-8 and mortality (Whalen et al., 2000). In another study, Gopcevic et al. collected blood and CSF samples at time of admittance from 20 adults who had sustained severe TBI, 10 of which died. They showed that plasma IL-8 was significantly lower in survivors versus non-survivors (71 and 111 pg/mL respectively), however, CSF IL-8 concentrations were not different. They also showed that IL-8 had a prognostic value for GCS, patient age, and Acute Physiologic and Chronic Health Evaluation (APACHE II; Gopcevic et al., 2007). Although plasma levels of IL-8 are considerably lower than CSF, several studies have found correlations between peripheral IL-8 and outcomes. Mussack et al. (2002) measured serum IL-8 at intake and 12 h later in 20 TBI patients and found a significant correlation of increased IL-8 levels 12 h after admission with outcomes assessed by GOS. In another study, serum concentrations of IL-8 72 h after TBI were significantly higher in non-survivors versus survivors (Kushi et al., 2003a). Most recently, Lo et al. took blood samples from 28 pediatric TBI patients at precisely 24 h following injury and correlated serum biomarker levels with unfavorable outcomes 6 months later (GOS $< 4$). Increased serum IL-8 was found to correlate with unfavorable outcome. Furthermore, when combined with GCS and increased specificity, an sensitivity was observed (Lo et al., 2010). The weakness of this study was that only 4 patients had an unfavorable outcome, nonetheless, it demonstrates the potential for using paired markers to predict outcomes with greater accuracy.

Our group has demonstrated that in CSF samples from 21 severe TBI patients, MCP-1 concentrations increased to 19,000 pg/mL on day 1, falling to approximately 3000 pg/mL from day 3 onward (Semple et al., 2010a). The rapid peak in CCL2 levels in CSF and its elevated expression in the CSF for several days merit investigation of this cytokine as a potential biomarker for TBI.

Upregulation of both IL-8 and MCP-1 at mRNA and protein level in post-mortem human brain, underlining the relevance of the chemokine network in human TBI (Frugier et al., 2010). Specifically, a 140-fold increase in IL-8 mRNA was detected in the injured brain compared to control, being the mediator with the strongest increase, while MCP-1 mRNA was increased by almost 20-fold (unpublished data). Overexpression of chemokines in these human samples was associated with tissue infiltration of CD68-positive macrophages and GFAP-positive reactive astrocytes (Frugier et al., 2010). Combined, these experimental studies provide compelling evidence that signaling through chemokine networks contributes to secondary tissue and neurological damage and could be considered in future studies assessing their meaning as biomarkers of TBI.

## OTHER CYTOKINES

The cytokines described above are the best characterized in terms of their role in neuronal injury and their potential as markers for TBI, however, there are a large number of less-well characterized immune modulators that could be useful TBI biomarkers.

Another potential biomarker is GM-CSF, a pro-inflammatory cytokine that is expressed in the CNS by neurons, astrocytes, and microglia (Franzen et al., 2004). GM-CSF is secreted by vascular endothelial cells, it crosses the BBB and can be detected in CSF (Coxon et al., 1999). We have found this cytokine to be significantly upregulated in post-mortem tissue from TBI patients that died 6–122 h after injury (Frugier et al., 2010). More recently we have found that GM-CSF is more highly expressed in the CSF of patients suffering from secondary hypoxia versus normoxic TBI patients, and is also increased in diffuse TBI versus focal TBI (unpublished results). The different levels of expression of this cytokine between injury types merits further investigation of this molecule as a TBI biomarker.

Also of potential interest is endothelial monocyte-activating polypeptide II precursor (p43/pro-EMAP II), a pro-inflammatory cytokine linked with apoptosis (Knies et al., 1998; van Horssen et al., 2006). In a high-throughput immunoblotting screen of 998 proteins in rats 24 h after ischemic injury versus penetrating TBI differential expression was found in only the cytokine p43/pro-EMAP II (Yao et al., 2008). In a subsequent study, tissue, blood, and CSF concentrations of this cytokine were shown *in vivo* to discriminate between ischemic brain injury and TBI modalities (Yao et al., 2009). Specifically, Yao et al. found that p43/pro-EMAP II is constitutively expressed in the brain of naïve rats, but significantly increases in CSF and plasma 24-h after penetrating ballistic-like brain injury, whereas a significant decrease was found in CSF and plasma following middle cerebral artery occlusion. Western blotting of brain tissue at 6, 24, 48, and 72 h showed similar increases in p43/pro-EMAP II expression at all time-points post-TBI, and significant decreases in expression following ischemic brain injury. Immunohistochemistry revealed that changes in p43/pro-EMAP II levels were due to changes in neuronal expression and decreases did not represent neuronal loss. Taken together, these data show that the little-studied p43/pro-EMAP II cytokine has potential to be a useful brain-specific biomarker.

## OTHER MARKERS OF INFLAMMATION

In addition to cytokines, several other molecules could potentially be useful as biomarkers of brain injury. For example, activation of the JAK/STAT pathway by IL-6 regulates GFAP expression. It is well established that GFAP expression increases in serum following TBI (Missler et al., 1999; van Geel et al., 2002). In severe TBI patients, serum GFAP levels have been shown to be able to predict mortality and outcome (Pelinka et al., 2004a,b; Nylen et al., 2006; Vos et al., 2010). More recently, GFAP-BDPs in serum of mild and moderate TBI patients within 4-h of injury has been found to correlate with injury severity (GCS), and maybe be associated with CT lesions (Papa et al., 2012). In a recent study it was found that combining measurements of GFAP in CSF and serum with the IMPACT Outcome Calculator a significant improvement in outcome prediction could be achieved (Czeiter et al., 2012). In addition, the expression of αII-spectrin breakdown product 145 (SBDP145) was measured in CSF and correlated significantly with 6-month mortality (Czeiter et al., 2012). Another study measured SBDPs in the CSF of severe TBI patients at the time of admission and every 6 h thereafter for up to 7 days (Mondello et al., 2010). It was shown that in addition to an increased expression of SBDPs in all TBI patients versus controls, there was a significant correlation of SBDP145 with injury severity (assessed by GCS). Furthermore, levels of SBDP145 and SBDP120 were significantly higher in patients that died, suggesting that these markers may be able to predict mortality (Mondello et al., 2010). Another marker of note is the microtubule-associated proteins (MAP-2), which are neuronal specific proteins found in dendrites (Drewes et al., 1998). Laboratory studies in models of ischemic and traumatic injury have established that MAP-2 expression is lost from injured brain regions, and increases in MAP-2 expression can be detected in serum shortly after injury (Kitagawa et al., 1989; Posmantur et al., 1996; Park et al., 2012). More recently, it has been shown that serum MAP-2 expression measured 6 months after severe TBI is still elevated. Furthermore, increased serum MAP-2 expression correlates with better outcome (GOSE), and was found to be higher in non-vegetative state patients versus vegetative state patients (Mondello et al., 2012). This suggests that MAP-2 has potential as a marker for outcome prediction, and increased serum MAP-2 expression may signal the emergence of higher cognitive function in severe TBI patients. Lastly, the inflammasome is responsible for the production of mature IL-1β and IL-18, and may therefore provide us with useful brain injury biomarkers. In a recent study, CSF was collected from 23 patients who had suffered a moderate or severe TBI, and levels of inflammasome proteins were measured (Adamczak et al., 2012). Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1, and Nacht leucine-rich-repeat protein-1 (NALP-1) were all elevated in the CSF of TBI patients versus controls. Furthermore, all 3 proteins correlated significantly with outcome (GOS at 5-months; Adamczak et al., 2012).

## COMPARABLE STUDIES IN SPINAL CORD INJURY

Development of biomarkers for CNS injury has focused almost exclusively on brain injury, however, markers for spinal cord injury (SCI) are also much needed. MRI after SCI can be used to detect hemorrhage, transaction, and lesions, but is not always the best method for predicting outcomes. Neurological assessment has been shown to be predictive for outcome, but cannot be administered within the first critical hours of injury (Lammertse et al., 2007). Biomarkers for SCI could allow clinicians to make earlier prognoses and decide on the best course of treatment. Research into biomarkers of SCI is very limited and has focused almost exclusively on S100B and NSE. Laboratory research has shown that in the CSF, expression of S100B, NSE, and neurofilament protein (NFL) are increased (Skouen et al., 1999; Nagy et al., 2002; Cao et al., 2008). A similar increase can be measured in the serum of animals subjected to an experimental SCI (Ma et al., 2001; Loy et al., 2005; Cao et al., 2008). There is some evidence from these studies that NSE and S100B can distinguish between injury severities. Both NSE and S100B expression was shown to correlate with injury severity in a rat weight-drop model of SCI (Cao et al., 2008). However, a similar study failed to find a difference in serum or CSF NSE expression between graded injury levels (Loy et al., 2005). Human studies into SCI biomarkers have focused on detecting ischemic injury in patients undergoing thoracoabdominal aortic aneurism surgery (van Dongen et al., 1998, 1999; Kunihara et al., 2001; Winnerkvist et al., 2007), and other studies have looked at S100B or NSE after surgery for lumbar disk herniation (Brisby et al., 1999), spinal epidural empyema (Marquardt et al., 2004b), or paresis due to spinal metastasis (Marquardt et al., 2004a). Data from these studies is inconclusive with regards to either S100B or NSE being useful as markers of ischemic injury. While several studies have reported increases in expression of these biomarkers, little is known with regard to how this relates to outcome. In the case of spinal epidural empyema or paresis due to spinal metastasis, longer periods of elevated serum S100B were related to unfavorable outcome (Marquardt et al., 2004a,b). Lastly, two studies have assessed biomarkers for human traumatic SCI. The first measured GFAP and NFL expression in CSF of patients with traumatic SCI, and although both markers were increased, no statistical analysis was done and only six patients were included (Guez et al., 2003). The second study took CSF from 27 patients with complete or incomplete SCI and measured protein expression of IL-6, IL-8, MCP-1, S100B, and GFAP (Kwon et al., 2010). All of the markers were increased in SCI patients, and there were significant differences in expression of each marker between injury severities. Furthermore, a model was developed that accurately predicted injury severity and outcome at 6-months post-injury. The results of this study suggest that biomarkers may be useful in SCI patients.

## DISCUSSION

Brain injury biomarkers promise to improve patient diagnosis, management and outcomes, and aid in the development of novel therapeutics. The now well accepted inflammatory response that occurs in the injured brain has the potential to offer clinicians a number of markers that could provide specific information on the injury. Experiments in animal models of TBI have revealed a plethora of inflammatory mediators that are expressed in the brain following injury. Many of these exhibit rapid changes in expression, reaching peaks of over 1000 orders of magnitude greater than physiological levels within hours of injury. The magnitude, timing, and duration of expression of these mediators might be able to provide not only information about the injury but also of

the complexity deriving from the combination of multiple insults. For example, in a rat Marmarou model of diffuse brain injury, TNF production is of a higher magnitude, and IL-1β expression is heightened and prolonged, when the injury is followed by a 30-min period of hypoxia (Yan et al., 2011). This suggests that cytokines may indeed reveal various degrees of brain damage with overlapping insults.

Translating information from animal studies into clinically relevant concepts can prove challenging, since human TBI is a very varied condition that is often accompanied by additional peripheral injuries, especially if we consider that animal models mostly reproduce a single form of TBI. Additionally, the human brain is gyroencephalic, and with a larger mass than the rodent brain it also presents a different ratio of white to gray matter. The development of inflammatory mediators as reliable markers of brain injury is jeopardized by the fact that peripheral injuries induce an immune response that may mask or be indistinguishable from the inflammatory response occurring in the brain. Indeed, several studies have noted that in the presence of multiple injuries, markers of inflammation cannot discriminate for the presence of brain injury (Hensler et al., 2000; Dziurdzik et al., 2004; Shiozaki et al., 2005; Hergenroeder et al., 2010). To this end, models of so called "poly-trauma" are being developed, which combine experimental TBI with peripheral injuries (Maegele et al., 2005; Weckbach et al., 2012). These can then be used to search for specific brain injury markers. Another consideration is that in most animal studies cytokine concentrations are measured directly in homogenized brain tissue rather than in CSF or blood samples. While this gives very useful description of the specific response of the brain to injury, such measurements are not possible in the clinic and if done they would have little relevance when considering the definition of a biomarker for early diagnostic and prognostic significance. An additional consideration should be differences in immune activation occurring in the species utilized in modeling TBI. Higher brain TNF levels have been reported in rats as compared to mice in equivalent severity of cerebral ischemia (Schroeter et al., 2003), so different responses between rodents and humans must be also expected. Even within the human population there is evidence that polymorphisms in cytokine genes could affect not only outcome, but also the subject level of cytokine synthesis in response to injury (Hadjigeorgiou et al., 2005; Uzan et al., 2005). Finally, the redundancy inherent in the inflammatory response is important to consider, since it makes it quite likely that in a genetically diverse population of humans a degree of variability will be observed in cytokine responses.

In studies that have concomitantly measured cytokine levels in CSF and serum differences in concentration and even timing of peaks has been observed (Kossmann et al., 1995; Csuka et al., 1999; Shiozaki et al., 2005). This undoubtedly reflects the compartmentalization of the CNS from the periphery and the limited diffusion of cytokines out of the brain parenchyma and vice versa. Changes in BBB compliance following TBI are also known to occur, and may affect the CSF:serum ratio of some cytokines (Kossmann et al., 1995; Csuka et al., 1999), as well as sequestration of cytokines by the liver (Wu and Pardridge, 1999). Nonetheless, many groups have been able to successfully detect significant increases in inflammatory markers in the blood following TBI and established associations or correlations with other injury parameters and outcomes. Among the cytokines, perhaps the most promising to date is IL-6, since 100-fold increases can be readily measured in serum following TBI (Winter et al., 2004; Chiaretti et al., 2005). IL-6 levels have been correlated with ICP (Hergenroeder et al., 2010), outcome (Arand et al., 2001; Woiciechowsky et al., 2002), but not in multi-trauma patients. To overcome this problem sampling for cytokine biomarkers could be done in CSF or in the brain parenchyma itself by microdialysis to get a CNS specific measurement of the immune response. Since ICP monitoring and management is common in TBI patients, CSF samples could be obtained from severely injured patients. Changes in CSF cytokine concentrations can be orders of magnitude greater than serum concentrations, and do not appear to be affected by peripheral injuries. However, data is still mixed with regard to the ability of cytokines to distinguish injury severity and type, predict or correlate with ICP, or to predict outcome characteristics. Further study of the cellular origin and biochemical meaning of raised or lowered level of a specific cytokine may help to interpret these data.

Given the problems discussed above, improved sensitivity may be achieved by combining a multitude of biomarkers with conventional neuroimaging techniques, and neurological scoring (e.g., GCS, GOS/E). Novel cytokine biomarkers could be measured in parallel with "classical" TBI biomarkers such as S100B and NSE to improve sensitivity. We have shown that CSF concentrations of sICAM-1 correlate well with tissue and BBB damage, giving an indication of the degree of immunologic activation in the injured CNS (Pleines et al., 1998), and in a subsequent publication measured sICAM-1 together with well known TBI biomarkers (Pleines et al., 2001). In the latter publication we showed that mean CSF protein concentrations of S100B correlate with IL-6, contusion size assessed by CT, and GOS, while serum S100B correlates with contusion size and GOS. In this study we also showed that NSE serum levels correlate with IL-6, and that NSE levels in CSF correlate with sICAM-1 and contusion size (but not GOS). Taken together, the correlation of serum S100B with contusions size and outcome shows that it reflects the extent of injury well, but NSE and cytokine biomarkers give a better indication of the degree of inflammation in the brain. Other groups have gone one to show that by combining pairs of biomarkers including IL-6, IL-8, S100B, and NSE, a higher degree of outcome predictability can be achieved versus any single biomarker (Winter et al., 2004; Berger et al., 2009; Lo et al., 2009). Similarly, combining GCS score with a single biomarker such as IL-8 also improves outcome predictability (Lo et al., 2010). Recently developed methods for predicting outcomes based on age, motor score, pupillary reactivity, and CT characteristics (IMPACT, Steyerberg et al., 2008) have been shown to benefit from the inclusion of brain injury biomarkers (Czeiter et al., 2012). Although inflammatory markers were not included in that study, several cytokines have been shown to have power to predict outcome, including IL-1β, IL-6, IL-8, and IL-10 (Bell et al., 1997; Whalen et al., 2000; Arand et al., 2001; Mussack et al., 2002; Woiciechowsky et al., 2002; Kushi et al., 2003b; Chiaretti et al., 2005; Shiozaki et al., 2005; Gopcevic et al., 2007; Kirchhoff et al., 2008).

In addition to being useful in prediction of changes in ICP, mortality, or 6 month outcomes, biomarkers could be used to categorize patients based on specific pathophysiological processes occurring in the injured brain. This information could help to provide individualized treatment based on the specific type or severity of injury. Using a rat model of diffuse injury we have shown that an additional hypoxic insult enhances cortical production of the cytokines IL-1β, IL-6, and TNF (Yan et al., 2011). In human studies, correlations have been found between injury severity and concentrations of a specific cytokine, such as IL-1β (Aly et al., 2006), IL-6 (Kossmann et al., 1996; Kalabalikis et al., 1999; Woiciechowsky et al., 2002; Chiaretti et al., 2005), and IL-10 (Neidhardt et al., 1997). An improved understanding of the roles of these cytokines in the secondary injury process may pave the way for targeted treatment strategies tailored specifically to the patient. Measurement of these cytokines could also be used to track the effects of a potential pharmacological treatment. For example, methylprednisolone (MP) has anti-oxidant and anti-inflammatory effects and is used in the treatment of SCI. Administration of MP to rats subjected to experimental SCI or subjected to an inflammatory stimulus has been found to reduce TNF expression (Buttini et al., 1997; Xu et al., 1998), and expression of other cytokines (Fu and Saporta, 2005). Another potential treatment is the tetracycline antibiotic, minocycline, which has been found to have anti-inflammatory effects. We have shown that IL-1β expression is significantly reduced in mouse brain subjected to CHI when minocycline is administered (Bye et al., 2007). Although interestingly, there was no significant reduction in other cytokines, including TNF, IL-6, G-CSF, MCP-1, and MIP-2.

The future for inflammatory mediators as biomarkers for use in TBI is still uncertain, in large part due to their lack of specificity. However, development of animal models of multi-trauma, the use of two or more markers, and new sampling techniques may overcome this problem. There is also a need to gain a more complete understanding of the temporal expression profile of each cytokine in specific types and severities of injury, since data is currently limited. The use of multiplex assays now allows for simultaneous measurement of several cytokines in brain samples and is providing useful information in this regard (Yan et al., 2011; Dalgard et al., 2012). In addition, the use of microdialysis technology in patients, while invasive and expensive, has potential to provide us with continual cytokine concentrations within the brain parenchyma itself (Winter et al., 2002; Helmy et al., 2007). Intraparenchymal measurement of this kind negates the need to consider BBB disturbances and may be more biologically relevant.

In conclusion, the monitoring of the inflammatory process has the potential to provide specific information on the injury and make predictions about probable outcomes. Several cytokines have shown potential in this area, but a more complete understanding of their specific roles and expression profiles is needed.

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# Microglia activation as a biomarker for traumatic brain injury

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Traumatic brain injury (TBI) has become the signature wound of wars in Afghanistan and Iraq. Injury may result from a mechanical force, a rapid acceleration-deceleration movement, or a blast wave. A cascade of secondary cell death events ensues after the initial injury. In particular, multiple inflammatory responses accompany TBI. A series of inflammatory cytokines and chemokines spreads to normal brain areas juxtaposed to the core impacted tissue. Among the repertoire of immune cells involved, microglia is a key player in propagating inflammation to tissues neighboring the core site of injury. Neuroprotective drug trials in TBI have failed, likely due to their sole focus on abrogating neuronal cell death and ignoring the microglia response despite these inflammatory cells' detrimental effects on the brain. Another relevant point to consider is the veracity of results of animal experiments due to deficiencies in experimental design, such as incomplete or inadequate method description, data misinterpretation, and reporting may introduce bias and give false-positive results. Thus, scientific publications should follow strict guidelines that include randomization, blinding, sample-size estimation, and accurate handling of all data (Landis et al., 2012). A prolonged state of inflammation after brain injury may linger for years and predispose patients to develop other neurological disorders, such as Alzheimer's disease. TBI patients display progressive and long-lasting impairments in their physical, cognitive, behavioral, and social performance. Here, we discuss inflammatory mechanisms that accompany TBI in an effort to increase our understanding of the dynamic pathological condition as the disease evolves over time and begin to translate these findings for defining new and existing inflammation-based biomarkers and treatments for TBI.

**Keywords: head trauma, microglia, inflammatory response, secondary cell death, anti-inflammatory therapy, brain imaging**

## INTRODUCTION

Traumatic brain injury (TBI) is characterized by a damage to the brain as a result of a violent impact, blow or jolt to the head that causes the brain to strike the inside of the skull or when an object perforates the skull and reaches brain tissue. The most recent estimates of the incidence and prevalence of TBI indicate that each year 235,000 Americans are hospitalized for non-fatal TBI, 1.1 million are treated in emergency, and 50,000 die (Corrigan et al., 2010). Common features of TBI include bruising, torn tissues, bleeding, and physical damage to the brain resulting in long term complications or death. It can be classified based on its severity, anatomical areas affected, and causative forces. Depending on the extent of damage to the brain, TBI varies from mild to moderate to severe. Serious secondary events may also occur, such as oxidative stress, massive edema, and alterations of endogenous neurotransmitter mechanisms, as depicted in **Figure 1**. In the case of mild TBI, the patient may remain conscious or faint for a few seconds or minutes. Characteristic symptoms of mild TBI include headache, confusion, lightheadedness, dizziness, blurred vision or tired eyes, ringing in the ears, bad taste in the mouth, fatigue or lethargy, a change in sleep patterns, behavioral or mood changes, and trouble with memory, concentration, attention, or thinking (National Institute of Neurological Disorders and Stroke, National Institutes of Health). In moderate to severe TBI similar symptoms may occur, but with worse manifestations. For example headaches may become intermittent, repeated vomiting or nausea, seizures, inability to awaken from sleep, dilation of one or both pupils of the eyes, slurred speech, weakness or numbness in the extremities, loss of coordination, and increased confusion, restlessness, or agitation. War-related TBI is usually associated with injury to the brain due to an improvised explosive device (IED) blast during military conflicts (Bogdanova and Verfaellie, 2012; Duckworth et al., 2012; Goeller et al., 2012). When a frontal blast wave encounters the head, a shock wave is transmitted through the skull, cerebrospinal fluid (CSF), and tissue, causing negative pressure at the contrecoup that may result in cavitation (Goeller et al., 2012).

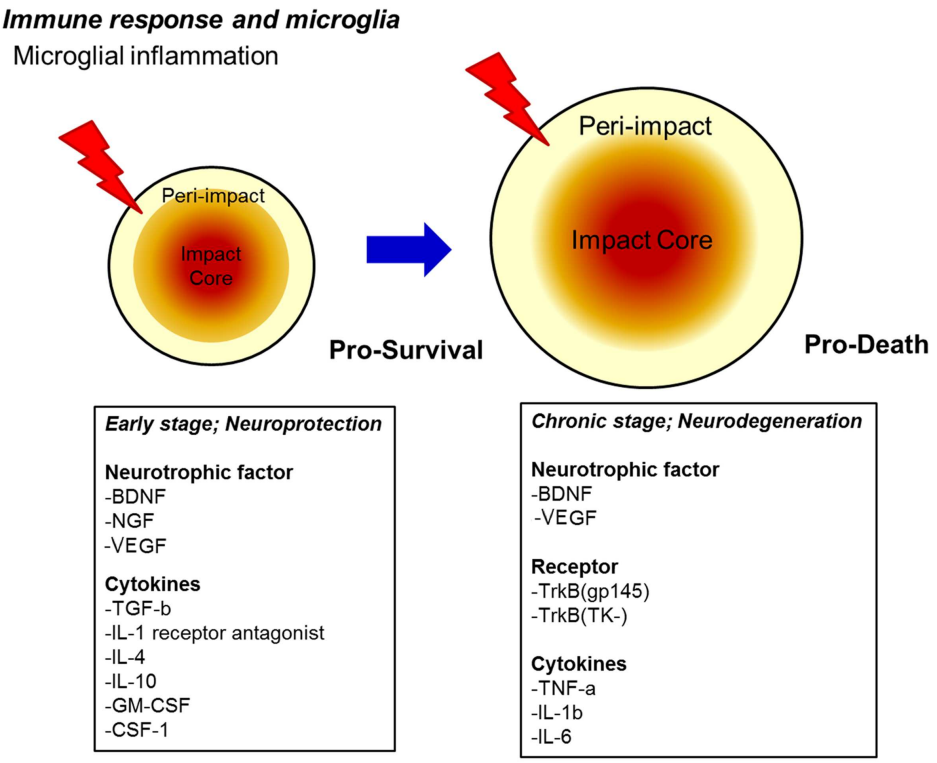


**FIGURE 1 | Proposed secondary cell death mechanism after TBI.** A sensitive balance in neurotrophic factor secretion and cytokine expression dictates the fate of injured neurons towards pro-survival (early stage) and pro-death (chronic stage) cellular processes after TBI. Upregulation of neurotrophic factors and cytokines may renderneuroprotectionin the early stage, whereas downregulation of neurotrophic factors coupled with continued upregulation of specific pro-death cytokines, as well as the onset of oxidative stress, massive edema, and alterations in endogenous neurotransmitter function may promote neurodegeneration in the chronic stage.

Patients with varying severity of TBI often struggle with physical and cognitive impairments for months, or years; and some never reach full recovery. An estimated 43.3% of Americans have residual disability 1 year after injury (Corrigan et al., 2010). Although TBI is typically believed to be a static pathological insult from a single event, previously unrecognized clinical symptoms can arise many years after the initial injury (Giunta et al., 2012). The most recent estimate of the prevalence of US civilian residents living with disability following hospitalization with TBI is 3.2–5.3 million (Corrigan et al., 2010; Coronado et al., 2011). Thus, greater efforts should center on this sector of the population living and aging with post-TBI sequelae.

In this mini-review, we bring attention to microglia, which possess a double-edge sword function, in that microglia can mount both pro-survival and pro-death actions after TBI occurrence. We propose new and present inflammation-based biomarkers that may enhance regenerative abilities and decrease degenerative events associated with microglial response for the treatment of TBI. Microglia exert neuroprotection by sequestering, via phagocytosis, foreign bodies that aberrantly penetrate the brain (Noda et al., 2011; Voss et al., 2012). Unfortunately during aging, microglial cells also display reduced phagocytic capacity (Fiala et al., 2005; Kohman, 2012). The challenge for developing therapies targeting microglial function is to manipulate microglia activation toward a reparative process that could retard, or even halt the progressive pathological symptoms of TBI and its co-morbidity factors.

## IMMUNE RESPONSE AND MICROGLIA

Activation of the immune system in the central nervous system (CNS) has become increasingly recognized as a key component of the normal process of aging, but also of the pathological onset and progression of many neurological disorders including TBI and neurodegenerative diseases. There are three phenotypic states of microglia-based on developmental and pathophysiologic studies: (*i*) resting, ramified; (*ii*) activated non-phagocytic [or antigen presenting cell (APC)-like] found in areas involved in CNS inflammation; and (*iii*) reactive, in which phagocytic microglia is present in areas of trauma or infection (Frei et al., 1987; Suzumura et al., 1987; Williams et al., 1992; Panek and Benveniste, 1995; Walker et al., 1995). With respect to activation, macrophages and microglia are able to polarize into two major subtypes, categorized as M1 or M2 (Mosser, 2003; Gordon and Taylor, 2005). This "classical" or *M1* subtype excessively secretes proinflammatory cytokines and promotes cell-mediated immunity (Mosser, 2003; Gordon and Taylor, 2005). It is marked by production of high levels of interferon-gamma (IFN-γ), tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-12, and low levels of IL-10. The *M1* phenotype

may be activated when microglia contact HIV proteins (such as transactivator of transcription [Tat] bind toll-like receptors 3 or 4 as well) (Suh et al., 2009). "Alternatively activated" or *M2* microglia tend to dampen (Bruce-Keller et al., 2001) inflammation, clear cellular debris (including amyloid plaques), and produce very low levels of TNF-α, IL-1, IL-12, and high amounts of anti-inflammatory IL-10 and transforming growth factor (TGF)-β, and suppressor of cytokine signaling (SOCS) (Mosser, 2003; Gordon and Taylor, 2005; Qin et al., 2006; Akhtar et al., 2010). These two phenotypes, respectively, belong to the type *ii* or *iii* microglial states. Further, the factors which cause polarization to *M1* or *M2* reinforce the maintenance of that phenotype in a cycle-like manner.

The initial inflammatory response after TBI results in neuronal injury and disruption of the blood-brain barrier (Smith et al., 1997; Nagamoto-Combs et al., 2007; Namas et al., 2009). Microglial cells become activated within minutes, and resemble peripheral macrophages by acting as APCs releasing proinflammatory cytokines and chemokines (Town et al., 2005; Cao et al., 2012). Activated microglia also produce other neurotoxic products after injury such as nitric oxide (NO) and superoxide free radicals that generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). In animal models of cortical controlled impact (CCI); fluid percussion brain injury in rats; combined unilateral lesion of the primary motor cortex and of the lateral pre-motor cortex in rhesus monkeys, microglial cells remain in their activated state for at least 1 year, especially in the thalamic area (Smith et al., 1997; Nagamoto-Combs et al., 2007; Nagamoto-Combs and Combs, 2010; Jacobowitz et al., 2012; Jin et al., 2012). Human postmortem studies have shown microglial activation 17 years after TBI in subcortical brain areas (Ramlackhansingh et al., 2011). These accrued results suggest the persistence of a chronic inflammatory stage mediated by microglia.

A novel feature of activated microglial cells is the delicate cytokine profile they acquire upon brain insult. Microglial cells may share common markers for activated macrophages including CD68, CD45, and major histocompatibility complex II (MHC-II) (Town et al., 2005; Cao et al., 2012). The sensitive balance in cytokine expression may dictate the fate of injured neurons toward pro-survival or pro-death mechanisms, as illustrated in **Figure 2**.

Microglial cells exist in at least two functionally distinguishable states once activated – namely a phagocytic phenotype (innate activation) or the aforementioned antigen presenting phenotype (adaptive activation) that is seen post-TBI (Town et al., 2005; Giunta et al., 2012). When injury to the CNS occurs, activated microglial cells acquire a predominant proinflammatory profile.

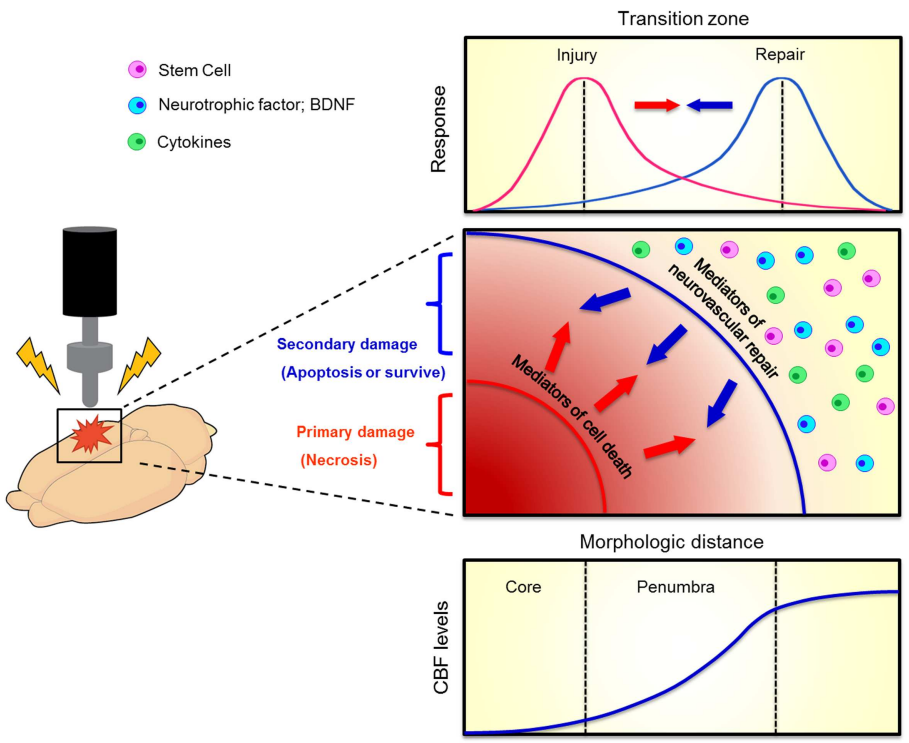


**FIGURE 2 | Evolution of penumbra after TBI.** The brain tissue surrounding the impacted core of TBI can become vulnerable to cell death due to spreading waves of pro-death cytokine mediators. This at-risk brain tissue corresponds to the penumbra which comprises the transition zone between injury and repair (top graphics). A therapeutic window exists for the repair process to abrogate the injury progression. When the brain cell faces damage, it suffers from two kinds of injuries, namely primary (necrosis) and secondary (apoptosis) cell death (middle graph). Neurovascular repair, such as transplantation of stem cells, upregulation of neurotrophic factors, and inhibition of pro-death cytokines,can rescue against the secondary cell death. The penumbra is traditionally defined as an area with mild to moderate reductions in cerebral blood flow (CBF, bottom graph). Such evolution of penumbra after brain injury was originally observed in stroke (Lo, 2008).

If microglia cells are challenged with certain pathogen-associated molecular patterns (particularly CpG-DNA), they activate a mixed response characterized by enhanced phagocytosis and proinflammatory cytokine production, as well as adaptive activation of T cells (Giunta et al., 2012). Among the repertoire of proinflammatory cytokines, IL-1-β and TNF-α play a pivotal role before, during, and after microglia activation. Once secreted, these cytokines can bind specific receptors to increase the amount of inducible nitric oxide synthase (iNOS). Also, they can act as molecular inducers of programmed cell death or apoptosis as shown in animal and human studies of neurodegenerative diseases like Alzheimer's disease (AD) (Heneka et al., 1999; Venters et al., 2000; Combs et al., 2001). In addition, after severe TBI, it has been shown that there is a pronounced increase of IL-6, IL-8, TNF-α, and IL-1-β mRNA in human post mortem tissue (Frugier et al., 2010).

Microglia not only express a gamut of proinflammatory cytokines (IL-1, IL-6, TNF-α), but also secrete a myriad of anti-inflammatory cytokines (IL-1 receptor antagonist, IL-4, IL-10) and neurotrophic factors brain-derived neurotrophic factor (BDNF, NGF, TGF-β). Of note, these neurotrophic factors are not exclusively secreted by activated microglia cells or macrophages; indeed these neurotrophic factors are synthesized and secreted by a myriad of cells (i.e., stroma cells, T cells, and astrocytes) within the CNS during inflammatory conditions (Murphy et al., 1977; Pál et al., 2012). Cytokines are small proteins expressed/secreted by microglia under inflammatory conditions. Important exogenous factors capable of cytokine induction in microglia are viral envelope proteins, bacterial cell wall components such as, lipopolysaccharides (LPS) and leukotriene A (LTA); also bacterial DNA, and prions (Heppner et al., 2001). Likewise, endogenous inducers act as inflammatory mediators, such as platelet-activating factor (PAF), lipids, serum proteins, or complement factors; additionally, disturbances in ATP and $[K^+]$ levels may cause microglia activation (Hanisch, 2002). Thus, shifting the cytokine profile of microglia toward a pro-survival phase (anti-inflammatory cytokines) may increase neuroprotection and regeneration of the CNS after TBI. In the subsequent sections, we discuss both pro-survival and pro-death functions of microglia and identify avenues for therapeutic development, as well as to propose potential biomarker approaches that will maximize the dynamic features of microglia. In the end, a multi-pronged strategy focusing on microglial function may reveal novel therapies and biomarkers for a better understanding of TBI treatment and pathology.

## HARNESSING MICROGLIAL PRO-SURVIVAL FUNCTIONS

Strategies designed to target specific molecules may be able to manipulate the cytokine profile of the activated microglia. There are several molecules that may be suitable to promote neuronal survival after damage. Among the candidates, granulocyte-macrophage colony stimulating factor (GM-CSF), and colony stimulating factor 1 (CSF-1) have demonstrated to be potent stimuli for microglia *in vitro* (Sawada et al., 1990; Suzumura et al., 1990; Streit et al., 2000). With the proper signaling cues, microglia may shift toward producing more of the neuroprotective substances (e.g., IL-10, IL-1ra, and TGF-β) soon after injury. The direct benefit of these molecules is immediate suppression of proinflammatory cytokines. TGF-β also has been shown to exert neuroprotective effects after injury, including improved function, decreased lesion size, and reduced iNOS production (Hamada et al., 1996; Tyor et al., 2002).

We propose that potential activated microglia-related biomarkers should possess the following features: (a) have high specificity and sensitivity for activated microglia, (b) stimulate microglia to polarize into the M2 phenotype, (c) promote synaptic, neuronal plasticity, and cell survival at a close or distal range from the area of injury, and (d) reduce the inflammatory response post-TBI (Mosser, 2003; Gordon and Taylor, 2005; Ben Achour and Pascual, 2010). Potential microglia-related biomarker to which above criteria could be applied to stimulate microglia into the M2 phenotype may be the chemokine fractalkine, CX3CL1, and its receptor CX3CR1. Both are constitutively expressed in the nervous system. The ligand is expressed exclusively by neurons and endothelial cells, whereas the receptor is s expressed by microglia, astrocytes, and neurons (Rancan et al., 2004). *In vitro* and *in vivo* models of neurological conditions and brain inflammation also reported how CX3CL1 reduces microglia toxicity and, consequently, neuronal damage (Zujovic et al., 2000; Mizuno et al., 2003; Cardona et al., 2006; Bhaskar et al., 2010; Noda et al., 2011; Pabon et al., 2011). Interestingly enough, CX3CL1 and CX3CR1 reduce brain damage in a rodent ischemic model via an adenosine-dependent mechanism (Cipriani et al., 2011). Microglial activation should not be linked only to deleterious effects. There are instances where activated microglia may have a protective role in TBI (Urrea et al., 2007). Case in point, at 3 h after moderate fluid percussion in rats, a new population of cells was recognized in the sub-ventricular zone of the traumatized hemisphere (Urrea et al., 2007). Double labeling confocal microscopy showed newly formed astrocytes, oligodendrocytes, and neurons co-localized with macrophages/microglia even after days on injury. These findings suggest that TBI stimulates a widespread cellular proliferation after injury, and that microglial activation may be involved in the observed focal neurogenesis in the dentate gyrus of the hippocampus (Urrea et al., 2007).

Most TBI animal studies indicate that extended microglial activation at the focal site of injury becomes detrimental over time (Hanisch and Kettenmann, 2007; Ramlackhansingh et al., 2011; Cao et al., 2012; Mannix and Whalen, 2012). Nevertheless, equally compelling studies suggest that persistent microglial activation in regions remote from focal injury might promote brain repair (Nagamoto-Combs et al., 2007, 2010; Thiel et al., 2010), possibly via neurotrophic factor secretion, especially BDNF, proximal and distal to the injured tissue (Krueger et al., 2011; Rostami et al., 2011; Cekic et al., 2012; Colak et al., 2012; Ma et al., 2012; Shi et al., 2012). For instance, in non-human primates even 6 months after injury microglial cells continue to release BDNF and its receptor subtypes TrkB[gp145] and TrkB[TK-] around the cortical lesion site and in the spinal cord (Nagamoto-Combs et al., 2007). Double labeling studies showed that a subpopulation of CD68-immunoreactive microglia/macrophage co-expressed BDNF in the cortex and spinal cord, and also TrkB[gp145] or TrkB[TK-] in the spinal cord; whereas cytokine expression of TNF-α, IL-6, and IL-1-β was less prominent at the 1, 6, and 12-month intervals, suggesting that immediate inflammatory responses had subsided (Nagamoto-Combs et al., 2007). Yet, in a CCI rat model there is a

decline in BDNF-mRNA and protein levels measured at 1–14 days post injury (Schober et al., 2012). Moreover, specific BDNF polymorphisms may not be involved in TBI pathology (Bagnato et al., 2012). Notwithstanding these inconsistencies, the findings are encouraging because they suggest that the prolonged microglial activation plays an important role in neurotrophic/tropic signaling, and identifying the appropriate growth factors (i.e., BDNF polymorphism) should facilitate in recovery process of the TBI brain. Thus, more studies are warranted to decipher the molecular cues released by activated microglia proximal and distal to the site of injury, and nurture such therapeutic molecules to be robustly and stably expressed not only at these brain regions vulnerable to secondary cell death, but also to the major impacted brain areas in order to sequester the extent of injured brain after TBI.

## INCREASING PHAGOCYTIC ACTIVITY OF MICROGLIA

An equally appealing function of microglia is their phagocytic activity which may rescue neurons from degeneration. Enhancing the phagocytic state of microglia at early stage post-TBI may retard cell death signals to spread to damaged neurons and neighboring cells (Jeon et al., 2012; Schafer et al., 2012; Tamashiro et al., 2012). Furthermore, there are many studies that have documented the process of cell autophagy as neuroprotective after TBI (Clark et al., 2008; Lai et al., 2008; Liu et al., 2008; Venkatesan et al., 2010). Indeed, nearby neurons and astrocytes close to the site of injury are capable of clearing out cell debris after brain injury (Zhang et al., 2008; Loov et al., 2012). An increase in autophagosomal formation proteins, such as microtubule-associated protein 1 light chain 3 (LC3) and beclin 1, has been detected in neurons and astrocytes at 1-h, 3-h, 32 days post-TBI (Zhang et al., 2008). However, autophagy may exacerbate the pathological manifestations of TBI (Bigford et al., 2009; Luo et al., 2011), likely due to aberrant clearance of healthy cells in addition to degenerating cells. Accordingly, for the phagocytic activity of microglia to attenuate the progression of TBI pathology, a regulatory mechanism should be devised to enhance the therapeutic autophagy, while blocking its deleterious side effects.

A potent approach to manipulating microglial phagocytic function is by stimulating neural progenitor cells (NPCs). Mouse NPCs possess a secretory protein profile distinct from other brain cells; specifically proteins that modulate microglial activation and phagocytosis (Mosher et al., 2012). That a close modulatory interaction exists between these two cell types at the protein level suggests that microglia and NPCs may influence each other functions and activity. Some of the factors secreted in large amounts by NPCs include tissue inhibitor of metalloproteinase type-1 (TIMP-1), vascular endothelial growth factor (VEGF), and haptoglobin, which are well known immunomodulatory proteins or regulators of microglia (Forstreuter et al., 2002; Hanisch and Kettenmann, 2007). Based on this knowledge, an envisioned drug for TBI may be potent immunomodulatory proteins that could foster the therapeutic phagocytic activity of microglia.

## REDIRECTING PRO-DEATH MICROGLIAL FUNCTIONS

Inactivating the pro-death inflammatory response of microglial cells is equally effective in combating the secondary cell death associated with TBI (Kapadia et al., 2008; Chen et al., 2011; Tsai et al., 2011). Recent pharmacologic strategies against TBI-induced secondary cell death employ inhibitors of oxidative stress and microglial activation. A high dose (100 mg/kg) pre-treatment with apocynin, an NADPH oxidase assembly inhibitor that retains proinflammatory profile of microglia, produces therapeutic potential against murine models of TBI (Choi et al., 2012; Zhang et al., 2012). One week after TBI, microglial activation remained, but ROS production was inhibited by apocynin in the hippocampal CA3 pyramidal neurons; they also found reduced BBB disruption, and neuronal rescue from cell death associated with TBI (Choi et al., 2012).

New therapies to attenuate exacerbation of microglia activation have emerged thanks to the study of potential biomarkers identified in models of acute spinal cord injury (SCI). Interestingly, an overlap in the cytokine profiles expressed by SCI rodents and human subjects has been recently demonstrated, in that rodent and human released in an SCI injury-dependent manner IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1) (Stammers et al., 2012). A promising neuroprotective approach is the use of the antibiotic minocycline. In SCI animal models, minocycline has shown therapeutic effects in reducing microglial activation, excitotoxicity, and neuronal and oligodendrocyte cell death associated with mitochondrial stabilization (Teng et al., 2004; Festoff et al., 2006; Yune et al., 2007; Lee et al., 2012). Of note, a recent study demonstrated increased motor recovery in human patients suffering from acute SCI after 7 days of minocycline treatment relative to patients treated with the placebo drug (Casha et al., 2012) In the field of stem cell therapy, studies have also shown that a chemokine/cytokine (i.e., inflammatory) response may actually guide the migration of stem cells from the periphery to the site of brain injury, thereby allowing efficient brain bioavailability of the grafted cells' secreted therapeutic molecules (Borlongan, 2011; Borlongan et al., 2011). Such inflammation-mediated cell migration suggest that a modest cytokine/chemokine upregulation aids stem cells in reaching their brain injured target areas. Recognizing the balance between proinflammatory and anti-inflammatory microglial function may provide new targets for arresting TBI-induced secondary cell death.

## CONTEMPLATING MICROGLIA-BASED BIOMARKERS FOR TBI

In parallel to developing treatments for TBI, utmost consideration for research investigations should be devoted to exploring biomarkers for TBI which will help optimize therapeutic intervention, in that the proper timing for treatment initiation will be guided by onset or peak time window of secondary cell death as may be captured by novel biomarker tools.

### CYTOKINE PROFILING

Cytokine profiling of microglial cells may lead to identification of specific proteins that regulate microglia (e.g., BDNF) and measuring these proteins in the blood or CSF may provide clues on the status of the TBI patient. Unraveling the cytokine and chemokines profile of activated microglia could lead to the identification of specific proteins that regulate microglia response after TBI (e.g.,

BDNF, NGF, and TGF-β). This approach would have to tackle the paracrine and autocrine roles, as well as interactions between cytokines, chemokines, and neurotrophic/tropic factors. Defining a cytokine profile for activated microglia in TBI can give us new insights on known neuroprotective approaches post-TBI. This knowledge may translate into the human clinical scenario by measuring these recognized proteins in the blood, plasma, or CSF and provide clues on the status of the TBI patient. For instance, cerebral microdialysis is a well-established laboratory tool that is increasingly used as a bedside monitor to provide on-line analysis of brain tissue biochemistry during neurointensive care (Tisdall and Smith, 2006). In a microdialysis study 12 patients suffering from diffuse severe TBI, defined as a post-resuscitation Glasgow Coma Score ≤8, were monitored over a period of 5 days. Their cerebral fluid, arterial and jugular venous plasma samples were screened for a cytokine and chemokine dataset using a principal component analysis and partial least squares discriminant analysis to demonstrate the pattern of production following TBI, distinct phases of the humoral inflammatory response and the differing patterns of response in brain and in peripheral blood (Helmy et al., 2012). Brain tissue microdialysis can become an established technique for monitoring acute and chronic TBI if future studies are capable of identifying an overlap in microglial cytokine profile against the microdialysis data analysis. Potential biomarkers identified in rat models of TBI that should be included in the repertoire of microglial profiling and overall brain tissue profiling by microdialysis may include epithelial/endothelial tyrosine kinase (Wu et al., 2012), poly(ADP-ribose) polymerase-1 (d'Avila et al., 2012), and myeloid differentiation primary response protein 88 (Li et al., 2011). In addition to this emerging cytokine profile, novel cytokines and associated proteins may be detected via high throughput screening assays (e.g., microRNA analysis) using blood, CSF, or tissue samples from TBI patients and animal models from time of impact and over different periods of secondary cell death evolution.

### DETECTING PHAGOCYTIC PROFILE

Detecting phagocytic profile of microglia may reveal close molecular and cellular association between NPCs and stem cells. In addition, measuring levels of NPCs or stem cells via imaging modalities (functional MRI) may reveal the phagocytic activity of microglia. The identification of potent immunomodulatory proteins in the phagocytic profile of microglia, NPCs, and stem cells in general could help us elucidate the overlap in modulatory and phagocytic functions among these cell types. It seems tangible to measure levels of NPCs or stem cells via imaging modalities (e.g., functional MRI) to provide real-time status of the phagocytic activity of microglia (Yu et al., 2010). Likewise, we could attempt to correlate already known phagocytic biomarkers (e.g., LC3) with those of inflammation and apoptosis to establish a causal relationship among these three critical cellular processes in the TBI brain. For example, in a lateral moderate fluid percussion injury model of TBI in adult rats, microarray analyses revealed apparent time-dependent expression changes in 23 apoptosis-related genes, including inflammatory cytokines such as IL-1-α, IL-1-β, and TNF which immediately increased at 3 h following the injury (Shojo et al., 2010). Thus, these time-dependent gene expression profiles elucidate the progression of the secondary cell death process of apoptosis, shown in this study as an ensuing event associated with inflammation (Shojo et al., 2010).

### ASSESSING THE LEVEL OF "PROINFLAMMATORY RESPONSE OF MICROGLIA"

Assessing the level of "proinflammatory response of microglia" could be measured via blood/CSF assays (Ziebell et al., 2011; Jin et al., 2012). The intent here is to visualize a threshold of pro-inflammation that is therapeutic (i.e., serving as signaling cue for stem cell migration from periphery to the injured brain), and has not reached a level that could invoke the deleterious neuroinflammatory response responsible for exacerbation of TBI pathological symptoms. For example, measuring *S*-nitrosoglutathione (GSNO), a nitrosylation-based signaling molecule, could reveal brain levels of peroxynitrite and oxidative metabolites, which when reduced levels are detected may indicate protection of the neurovascular unit integrity (Khan et al., 2009, 2011). Coincidentally, the detection of increased neurotrophic factors produced by constant low levels of GSNO treatment over time may represent enhanced synaptic plasticity (Khan et al., 2009, 2011). Accordingly, assessment of surrogate markers of GSNO involving peroxynitrite and oxidative metabolites, and neurotrophic factors may provide insights on neurovascular integrity and synaptic plasticity in TBI.

## CONCLUSION

The major pro-survival feature of microglia is their phagocytic activity. Identifying the signaling factors that nurture microglia to preserve their regenerative function after injury versus the predominating inflammatory activity (e.g., neuroinflammation) will provide insights into homeostatic mechanisms in maintaining a healthy brain. An appealing characteristic of microglia, which deserves more attention, is their migration to the site of injury. However, the migratory mechanisms thriving microglia to populate the CNS after arrival at the injured brain remain poorly understood. An in-depth examination of the molecular cues that regulate the anti-inflammatory response will guide the development of effective treatments to reduce detrimental effects of microglial activation and shift their function toward microglia-based therapies for TBI.

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# Application of blood-based biomarkers in human mild traumatic brain injury

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Traumatic Brain Injury (TBI) is a global health concern. The majority of TBI's are mild, yet our ability to diagnose and treat mild traumatic brain injury (mTBI) is lacking. This deficiency results from a variety of issues including the difficulty in interpreting ambiguous clinically presented symptoms, and ineffective imaging techniques. Thus, researchers have begun to explore cellular and molecular based approaches to improve both diagnosis and prognosis. This has been met with a variety of challenges, including difficulty in relating biological markers to current clinical symptoms, and overcoming our lack of fundamental understanding of the pathophysiology of mTBI. However, recent adoption of high throughput technologies and a change in focus from the identification of single to multiple markers has given just optimism to mTBI research. The purpose of this review is to highlight a number of current experimental peripheral blood biomarkers of mTBI, as well as comment on the issues surrounding their clinical application and utility.

**Keywords: peripheral blood, diagnostic markers, prognostic markers, pathophysiology, biological mechanisms, high throughput, post-concussion syndrome, chronic traumatic encephalopathy**

## INTRODUCTION

Traumatic brain injury (TBI) affects up to 10 million people globally (Hyder et al., 2007). Mild traumatic brain injury (mTBI) accounts for between 70 and 90% of all TBI cases, and has an estimated incidence rate of 653/100,000 people in Ontario alone (Ryu et al., 2009). mTBI has gained considerable notoriety during the past decade of conflict in Afghanistan and Iraq as a common source of morbidity from these wars. An estimated 10–20% of all veterans of these conflicts sustain mTBI, with blast injuries being the leading cause (Schneiderman et al., 2008; Kelly et al., 2012; Shively and Perl, 2012). Yet, mTBI is proportionally understudied compared to moderate and severe injury (Papa et al., 2008).

The underlying pathophysiology of mTBI remains undetermined, and as a result there are currently no efficient diagnostic, prognostic, or therapeutic strategies available clinically. Researchers have begun to investigate mTBI at the cellular and molecular level, as shortcomings in current brain imaging techniques and flawed clinical diagnostic approaches have increased the appeal of utilizing the peripheral blood to identify immune and damage related signaling between the brain and the periphery. Specifically, the goal of this approach is to uncover either a single marker or panel of markers to aid in early detection and diagnosis, as well as to predict patient outcomes. Furthermore, this method may help elucidate underlying biological mechanisms and provide greater insight into therapeutic strategies. However, the utility of such markers needs to be determined in the experimental phase through careful evaluation against specific clinical questions, including: whether/how they directly relate to disease mechanisms, prognosis, diagnosis, and the monitoring of therapeutic inventions. This validation will facilitate a positive transition within the "bench to bedside" process.

The determinants and modifiers of the clinical entity of concussion, mTBI, and post-concussion syndrome (PCS) include several factors such as environmental, psychosocial, and co-morbidities among others. These factors may be operative before and/or after trauma. The scope of this review is narrowly focused on the biological factors of presumed loss of cell integrity that can be detected using signature biochemical biomarker patterns in human peripheral blood, in terms of their specific utility and clinical relevance in relating to pathophysiology, diagnosis, and prognosis. Biomarker categorization will be based on a stratification originally put forth by the Biomarkers Definition Working Group (BDWG) (Biomarkers Definitions Working Group, 2001), and serve to aid in the guided development of translating important laboratory findings into meaningful clinical practice.

## mTBI DEFINITION AND TERMINOLOGY

In order to assess and treat mTBI clinically, a clear definition is necessary. In view of this, clinicians and researchers have historically struggled to agree on a singular mTBI definition and accepted terminology (Moser et al., 2007; Ruff et al., 2009; Marshall et al., 2012). This disagreement may stem from a variety of issues including the heterogeneity in both trauma mechanism and symptom presentation, and the difficulty in detecting signs and symptoms of injury (Greenwald et al., 2012). In addition, there are disputes over the use of the term mTBI as opposed to concussion; it is unclear if these terms can be used interchangeably, or if there is a difference in the underlying definition (Greenwald et al., 2012; Jeter et al.,

2012). It has been suggested that an mTBI that occurs in sports is typically referred to as a concussion (Moser et al., 2007). In an attempt at clarity and unification, a definition has been put forth by the American Congress of Rehabilitation Medicine (ACRM) that is endorsed by both the Centre of Disease Control and Prevention (CDC) and the World Health Organization (WHO). In summary, the ACRM defines an mTBI as:

> "A traumatically induced physiological disruption of brain function, as manifested by at least one of the following. . .loss of consciousness. . .any loss of memory for events immediately before or after the event. . .focal neurologic deficit that may or may not be transient, but where the severity of the injury does not exceed the following: loss of consciousness of approximately 30 min or less after 30 min, an initial Glasgow Coma Scale score of 13–15 and post-traumatic amnesia not greater than 24 h" (Greenwald et al., 2012).

## PERIPHERAL BLOOD AS A SOURCE FOR mTBI BIOMARKERS

Using peripheral blood for mTBI biomarker discovery potentially addresses a number of clinical concerns, such as current diagnostic, prognostic, and treatment approach pitfalls, the difficulty in creating representative animal models of human mTBI, and the inability to conduct biological studies on patients at the primary site of injury. For example, diagnosis and prognosis in moderate and severe TBI using conventional imaging techniques is informative (Shenton et al., 2012), but these techniques fail to detect the majority of mild brain injuries and provide little or no pathophysiological information related to injury mechanism (Papa et al., 2008; Bettermann and Slocomb, 2012). Furthermore, animal models of human mTBI, which have largely focused on the use of rodents, have been met with little success. This is due to a number of factors, such as the heterogeneity of the human clinical population from which it mimics, and the anatomical differences between the human and rodent brain (Marklund and Hillered, 2011). Lastly, the use of a biological correlate at or near the site of injury, such as the Cerebrospinal Fluid (CSF) is potentially advantageous due to its proximity to the brain and involvement in the central nervous system (CNS). However, the acquisition of CSF fluid is a relatively invasive procedure. By comparison, acquiring a blood sample from a patient population is more accepted in clinical practice, and can provide substantial information relating to specific neurological injury processes within the brain, the blood brain barrier (BBB), and neuroendocrine-immune signaling processes between the CNS and periphery.

## BIOMARKERS OF mTBI DIAGNOSIS

Ideally, a diagnostic biomarker should indicate the presence or absence of disease/injury, and more specifically, should be able to stage or classify its severity (Biomarkers Definitions Working Group, 2001). No clinically accepted TBI peripheral blood biomarkers currently exist (Bettermann and Slocomb, 2012). The spectrum of TBI is presently diagnosed and stratified through the Glasgow Coma Scale (GCS) rating system alongside presentation, neurological examination, and CT imaging (Sharma and Laskowitz, 2012). While the GCS can be effective in assessing neurocognitive state, and offers some prognostic information regarding patient outcome, it tells us little about the physiological source of these symptoms, and can be confounded by polytrauma, alcohol, and other drug use (Papa, 2012). In addition, clinical CT imaging often fails to detect moderate and mild TBI, causes potential exposure to harmful radiation, and is a relatively costly procedure (Bettermann and Slocomb, 2012).

Diagnosing mTBI through a blood-based test may circumvent the pitfalls of the current clinical diagnostic approach, potentially offering a specific and sensitive evaluation of presented neurological deficit that is based on objective, quantifiable biological changes directly related to trauma physiology (Topolovec-Vranic et al., 2011). However, this approach is confounded by a variety of factors, including a poor understanding of the underlying biological mechanisms of mTBI, as well as the difficulty in linking blood-based protein markers to a range of dynamic clinical symptoms that are difficult to objectively assess. Moreover, in specific populations such as military personnel, the relationship between combat-related mTBI and residual mTBI symptoms, post-traumatic stress disorder (PTSD) symptoms, and neurocognitive deficits remains unclear (Brenner, 2011; Miller, 2011). TBI occurrence and severity are difficult to ascertain in this population because of retrospective bias in determining relevant clinical variables, such as whether there was loss of consciousness or post-traumatic amnesia (Schneiderman et al., 2008; Kelly et al., 2012; Shively and Perl, 2012).

Numerous markers have been evaluated on their ability to diagnose mTBI, with modest success. The most widely studied marker in brain injury is Serum protein 100B (s100B), a low affinity calcium binding protein primarily expressed in glial cells and Schwann cells (Persson et al., 1987). s100B is a highly sensitive protein that can be found in both the CSF and blood within 6 h of mTBI (de Kruijk et al., 2001; Berger et al., 2002; Giacoppo et al., 2012). However, s100B has poor specificity, as it can be extracranially derived from various cell types elsewhere in the body (Bettermann and Slocomb, 2012). Furthermore, the function of this marker itself is not itself fully understood (Giacoppo et al., 2012), and thus should not be considered as a stand-alone marker for mTBI diagnosis. Similar conclusions have also been made about Neuron Specific Enolase (NSE), a neuronal damage marker. NSE was assumed to have high specificity to the brain, but was found be released into the serum as a result of hemolysis (Giacoppo et al., 2012; Papa, 2012; Żurek and Fedora, 2012), limiting its accuracy as a predictor of brain injury. Possibly more brain specific than NSE, however, is Ubiquitin Protein Hydrolase – 1 (UCH-L1), a marker of neuronal damage linked to TBI (Berger et al., 2012). UCH-L1 can be located in the serum of patients within 4 h of injury (Berger et al., 2012), but has yet to correlate with mTBI (Berger et al., 2012). Conversely, Myelin Basic Protein (MBP) measures axonal damage, and unlike s100B and NSE, has high brain specificity, but suffers from a delayed introduction into the blood stream (24–72 h post-injury) (Giacoppo et al., 2012).

Despite the limited success of various diagnostic mTBI biomarkers to date, current research provides reason for optimism. Recent work by Papa et al. (2012) has identified Glial Fibrillary Acidic Protein Breakdown Products (GFAP – BDP) elevated in the serum of mild and moderate TBI patients within a few hours of injury. Importantly, these increases were also correlated with GCS ratings, CT lesions, and neurosurgical interventions (Papa

et al., 2012). Furthermore, GFAP itself appears to show high specificity to brain tissue, as multi-trauma does not affect its serum levels (Pelinka et al., 2005). Although caution must be taken in the interpretation of all correlative analyses, these findings are promising.

While markers such as GFAP and UCHL-1 measure astrocytic and neuronal damage, respectively, an important issue is raised regarding "proof of concept." The pathophysiological mechanism(s) of trauma-induced injury in mTBI is unclear, and any single marker representing what may only be one "piece of the puzzle" has to be interpreted with caution. mTBI may encompass both neuronal and glial cell injury, with possible damage specific to axonal structures (Johnson et al., 2012) (see **Table 1** for a summary of blood biomarkers in mTBI). In view of this, multiple markers used to assess the spectrum of brain tissue injuries that are mechanistically correlated with clinical symptoms would likely increase diagnostic accuracy. Furthermore, specific symptoms (e.g., loss of consciousness, amnesia) and types of trauma (e.g., focal versus, diffuse, direct head impact versus acceleration/deceleration injury not specific to the head) may need to be examined separately with greater scrutiny in order to create more direct connections between specific biological markers observed in the blood after injury and their precise underlying etiology.

## BIOMARKERS OF mTBI PROGNOSIS

A prognostic biomarker is used to predict the clinical outcome of a disease or injury (Biomarkers Definitions Working Group, 2001; Petzold, 2007) and can be useful in guiding treatment strategies (Petzold, 2007). In mTBI, prognostic biomarkers generally have a twofold purpose: (1) to predict recovery; (2) to stratify risk for specific secondary pathological outcomes, such as PCS and chronic traumatic encephalopathy (CTE) (Bruns and Jagoda, 2009). However, prognostic markers may also have more precise predictive utility, such as aiding in the clinical decision to image patients (Biberthaler et al., 2006). Unfortunately, there are currently no accurate prognostic biomarkers of mTBI outcome, and more specifically, in risk stratification for the development of PCS and CTE. This problem is further compounded by the lack of diagnostic markers for CTE and PCS themselves (Lakhan and Kirchgessner, 2012).

The chronic affects of mTBI have received increasing media attention due to its impact on affected athletes and military personnel (Cancelliere et al., 2012). Among those who have had an mTBI, 50% will continue to experience cognitive, neurological, and behavioral symptoms such as headache, difficulty concentrating, anxiety, and depression (Begaz et al., 2006). This percentage drops to around 15% at the 1-year mark (Begaz et al., 2006), while some may continue to experience symptoms for years post-injury (NIH, 1999). This condition is known as PCS, and to date, little is known about its pathophysiological etiology (Nygren-de Boussard et al., 2004). Furthermore, chronic mTBI patients may also be at risk for CTE. CTE was originally identified over 80 years ago in "punch drunk" boxers, and presents as a neurodegenerative condition that worsens with age, quite often resulting in dementia, depression, memory loss, and even suicide. CTE may occur as a result of multiple brain injuries; 17% of those with repetitive head injuries go on to develop this syndrome (McKee et al., 2009).

To date, analysis of prognostic mTBI markers have been correlated to clinical decisions to image, as well as various clinical indices of recovery such as return to work (RTW) and the Glasgow Outcome Scale (GOS) (Bazarian et al., 2006; Beers et al., 2007; Metting et al., 2012). It was originally believed that s100B would be a useful clinical aid in predicting recovery and lowering the number of ill-advised CT scans (Ingebrigtsen and Romner, 1996). Unfortunately, recent assessments of this marker have revealed it is a poor predictor of intracranial risk (Morochovic et al., 2009), early neurological outcome (Piazza et al., 2007; Morochovic et al., 2009), and long-term post-concussion symptoms (Bazarian et al., 2006). In addition, increased levels of serum s100B have been noted in patients who have made a complete neurological recovery (Piazza et al., 2007). s100B has also been outperformed by GFAP in predicting long-term outcome (6 months), as reflected by the Glasgow Outcome Score Extended (GOSE) and RTW assessment (Metting et al., 2012). However, the authors of this study concluded that GFAP was still a weak overall predictor of outcome in mTBI (Metting et al., 2012). Also, NSE serum levels 1 month post mTBI are not correlated to outcome as reflected by the GOS (Meric et al., 2010), and the Tau protein has not been shown to correlate to PCS at 3 months (Ma et al., 2008) (see **Table 1**). Yet, the interpretation of these results must be approached cautiously, particularly in correlating mTBI recovery to assessments such as the GOSE and RTW. While possibly indicative of recovery, these correlates provide no objective and reliable pathophysiological determination. For example, it is quite possible that a patient suffering with PCS may RTW while not completely recovered, and conversely, some patients may have recovered well before they RTW. The GOSE and RTW assessments contain fairly ambiguous groupings such as "moderately disabled," "good recovery," and "previous work not resumed, but working on a lower level" (Metting et al., 2012). The utility of these types of scales, which are based on limited clinical symptom assessment, should be questioned, as the potential for subjective interpretation is high.

A greater number of longitudinal, multi-marker studies correlated with specific secondary symptoms (e.g., headaches, nausea, anxiety) may provide a useful kinetic background to identify candidate prognostic biomarkers. Beers et al. (2007), provide a useful framework through their study design, assessing multiple markers at multiple points in the acute period after trauma and at 6 months. Metting et al. (2012) also used a multi-marker approach, assessing GFAP and s100B as outcome markers in mTBI. More studies following this approach would be of use. Furthermore, diagnosing PCS and CTE still remain a challenge for clinicians, and until this is rectified, it will remain difficult to accurately predict patient outcome.

## BIOMARKERS OF mTBI PATHOPHYSIOLOGY

The fundamental pathophysiology of injury in mTBI is not understood, making it difficult to identify clinically functional biomarkers. Still, while many well-studied biomarkers have been criticized for their inability to suffice as stand-alone indicators of injury presence and outcome (Rothermundt et al., 2003; Giacoppo et al., 2012; Papa, 2012), investigating these markers has provided us with pivotal insight into the pathophysiology of mTBI. For example, clinically presented symptom clusters of mTBI are thought to

**Table 1 | Selected peripheral blood biomarkers of mTBI.**

| Marker | Biological roles | Diagnostic | Prognostic | Injury mechanism | Reference |
|---|---|---|---|---|---|
| s100B | Calcium binding protein found in astrocytes and some neuronal cells | Lacks specificity, elevated levels found in the serum of multi-trauma patients | Poorly related to outcome as measured by return to work (RTW) | Suggests astrocyte damage/activation as a cellular sequelae to primary insult, as well as possible BBB disruption | Bazarian et al. (2006), Biberthaler et al. (2006), Naeimi et al. (2006), de Kruijk et al. (2001), Metting et al. (2012), Nygren-de Boussard et al. (2004) |
| | Found elevated in serum acutely post mTBI | Some validity for diagnosis of intracranial lesions (IL) | Even highly elevated levels have been shown full recovery | | |
| NSE | Glycolytic enzyme, specific to the cytoplasm of neurons | Lacks sensitivity, and specificity; elevated levels found in blood resulting from hemolysis | Poor correlation between serum levels and GOS | Suggests acute neuronal damage | Meric et al. (2010), Naeimi et al. (2006), Berger et al. (2007), de Kruijk et al. (2001) |
| | Elevated post mTBI | | | | |
| GFAP/GFAP BDP | Protein found in glial cells, major part of the astroglial skeleton Elevated within 1-h post mTBI | Promising, BDPs have high specificity and sensitivity | Poor predictor of RTW or GOSE | Suggests astrocyte damage, possible BBB disruption | Papa et al. (2012), Metting et al. (2012) |
| MBP | One of two most abundant CNS proteins found in myelin | Detection of serum elevations may take up to 2–3 days, making it temporally unfavorable | Elevated serum levels may be related to poor outcome | Suggests structural axonal damage | Beers et al. (2007), Berger et al. (2005) |
| | Elevated in serum post mTBI | | | | |
| Tau | Microtubule associated proteins located in CNS axons | Correlated with mTBI | Poor outcome predictor using 3-months PCS assessment as well as RPCQ | Suggests hyperphosphorylation resulting in formation of CNS tangles "tauopathy" | Guzel et al. (2010), Ma et al. (2008), Bazarian et al. (2006), Bulut et al. (2006), Small et al. (2013) |
| | Found elevated in the serum within 6 h of mTBI | Unable to identify patients with IL found on CT scans | | | |
| UCH-L1 | Cytoplasmic protein found specifically in neurons | Not associated with pediatric mTBI | N/A | Suggests neuronal loss and disruption of the BBB | Berger et al. (2012) |
| SBDP145 | One of the all-spectrin breakdown products, found in presynaptic terminals and axons | Not associated with pediatric mTBI | N/A | Suggests cell necrosis | Berger et al. (2012) |

be associated with neuronal (de Kruijk et al., 2001) and glial cell damage/activation (Pelinka et al., 2004; Metting et al., 2012), often specifically pertaining to axon structures (Bulut et al., 2006; Berger et al., 2007; Guzel et al., 2010), developing into what has become known as diffuse axonal injury (DAI) (Johnson et al., 2012). These developments, as well as our present understanding of secondary injury in mTBI are based on both experimental studies, and a large existing body of research on moderate and severe TBI. From this it has been suggested that secondary injury results from a maladaptive healing response that amplifies the damage incurred

from primary injury. This is accomplished in a complex, multifaceted nature, involving a variety of biochemical cascades related to a disruption in energy metabolism, protein synthesis and degradation, and dysfunction at the level of neural synapse (Greve and Zink, 2009; Cederberg and Siesjö, 2010; Jaerve and Müller, 2012; Johnson et al., 2012; Kan et al., 2012). However, studies on the biological sequelae resulting from the primary insult in mTBI are still relatively scarce.

Very few potential markers related to the advent of secondary injury have been assessed. Among these is SBD145, a cleavage product of αII-spectrin that is indicative of cell necrosis (Berger et al., 2012). However, in a single study assessing this marker in mTBI patients, no correlation between blood levels of SBD145 and predictive outcome in pediatric mTBI was found (Berger et al., 2012). The Tau protein has also been implicated in mTBI secondary injury, and has been found elevated in the serum of mTBI patients (Guzel et al., 2010). The term "tauopathy" has been associated with other neurological disorders such as Alzheimer's and Parkinson's disease, and is thought to be involved in the etiology of CTE (McKee et al., 2009). Tauopathy refers to the hyperphosphorylation of the Tau protein, causing biochemical alterations which lead to the formation of axonal tangles, ending in disruption of neuronal communication (McKee et al., 2009; Guzel et al., 2010). In view of this, Small et al. (2013) recently demonstrated increased Tau deposits in retired NFL players with histories of cognitive and mood symptoms. Although this preliminary study was constrained by a small sample size, it is the first report to date to identify such findings in live humans at risk for CTE. Further studies assessing this possible mechanism would help not only in CTE pathology, but would provide pivotal information about the degenerative processes occurring post mTBI.

There is strong evidence to support that TBI pathophysiology involves systemic innate and adaptive immune responses that are intricately involved in a communicative process between the periphery and brain parenchyma (Morganti-Kossmann et al., 2007; Cederberg and Siesjö, 2010; Giacoppo et al., 2012; Papa, 2012). In addition, recent experimental data in animals and humans in TBI have uncovered immunoexcitotoxicity as a novel pathological mechanism leading to CTE (Blaylock and Maroon, 2011). Thus, in parallel with the necessity of understanding the molecular pathophysiology of cell damage, there is a need for clinical studies in mTBI assessing markers of inflammation, in particular, those involving the recruitment of immune cells into both cellular and microvascular brain structures (Kochanek and Hallenbeck, 1992; Clark et al., 1994).

## SUMMARY/FUTURE DIRECTIONS

Considering the health related and economic impact of mTBI (Dash et al., 2010; Giacoppo et al., 2012; Papa, 2012), an improved understanding of this condition is urgent. The prospect of using peripheral blood-based markers synergistically with current clinical diagnostic and prognostic assessments of mTBI is favorable for a variety of reasons: (1) it is more clinically accepted compared to other invasive procedures; (2) it is cost effective; (3) it may quickly and accurately provide specific information about the underlying pathophysiology of mTBI, which clinicians can then use in the diagnosis and formulation of treatment strategies.

Ultimately, the goal of biomarker research is to identify surrogate markers as an adjunct or replacement for specific clinical endpoints. By definition, the surrogate marker is one of achievement, as biomarkers are first considered "candidates" in hopes of acquiring the surrogate rank. However, in order to identify markers that may achieve this status, an in depth understanding of the cellular and molecular pathogenic mechanisms of mTBI is required. This point cannot be overlooked in any facet of biomarker research. Our current understanding of both primary and secondary mTBI pathology is poor, as is our understanding of PCS and CTE. Future research, whether experimental or clinical, would benefit by employing a more mechanistic based focus. Assessing a panel of markers will likely improve diagnostic and prognostic accuracy. Furthermore, in order to ensure proof of principle, specific mTBI symptoms need to be more directly linked to biological findings to establish causation. It will remain difficult to identify the mechanistic underpinnings of mTBI if biological findings are simply correlated to a cluster of symptoms. Controlling for specific symptoms/outcomes through greater cohort stratification may prove useful.

## PROSPECTIVE TRANSLATIONAL TECHNIQUES

High throughput "OMICs" technologies will continue to be invaluable moving forward with enhanced detection and characterization of novel blood-borne biomolecules. The field of proteomics shows great promise through its ability to characterize entire cellular environments ("Top-down approach") and identify novel proteins involved in pathological processes ("Bottom-up approach") (Colantonio and Chan, 2005). Wang et al. (2005) have published a very informative review on proteomic based research in TBI that captivates the essential importance and potential of this technology to the field.

Assessing the peripheral immune system in mTBI may potentially lead to invaluable information on the cause and/or consequence of secondary injury. Although severely understudied in mTBI, research in moderate and severe TBI has underlined neuroinflammation as an important process in secondary injury (Lenzlinger et al., 2001; Jaerve and Müller, 2012). In view of this, flow cytometry is a high throughput immunological technique with proven utility to elucidate pathological cell signaling pathways using human biological fluids, including whole blood, and is also used as a clinical diagnostic tool (Laerum and Farsund, 1981). Studies assessing the mechanisms underlying secondary injury in mTBI that incorporate conventional flow cytometry and imaging cytometry may yield important advancements to our understanding of both injury pathology and etiology of PCS and CTE.

In moving forward, it will be important for mTBI research to focus on elucidating pathophysiology. It would appear that a single marker will not achieve this, and to date, the search for biological indicators of mTBI has been met with limited success. However, there is much reason for optimism with regard to the ultimate potential of blood-based biomarkers. Recent data on such markers as GFAP-SBP and Tau have proved hopeful (Papa et al., 2012; Small et al., 2013). These markers may provide pivotal information into the underlying pathology behind mTBI and CTE respectively. Furthermore,

advances in high throughput "OMICs" techniques such as mass spectrometry and imaging cytometry provide real potential in uncovering the biological mechanisms underlying mTBI and its chronic sequelae. These techniques will no doubt be intrinsically involved in the entire translational process of mTBI research, from the elucidation of pathophysiological mechanisms to the clinical implementation of validated diagnostic and prognostic biomarkers.

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# The potential for bio-mediators and biomarkers in pediatric traumatic brain injury and neurocritical care

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The use of biomarkers of brain injury in pediatric neurocritical care has been explored for at least 15 years. Two general lines of research on biomarkers in pediatric brain injury have been pursued: (1) studies of "bio-mediators" in cerebrospinal fluid (CSF) of children after traumatic brain injury (TBI) to explore the components of the secondary injury cascades in an attempt to identify potential therapeutic targets and (2) studies of the release of structural proteins into the CSF, serum, or urine in order to diagnose, monitor, and/or prognosticate in patients with TBI or other pediatric neurocritical care conditions. Unique age-related differences in brain biology, disease processes, and clinical applications mandate the development and testing of brain injury bio-mediators and biomarkers specifically in pediatric neurocritical care applications. Finally, although much of the early work on biomarkers of brain injury in pediatrics has focused on TBI, new applications are emerging across a wide range of conditions specifically for pediatric neurocritical care including abusive head trauma, cardiopulmonary arrest, septic shock, extracorporeal membrane oxygenation, hydrocephalus, and cardiac surgery. The potential scope of the utility of biomarkers in pediatric neurocritical care is thus also discussed.

**Keywords: cerebrospinal fluid, abusive head trauma, shaken baby syndrome, neuron specific enolase, S100β, GFAP, myelin basic protein, UCH-L1**

## INTRODUCTION

Serum or cerebrospinal fluid (CSF) biomarkers of brain injury have been suggested as possible diagnostic adjuncts in neurocritical care since the groundbreaking work from the laboratory of Per Vaagenes (Kjekshus et al., 1980; Vaagenes et al., 1980, 1984, 1986, 1987, 1988; Bohmer et al., 1983; Vaagenes, 1986) in experimental and clinical cardiac arrest (CA), anoxic brain injury, stroke, and open heart surgery over 30 years ago. Earlier work in the 1960s concluded that serum creatine kinase, aspartate aminotransferase, and lactate dehydrogenase were not helpful as brain injury biomarkers (Dubo et al., 1967). However, in an often overlooked but remarkably prescient series of reports, his team used creatine phosphokinase brain band (CPK-BB) as the primary biomarker, along with CSF lactate dehydrogenase and aspartate aminotransferase and found that levels of these markers increased in CSF across these conditions. The studies included canine models of CA, and patients with CA, anoxic brain injury, cardiac surgery, or stroke. This was remarkably translational work for the early 1980s. They also explored the possible theragnostic utility of CSF biomarkers in patients treated with hypothermia vs. normothermia after CA (Vaagenes, 1986). Vaagenes called this approach "*a chemical biopsy of the brain*" and indicated that it may be clinically useful in prognosticating or in determining appropriate "levels of care." Although the development of brain injury biomarkers is certainly challenging, it is unclear why we did not listen more carefully to him and further pursue this line of investigation 30 years ago.

The use of biomarkers of brain injury in pediatric neurocritical care has been explored for at least 15 years. We believe that studies in pediatric populations and applications are essential. Although in many cases, serum biomarkers of brain injury perform similarly in adult and pediatric applications, it is important to recognize that some biomarkers, show important age dependent differences in normal values in biological samples including CSF or serum. The most well recognized in this regard is S100B which exhibits high levels during infancy. These developmental increases also appear to be somewhat variable in magnitude and thus mandate that need for age matched controls when using this biomarker (Portela et al., 2002; Gazzolo et al., 2003). Thus, it is important to include the full spectrum of pediatric age groups when testing new pediatric biomarkers. Similarly, some disease processes exhibit age dependent second injury mechanisms such as the propensity toward neuronal apoptosis early in development. Brain injury biomarkers can have unique applications in pediatric traumatic brain injury (TBI) such as in detection of clinically silent brain injury in abusive head trauma (AHT) (Berger et al., 2006b). Finally, the nature of brain injury and its time course vary greatly across the spectrum

of insults seen in pediatric neurocritical care and it is likely that the serum or CSF biomarker signature generated for each insult will differ. Our prior study of serum brain injury biomarker levels in infants and children across neurological diseases in the PICU confirmed that fact (Berger et al., 2006a), and represents an initial step in this regard. These issues mandate the development and testing of brain injury bio-mediators and biomarkers specifically in pediatric applications.

In this review, we will begin with studies of bio-mediators and biomarkers of brain injury in pediatric TBI and then broaden the discussion to other key disease processes associated with brain injury in the pediatric intensive care unit (ICU). This includes AHT, CA, and other pediatric neurocritical care conditions where brain injury biomarkers are showing promise.

### EARLY STUDIES ON BIO-MEDIATORS AND BIOMARKERS OF BRAIN INJURY IN PEDIATRIC TBI

Bell et al. (1997b) examined CSF levels of the cytokines interleukin-6 (IL-6) and IL-10 in infants and children after severe TBI (Glasgow coma scale score $< 8$) and reported marked increases of both vs. controls. The levels of IL-6 in CSF were similar to the levels of IL-6 in serum in separate children with septic shock (Bell et al., 1997a), highlighting the surprising magnitude of the "inflammatory response" in brain after TBI, and suggesting that IL-6 might be useful as a biomarker of brain injury after TBI. Most of the early work on biomarkers of brain injury in children focused on TBI which is logical given its prevalence in children, and the availability of CSF as a biological sample source with the use of CSF diversion in the treatment of patients with severe TBI including AHT (Kochanek et al., 2012a,b). In general, two lines of research have been pursued: (1) studies of "bio-mediators" in CSF of children after TBI to explore the secondary injury cascade in an attempt to identify potential therapeutic targets and (2) studies of the release of structural proteins into the CSF, serum, or urine in order to diagnose, monitor, and/or prognosticate in patients with TBI. Although there is overlap between what constitutes a bio-mediator vs. a biomarker, the use of this construct to categorize studies is helpful. Among those studies, we published several seminal reports such as the aforementioned study on IL-6, the first use of CSF biomarkers to examine the molecular footprints of apoptotic neuronal death (Bcl-2, cytochrome *c*) after pediatric TBI (Clark et al., 2000; Satchell et al., 2005), and the first studies targeting use of serum biomarkers to aid in making the diagnosis of silent brain injury in infants with AHT (Berger et al., 2006b, 2009). We will discuss these and other recent studies on bio-mediators and biomarkers in pediatric TBI. Finally, in 2006, Berger et al. (2006a) published a study on the potential utility of three different serum biomarkers [neuron specific enolase (NSE), S100β, and myelin basic protein (MBP)] in three common pediatric neurocritical care diseases, namely, TBI, AHT, and cardiopulmonary arrest. Subsequent to that publication, other groups have published promising reports on the potential utility of these and several other serum biomarkers to identify brain injury in important diseases encountered in the pediatric ICU including recent reports on septic shock, extracorporeal membrane oxygenation (ECMO), hydrocephalus, and cardiac surgery (Cengiz et al., 2008; Hsu et al., 2008; Bembea et al., 2011; Bhutta et al., 2012). The potential scope of the utility of biomarkers in pediatric neurocritical care will thus also be discussed. An overview of the topics addressed in this review is provided in **Figure 1**.

## DEFINING THE EVOLUTION OF SECONDARY DAMAGE IN PEDIATRIC TBI USING CSF "BIO-MEDIATORS"

The concept that CSF could be used to assess bio-mediator substances involved in secondary injury mechanisms was suggested as early as 1949 as shown for the neurotransmitters acetylcholine and serotonin by Tower and McEachern (1949) and Sachs (1957) in studies focused on clinical TBI (reviewed by Hayes et al., 1992). An early review on the use of CSF bio-mediators of brain injury in pediatric TBI provided initial rationale for the use of this approach (Kochanek et al., 2000) and the potential value of this line of investigation has gained support. Although the control of intracranial hypertension after severe TBI is important to prevent secondary brain ischemia and herniation, recent studies have suggested the need for additional therapies targeting other mechanisms of secondary damage. A multi-center randomized controlled trial (RCT) of decompressive craniectomy in adults with severe TBI (Cooper et al., 2011) showed that despite better control of raised ICP with surgical decompression, outcomes were worse vs. medical management – which unlike surgery, may be treating both ICP and other secondary injury mechanisms. Similarly, Mehta et al. (2010) reported that despite highly successful control of ICP in infants with severe TBI, ∼50% of children $<2$ years of age still had unfavorable long-term outcomes. Taken together, these studies suggest that we need to define the pivotal molecular secondary injury pathways after TBI and target them with novel therapies. In a number of studies, we have used CSF bio-mediators for this purpose and suggest potential therapeutic targets. Selected studies are discussed below.

### BIO-MEDIATORS OF NEURONAL DEATH

Early work in TBI suggested that neuronal death resulted from necrosis either from the primary impact or secondarily from ischemia-reperfusion during intracranial hypertension (reviewed in Kochanek et al., 2000). However, brain tissue samples from adults with severe TBI suggested that the molecular footprints of apoptosis including Bcl-2, Bcl-xl, Bax, and/or cleavage of caspase-3 were detectable in the initial days after severe TBI (Clark et al., 1999). Subsequently, Clark et al. (2000) showed that increases in CSF levels of the anti-apoptotic protein Bcl-2 were seen in infants and children early after severe TBI and were correlated with survival. Satchell et al. (2005) followed up on that study and reported that CSF levels of the pro-apototic protein cytochrome *c* were increased in infants with severe TBI. Cytochrome *c* levels were increased vs. control, and associated with mortality and AHT as an injury mechanism. This suggested that victims of AHT might represent a specific target population for the use of anti-apoptotic therapies after pediatric TBI. However, it is difficult to determine whether effects attributed to AHT are occurring independent of young age, since most infants with severe TBI are victims of AHT. Caspase-3 levels are also known to be much higher early in development than in older children (or adults) based on pre-clinical studies (Yakovlev et al., 2001). In addition, female gender was associated with increased levels of cytochrome *c* after severe TBI

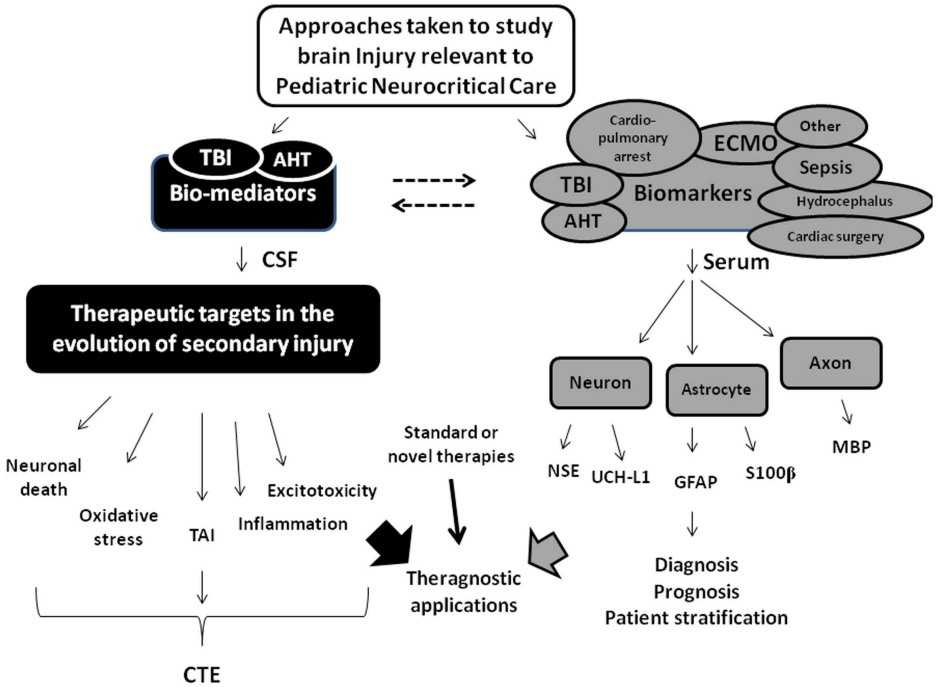


**FIGURE 1 | Overview of the approaches taken by our research team and others in the application of bio-mediators and biomarkers of brain injury to study pediatric neurocritical care**. Two basic strategies have been utilized, namely (1) studies using bio-mediators to study evolution of secondary injury and define new therapeutic targets (shown in black), and (2) studies of biomarkers largely of structural origin released from injured or dying cells representing three major cellular components in the brain (i.e., neurons, astrocytes, and axons) to serve as diagnostic or prognostic adjuncts. For the studies of bio-mediators of secondary injury, this approach has been largely carried out in traumatic brain injury (TBI) using assessment of cerebrospinal fluid (CSF) that is drained as part of standard of care to reduce intracranial pressure. In those studies, five major secondary injury pathways including neuronal death, oxidative stress, traumatic axonal injury (TAI), inflammation, and excitotoxicity have received the most investigation using this approach. These pathways can ultimately lead to long-term disabilities and/or chronic traumatic encephalopathy (CTE). For the studies of biomarkers (shown in gray), although many biomarkers have been examined, five have been used in the majority of studies including the neuronal markers neuron specific enolase (NSE) and ubiquitin C-terminal hydrolase-L1 (UCH-L1), the astrocyte markers S100β and glial fibrillary protein (GFAP), and the axonal injury marker myelin basic protein (MBP). Both approaches have theragnostic applications. The dotted lines reflect that recognition that some "bio-meditros" can serve as biomarkers and vice versa. Please see text for additional details. AHT, abusive head trauma; ECMO, extracorporeal membrane oxygenation.

in infants and children, which is consistent with the predominance of apoptosis as a cell death pathway after exposure of female vs. male neurons to neurotoxins in cell culture (Du et al., 2009). Increases in the CSF levels of cytochrome *c* after pediatric TBI and its association with AHT and female gender were confirmed in a study examining biomarkers of apoptosis vs. necrosis (Au et al., 2012). These studies suggest that apoptotic neuronal death may represent a therapeutic target in pediatric TBI, particularly in infants. Studies in experimental models of TBI suggested that levels of cleavage products of αII Spectrin might be able to aid in differentiating apoptotic vs. necrotic neuronal death mechanisms in TBI (Pike et al., 1998a,b). αII Spectrin is cleaved by either calpain during necrosis to 145 and 150 kDa degradation products or by caspase-3 during apoptosis to a 120 kDa degradation product. And this approach has also been used to estimate the time course and necrotic vs. apoptotic neuronal death in adult patients with severe TBI using CSF analysis (Pineda et al., 2007). A predominantly necrotic profile was seen in adults in the initial 5 days after injury. Monitoring markers of neuronal apoptosis after TBI thus could be particularly important in children and is an area for future clinical work. Studies in experimental models of TBI suggest that other neuronal death pathways may play important roles including autophagy, necroptosis, and pyroptosis (You et al., 2008; Du et al., 2009; Adamczak et al., 2012). Unique biomarkers of these processes are also needed to define the quantitative contribution of these pathways to the evolution of neuronal death after TBI and other disorders in neurocritical care. The studies on neuronal death mechanisms in pediatric TBI also highlight the fact that pediatric TBI includes the special condition of AHT. Although TBI resulting from motor vehicle accidents, falls, and other mechanisms seen in both children and adults produces heterogeneous pathologies, AHT adds considerably to this problem. In addition to routine TBI presentations such as contusion, subdural hematoma, or diffuse axonal injury, AHT often presents with unique pathologies (Ichord et al., 2007). For example, in many cases the CT findings are consistent with hypoxic ischemic encephalopathy (HIE) – possibly from apnea at the scene, delay in presentation, or cervical nerve root injury. In addition, AHT is often repetitive, and thus both acute and chronic TBI can be superimposed. Given these factors, serum and CSF bio-mediators

and biomarkers of brain injury may have special value in AHT – as shown in a number of studies discussed in this review.

## OXIDATIVE STRESS BIO-MEDIATORS AND BIOMARKERS AFTER PEDIATRIC TBI

Another mechanism that may represent an important therapeutic target is oxidative stress. Bayır et al. (2002) published the first comprehensive report on CSF markers of oxidative stress after severe TBI in children. Strong evidence for major losses of antioxidants such as ascorbate was seen along with increases in levels of markers of oxidative damage such as F2-isoprostane. During the initial week after injury, a progressive reduction of CSF levels of ascorbate was noted. This suggests ongoing oxidative stress in children after severe TBI. Mitochondrial dysfunction was shown to occur in brain tissue samples from patients with severe TBI (Verweij et al., 2000) and may serve as a key source for free radicals (Kagan et al., 2004, 2009). In an experimental model of pediatric TBI, selective oxidation of the mitochondrial lipid cardiolipin was seen early after injury, suggesting that mitochondria are an initial source of free radicals (Bayır et al., 2007). Given that cardiolipin oxidation is intimately linked to release of cytochrome *c*, oxidative stress may be critically linked to apoptotic neuronal death after TBI (Kagan et al., 2004, 2009). Antioxidants that target mitochondria may thus represent a logical strategy to target apoptotic neuronal death, which could be particularly important in pediatric TBI. Consistent with that hypothesis, mitochondrial targeting has shown impressive success in experimental models of pediatric TBI (Ji et al., 2012). CSF levels of antioxidants or oxidized cardiolipin, as assessed by oxidative lipidomics, might also represent excellent biomarkers for theragnostic use and merit future study (Tyurin et al., 2008; Kagan et al., 2009; Ji et al., 2012). Finally, in a theragnostic application focused on oxidative stress in pediatric TBI, mild therapeutic hypothermia markedly attenuated the increase in CSF levels of markers of oxidative stress, suggesting that hypothermia mitigates this mechanism in patients (Bayır et al., 2009).

## BIO-MEDIATORS OF NEUROINFLAMMATION

Another secondary injury mechanism that has been studied in pediatric TBI using CSF levels of bio-mediators is inflammation. Early work on CSF bio-mediators in pediatric TBI focused on inflammatory cytokines (Bell et al., 1997b). Subsequently, CSF levels of a number of inflammatory mediators were measured (Whalen et al., 2000; Amick et al., 2001; Robertson et al., 2001b; Han et al., 2002; Tong et al., 2004; Buttram et al., 2007; Fink et al., 2008; Salonia et al., 2010). A complete description of those studies is beyond the scope of this review; however, several points are noteworthy. First, severe TBI is consistently accompanied by a robust increase in CSF levels of cytokines and chemokines, particularly IL-6 and IL-8 (Bell et al., 1997a,b; Whalen et al., 2000; Amick et al., 2001; Buttram et al., 2007). Second, the inflammatory response is complex and contributes detrimental and beneficial effects depending on timing (Scherbel et al., 1999). It thus represents a perplexing therapeutic target. Third, multiplex technology has been useful to study cytokines and chemokines after TBI – allowing multiple mediators to be quantified in a single sample. A multiplex approach was used by Buttram et al. (2007) to test the effect of therapeutic hypothermia on CSF levels of cytokines and chemokines after severe TBI in children. Many inflammatory mediators were increased, but, surprisingly hypothermia had only modest effects on them. Cellular effectors of neuroinflammation include microglia, macrophages, and T-lymphocytes, and additional CSF markers are needed to determine if aspects of the inflammatory process can be therapeutically targeted in pediatric TBI.

## BIOMARKERS AND BIO-MEDIATORS OF TRAUMATIC AXONAL INJURY

Traumatic axonal injury (TAI) represents a mechanism of secondary damage that has been receiving increased attention recently, particularly as new imaging modalities are revealing the scope of this process (Tong et al., 2004; Babikan et al., 2005; Galloway et al., 2008). TAI was once believed to represent largely a primary injury process, however, the importance of "secondary axotomy" resulting from calcium accumulation and mitochondrial failure in axons has gained support (Smith et al., 2013). In pediatric TBI, Su et al. (2012) reported on this pathway using CSF levels of MBP, showing marked and sustained increases in this biomarker. The levels were on the order of ∼1000-fold higher than control, suggesting a major contribution of TAI. Drugs targeting TAI have not been tested in pediatric TBI, although calpain antagonists, cyclosporine-A, and FK506 have shown promise in experimental models (Smith et al., 2013). In the study by Su et al. (2012) mild hypothermia did not reduce CSF levels of MBP after severe TBI. Therapies that target TAI are needed and theragnostic use of a TAI biomarker such as MBP is logical. Pre-clinical studies suggest that there may be more injury of unmyelinated than myelinated axonal fibers (Reeves et al., 2005), and thus, new CSF biomarkers of unmyelinated axons are needed.

Excitotoxicity is a widely accepted secondary injury mechanism early after TBI. It could underlie early post-traumatic seizures and subclinical status epilepticus which are important in infants and young children (Liesemer et al., 2011). Early work on excitotoxicity in pediatric TBI was carried out by Ruppel et al. (2001) who reported marked increases in CSF levels of glutamate and other excitatory amino acids after severe injury. The increases peaked early in most patients and were associated with AHT. Robertson et al. (2001a) showed that the increases in CSF glutamate were coupled to retaliatory increases in levels of the endogenous anticonvulsant adenosine. Excitotoxicity may also mediate synaptic injury and one study showed marked increases in CSF levels of the synaptic protein α-synuclein after severe TBI in children (Su et al., 2010). α-Synuclein levels were increased ∼5-fold early after injury vs. control and progressed to levels ∼10-fold higher over the first week. A hot area of research in TBI is in defining the link between acute injury and the development of chronic traumatic encephalopathy (CTE) (DeKosky et al., 2010). TBI is linked to a variety of neurodegenerative diseases including Parkinson's disease (PD). Deposition of α-synuclein aggregates in Lewy bodies in PD suggests a link to this mechanism. Although this is an area of intense study in adults, particularly with mild repetitive TBI, there has been little study of this association in children. This is a vital area of future research for TBI biomarkers in pediatrics given the role of sports concussion and its link to CTE.

## SERUM BIOMARKERS IN PEDIATRIC TBI AND CARDIOPULMONARY ARREST

### DIAGNOSIS AND PROGNOSIS IN TBI

Building on the work in CSF, studies on the potential application of serum biomarkers of brain injury in pediatric neurocritical care began to emerge and initially focused on TBI and cardiopulmonary arrest. These conditions represent two of the most common disease processes encountered in pediatric neurocritical care and were thus logical targets for initial work on serum biomarkers. For diagnostic and prognostic indications, the approach focused on the use of proteins that are largely structural in nature and as unique as possible to the CNS. Most of the studies in pediatrics have centered around five biomarkers, namely, the neuronal markers NSE and ubiquitin C-terminal hydrolase-L1 (UCH-L1), the astrocyte markers S100β and glial fibrillary protein (GFAP), and the axonal injury marker MBP. After demonstrating robust increases in NSE and S100β in CSF in infants and children with severe TBI (Berger et al., 2002), Berger et al. (2005) measured serum levels of NSE, S100β, and MBP in 100 infants and children with TBI in cases of varying severity. All three biomarkers showed significant increases vs. controls, with sensitivity and specificity of initial values, for example, of 71 and 64% (NSE) and 77 and 72% (S100β). This suggested promise for the use of these serum biomarkers as diagnostic adjuncts in severe pediatric TBI. The biomarkers were also increased in many children who presented with a GCS score of 15 suggesting possible utility across injury severities – although a comprehensive study of serum biomarkers in mild TBI in children remains to be completed. Fraser et al. (2011) also explored the potential use of the biomarker GFAP in severe TBI in children. Serum GFAP levels measured on day 1 correlated with Pediatric Cerebral Performance Category scores assessed at 6 months. GFAP may also thus represent a potentially useful serum biomarker of brain injury in pediatric neurocritical care. Finally, Berger et al. (2012) recently studied the potential utility of serum levels of UCH-L1 and αII-SDP in pediatric TBI. UCH-L1 and αII-SDP levels were increased in cases of moderate or severe (but not mild) TBI and were correlated with Glasgow outcome scale score. These correlations were stronger than those for NSE, S100β, and MBP. Taken together, these studies suggest promise for a number of serum biomarkers in diagnostic and prognostic applications across the injury spectrum in pediatric TBI.

### DIAGNOSTIC ADJUNCT IN AHT

An important subgroup of patients with TBI for potential utility of serum biomarkers is cases of AHT – particularly infants with mild injury in whom the diagnosis may be missed and confused with conditions such as colic or gastroenteritis (Jenny et al., 1999). Based on a series of reports, NSE and MBP were shown to be the most potentially useful as screening tools to identify brain injury in well-appearing infants with clinically silent AHT (Berger et al., 2006b). Those studies led to the development of an NIH-funded prospective case-control study on the use of serum biomarkers for this purpose that has now entered nearly 900 infants. Studies are also ongoing examining the utility of GFAP and UCH-L1 in this setting. We also carried out a study of the application of proteomics (2-dimensional gel electrophoresis) on the injury response in AHT and compared it to non-abusive mechanisms of TBI in infants and young children (Gao et al., 2007). Several unique aspects of the proteomic injury profile were seen in AHT, notably, a reduced acute phase response. Infants who were victims of AHT had CSF proteomic profiles with reduced levels of acute phase reactants such as haptoglobin and complement components vs. children with TBI from other causes such as motor vehicle accidents. This could reflect a delay in presentation or represent a consequence of repeated injury often seen in cases of AHT. We also used a Multiplex approach in an attempt to define a combination or panel of serum biomarkers with high sensitivity and specificity to detect silent brain injury in infants with AHT (Berger et al., 2009). In that study, vascular cellular adhesion molecule (VCAM) and IL-6, used together, could discriminate the AHT vs. control with a sensitivity and specificity of 87 and 90%, respectively, when evaluated in an appropriate pediatric population to target missed AHT. Further studies using combinations or panels of biomarkers are needed in AHT and across the relevant diseases in pediatric neurocritical care.

### SERUM BIOMARKERS OF BRAIN INJURY IN PEDIATRIC CARDIOPULMONARY ARREST

We also carried out, to our knowledge, the first comparative study of serum levels of NSE, S100β, and MBP in critically ill infants and children after TBI, AHT, and cardiopulmonary arrest (Berger et al., 2006b). Distinct temporal profiles were seen for each of these conditions. TBI showed the largest acute increases in serum biomarker levels likely reflecting immediate damage from the primary injury. In cardiopulmonary arrest and AHT, delayed increases in the neuronal death marker NSE suggested its (or other neuronal death markers) potential utility for prognostic and theragnostic applications, and for the need to evaluate therapies targeting delayed neuronal death in cardiopulmonary arrest and AHT. Several studies in neonatal HIE from birth asphyxia have quantified serum biomarkers including S100β and NSE (Massaro et al., 2012; Roka et al., 2012). In 25 infants treated with either hypothermia or normothermia, serum S100β levels were lower in the hypothermia group and both S100β and NSE levels were higher in infants with worse outcome (Roka et al., 2012). In a larger study of 75 infants with neonatal encephalopathy and treated with hypothermia, S100β and NSE were again shown to be higher in patients with unfavorable outcome (Massaro et al., 2012). This suggests that these biomarkers are useful even if hypothermia is used in the treatment regimen. The astrocyte marker GFAP has also been shown to be increased in serum early after injury in neonates with HIE (Ennen et al., 2011). In preliminary studies, we reported use of three serum biomarkers NSE, S100β, and MBP as aids in prognostication in pediatric CA and observed outstanding performance based on receiver operator characteristic analysis (Fink et al., 2011). Several time points were employed, but 24 h values for NSE and S100β with cut points of 0.008 or 53.10 ng/mL exhibited high probability for classifying good vs. poor outcome in infants and children. This finding was seen despite the fact that there was heterogeneity in the etiologies of the arrests. Studies of the effect of mild hypothermia on biomarker levels and outcome are also ongoing including assessment of the efficacy of 24 vs. 72 h of hypothermia. Studies of the potential utility of UCH-L1 in

pediatric CA are also ongoing. For additional discussion of serum biomarkers across adult and pediatric TBI and CA, the reader is referred to a prior review (Kochanek et al., 2000).

## BRAIN INJURY BIOMARKERS ACROSS OTHER PEDIATRIC NEUROCRITICAL CARE DIAGNOSES

There have been a number of new applications of brain injury bio-mediators and biomarkers in pediatric neurocritical care. We will highlight several recent and promising studies in this regard in septic shock, ECMO, hydrocephalus, and cardiac surgery.

### SERUM BIOMARKERS OF BRAIN INJURY IN PEDIATRIC SEPTIC SHOCK

Hsu et al. (2008) assessed serum levels of S100β, NSE, and GFAP over the initial week of presentation in 24 children with septic shock and reported substantial (∼10 and 20-fold) increases in S100β and NSE respectively, despite lack of focal neurological deficits on exam. However, continuous EEG revealed moderate to severe encephalopathy in the patients. Biomarker levels were low early after sepsis and peaked at 5–7 days, contrasting TBI or CA. It is unclear whether these increases reflect permanent or transient damage, are associated with any long-term neurological morbidity, or reflect increases from extracerebral sources (Redl et al., 2008). However, this study should serve as an excellent foundation for future work in this area.

### SERUM BIOMARKERS OF BRAIN INJURY DURING EXTRACORPOREAL MEMBRANE OXYGENATION

Bembea et al. (2011) explored the use of plasma GFAP levels in 22 pediatric patients treated with ECMO for respiratory failure, cardiac failure, CA, or sepsis. Infants admitted to the ICU but without neurological injury served as controls. Seven infants treated with ECMO developed neurological complications including intracranial hemorrhage, cerebral edema, or brain death. Peak GFAP levels were ∼50-fold higher in these infants. Several temporal patterns were seen including progressive increases, or increases at single time points. The extracorporeal-CPR group was at highest risk for brain injury and increased plasma GFAP levels. A commentary on this report suggested the need for rigorous biokinetic analyses and the development of standardized assays for GFAP (Hayes et al., 2011). Children on ECMO are a perfect group for use of serum brain injury biomarkers given the difficulty in routine brain imaging during ECMO.

### CSF BIOMARKERS IN PEDIATRIC HYDROCEPHALUS

Cengiz et al. (2008) studied the application of CSF biomarkers of brain injury to another common diagnosis in pediatric neurocritical care, namely hydrocephalus. CSF levels of the neuronal injury marker cleaved-tau protein were assessed in 11 children with hydrocephalus requiring shunt placement or revision vs. values in controls. Cleaved-tau is a marker of neuronal damage or turnover formed by the proteolytic cleavage of the structural protein microtubule associated protein-tau (MAP-tau). Cleaved-tau CSF levels were increased in patients with hydrocephalus and correlated with duration of symptoms; ∼75% of the patients had signs of increased ICP before surgery. Tau-cleavage products are promising biomarkers of CTE and thus this study may represent a valuable early report on this topic in children relevant to TBI.

### SERUM BIOMARKERS OF BRAIN INJURY–THERAGNOSTIC APPLICATION IN CARDIOPULMONARY BYPASS

Finally, several groups have tested serum biomarkers of brain injury in the setting elective cardiac surgery in children (Abdul-Khaliq et al., 2000; Ali et al., 2000; Matheis et al., 2000; Lindberg et al., 2003; Lardner et al., 2004; Liu et al., 2009; Bhutta et al., 2012). Although a complete review of those studies is beyond the scope of this review, several studies have explored the theragnositc use of brain injury biomarkers after cardiac surgery in children. In an RCT of ketamine (2 mg/kg IV, $n = 13$) vs. placebo ($n = 11$) before surgery in infants, plasma levels of NSE, S100β, cytokines, and C-reactive protein were assessed (Bhutta et al., 2012). C-reactive protein levels were lower with treatment, although whether this reflected differences in brain injury was unclear. Treatment reduced injury as reflected by choline and glutamate plus glutamine/creatine levels assessed by magnetic resonance spectroscopy (MRS) in frontal white matter, but no differences between groups were seen on behavioral testing post-operatively. A combination of serum biomarkers with MRS may represent a useful theragnostic approach in acute brain injury. This strategy is being used to study the effect of 24 vs. 72 h of hypothermia in pediatric CA (Fink et al., 2011). Matheis et al. (2000) used serum levels of S100β to show increased oxidative injury after uncontrolled vs. controlled re-oxygenation after cardiac surgery in infants. Abdul-Khaliq et al. (2000) used S100β to study the effect of treatment with sodium nitroprusside in 25 neonates after cardiac surgery and reported reductions in serum levels of this biomarker with treatment. Similar approaches have been taken for other therapies after cardiac surgery in children including corticosteroids (Lindberg et al., 2003).

## CONCLUSIONS

It is an exciting time for biomarker development and exploration of bio-mediators in pediatric neurocritical care and rewarding that after over 15 years of work in this area, use of these tools may become standardized and incorporated into routine clinical use for diagnosis, prognosis and other aspects of patient management. Assessment of bio-mediators and biomarkers in CSF and serum is also helping to define therapeutic targets and provide theragnostic value in monitoring treatment efficacy. Brain injury biomarkers may also guide patient stratification for clinical trials – to help define the best sample for future RCTs or help show treatment effects. This could be important given the many failures of trials in TBI and the heterogeneity of this and other conditions in pediatric neurocritical care. We look forward to the development of point of care technology for brain injury biomarker applications in pediatric neurocritical care.

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# Amyloid-β peptides and tau protein as biomarkers in cerebrospinal and interstitial fluid following traumatic brain injury: a review of experimental and clinical studies

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Traumatic brain injury (TBI) survivors frequently suffer from life-long deficits in cognitive functions and a reduced quality of life. Axonal injury, observed in many severe TBI patients, results in accumulation of amyloid precursor protein (APP). Post-injury enzymatic cleavage of APP can generate amyloid-β (Aβ) peptides, a hallmark finding in Alzheimer's disease (AD). At autopsy, brains of AD and a subset of TBI victims display some similarities including accumulation of Aβ peptides and neurofibrillary tangles of hyperphosphorylated tau proteins. Most epidemiological evidence suggests a link between TBI and AD, implying that TBI has neurodegenerative sequelae. Aβ peptides and tau may be used as biomarkers in interstitial fluid (ISF) using cerebral microdialysis and/or cerebrospinal fluid (CSF) following clinical TBI. In the present review, the available clinical and experimental literature on Aβ peptides and tau as potential biomarkers following TBI is comprehensively analyzed. Elevated CSF and ISF tau protein levels have been observed following severe TBI and suggested to correlate with clinical outcome. Although Aβ peptides are produced by normal neuronal metabolism, high levels of long and/or fibrillary Aβ peptides may be neurotoxic. Increased CSF and/or ISF Aβ levels post-injury may be related to neuronal activity and/or the presence of axonal injury. The heterogeneity of animal models, clinical cohorts, analytical techniques, and the complexity of TBI in the available studies make the clinical value of tau and Aβ as biomarkers uncertain at present. Additionally, the link between early post-injury changes in tau and Aβ peptides and the future risk of developing AD remains unclear. Future studies using methods such as rapid biomarker sampling combined with enhanced analytical techniques and/or novel pharmacological tools could provide additional information on the importance of Aβ peptides and tau protein in both the acute pathophysiology and long-term consequences of TBI.

**Keywords: traumatic brain injury, biomarkers, Alzheimer's disease, amyloid beta, tau, cerebrospinal fluid, microdialysis**

## INTRODUCTION

In the United States, around 1.4 million people sustain a traumatic brain injury (TBI) annually (Zohar et al., 2011; Sivanandam and Thakur, 2012) and younger individuals are predominately affected (Fins, 2003; Kovesdi et al., 2010). Depending on the severity of the injury, survivors can experience significant impairments in cognition and display marked personality changes, which can have a negative impact both on the patient and the society (Magnoni and Brody, 2010; Sivanandam and Thakur, 2012). The pathophysiology of TBI is complex and involves multiple cellular and biochemical changes generated by the initial impact, leading to a disease process which exacerbate the injury for a prolonged period of time. This secondary injury process involves inflammatory cascades and heterogenous cell death pathways including apoptosis, autophagia, and necrosis (Kovesdi et al., 2007; Loane et al., 2009; Marklund and Hillered, 2011; Sivanandam and Thakur, 2012). Due to individual patient factors and initial injury characteristics, TBI produces either a focal lesion (cortical contusions, epi-subdural, or intracerebral hemorrhages), diffuse injury (diffuse axonal injury, DAI, and/or diffuse brain swelling; Strich, 1956; Yarnell and Ommaya, 1969; Gennarelli et al., 1982; Adams et al., 1989; Povlishock et al., 1992), or a mixture thereof (Saatman et al., 2008). There are substantial differences among these injury types and clinical TBI characteristics are markedly heterogeneous.

Importantly, wide-spread injury to white matter tract axons has emerged as a crucial contributor to the morbidity observed in TBI survivors (Smith and Meaney, 2000; Smith et al., 2003c; Czeiter et al., 2008). In injured axons, amyloid precursor protein (APP) accumulates mainly due to a TBI-induced disruption of axonal transport (Pierce et al., 1996). In addition, increased neuronal APP expression has also been observed in human and animal models and across the spectrum of severe TBI (Otsuka et al., 1991; Sola et al., 1993; Lewen et al., 1995; Pierce et al., 1996; Murakami et al., 1998; Ciallella et al., 2002; Itoh et al., 2009). Thus, elevated APP levels in injured axons may be due to a combination of increased neuronal expression and accumulation due to

disrupted axonal transport. When APP is proteolytically cleaved by β- and γ-secretases, amyloid-β (Aβ) peptides of various lengths can be produced by normal cell metabolism and be released from the presynaptic ending of the axon in the uninjured brain (Price et al., 1995; Blennow et al., 2006; Masters et al., 2006). Experimental TBI results in increased gene and protein expression of β-secretase 1 (*BACE1*; also named β-site APP cleaving enzyme 1), the major β-secretase involved in the production of Aβ from APP in neurons (Cai et al., 2001; Blasko et al., 2004; Loane et al., 2009; Yu et al., 2012a). Although the γ-secretase presenilin-1 and BACE1 were not co-transported with APP in the sciatic nerve (Lazarov et al., 2005), BACE1 protein was found to co-accumulate with APP in injured axons following TBI in the pig (Chen et al., 2004) and in patients dying within weeks post-injury (Uryu et al., 2007). Additionally, presenilin-1 may also co-accumulate with APP in injured axons (Uryu et al., 2007). As will be discussed in the subsequent paragraphs, an association between APP accumulation and Aβ formation in injured axons, post-injury plaque deposition and the development of Alzheimer's disease (AD) has not been firmly established. However, Aβ was found to co-accumulate with APP in injured axons up to 6 months post-injury in a miniature swine TBI model and at autopsy up to 3 years following human TBI (Chen et al., 2004, 2009). Combined, these reports argue that TBI may result in an increased production of Aβ peptides from APP. Since increased Aβ peptide generation may have neurotoxic properties and aggregate into plaques and oligomers (*vide infra*), it may have important implications in the secondary injury cascade post-TBI.

Alzheimer's disease, the most common neurodegenerative disease, affects more than 25 million people worldwide and shows a rapidly increasing prevalence (Blennow et al., 2006). AD is primarily characterized by progressive cognitive impairments including loss of episodic memory and language, impaired judgment, decision-making, and orientation. Neuropathology is diagnostic and extracellular plaques of Aβ peptides and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau proteins are typically found in the brains of AD patients (Blennow et al., 2006; Trojanowski et al., 2010; Kennedy et al., 2012; Weiner et al., 2012). More than two decades ago, it was postulated that a single, severe TBI may result in dementia with early onset (Clinton et al., 1991). Specifically, TBI was suggested to be an independent risk factor for AD in many studies (Clinton et al., 1991; Gualtieri and Cox, 1991; Mortimer et al., 1991; Breteler et al., 1992; Mayeux et al., 1993; Guo et al., 2000). A re-analysis of 11 case control studies (Mortimer et al., 1991) and results from a cohort of 548 injured WWII veterans (Plassman et al., 2000) found that the risk for developing AD following TBI can be increased up to 4.5-fold. The association between AD and TBI was further strengthened by clinical and experimental studies demonstrating that in brain tissue from TBI survivors or from brain-injured animals, pathological findings with a resemblance to those of AD were observed (Guo et al., 2000; Johnson et al., 2010; Magnoni and Brody, 2010). A genetic factor for AD, the ε4 allele of the lipid transport apolipoprotein E (Apoε4) seems to worsen the prognosis following TBI and predispose to the formation of Aβ plaques in AD (Nicoll et al., 1995; Kim et al., 2009). These reports argue that TBI may be a risk factor for the long-term development of AD (Mortimer et al., 1991; Plassman et al., 2000; Fleminger et al., 2003; Johnson et al., 2010; Magnoni and Brody, 2010).

Despite this suggested link between TBI and AD, numerous unanswered questions remain. For instance, is the increased risk of AD after TBI a direct consequence of cascades initiated at the time of impact, reflected by initial changes in Aβ and tau levels in brain, cerebrospinal fluid (CSF), and/or interstitial fluid (ISF)? Alternatively, does the TBI-AD link merely reflect a hastened cognitive decline and/or a reduced cognitive reserve induced by TBI? Specifically, in recent *in vivo* studies, Aβ and/or tau have been analyzed as biomarkers in both the experimental and clinical TBI setting in the CSF or in the ISF using microdialysis (MD). Although the analysis of phospho-tau and Aβ peptides is crucial in the diagnosis of AD (Mattsson et al., 2012), the interpretation of tau and Aβ peptides following TBI is unclear. Compared to most AD models, the data on Aβ and tau formation following experimental TBI are, to some extent, highly heterogeneous and AD pathology has not been robustly confirmed. In fact, rodent TBI models have been unable to show the hallmark findings of NFTs and Aβ plaques post-TBI. Regardless, since tau and Aβ levels may markedly influence the pathophysiology of TBI, both acutely and at long-term, they can potentially be used as biomarkers. In this review, we focus on the available evidence for increased Aβ and tau pathology in injured brain tissue and the use of Aβ peptides and tau as potential biomarkers in the CSF and ISF following TBI.

## Aβ AND TAU HISTOPATHOLOGY FOLLOWING TBI-ANIMAL STUDIES

Due to the heterogeneity of clinical TBI, numerous animal models exist (Marklund and Hillered, 2011). To date, most TBI studies evaluating tau and Aβ have used the focal controlled cortical impact (CCI) model, and only infrequently have models of diffuse TBI producing wide-spread axonal injury been evaluated (**Tables 1** and **2**). In initial TBI studies in rats, immunohistochemical analysis (IHC) revealed accumulation of APP in injured axons although Aβ peptides were not detected (Lewen et al., 1995; Pierce et al., 1996). Instead, mice overexpressing human APP [APP-yeast artificial chromosome (APP-YAC mice), PDAPP, and recently 3xTg-AD mice] displaying Aβ plaque pathology were developed and studied using the CCI model (Murai et al., 1998; Nakagawa et al., 1999, 2000; Hartman et al., 2002; Uryu et al., 2002; Conte et al., 2004; Tran et al., 2011a, 2012) (**Table 1**). Non-transgenic mice "knocked-in" with the human Aβ coding sequence to their endogenous APP gene ($APP^{NLh/NLh}$) have also been developed (Abrahamson et al., 2006, 2009). Although these models failed to mimic the formation of Aβ plaques similar to that observed in humans, findings such as exacerbated cell death and brain atrophy in APP-overexpressing mice were noted post-TBI (Smith et al., 1998). Since a *decreased* plaque load was found in aged plaque-forming PDAPP transgenic mice following TBI, plaque pathology may be potentially reversible (Nakagawa et al., 2000).

When rats were evaluated in the impact/acceleration and lateral fluid percussion injury models, both showing wide-spread axonal injury, long-term accumulation of Aβ in injured axons was noted although not Aβ plaques (Iwata et al., 2002; Stone et al., 2002; Tian et al., 2012). Although recent studies using various Enzyme-Linked Immunosorbent Assay (ELISA) and

**Table 1 | Animal studies on traumatic brain injury (TBI) and Aβ.**

| Reference | Type of animal | Age | Animal model | Aβ detection technique | Aβ peptide | Time post-injury | Plaque formation | Major findings |
|---|---|---|---|---|---|---|---|---|
| Murai et al. (1998) | APP ↑↑mice | 12–15 m | CCI | IHC, ELISA | ↓ Aβx-40, Aβx-42/43 ↔ | 1 w | No | APP-overexpressing mice were unaltered in lesion volume and behavior. Punctate cortical Aβ-IR↑ by TBI |
| Smith et al. (1998) | PDAPP mice | 4 m | CCI | IHC, ELISA | Sevenfold ↑ Aβ40, threefold ↑ Aβ42 | 2 h | No increase by TBI | In PDAPP mice, both Aβ40 and Aβ42 levels were increased early post-TBI associated with ↓ cognition and ↑ neuronal cell death |
| Nakagawa et al. (1999) | PDAPP mice | 4 m | CCI | IHC | ↓ Aβx-40, Aβx-42 | 5–8 m | No | Exacerbated hippocampal atrophy in PDAPP mice post-injury. TBI reduced Aβx-40 and Aβx-42 burden in the transgenic mice |
| Nakagawa et al. (2000) | PDAPP mice | 24 m | CCI | IHC | ↓ Aβ | 1–16 w | Reduction by TBI in PDAPP mice | Hippocampal atrophy worse after TBI in PDAPP mice. Aβ plaque burden reduced by TBI |
| Hartman et al. (2002) | PDAPP ± human APOE4 | 8–9 m | CCI | IHC | ↑ Aβ-ip | 3 m | In PDAPP/ APOE4+ mice only | AP deposits in PDAPP mice (diffuse plaques). TBI accelerates Aβ deposition in PDAPP mice in Apoe4 presence neuron loss ↔ |
| Uryu et al. (2002) | Tg2576 and wild-type mice | 9 m | CCI | IHC, ELISA | ↑ Aβ | 9–16 w | No | Aβ burden mildly increased in both single and repetitive mTBI mice compared to sham-injured controls |
| Conte et al. (2004) | Tg2576 mice | 11 m | CCI | IHC | ↑ Aβ40 and Aβ42 | 8 w | No | Vitamin E attenuated learning deficit and TBI-induced Aβ increases following repetitive mild TBI |
| Abrahamson et al. (2006) | $APP^{NLh\ NLh}$ mice | 3 m | CCI | IHC, ELISA, WB | ↑Aβ40, ↑↑Aβ42 | 3 h–14 d | No | A caspase inhibitor attenuated the TBI-induced increase in APP and Aβ and improved histological outcome |
| Abrahamson et al. (2009) | $APP^{NLh\ NLh}$ mice | 3 m | CCI | ELISA | ↑ Aβ40, Aβ42 ∼two to threefold increase | 3–7 d | No | Simvastatin attenuated TBI-induced increases in hippocampal Aβ levels and improved behavioral outcome |
| Loane et al. (2009) | BACE1 KO mice | 11–12 m | CCI | ELISA | ↑ Aβx-40 | 1–7 d | No | Genetic (β-) of pharmacological (γ-) inhibition of secretases improved motor, cognitive, and histological outcome |
| Tran et al. (2011b, 2012) | 3xTg-AD mice | 5–7 m | CCI | IHC, ELISA, WB | ↑ Aβ, Aβ40 | 1–24 h | No | Intra-axonal Aβ accumulation early and increased Aβ in ipsilateral hippocampus |
| Mannix et al. (2011) | BACE1 KO mice | 2–3 m | CCI | ELISA | ↑ Aβ1-40 | 23 d | No | Young BACE1 KO had lower Aβ1-40 pre- and post-injury and markedly impaired behavioral outcome |
| Yu et al. (2012b) | WT mice | 7 w | CCI | IHC, ELISA, WB | Aβ oligomers Aβ42 | 3 d | No | Levels of Aβ42 and Aβ oligomers were found to be significantly increased in the hippocampus after TBI. Lithium attenuated TBI-induced Aβ load and functional deficits |

*(Continued)*

**Table 1 | Continued**

| Reference | Type of animal | Age | Animal model | Aβ detection technique | Aβ peptide | Time post-injury | Plaque formation | Major findings |
|---|---|---|---|---|---|---|---|---|
| Schwetye et al. (2010) | PDAPP, Tg2576 mice | 3–6 m | CCI | MD, ELISA | Baseline Aβ1-x ↑ in transgenics, ↓ Aβ1-x after TBI | 2–24 h | No | Aβ levels in interstitial fluid were immediately decreased by 25–50% in the ipsilateral hippocampus following TBI |
| Smith et al. (1999) | Miniature swine | 4 m | RA | IHC | ↑ Aβ | 3–10 d | Diffuse plaques in 1/3 | Accumulation of Aβ and tau together with, e.g., APP in injured axons. Few plaques in white matter tracts and layer III in cortex |
| Iwata et al. (2002) | Rats | 3–4 m | LFP | IHC, WB, ISH | ↑ Aβ | 1 m–1 y | No | Accumulation of Aβ and strong immunoreactivity in injured axons |
| Stone et al. (2002) | Rats | N/A | I/A | IHC | ↑ Aβ | 48 h | No | Aβ formation in foci of axonal injury |
| Chen et al. (2004) | Miniature swine | 6 m | RA | IHC, WB, ELISA | ↑ Aβ, APP | 3 d–6 m | Yes, in gray and white matter | Co-accumulation APP and Aβ peptide in injured axons |
| Tian et al. (2012) | Rats | N/A | WD | IHC, ELISA, WB | ↑ Aβ42 | 14 d | No | TBI increased Aβ42 expression-Aβ42 deposits attenuated by intranasal NGF |

*In the vast majority of rodent studies, the CCI model of focal TBI was used and only rarely were TBI models with a higher degree of axonal injury evaluated. The age of the animals, genetic profile and modification, time post-injury, and analytical techniques may all have contributed to the inconclusive or negative findings in many of these studies. Amyloid plaque formation was consistently observed in pig models although displaying smaller and more diffuse plaques compared to human TBI. Thus, currently available animal models may not perfectly mimic the plaque-forming capacity observed in a subset of TBI patients.*

*APP, amyloid precursor protein; BACE1, beta-secretase 1; CCI, controlled cortical impact; d, post-injury day; ELISA, enzyme-linked immunosorbent assay; F, female; HC, hippocampus; I/A, impact-acceleration; IHC, immunohistochemistry; IR, immunoreactivity; ISH, in situ hybridization; LFP, lateral fluid percussion; M, male; MD, microdialysis; m, months; N/A, data not available; NGF, nerve growth factor; PDAPP, platelet-derived amyloid-beta precursor protein; RA, rotational acceleration; WB, western blotting; WD, weight drop; wt, weight. All rats in this Table are Sprague–Dawleys.*

**Table 2 | Animal studies on traumatic brain injury (TBI) and tau.**

| Reference | Animal | Age | Animal model | Tau detection technique | Tau type | Time detected | Major findings |
|---|---|---|---|---|---|---|---|
| Hoshino et al. (1998) | Rat | 3 m | LFP | IHC | P-tau | 6 m | Six months after TBI, numerous neurons were immunoreactive for P-tau or Aβ |
| Smith et al. (1999) | Pig | 4 m | RA | IHC | T-tau | 3–10 d | Accumulations of T-tau and NF-rich inclusions were found in neuronal perikarya. Tau accumulated in most axonal bulbs |
| Liliang et al. (2010a) | Rat | N/A | WD | ELISA, WB | T-tau | 1–6 h | T-Tau levels ↑↑ at 1 h, returned to baseline by 6 h post-injury. Tau levels were higher in the severe TBI group compared to the mild TBI group |
| Genis et al. (2000) | ApoE-deficient mice | 4 m | WD CHI | WB | P-tau, T-tau | 4–24 h | P-tau increased at baseline in transgenics. In WT controls, P-tau ↑ at 4 h post-TBI, returned to baseline at 24 h. Minimal increase in P-tau in transgenics, clearly less than in WT controls |
| Yoshiyama et al. (2005) | T44tauTg and WT non-Tg mice | 12 m | Mild repetitive | BC, IHC, WB | NFT* | 9 m | Behavioral outcome not impaired 6 months post-TBI. Only one Tg T44 mouse only showed extensive NFTs and cerebral atrophy |
| Gabbita et al. (2005) | Adult rat[1] | Adult | CCI | ELISA, IB | C-tau | 6–168 h | C-tau levels was increased at 6 h post-TBI, peaked at 168 h post-injury. Elevated brain C-tau levels associated with TBI-induced tissue loss |
| Tran et al. (2011a) | 3xTg-AD and wild-type B6/SJL mice | 5–7 m | CCI | ELISA, IHC, WB | P-tau | 24 h–7 d | In 3xTg-AD mice, TBI resulted in increased intra-axonal phospho-tau immunoreactivity after TBI |
| Tran et al. (2011b) | 3xTg-AD, APP/PS1, TauP301L mice | 2–6 m | CCI | IHC, WB | T-tau, P-tau | 1–24 h | Increased tau pathology early in 3xTg-AD and TauP301L mice with a peak at 1 and 24 h post-TBI. Increase in contralateral hippocampus beginning at 12 h post-TBI. P-tau increased in fimbriae and fornix |
| Tran et al. (2012) | 3xTg-AD mice | 5–7 m | CCI | WB, IHC, HP | P-tau | 24 h | Abnormal co-accumulation of several phosphorylating kinases with tau at 24 h post-TBI. A JNK inhibitor reduced P-tau accumulation in axons |
| Yu et al. (2012b) | WT mice | 7 w | CCI | IHC, WB, ELISA | P-tau | 3 d | P-Tau was increased in the thalamus post-TBI; lithium administration reduced P-tau at 3 d post-TBI, resulting in improved spatial learning |
| Ojo et al. (2013) | h-Tau mice | 18 m | Repetitive mTBI | IHC | P-tau | 21 d | Increased P-tau by repetitive, 48 h apart, mTBI although not a single mTBI |

*In both focal and diffuse TBI models did the levels and expression of tau consistently increase post-injury.*

*APP, amyloid precursor protein; BC, biochemical analysis; CCI, controlled cortical impact; C-tau, cleaved-tau; ELISA, enzyme-linked immunosorbent assay; HP, histopathological analysis; h-tau, mice overexpressing human tau; IB, immunoblotting; IHC, immunohistochemistry; LFP, lateral fluid percussion; m, month; P-tau, phosphorylated tau; PS1, presenilin-1; JNK, c-Jun N-terminal kinase; RA, rotational acceleration; T-tau, total tau; N/A, data not available; NFT, neurofibrillary tangles; TBI, traumatic brain injury; w, week; WB, western blotting; WT, wild-type; CHI, closed head injury; WD, weight drop; mTBI, mild traumatic brain injury; [1]Age not mentioned; *NFTs were observed in one mouse only suggesting this study was negative for producing a tauopathy post-injury.*

immunohistochemical detection methods have shown increased Aβ load in wild-type animals (Loane et al., 2009; Mannix et al., 2011; Tian et al., 2012), the vast majority of rodent TBI mice models failed to replicate the Aβ plaque formation observed in humans (see **Table 1**). To date, only in PDAPP-human APOε4 transgenic mice was TBI found to accelerate Aβ plaque formation (Hartman et al., 2002). Since the rodent Aβ sequence differs from the one in humans at amino acid positions 5, 10, and 13 (Selkoe, 1989), poor immunohistochemical detection techniques and less aggregating properties of mouse Aβ was suggested (Smith et al., 1998). At present, improved immunohistochemical methods have alleviated this problem of Aβ detection in rodents and additionally, APP transgenic animals carry the human sequence. The increased Aβ load noted in some animal TBI models may be dependent on

the evaluated Aβ species, time span post-injury, and age of the animal. It is also plausible that Aβ formation is more extensive in TBI models with a higher degree of axonal injury.

Although rodent TBI models produce pathology similar to that observed in humans, there are obvious differences in anatomy as well as gray-white matter ratio, and rodents are also lissencephalic (Morales et al., 2005; Marklund and Hillered, 2011). Thus, high-order species may have advantages in terms of clinical relevance and AD-like pathology was evaluated in a rotational acceleration DAI model in miniature swine (Meaney et al., 1995; Smith et al., 1997; Johnson et al., 2010). Although a smaller number of Aβ plaques compared to TBI patients was observed, this model produced Aβ accumulation in injured axons in addition to plaque formation (Smith et al., 1999; Chen et al., 2004). Furthermore, diffuse Aβ plaques in both gray and white matter were identified (Smith et al., 1999) and APP co-accumulated with Aβ post-injury (Chen et al., 2004) (**Table 1**). This swine model appears suitable for the study of Aβ pathology following TBI, particularly in relation to axonal injury.

The microtubule-associated protein tau has six isoforms in humans and is a normal constituent primarily of axons. In pathological conditions such as TBI, tau can be hyperphosphorylated (P-tau) and aggregate which is needed for the formation of NFTs (Geddes et al., 1999; McKee et al., 2009; Ojo et al., 2013). Tau dissociated from microtubuli can disperse not only by interneuronal transfer but also via glial to glial spread (Genis et al., 2000; Tran et al., 2011a,b), be involved in Aβ-induced neurotoxicity (Rapoport et al., 2002) and also be neurotoxic by itself (Farias et al., 2011). The formation of NFTs has been observed both following repetitive mild human TBI and many years following a single severe TBI in a subset of patients in addition to its crucial role in AD. Tau formation has been evaluated in numerous experimental TBI studies using Western Blot, ELISAs, and immunohistochemistry (Hoshino et al., 1998; Smith et al., 1999; Genis et al., 2000; Ikonomovic et al., 2004; Gabbita et al., 2005; Yoshiyama et al., 2005; Uryu et al., 2007; Liliang et al., 2010a,b; Tran et al., 2011a,b, 2012; Rostami et al., 2012; Yu et al., 2012b; Ojo et al., 2013). The vast majority of rodent studies have used focal TBI models and evaluated changes in total tau (T-tau), cleaved-tau (C-tau), and/or P-tau within the first post-injury weeks (**Table 2**). Importantly, these rodent models have not been able to reproduce the NFT pathology observed in AD.

Several studies have used transgenic mice in the study of tau pathology following TBI (Genis et al., 2000; Yoshiyama et al., 2005; Tran et al., 2011a,b, 2012; Yu et al., 2012b). Although both wild-type and Apoε-deficient mice showed tau hyperphosphorylation post-injury following closed head injury, it was more marked in wild-type controls (Genis et al., 2000). These important findings need to be reproduced also in other TBI models. Importantly, accumulation of phosphorylated tau over time may influence neuronal structure and synaptic properties (Dickstein et al., 2010). Due to the increasing interest in the long-term sequelae of mild repetitive TBI in humans (e.g., concussions in sports), repeated mild TBIs in mice have been evaluated. Although NFTs or behavioral deficits were not induced by repeated mTBI in transgenic mice expressing the shortest human tau isoform (Yoshiyama et al., 2005), increased P-tau without NFT formation was observed following repeated mTBI in aged mice overexpressing human tau (Ojo et al., 2013).

In summary, the swine, wild-type rodents, and transgenic mice TBI models thus consistently showed increased tau protein levels post-injury without producing the NFTs observed in AD. Importantly, most animal TBI studies negative for NFT formation have only used short-term survival whereas NFT was only observed in patients surviving for many years following severe TBI although not in patients dying within 4 weeks of the injury (Smith et al., 2003a; Johnson et al., 2012).

## Aβ AND TAU HISTOPATHOLOGY FOLLOWING TBI-HUMAN STUDIES

In approximately 30% of patients dying early from TBI, Aβ plaques was identified at autopsy across all age groups (Gentleman et al., 1993, 1997; Roberts et al., 1994; Horsburgh et al., 2000; Smith et al., 2003b; Uryu et al., 2007; Chen et al., 2009). Diffuse Aβ plaques have been also observed by immunohistochemistry in surgically removed focal injuries within days post-injury (Ikonomovic et al., 2004; DeKosky et al., 2007). Aβ plaques have also been found in injured axons of DAI patients dying <9 days post-injury (Smith et al., 2003c). Importantly, wide-spread Aβ pathology can remain for many years in the brains of survivors of moderate to severe TBI (Johnson et al., 2010, 2012). Contrary to the diffuse plaques observed acutely, these long-term Aβ plaques were more often fibrillary (Johnson et al., 2012). Since Aβ plaques are found in only ∼30% of TBI patients, the development of neurodegeneration and/or AD likely has a multifactorial basis including altered expression of, e.g., the Aβ-degrading enzyme neprilysin gene which is related with some forms of AD (Helisalmi et al., 2004). Notably, neprilysin gene polymorphism was linked to the occurrence of Aβ plaques following TBI (Johnson et al., 2009), raising the possibility to screen individuals with a high risk of TBI such as participants in contact sports or soldiers.

Numerous clinical reports have reported tau pathology, in particular an accumulation of NFTs, in the brains of athletes who sustained several concussions during their career. This entity has been named chronic traumatic encephalopathy (CTE) (Corsellis et al., 1973; Roberts et al., 1990; Dale et al., 1991; Geddes et al., 1999; McKee et al., 2009, 2013). Although these findings have also been observed in athletes from a variety of different sports including American football or ice hockey, they have been classically seen in the brains of up to 17% of former professional boxers (previously named *dementia pugilistica* or "punch-drunk" syndrome) (Roberts et al., 1990). Common symptoms in CTE include memory loss, Parkinson-like movements, and dementia (Roberts et al., 1990; Jordan et al., 1995; McKenzie et al., 1996; McKee et al., 2009; Nowak et al., 2009). In CTE, the vast majority of cases display wide-spread NFTs and Aβ pathology is much less frequently observed (McKee et al., 2013). Recently, the largest cohort of individuals to date with a history of repeated concussions was analyzed where wide-spread tauopathy was observed (McKee et al., 2013). Although these reports and others suggest that repeated concussions/mTBI should be regarded very seriously, the number of examined individuals is still low and the incidence of CTE, its risk factors, and the contribution of other co-variables has yet to be defined.

Tau pathology, including high density and wide-spread NFTs, was also observed in patients who suffered a single, severe TBI 1–47 years previously (Johnson et al., 2012). In this study, 39 patients with a single, severe TBI surviving for more than 1 year post-injury were compared to 47 age-matched controls. Mean survival was 8 years and NFTs were present in 34% of patients <60 years old compared to 9% of controls of similar age. Additionally, the NFTs in TBI patients were commonly observed in superficial cortical layers, in depths of the sulci, and clusters were observed in the cingulate gyrus, the insular cortex, and the superior frontal gyrus. In contrast, NFTs were rarely observed outside the transentorhinal cortex and the CA1 in controls (Johnson et al., 2012). This study was the first to observe NFT at long-term following a single, severe TBI in humans although additional studies including a larger number of patients are required for confirmation of these findings. The long delay between the injury and the NFT analysis and the large age span in this patient cohort add to the inherent variability and many potential co-variables may have contributed to the formation of NFTs (Johnson et al., 2012). The process of delayed NFT formation in human TBI, if at all present, remains to be defined. Early following severe TBI, total and phospho-tau protein was found to accumulate in both neuronal cell bodies and axons post-TBI in a subset of patients (Smith et al., 2003a; Uryu et al., 2007) although without clear NFT pathology. In surgically resected brain tissue early post-injury, diffuse neuronal tau immunostaining was observed in most patients, although only 2/18 patients showed NFTs (Ikonomovic et al., 2004). In addition, NFTs were not found in TBI patients who died within 4 weeks from injury (Smith et al., 2003a), suggesting that the mechanisms leading to NFT and/or CTE pathology requires a prolonged time post-injury to develop.

Thus, numerous animal and human observations support a link between AD and TBI. However, there are substantial clinical and histopathological differences between AD and TBI (Johnson et al., 2010). In the brains of CTE victims, P-tau immunoreactive NFTs are found superficially in wide-spread cortical regions (Hof et al., 1992; McKee et al., 2009) in contrast to AD where NFT are predominately observed in deep cortical layers. Additionally, typical neuritic plaques with a dense core of fibrillar Aβ represent a typical finding in AD patients, whereas diffuse Aβ plaques with non-fibrillary Aβ are observed early in TBI (Horsburgh et al., 2000; Johnson et al., 2010). The Aβ plaques observed in AD develop over several years and are typically seen in older individuals in contrast to TBI, where Aβ plaques have been demonstrated as early as 2 h post-injury and in young patients as well (Roberts et al., 1994; Ikonomovic et al., 2004; Johnson et al., 2010). Additionally, TBI Aβ plaques appear more in the gray matter in contrast to AD (Smith et al., 2003b) and it is unclear whether the diffuse TBI-induced Aβ plaques progress into the more solid and dense plaques characteristic of advanced AD (Horsburgh et al., 2000; Chen et al., 2009; Johnson et al., 2010, 2012). Several years following a single, severe TBI, fibrillary Aβ plaques have been observed, implying that TBI may accelerate the pathophysiological process leading to AD. These data suggest that the mechanisms leading to an increased risk for neurodegeneration and AD following TBI are highly complex.

## RATIONALE OF Aβ PEPTIDES AS BIOMARKERS FOLLOWING TBI

*In vitro* and animal AD models indicate that Aβ accumulation, in particular the soluble oligomeric form, may be a crucial initiating factor in AD (LaFerla et al., 2007; Gouras et al., 2010) preceding tau-related neurotoxicity (Hardy and Selkoe, 2002). However, both *in vitro* and *in vivo* animal studies demonstrate that extracellular Aβ concentrations are regulated by neuronal metabolism and synaptic activity (Cirrito et al., 2005, 2008). The majority (80–90%) of generated Aβ peptides consist of the 40-amino acid long peptide Aβ1-40 (Aβ40). The longer Aβ1-42 (Aβ42) proteolytic variant is more hydrophobic and tends to aggregate into plaques (Brody et al., 2008). In the experimental setting, Aβ may be synaptotoxic (Claeysen et al., 2012; Koffie et al., 2012), neurotoxic (Walsh et al., 2002), disrupt cellular membranes (Berman et al., 2008), interfere with mitochondrial function (Parihar and Brewer, 2010), activate NMDA receptors (Texido et al., 2011), or activate microglia (Stalder et al., 1999). Importantly, both endogenously and exogenously elevated Aβ may lead to neuronal death and behavioral dysfunction (Mattson, 2004). Since Aβ peptides co-accumulate with APP (Smith et al., 1999, 2003b; Uryu et al., 2007), damaged axons may be a key source of Aβ, released into the surrounding tissue due to lysis or leakage (Smith et al., 2003c).

Therefore, since neuronal/axonal Aβ peptides, released from normal neuronal activity and/or from increased production via injury-induced accumulation of APP, are implicated in the secondary injury process, Aβ peptides sampled from CSF (**Table 3**) or ISF (**Table 4**) are of interest as biomarkers in TBI.

## CSF BIOMARKERS OF Aβ PATHOLOGY FOLLOWING TBI

In the human CSF, Aβ peptides are found throughout life in their soluble forms. Studies of AD patients have shown that low CSF Aβ42 concentrations correlate with a high number of brain plaques (Strozyk et al., 2003). Additionally, some studies have found increased diagnostic accuracy of the Aβ42/Aβ40 ratio compared to Aβ42 alone (Hansson et al., 2007).

When the antibodies R165, which specifically recognize Aβ42 and R163, reacting only with Aβ40, were used in combination with Western Blot and ELISA, CSF Aβ40 and Aβ42 levels were found to be increased early following severe TBI (Raby et al., 1998; Emmerling et al., 2000) in contrast to normal, (∼50 pg/ml), plasma levels. On the contrary, decreased CSF Aβ40 and Aβ42 concentrations have also been observed (Franz et al., 2003; Kay et al., 2003) and associated with poor clinical outcome (Franz et al., 2003). In lumbar CSF, the Aβ40, Aβ42, and total Aβ levels are highly correlated and may fluctuate markedly over time when serial taps are used (Bateman et al., 2007). Similar studies in TBI, where CSF samples are frequently obtained from ventricular CSF, are lacking.

The driving force of Aβ peptides from brain parenchyma into the interstitial and intraventricular compartments are yet incompletely understood following TBI and may be related to the presence of cerebral edema and the function of the blood-brain and brain-CSF barriers (Brightman and Kaya, 2000; Iliff et al., 2012). The CSF levels of Aβ40 and Aβ42 in controls and AD patients differ markedly among published studies (Mehta et al., 2000; Frankfort et al., 2008), similar to the observations in the available TBI studies (**Table 3**). Thus, it is plausible that the evaluation method, time

**Table 3 | Amyloid β and tau levels in cerebrospinal fluid (CSF) in patients with traumatic brain injury.**

| Reference | Patients (*N*) | Age (years) | Sample period | Aβ1-40 | | Aβ1-42 | | Tau | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | TBI | Control | TBI | Control | TBI | Control |
| [a]Raby et al. (1998), Emmerling et al. (2000) | 6, severe TBI | 35.5 (19–51) | 3 w | 0.94 ± 0.08 ng/mg | 1.59 ± 0.53 ng/mg | 1.17 ± 0.11 ng/mg | 0.38 ± 0.2 ng/mg | 2308 ng/ml | N/A |
| [b]Zemlan et al. (1999) | 15, severe TBI | 32.4 ± 14.1 | 1–8 dpi | N/A | N/A | N/A | N/A | C-tau: 1519 ± 3019 pg/ml | C-tau: 0–31 pg/ml |
| [b]Zemlan et al. (2002) | 28, severe TBI | 35.1 (18–75) | 1–7 dpi | N/A | N/A | N/A | N/A | C-tau ventricular: d 1: 3205 pg/ml d 3: 556 pg/ml | C-tau lumbar: 75 ± 86 pg/ml |
| [c]Franz et al. (2003) | 29, severe TBI *n* = 15 vCSF *n* = 14 lCSF | 41 (15–72) | 1–284 dpi | N/A | N/A | 167 (120–477) pg/ml | 284 (172–564) pg/ml; 388 (256–768) pg/ml | 1756 (35–5720) pg/ml | 193 (16–326) pg/ml[1]; 109 (69–159) pg/ml[1] |
| [d]Olsson et al. (2004) | 28, severe TBI | 41 (15–81) | 0–11 dpi | N/A | N/A | 96 (79–196) pg/ml (d 7–11) | N/A | N/A | N/A |
| [d]Ost et al. (2006) | 39, severe TBI | 49 (16–82) | 0–14 dpi | N/A | N/A | N/A | N/A | T-tau (d 2–3): 682 and IQR 1155 pg/ml and 8500 and IQR 7630 pg/ml[2] | T-tau: 677 and IQR 308 pg/ml |
| [b]Zetterberg et al. (2006) | 14, boxers | 22 ± 3.8 | 7 dpi–3 m | 19400 ± 50 ng/L | 19300 ± 2740 ng/L | 858 ± 128 ng/L | 773 ± 133 ng/L | T-tau: 449 ± 176 ng/L; P-tau: 37.9 ± 10.2 ng/L | T-tau: 325 ± 97.7 ng/L; P-tau: 46.4 ± 14.5 ng/L |
| [a]Neselius et al. (2012) | 30, boxers | 22 (17–34) | [a]1–6 dpi; [b]> 14 dpi | N/A | N/A | [a]306 ± 52 ng/L; [b]294 ± 54 ng/L | 297 ± 039 ng/L | [a]T-tau: 58 ± 25 ng/L; [b]T-tau: 49 ± 21 ng/L | T-tau: 45 ± 17 ng/L |

*Both Aβ and tau levels following TBI show a large variability due, e.g., to the heterogeneity of the study protocols, patient cohorts, analytical techniques, and post-injury time point.*

*[1]Control values included patients with dementia and headache; [2]survivors versus non-survivors; dpi, day post-injury; NPH, normal pressure hydrocephalus; l, lumbar; N/A, not available; v, ventricular; CSF, cerebrospinal fluid. Data presented as [a]Means and range; [b]means ± Standard Deviations; [c]medians and range; [d]medians and interquartile range (IQR). ELISA was used to determine Aβ levels in all studies.*

**Table 4 | Amyloid β and tau levels in interstitial fluid (ISF) in patients with traumatic brain injury-microdialysis (MD) studies.**

| Reference | Patients (*N*) | Type of injury | Catheter location | Catheter | Sample interval | Analyte | Analysis method | ISF levels (pg/ml) | ISF tau levels (pg/ml) | Major findings |
|---|---|---|---|---|---|---|---|---|---|---|
| Brody et al. (2008) | 19 | Severe TBI ($n=12$); SAH ($n=6$); unruptured aneurysm ($n=1$) | Frontal in most patients | CMA70 ($n=6$) CMA71 ($n=13$) | Aβ1-x:1, every 2 h; Aβ 1–40 and Aβ 1–42, every 8 h | Aβ1-x Aβ 1–40 Aβ 1–42 | ELISA | Not reported; estimated from Figures: Aβ42; most MD samples between 10 and 60 pg/ml Aβ1-x: median 1000 pg/ml | N/A | A positive correlation between changes in brain interstitial fluid Aβ concentration and neurological status was found |
| Marklund et al. (2009) | 8 | Severe TBI, focal/mixed ($n=5$), DAI ($n=3$) | Frontal ($n=6$); peri-C ($n=2$) | CMA71 | Every 1 h | Aβ40, Aβ42, T-tau | ELISA | Aβ42 (median and range): 167 pg/ml (31–295) | T-tau: 2881 ± 1774 pg/ml (121–6500) Means ± SD and range | High levels of Aβ42 in ISF post-injury. Aβ42 levels were higher in DAI patients. Tau protein levels were higher in patients with focal/mixed disease |
| Magnoni et al. (2012) | 16 | DAI ($n=8$), EML ($n=7$), nEML ($n=1$) | Frontal ($n=10$); peri-C (n = 6) | CMA71 | Every 1–2 h, every 4–6 h for most patients | Aβ1-x, T-tau, NF-L | ELISA | First 24 h (median and range): peri-C Aβ1-x: 270 pg/ml (83–417); non-C Aβ: 1023 pg/ml (778–1968) | First 24 h: peri-C T-tau: 15950 pg/ml (11390–27240); non-C T-tau: 3469 pg/ml (1684–8691)[1] | Patients in the pericontusional group had lower Aβ and higher tau levels compared to patients in the non-contusional group. Initial tau levels were inversely correlated with initial Aβ levels. *In vitro* recovery for αβ was 30 and 1–2% for tau |

*Since the normal, injured tau, and Aβ peptide levels in the injured human brain are unknown it is yet difficult to establish the magnitude of TBI-induced alterations. Both increased and decreased Aβ peptide levels have been suggested depending on injury site and catheter location. Aβ peptide levels may be increased due to their formation in injured axons and also be related to the level of consciousness and degree of neuronal activity. Interstitial tau levels may be higher in patients with a focal disease and be inversely correlated with Aβ peptide levels. It appears that MD is a useful tool for the study of Aβ and tau dynamics in the injured human brain following TBI.*

*Aβ, beta amyloid; DAI, diffuse axonal injury; ELISA, enzyme-linked immunosorbent assay; EML, evacuated mass lesion; nEML, non-evacuated mass lesion, MD, microdialysis; N/A, not available; NF-L, neurofilament light chain; Non-C, non-contusional; Peri-C, pericontusional; SAH, subarachnoidal hemorrhage; TBI, traumatic brain injury; SD, standard deviations.*

post-injury and TBI severity, Apoε4 and neprilysin gene status, the presence of TBI-induced Aβ plaques, and yet undetermined factors may all influence Aβ levels in CSF. Future studies combining CSF with ISF levels correlating tissue and behavioral outcome in addition to the analysis of yet other Aβ peptide species are needed to determine the clinical value of CSF Aβ peptide levels as biomarkers.

## INTERSTITIAL FLUID BIOMARKERS OF Aβ PATHOLOGY FOLLOWING TBI

Microdialysis sampling of the ISF has been used for more than two decades for neurochemical monitoring of the human brain (Hillered and Persson, 1999; Bellander et al., 2004; Hillered et al., 2005). MD may be considered mainly a focal sampling method in contrast to CSF sampling, which reflects more global events (Hillered et al., 2005). Aβ peptides are regarded normal constituents of human ISF (Seubert et al., 1992), possibly reflecting a physiological secretion from neuronal metabolism (Hong et al., 2011). In the pathogenesis of AD, Aβ can aggregate into insoluble species and Aβ oligomeric forms, which have been shown to be cytotoxic and influence synaptic function (Funke, 2011; Hard, 2011). Although initial Aβ aggregation can occur intracellularly and/or extracellularly (Meyer-Luehmann et al., 2003; Gouras et al., 2010), a large amount of the required Aβ peptides comes from a pool of soluble Aβ in the ISF (Cirrito et al., 2008; Funke, 2011).

To investigate the dynamics of soluble Aβ, hippocampal MD was used in awake transgenic mice before and during the process of Aβ plaque formation (Hong et al., 2011). They found that diffusible forms of Aβ, predominantly Aβ42, came from a large reservoir of less soluble Aβ42 in brain parenchyma and decreased in ISF during deposition of Aβ (Hong et al., 2011). Additional *in vitro* and *in vivo* MD experiments were able to demonstrate a linear correlation between neuronal activity and the interstitial Aβ concentrations (Kamenetz et al., 2003; Cirrito et al., 2005, 2008). Following TBI, decreased electroencephalographic (EEG) activity in the hippocampus occurred concomitantly with decreased MD hippocampal Aβ levels, supporting the hypothesis that a TBI-induced reduction in neuronal activity may lead to reduced ISF Aβ levels (Schwetye et al., 2010).

For human use, most MD studies evaluate either the 20 or the 100 kDA cut-off MD catheters (Hutchinson et al., 2005; Hillman et al., 2006). Since Aβ40 or Aβ42 peptides have a molecular weight (MW) of ∼4.5 kDa, both catheters could be used. However, if T-tau (*vide infra*) is also evaluated, the 100 kDa catheter needs to be used due to the 48–67 kDa MW of tau proteins (Ost et al., 2006). Cerebral MD has recently been used in humans with severe TBI for the study of interstitial Aβ changes (Brody et al., 2008; Marklund et al., 2009; Magnoni and Brody, 2010; Magnoni et al., 2012) (**Table 4**). In an early study, MD and an Aβ1-x ELISA was used to analyze every Aβ peptide species from amino acid 1–28 or higher (Brody et al., 2008). A key finding was that ISF Aβ peptides levels were lower than in ventricular CSF explained by a 30% MD recovery. When Aβ1-x levels were compared to Aβ40 and Aβ42 in pooled 8 h-samples, the latter were 2.5 and 35 times lower, respectively, suggesting that most Aβ peptides in the injured human brain are neither Aβ40 nor Aβ42. Finally, in most patients did the ISF Aβ levels increase over time and the level of consciousness correlated well with ISF Aβ levels, implying a link to synaptic activity (Brody et al., 2008). An additional study from the same group (Magnoni et al., 2012) showed that although the MD Aβ levels were lower when the MD catheter was placed in the pericontusional tissue compared to a non-contusional area, pericontusional Aβ levels increased more substantially over time. Another MD study analyzed ISF Aβ40 and Aβ42 levels in patients with severe TBI where higher Aβ42 levels were found in patients with diffuse TBI compared to focal TBI patients (Marklund et al., 2009). Notably, MD Aβ40 levels were above detection level in only half of the patients in this study (Marklund et al., 2009).

These studies indicated that MD is a useful tool to study Aβ dynamics in the injured brain following TBI. Given the lack of baseline, uninjured control Aβ values, alterations in the Aβ peptides levels following TBI should be interpreted with caution. It has been hypothesized that reduced Aβ production may be due to neuronal loss and/or decreased synaptic activity (Cirrito et al., 2005, 2008; Brody et al., 2008; Magnoni and Brody, 2010) and may be increased by axonal injury (Marklund et al., 2009). Although it has been speculated that toxic Aβ byproducts such as oligomers and protofibrils initiate cascades ultimately leading to neurodegeneration and dementia (Magnoni and Brody, 2010), available evidence is insufficient to imply a causative role for the early post-injury Aβ changes. Moreover, it should be stressed that brain ISF is not in full equilibrium with the CSF (Fishman, 1992; Brody et al., 2008) and the half life of Aβ in brain tissue has not been established. Larger patient series are needed to investigate their relationship with clinical outcome and discern possible differences between injured and uninjured brain regions as well as between focal and diffuse TBI.

## TAU AS A BIOMARKER FOLLOWING TBI

Total tau is present in abundance in the central nervous system and in particular in unmyelinated axons and cortical interneurons (Trojanowski et al., 1989; Sivanandam and Thakur, 2012). Its biological activity is regulated by phosphorylation (P-tau), which has been associated with various neuropathologies (Alonso et al., 2001; Feijoo et al., 2005; Morris et al., 2011). Following human TBI, C-tau is considered a reliable biomarker of neuronal injury (Shaw et al., 2002; Zemlan et al., 2002; Gabbita et al., 2005) and has been suggested to be an indicator of axonal injury (Trojanowski et al., 1989; Wilhelmsen, 1999; Zemlan et al., 1999; Emmerling et al., 2000; Franz et al., 2003; Ost et al., 2006; Zetterberg et al., 2006; Magnoni et al., 2012; Sivanandam and Thakur, 2012). NFTs are formed by abnormal, phosphorylated tau filaments and CSF tau are commonly increased 3–4 times in AD (Blennow and Hampel, 2003; Selkoe and Schenk, 2003; Sivanandam and Thakur, 2012). Tau levels can be markedly increased in the CSF after TBI (**Table 3**) and show promise also as a specific serum biomarker in the human (Liliang et al., 2010b) and experimental setting (Rostami et al., 2012).

There is evidence to support that P-tau is important in the development of neurodegeneration (see previous section). Apoε deficiency and TBI have both been associated with hyperphosphorylation of a tau protein domain (Genis et al., 2000; Sivanandam and Thakur, 2012) (**Figure 1**). Additionally, tau misprocessing can be caused by abnormal accumulation of Aβ and tau *per se* may

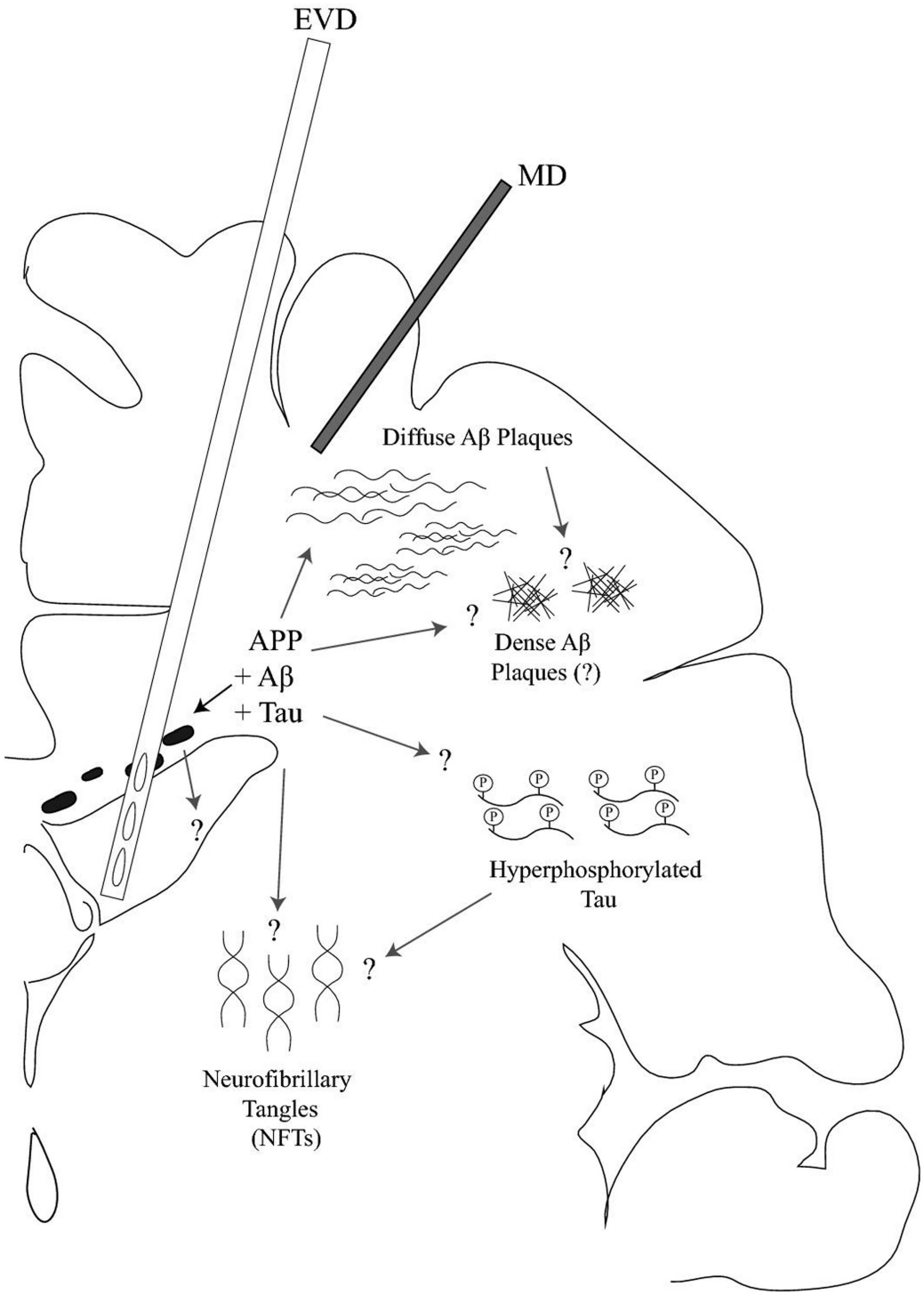


**FIGURE 1 | Schematic drawing of interstitial fluid (ISF) and cerebrospinal fluid (CSF) sampling of tau protein and amyloid-β (Aβ) peptides following traumatic brain injury (TBI) on a coronal brain section**. An external ventricular drainage (EVD) and a microdialysis (MD) catheter are placed into the frontal horn of the ventricular system and superficial cortex, respectively. Initially, TBI results in an accumulation of amyloid precursor protein (APP) that, following its degradation, may lead to intra-axonal amyloid-β (Aβ) accumulation and plaque formation in the brain parenchyma. Following TBI, early Aβ plaques are typically of the diffuse type in contrast to those observed in Alzheimer's disease whereas dense plaques may be observed in patients surviving for many years post-injury. Alternatively, Aβ peptides may also be produced by normal neuronal activity and be reduced by TBI. Neurofibrillary tangles (NFTs) can also be formed after TBI as a consequence of hyperphosphorylated tau. In humans, NFT formation does not appear to occur acutely and has mainly been observed beyond 4 weeks post-injury following a single, severe TBI. However, hyperphosphorylated tau aggregations can be observed as a characteristic observation following repetitive mild TBI. The question marks illustrate the unknown features of Aβ and tau accumulation, their release into the CSF or ISF, or the dynamic distribution between the CSF and ISF levels of Aβ and tau.

mediate Aβ cytotoxicity in AD (Le et al., 2012), adding to the complexity of tau and Aβ changes following TBI.

## TAU IN CSF AND ISF FOLLOWING TBI

Previous studies have consistently shown that tau CSF levels, which have been closely linked with the presence of axonal injury, increased intracranial pressure, and clinical outcome, are increased in TBI patients compared to normal controls (Zemlan et al., 2002; Franz et al., 2003; Ost et al., 2006; Zetterberg et al., 2006; Liliang et al., 2010b; Magnoni et al., 2012) (**Table 3**). The results are different in milder forms of TBI, including boxing, since tau levels are only slightly increased or even unchanged (Zetterberg et al., 2006; Neselius et al., 2012). When evaluating tau as a biomarker following TBI, it must be considered that ventricular CSF typically has higher tau levels than lumbar CSF (Blennow and Nellgard, 2004).

Only recently has tau also been analyzed in the ISF (**Table 4**). Using MD, ISF T-tau levels were clearly above the detection limit in all patients and were higher in patients with a focal/mixed TBI compared to DAI patients (Marklund et al., 2009). The ISF tau levels were comparable to those previously measured in ventricular CSF post-TBI (Franz et al., 2003; Ost et al., 2006). Recently, MD tau levels were found to be markedly higher in TBI patients with the MD probe placed in the pericontusional area compared to when the MD probe was placed in a brain region without contusions. Additionally, high initial ISF tau levels correlated with poor clinical outcome (Magnoni et al., 2012). The MD recovery of tau is likely low, estimated to be 1–2% (Magnoni et al., 2012), since hyperphosphorylation markedly decreases the solubility of tau (**Table 4**). Although T-tau has commonly been analyzed as biomarkers, the phosphorylation status of tau is likely more important in the pathophysiology of TBI to date.

## CONCLUSION AND FUTURE DIRECTIONS

The current literature on early and late CSF, ISF, and brain tissue changes of Aβ peptide levels and tau following TBI was reviewed. To define the precise relation between Aβ and tau levels in brain tissue, CSF and/or blood and clinical disease remains an important scientific challenge due to the association between TBI and the risk of developing neurodegeneration and AD. Available experimental and clinical evidence implies a complex relationship between increased tau protein release, Aβ peptide deposition, and NFT and Aβ plaque formation following TBI. Rodent studies, perhaps most importantly those carried out in transgenic mice, have provided important mechanistic information and shed light into many aspects of tau and Aβ formation following TBI although without consistently mimicking the histopathological findings observed in humans. TBI severity, the used species and model, choice of analytical technique, and the inherent difference between human and rodent brain may contribute to the inconsistent results obtained using experimental TBI models. On the other hand, the swine TBI model appears to produce Aβ pathology more closely resembling the human situation. Only biomarker analysis of Aβ peptides and tau may not be sufficient to elucidate the complex cellular, biochemical, genetic (e.g., neprilysin and Apoε4), and metabolic cascades ultimately predisposing TBI victims to an increased risk for AD. It appears likely that TBI accelerates the process leading to AD, although the mechanisms and relation to the acute injury cascade remain largely unknown. Possibly, many additional *in vitro* and *in vivo* experiments dissecting various aspects of the tau/Aβ cascade are needed. It is expected that the increased use of tau and Aβ peptides as biomarkers in the clinical setting will enhance our understanding of the link between TBI and the later development of AD.

Available studies show that Aβ and tau can be analyzed in interstitial and CSF although the analysis methods and the resulting biomarkers levels differ markedly among studies. The studies are mainly observational and long-term follow up data is frequently lacking. However, robust data exist for tau, showing elevated levels in the CSF and the ISF and a correlation between tau levels in both compartments and long-term outcome was also suggested. Emerging data suggest that tau is promising as a biomarker also in peripheral blood. The interpretation of post-injury Aβ levels is currently more complicated. Aβ peptides are produced both by normal neuronal metabolism and by enzymatic processing of accumulated APP in injured axons following TBI. Thus, their levels may be related to the level of consciousness, the presence of axonal injury or both and be reduced in the vicinity of cortical contusions. Importantly, increased Aβ peptide levels, particularly the longer and fibrillary ones, can also be neurotoxic *per se* (Brody et al., 2008; Marklund et al., 2009; Magnoni et al., 2012). Different analysis methods also render comparisons between studies difficult. Although the Aβ1-42 peptide is important in AD and has attracted much interest in TBI, other subspecies may also be highly relevant and much recent interest is directed toward Aβ oligomers and protofibrils (Magnoni and Brody, 2010).

Then, what is the current and future potential of tau protein and Aβ peptides as biomarkers and what can they tell us about the possible neurodegeneration occurring post-TBI? Ideally, the levels of a biomarker should closely correlate with a biological or pathogenic process (Czeiter et al., 2012) or be used as surrogate end-points. Obviously, the chronic sequelae of TBI survivors are crucial. However, at the current level of knowledge, the correlation between early Aβ and tau biomarker findings and the later development of AD is weak. Interestingly, it has been shown that acute Aβ accumulations can be reversed following TBI (Smith et al., 1998). Moreover, the vast complexity and variability in the used TBI models do not allow clear conclusions or extrapolation of the experimental results into clinical practice to date. Instead, available evidence suggests that Aβ and tau could be used as injury markers or in mechanistic studies. In future studies, correlation of levels in ISF, CSF, and/or serum with advanced neuroimaging such as diffuse tensor imaging or Positron Emission Tomography (PET) using, e.g., Pittsburgh Compound B (Quigley et al., 2011) preferably using rapid biomarker sampling combined with enhanced analytical tools could provide additional information. Long-term and serial biomarker determination would also be of importance where potential differences in the biomarker levels in lumbar versus ventricular CSF could be evaluated. BACE1 inhibitors, γ-secretase inhibitors, statins, and neprilysin replacement therapy are emerging treatment possibilities for AD which could also play key roles in the future study of TBI. Combined

with biomarker analysis, these pharmacological tools could provide crucial information related to the importance of tau and Aβ peptides in the pathophysiology and long-term consequences of TBI.

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# Assessing neuro-systemic & behavioral components in the pathophysiology of blast-related brain injury

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Among the U.S. military personnel, blast injury is among the leading causes of brain injury. During the past decade, it has become apparent that even blast injury as a form of mild traumatic brain injury (mTBI) may lead to multiple different adverse outcomes, such as neuropsychiatric symptoms and long-term cognitive disability. Blast injury is characterized by blast overpressure, blast duration, and blast impulse. While the blast injuries of a victim close to the explosion will be severe, majority of victims are usually at a distance leading to milder form described as mild blast TBI (mbTBI). A major feature of mbTBI is its complex manifestation occurring in concert at different organ levels involving systemic, cerebral, neuronal, and neuropsychiatric responses; some of which are shared with other forms of brain trauma such as acute brain injury and other neuropsychiatric disorders such as post-traumatic stress disorder. The pathophysiology of blast injury exposure involves complex cascades of chronic psychological stress, autonomic dysfunction, and neuro/systemic inflammation. These factors render blast injury as an arduous challenge in terms of diagnosis and treatment as well as identification of sensitive and specific biomarkers distinguishing mTBI from other non-TBI pathologies and from neuropsychiatric disorders with similar symptoms. This is due to the "distinct" *but shared* and partially identified biochemical pathways and neuro-histopathological changes that might be linked to behavioral deficits observed. Taken together, this article aims to provide an overview of the current status of the cellular and pathological mechanisms involved in blast overpressure injury and argues for the urgent need to identify potential biomarkers that can hint at the different mechanisms involved.

**Keywords: biomarkers, blast injury, brain injury, neurotrauma, blast overpressure, mild TBI, PTSD, neuropsychiatry**

## INTRODUCTION

Traumatic Brain Injury (TBI) represents a major public health problem with an over 150,000 military personnel diagnosed with form of mild traumatic brain injury (mTBI), due to the exposure to blast resulting in a wide range of neurological and psychological symptoms (1, 2). Blast-related brain injuries can be provocatively described as "a silent epidemic of an invisible wound." Current Explosive mechanisms [improvised explosive devices (IEDs), landmines, and rocket-propelled grenades (RPGs)] are believed to account for 56–78% of Operation Enduring Freedom (OEF), Operation Iraqi Freedom (OIF), and Operation New Dawn (OND) related injuries (3, 4). This has led to labeling the blast-induced TBI (bTBI) as the signature brain injury for combat troops in today's military (5, 6).

Between 2000 and 2010, the Department of Defense (DoD) reported ∼200,000 head injuries as a consequence of combat-related incidents as well as events occurred in a non-deployed environment (civilian injuries) (7). However, even this number may be an underestimate due to the fact that the majority of blast-related mTBIs go misdiagnosed and untreated as a consequence of in-appropriate approaches of screening, invalidated diagnostic criteria or specific detectable abnormalities, and lack of diagnostic tools. Acute blunt penetrating injuries comprised 2.8% of this total, the rest were classified as mTBI (7).

Out of more than 8,000 cases of TBI reviewed by the Defense and Veterans Brain Injury Center, ∼50% were related to blast-related barotrauma (8). The clinical features observed in mTBI resulting from blast exposure vary, these include: headache, fatigue,

tinnitus, and irritability which have been highly recognized in recent conflicts. Blast overpressure (BOP) injury has been considered the main cause of both morbidity and mortality in neurotrauma (9, 10). Furthermore, blast TBI has been the center for military medical concern in the context of polytrauma, since blast-induced injury, due to its complex components (*primary*, *secondary*, *tertiary*, and *quaternary* injuries) is often accompanied by hemorrhagic blood loss, multiple fractures, burns, and systemic injury coupled with TBI (11–13).

The recognition of the high incidence and impact of bTBI; in addition, to the need for a more accurate diagnosis and effective therapeutic interventions, led to an impressive number of experimental and human blast injury studies aiming at investigating the complex interconnected pathways involved in the blast-induced neuropathological/behavioral changes.

This review will focus on three major questions: (i) What is the experimental and human evidence that blast is associated with progressive alterations in the brain and via what mechanism(s) they are mediated? (ii) What is the relation between blast-induced brain injury and the development of neuropsychological disorders such as post-traumatic stress disorder (PTSD)? (iii) What are the biochemical markers that can identify, track and predict the injury and symptoms observed in patients exposed to blast injury?

## BIOMECHANICS OF BLAST INJURY

Blast overpressure-induced injury results from an explosion characterized by an abrupt release of energy in such a short period of time within a small volume creating a non-linear shock and pressure wave (14). The blast shock wave of the primary blast is solitary supersonic pressure wave (peak overpressure) characterized with a rapid (sub-milliseconds–milliseconds) increase in pressure followed by sharp fall in pressure, often to sub-atmospheric levels before returning to ambient pressure (15, 16). This is coupled with the "blast wind" (forced super-heated air flow) that gives rise to a very large volume of gas that may throw victim's body against other objects. Blast wind, along with the shock wave are the main components of the "blast wave" (17, 18). Blast waves comprise the shock front followed by the blast wind (19). Blast waves impinge on the head-brain complex while mechanical pressure pulses in the brain; the severity of the injury is dependent upon the magnitude and duration of the pressure cycle (20). The net loading at a material point in the brain comprised of a direct transmissive load and deflection-induced indirect loads. The pressure pulse in the brain is governed by the acoustic impedance mismatches between the head and the brain, and the flexural rigidity of the skull (20).

Blast can cause four different types of insults: (i) the *primary injury* resulting from the BOP waves due to the shock-wave overpressure or/and under pressure. This event is usually associated with contusion, edema, hemorrhage, and diffuse axonal injury (DAI) (11, 17, 21, 22). (ii) The *secondary injury* that is due to shrapnel or hard objects propelled at the body. (iii) The *tertiary insult* involves head translation/rotation coupled with acceleration/deceleration due to blunt impact arising from blast wind and finally (iv) the *quaternary insult* resulting from thermal burns or the probable use of toxic gases or chemicals.

Compared to previous past conflicts, the majority of war zone wounds have been attributed to secondary blast injury (shrapnel propelled by explosions), while tertiary and quaternary blast injuries were related to terrorist-linked acts involving structural collapse and the use of toxic material. Previous studies on primary injury (BOP) have traditionally focused on gas-containing hollow organs such as the lungs and gastrointestinal tract (14, 23).

In one study by Clemedson discussing blast injury, the term "blast injury" has been used to describe the biophysical and pathophysiological events post exposure to high explosion or the shock wave associated with it (24). The greatest interest was devoted to study the peak pressure, as well as the impulse relevant to pulmonary injuries produced (25–28). Interestingly, on the pathophysiology focused on the sudden alteration in the body ambient pressure, primarily in gas-air filled organs including the lungs, intestines, or in tissues with different specific weight such as the ear and intestines; this occurred at the interface between media with very large differences in density (16, 24, 29, 30).

Furthermore, BOP can induce a mild form of brain injury with significant neurological conditions involving cerebral edema, neuroinflammation, and vasospasm along with DAI and neuronal death. This neuronal injury phase is followed by a series of complex neuropsychiatric symptoms which may include memory loss and behavioral changes (5, 13, 31–33). As such, exposure to complex blast waves can be viewed as the inducer of multitude of injuries or even polytrauma involving several organ injuries interaction that exacerbates blast insult outcome (13). Finally, blast wave propagation to the brain parenchyma is another controversial mechanism which may involves both direct propagation through the skull or in an indirect propagation via blood vessels which has a direct implication on vascular disturbance (31, 34).

Blast wind passage to the skull causes acceleration/rotation to the brain comprising the direct injury. Indirect injury involves the compression of the abdomen and chest transferring kinetic energy to the body's biofluid. This rippling effect generates oscillating waves from blood to the brain distant from the contact point. In turn, this kinetic energy transfer will induce functional and morphological changes in brain structures which represent a distinct complex feature of blast-induced brain injury not present in other traditional brain injury models (21, 31, 35). The complex mechanism of blast injury involves consequences of primary blast effects on autonomous nervous system. Taken together, it should be comprehended that the mechanics of neurotrauma due to blast injuries are quite different from that of other types of injuries arising from motor vehicle accidents (blunt) or penetrating injuries (ballistics).

## NEUROPATHOLOGICAL ALTERATION IN BLAST INJURY

Experimental studies of primary blast brain injuries (though limited) have shown evidence of altered cellular, molecular and biochemical processes, and behavioral outcomes. For instance, different studies have shown a heterogeneous profile of brain-associated cellular impairments including: elevation in β-amyloid precursor protein, altered expression of protooncogenes *c-Myc*, *c-Fos*, and *c-Jun* and impaired axonal transport along with oxidative stress with elevated nitric oxide generation (8, 33, 36–44). In addition, neuronal injury and glial activation (discussed later) coupled with elevation of biochemical markers such as, neuron specific enolase (NSE), ubiquitin C-terminal hydrolase 1 (UCH-L1), and

glial fibrillary acidic protein (GFAP) have been also reported. Other studies have shown evidence of axonopathy, edema, and hypertrophic astrogliosis with pronounced altered gene expression post-injury event (40, 44–46). However, there were a lot of ambiguity in the overpressure and duration utilized and the methods used to measure these parameters which were often unclear and not standardized (33, 43, 47).

Furthermore, such heterogeneous neural profile has been attributed to several factors including the suitable experimental model systems that can closely mimic "composite" primary, secondary, tertiary, and quaternary components of blast exposure, the lack of standardized blast wave instruments, different body localization and body armor, and the use of different animal species (31, 32, 41, 48) (see **Table 1**).

Several studies have been performed to assess neuropathological effect of BOP coupled with other comorbid factors (17, 29, 47–51). In these studies, several parameters were varied (different blast injury models, intensity, animal species used) or other modifications were included (protective vests, stressors, and animal localization).

One representative study is that of Kamnaksh et al. where they assessed different stressors and their contribution to blast injury. These stressors included transportation and blast sound with or without blast injury. Of interest, all groups exhibited increased anxiety, while injured and blast noise-exposed rats showed elevated corticosterone, interferon-c (IFN-c), and interleukin-6 (IL-6) in the amygdala and hippocampus. Injured animals showed elevated Iba1, GFAP, and apoptotic immunoreactivity (52). These data demonstrate that exposure to biological stressors can lead to behavioral changes and trigger specific neuropathological alteration even in the absence of detectable injury.

Pun et al. using a rat model, assessed the effects of a single sublethal blast over pressure (BOP) exposure (48.9–77.3 kPa) in an open-field set up. Histopathological analysis of inflicted brains revealed "darkened" and shrunken cortical neurons with narrowed vasculature at day 1 post-injury. Signs of recovery were demonstrated at days 4 and 7 post-blast exposure. Oligodendrocytes and astrocytes showed TUNEL-positivity in the white matter at day 1. Acute axonal damage was observed in the white matter as indicated by elevated amyloid precursor protein immunoreactivity with no sign of macrophages/microglia change. Major gene changes were observed at day 1 and 4 post-blast pointing toward signs of repair at day 4 and 7. These findings suggest that the BOP levels in the study resulted in mild cellular injury and white matter perturbations (47). In another study by Koliatsos et al. primary (BOP) wave effect of mild BOP (68, 103, and 183 kPag) was compared to secondary and tertiary effects. Using a shock tube generating shock waves, the effects of blast on parenchymatous organs including brain, were evaluated. The main injuries in non-brain organs included hemorrhages in the lung interstitium, hemorrhagic infarcts in liver, spleen, and kidney. Neuropathological changes and behavioral outcomes were evaluated at mild blast intensity showing signs of multifocal axonal injury in the cerebellum, the corticospinal system, and optic tract. These findings were accompanied with prolonged behavioral and motor abnormalities (deficits in social recognition, spatial memory, and in motor coordination). Interestingly, shielding of the torso ameliorated axonal injury and behavioral deficits (50).

In a different study, de Lanerolle et al. used a swine model to assess different scenarios of blast exposure including: simulated free field (blast tube), high-mobility multipurpose wheeled vehicle surrogate, and building 4-walled structure. Of interest, histological changes in the three blast scenarios showed minimal neuronal injury with fiber tract demyelination and intra-cranial hemorrhage. Neuropathological changes involving increased astrocyte activation coupled with proliferation and periventricular axonal injury detected were observed with β-amyloid precursor protein (53).

Long et al. assessed blast-induced physiological, neuropathological, and neurobehavioral changes coupled with Kevlar protective vest encasing the thorax and part of the abdomen using a compression-driven shock tube (at 126- and 147-kPa). Kevlar vest effect reduced air blast mortality and also ameliorated the widespread fiber degeneration in rat brains. BOP was shown to induce abnormal neurologic and neurobehavioral performance along with cardiovascular disruptions involving hemorrhagic hypotension with disruption in cardio-compensatory resilience (reduced peak shed blood volume, etc.) (10). Similarly, Rafaels et al. using a male ferrets with protected thorax and abdomen, evaluated intra-cranial hemorrhage and cardiorespiratory coupling at different ranges of blast exposures. Increasing severity of blast exposure demonstrated increasing apnea immediately after blast accompanied by hemorrhages in proximity to the brain stem (51).

In an interesting study, Garman et al. characterized the neuropathological changes produced by a single blast exposure in rats with body shielding using a helium-driven shock tube (exposure of 35 Psi with left side-head-only exposure) (54). Neuropathological analysis was conducted at various time points (24 h, 72 h, or 2 weeks post-blast). Multifocal axonal degeneration was present in all blast-exposed rats at all-time points coupled with diffused axonal injury in the cerebellar and brainstem white matter tracts. In addition, reactive microglial activation was also identified despite subtle GFAP, ED1, and Iba1 staining. Finally, increased blood–brain barrier (BBB) permeability was seen at 24 h. Findings from this study indicated axonal, dendritic, neuronal, and synaptic degeneration in the initial 2 weeks post exposure with body shielding. Over time, there was also evidence of progression of the axonal degenerative process characterized by increased axonal fragmentation similar to the process of DAI that follows TBI which is suggestive of a therapeutic window in the immediate post-blast period (54).

In conclusion, these different blast studies presented distinguished heterogeneous results (summarized in **Table 1**); and provided different insights into the associated neuropathological changes occurring post-blast exposure. These findings highlight the challenges encountered in modeling experimental blast injury and translating the findings into preclinical brain injury studies to be evaluated and verified clinically (discussed in different sections).

## NEURONAL INJURY MECHANISMS

The exact mechanism by which BOP mediates neuronal injury has not been fully elucidated (47). The neuropathological changes

**Table 1 | Recent major studies on experimental blast injury with different parameters assessed (behavioral, neuropathological, and biomarker changes)**.

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Abdul-Muneer et al. (102) | Rat/primary blast/shock tube/123 kPa | 1/6/6/24/ 48 h/8 days | One or two (24 h between intervals) | None | | Vascular damage, BBB leakage, neuroinflammation MMPs changes, AQP-4, oxidative stress (4HNE-3-NT), and edema; S100B and NSE (serum) |
| Ahmed et al. (136) | Rat/compressed air-driven shock tube/138 kPa | 1, 3, 7, 14, 26, 36, and 42 days | Single or five (24 h between each blast) | Repeated vs. single blast comparison | | Oxidative stress, vascular abnormalities, neuronal, and glial cell death |
| Arun et al. (137) | Mouse/A compressed air-driven shock tube/21 psi | 6 or 24 h | Three blast (1.5 min) | Mice restrained in the prone position with a tautly-drawn net | | Initial decrease and later increase GFAP and total tau proteins (liver, spleen, brain, and plasma) |
| Zou et al. (138) | Rat/5 kg TNT and PETN detonation: 3 m distance (high exposure, 480 kPa) and 2 m distance (low injury, 180 kPa) | 24, 72 h and 2 weeks | Single | None | | Retina injury: blast-dependent increase in VEGF, iNOS, eNOS, nNOS, AQP4, GFAP, elevated inflm cytokines, and chemokines |
| Prima et al. (139) | Rat/composite blast with head acceleration and Primary blast with no acceleration/ 230–380 kPa | 6 h and 1 and 7 days | Single | Primary blast vs. composite' blast animals are body armored | | Thrombin generation (TG) serum integrin α/β, sE-selectin, sICAM-1, and matrix metalloproteinases MMP-2, MMP-8, and MMP-13 |
| Tumer et al. (104) | Rat/compressed air-driven shock tube ∼2 m distance/358 kPa for 10 ms/noise level noise level (100–105 dB) | 6 h | Single | None | | Increased oxidative stress; activation of the sympatho-adrenal medullary axis; (TH), dopamine-β hydroxylase (DβH), neuropeptide Y (NPY) plasma norepinephrine (NE); diffused neuronal injury |
| Genovese et al. (135) | SD-rat/shock tube airblast exposure 74.5 kPa | Every 7 days for 8 weeks | 1/day for 3 days | None | Conditioned fear/PTSD | Neuronal pathology |
| Huber et al. (131) | Mouse/compressed gas-driven shock tube | 24 and 30 days | Single | None | | Elevation of multiple phospho-, cleaved-tau, and (MnSOD or SOD2) levels |
| Sajja et al. (140) | Rat/helium shock tube/117 kPa | 7.5 ms | 24, 48 h | Magic angle spinning 1H MRS analysis | | Elevated *N*-acetyl aspartate, glutamate, and increased GFAP, Bcl-2, Bax, caspase-3, signs excitotoxicity (glutamate/creatine; hippocampal neuronal loss; mitochondrial distress |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Skotak et al. (141) | Rat/helium driven shock tube/(130, 190, 230, 250, and 290 kPa) | 24 h | Single | Biomechanical loading assessed with pressure gauges (thorax, cranial space, and nose) | | Diffuse blood-brain barrier breakdown in brain parenchyma; fatality; lung hemorrhage; no evident neuronal injury |
| Valiyaveettil et al. (34) | Mouse/blast over-pressure/20.6 psi | 4, 24, and 72 h | Three times (1–30 min) | None | | Platelet serotonin decreased at 4 h post blast; increase in the plasma serotonin levels. Increase in blood, plasma, and brain myeloperoxidase enzyme activity. Constriction of blood vessels of the brain |
| Takeuchi et al. (142) | Rats/laser-induced shock waves/0.5–1, 0.5 J/cm$^2$ | 14 days | Single | None | | Decrease in the CB (cingulum bundle) axonal density |
| Turner et al. (143) | Rats/tabletop shock tube/31, 50, 72, and 90 psi | 72 h | Single | Thorax and abdomen protection | | Neural degeneration; increased glial activation (GFAP); extensive intracranial bleeding leading to death |
| Tweedie et al. (144) | Mouse/concussive head trauma (weight drop with metal protection)/ explosion shock wave pressure (7 m distance ∼2.5 psi–17.2 kPa) | 7 days | Single | Comparison between mild TBI and blast injury | Altered cognitive and emotional behaviors (Y maze, novel object recognition passive avoidance/elevated plus maze cognition and anxiety | Altered hippocampal gene expression |
| Cho et al. (134) | Mouse/bast chamber (compression wave attached to a PVC tube)/94, 123, and 181 kPa | 7, 14, 28 days and 3 months | Single | Body is protected with fiberglass screen mesh/hearing loss model | | Decreased spiral ganglion neurons (SGNs) and afferent nerve synapses, loss of outer hair cells (OHCs), tinnitus, hearing loss |
| Yeoh et al. (103) | SD rat, rifle primary shock tube (145, 232, and 323 kPa) | 5 min and 24, 48 h | Single | None | | IgG assessment cardiovascular injury due to primary blast injury is distinct from a typical TBI |
| Cho et al. (134) | Male SD rat, shock tube 129.23 ± 3.01 kPa for 2.5 ms | 4, 24, 48 h and 2 weeks post BOP | Single | None | Short term memory | Immunological assessment (TMF-$\gamma$, MCP-1) neuronal loss |
| Ahlers et al. (145) | Rat/pneumatically driven shock tube at 116.7, 74.5, and 36.6 kPa | 6, 24 h and 1 week | Single or 12 blasts (24 h at 36.6 kPa) | Three body orientation (sideway, facing away vs. frontal) | Morris water maze task 116.7 kPa demonstrated transient alteration or loss of consciousness, 74.5 kPa demonstrated anterograde memory deficits | Subdural hemorrhage and cortical contusions |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Ahmed et al. (146) | Swine/blast overpressure/mild (24–37 psi) or moderate (40–52 psi) | 6, 24, 72 h and 2 weeks | Single | None | | CSF biomarkers (CK-BB NFH, GFAP, S100B, VEGF, Claudin 5, and NSE); neuronal and glial cell damage, altered vascular permeability, and inflammation |
| Balakathiresan et al. (123) | Rat/air-driven shock tube 120 kPa | 3 and 24 h | Short interval (three times – 2 h), long interval (three times – 24 h each) | None | | CSF and serum miRNAs (let-7i) |
| Hines-Beard et al. (147) | Mouse/primary ocular blast injury; pressurized air tank with paintball gun/23.6, 26.4, and 30.4 psi) | 3,7, 14, and 28 days | | Visual acuity deficit detected in 30 psi group eyes via optokinetics | | Retinal damage was present in the eyes from the 30 psi group-corneal edema, corneal abrasions, at optic nerve avulsion |
| Bir et al. (148) | Rat/gas-driven shock tube, 90, 103, 117, 193, and 159 kPa | 24, 48, and 72 h | Single | None | | MRI analysis showed hippocampal reduction in the Cerebral Blood Flow |
| Kovesdi et al. (150) | Rat/shock tube/20.6 psi | 8 and 45 days | Single | Minocycline (50 mg/kg i.p. NSAID); mitigate neurobehavioral changes/body protection | Impaired memory and increased anxiety. (open field, elevated plus maze, and Barnes maze) minocycline showed neuroprotection | Elevated brain and Serum: CRP, MCP-1, NFH, NSE, Tau, GFAP, MBP, S100B, CRP, MCP-1, TLR-9, Claudin 5, and AQP4 |
| Li et al. (95) | Macaca fascicularis/120 kg of TNT/80 and 200 kPa | 3 days and 1 month | Single and double (3 days interval at 80 kPa) | Monkey Cambridge neuropsychological test automated battery motor coordination and working memory | | Increased (AQP-4) white matter degeneration, astrocyte hypertrophy; MRI revealed ultrastructural in Purkinje neurons in the cerebellum and hippocampal pyramidal neurons |
| Rafaels et al. (51) | Ferrets/8′ shock tube/variable peak overpressure (98–818 kPa range) | 1–5 h | Direct recording | Head exposure/thorax and abdomen protection | | Apnea; brain bleeding; fatality |
| Shridharani et al. (153) | Pigs/compressed-gas shock tube/variable (107–740 kPa range) | 1.3–6.9 ms | Direct recording | Heads exposed/lungs and thorax protected (ballistic protective vests) | | Apnea intracranial pressures indicates pressure attenuation by the skull up to a factor of 8.4 |
| Sundaramurthy et al. (96) | Rat/Nebraska's shock tube/100, 150, 200, and 225 kPa) | NA | Single | Variable *Animal Placement Location* along the shock tube (i.e., inside, outside, and near the exit) | | Surface and intracranial pressure elevation linearly with the incident peak overpressures |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Svetlov et al. (92) | Rat, external shock tube (230–380 kPa) | 1 and 7 days post trauma | Single | Primary and composite blast | | Persistent gliosis accumulation of GFAP/CNPase in circulation as well as IL-1/IL-10 fractalkine, orexin A, VEGF-R, NRP-2 increased after primary, and composite; integrin-α/β, ICAM-1, L-selectin, NGF-β increased after primary blast |
| Elder et al. (154) | Rat/air blast shock tube (WRAIR)/74.5 | 4.5 months | Three times (24 h) | Anxiety and fear; locomotor activity, MWM, rotarod, elevated zero arm, predator scent exposure; movement restricted with shielding; contextual and cued fear conditioning | | Elevation in the amygdala of the protein stathmin 1 (proteomic changes) |
| Dalle Lucca et al. (155) | Rat/compressed air-driven shock tube/120 kPa | 0.5, 3, 48, 72, 120, and 168 h | Two | None | | Hemorrhage and edema in the brain cortex; elevated TNF-α, C3/C5b-9, and AQP-4; increased leukocyte infiltration |
| Arun et al. (22) | *In-vitro* 96 well plates-SH-SY5Y human neuroblastoma cells bTBI model/compressed air-driven shock tube (13.68, 18.03, and 21.05 psi) | 24 h | Sing1e or three times (2 min intervals at 21.05 psi) | Plate orientation (horizontal vs. vertical) | | Decreased ATP levels, increased LDH, and ROS; downregulation of CyPA protein |
| Chavko et al. (62) | Rat/air-driven shock tube/36 kPa point-pressure measurements of cerebral ventricles | ∼2.94 ms | Single | Head orientation (head facing blast, right side exposed, head facing away) | | Pressure wave propagation and head orientation dependence |
| Kuehn et al. (156) | Rat/cranium only blast injury apparatus/137.9–515 kPa | 24 h and 7 and 10 days | Single | None | Accelerating rotarod; apnea | H&E staining subarachnoid hemorrhages; brain injury (caspase-3, and β-amyloid precursor protein (β-APP), IgG labeling, and Fluoro-Jade C); cardiac arrest; vasogenic edema |
| Cernak et al. (157) | Mouse/helium modular, multi-chamber shock tube/mild (183 kPa) moderate (213 kPa), severe (295 kPa) | 1–5, 7, 10, 14, 21, and 30 days | Single | Supine vs. prone position) | Motor, cognitive, and behavioral) outcomes, assessed via : rotarod, anxiety learning, and memory via active avoidance procedure | Inflammation elevated in tissue CCL, osteopontin, MRP8, ED1, and GFAP at different time points |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Koliatsos et al. (50) | Mouse/helium multi chamber shock tube/high (25–45 psi), low (2.1 psi) | 3, 5 days (biochem testing) and 7–14 (behavioral) | Single | Either Head or Torso Covered | Rotarod, Y maze open field social and spatial recognition memory and motor deficits | Axonal swellings (injury), APP, but degeneration staining 7–14 days after exposure |
| Kovesdi et al. (149) | Rat/compression-driven shock tube/20.6 psi | 15, 44, 66 days (behavioral) and 66 days (biochemical) | Single | Enriched environment (EEN) contribution | Memory problems, increased anxiety, and depression; improved spatial memory in EEN | Axonal degeneration; elevation in IL-6, IFN$\gamma$ VEGF, and tau protein levels; hippocampal GFAP and DCX |
| de Lanerolle et al. (53) | Swine/explosive blast levels in three scenarios: simulated free field (35 psi), high-mobility, vehicle (65 psi), and building setup (63 psi) | 72 h and 2 weeks | Single | Blast varied settings: blast tube, high mobility; multipurpose wheeled vehicle, and four-sided structure | | Little neuronal injury, fiber tract demyelination, or intracranial hemorrhage observed; increased astrocyte activation; bulbs positive for BAPP |
| Pun et al. (47) | Rat/120 kg of 2,4,6-trinitrotoluene (TNT)/48.9 kPa (7.1 psi) or 77.3 kPa (11.3 psi) at 24 or 40 m | 1, 4, and 7 days | Single | Concrete block was placed between the animals and the explosive source at a distance of 1.5 m from the animals | | Cortical neurons were "darkened" and shrunken with narrowed vasculature (day 1, not at 4–7 days); no Iba-1 change; TUNEL-positive cells in the white matter of the brain (day 1); an increase in APP in the white (acute axonal damage); genomics analysis showed signs of repair at day 4 and 7 post-blast |
| Reneer et al. (151) | Rat/multi-mode shock tube, the McMillan blast device (compressed air/ helium driven tube mode, or oxyhydrogen – RDX explosives mode/ 100, 150, and 200 kPa) | 3 min post blast | Single | Two overpressure modes (air vs. explosives), Kevlar vest body protection | | Rats exposed to compressed air-driven blasts had more pronounced vascular damage than those exposed to oxyhydrogen-driven blasts of the same peak overpressure |
| Risling et al. (152) | Rat/blast tube with pressure wave/130 and 260 kPa | 2 h, 1, 3, 5 days, and 3 weeks | | Three groups comparison – (1) fixed no head acceleration forces; (2) controlled penetration of a 2-mm thick needle; and (3) high-speed sagittal rotation angular acceleration | | Diffuse axonal injury (DAI) in penetration and rotation models; genomics changes in the expression in a large number of gene families cell death, inflammation, and neurotransmitters in the hippocampus (acceleration and penetration injuries); downregulation of genes involved in neurogenesis and synaptic transmission |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Rubovitch et al. (93) | Mouse/open field explosives ∼500 g TNT detonation (1 m elevated)/5.5 and 2.5 psi | 30 days | | Mice in plastic net 4 or 7 m; MRI and DTI analysis | Significant decrease in cognitive and behavioral (Y maze; hippocampal function and spatial memory; novel object recognition task | Increased BBB permeability; 1 month post-blast; increase in fractional anisotropy (FA); no visible organ damage; and elevated MnSOD2 |
| Connell et al. (158) | Female Guinea pig/2.5-cm strips of shock tubing/(23, 41, and 64 kPa | 30 min | | *Ex vivo* model of spinal cord white; shock tubing (explosive lining of 0.1 grain/foot composed of tetranitramine and aluminum) | | Nervous tissue compression, and increased axonal permeability |
| Garman et al. (54) | Rat/helium-driven shock tube/35 psi (4 ms) | 24, 72 h and 2 week | | Head exposure with body armor | | Increased blood–brain barrier permeability; elevated APP, GFAP, Iba1, ED1, and rat IgG. |
| Gyorgy et al. (122) | Pig/compression-driven shock tube/∼20, 20–40, and ∼40 psi | 6, 24, 72 h and 2 week | | None | | Serum elevation of S100B, MBP, and NF-H, but not NSE |
| Readnower et al. (44) | Rat/air-driven shock tube/120 kPa | 3, 24 h and 5 days | Single | None | BBB breakdown: At 3 and 24 h post exposure; increase in IgG staining in the cortex; brain oxidative stress: (4-HNE) and (3-NT) were significantly increased at 3 h post exposure and returned to control levels at 24 h post exposure; and microglia activation: at 5 days | |
| Cheng et al. (159) | Rat/electric detonator with the explosive equivalent of 400 mg TNT (100, −400 kPa) (distance of 5, 7.5, and 10 cm) | 1, 2, 3, 5, and 7 days | Single | Head orientation(frontal, parietal, and occipital head exposure) | 87% Rats developed apnea, limb seizure, poor appetite, and limpness | Diffuse subarachnoid hemorrhage and edema; cortical capillary damage; and tissue water and NSE |
| Cai et al. (160) | Rat/5 g compressed dynamite stick (75 cm from chest) | 3, 6, 12 h and 1, 2, 3, 7 days | Single | Blast vs. burn-blast | | Serum neutrophil elastase (NE); water lung content |
| Long et al. (10) | Rat/compression-driven shock tube/126 and 147 kPa | 24 h | Single | Kevlar – protective vest (thorax – abdomen) | MWM testing beam walking and spatial navigation(disrupted neurologic neurobehavioral performance) | Heart rate, MAP, brain axonopathy, and widespread fiber degeneration |
| Säljö et al. (42) | Rat shock tube/10, 30, and 60 kPa (4 ms) | 0.5, 3, 6, and 10 h and 1, 2, 3, 5, and 7 days | Single | Morris water maze: impaired cognitive function: 48 h post injury | | Dose-dependent rise in intracranial pressure ICP in rats exposed to blast and an increasing time delay in elevation with decreasing intensity of exposure. the ICP returned to control levels after 7 days |

*(Continued)*

**Table 1 | Continued**

| Reference | Animal model/ device used-BOP intensity | Time point assessment post injury | Repeats of blast and time between exposure | Additional variables studied | Behavioral assessment (if available) | Neuro, systemic, and other organ-specific pathology/ biomarkers parameters |
|---|---|---|---|---|---|---|
| Säljö et al. (41) | Pig – Howitzer (9 and 30 kPa); Bazooka (42 kPa); automatic rifle (23 kPa) Rat/shock tube (8.7 kPa) | 3 and 7 days | Three (exposure in air; 15 min intervals) two (exposure under water; 6–7 min) | Comparison of pressure time of different blast overpressure in: air, underwater, and localized blast | | In pig study: small parenchymal and subarachnoid hemorrhages, predominately in the occipital lobe, cerebellum, and medulla oblongata; no observation in rat study |
| Cernak et al. (45) | Rat/large-scale BT-I shock tube/3389 kPa and small-scale BT-III shock tube (440 kPa) | 3, 24 h and 5 days | Single | Protected head vs. whole body exposure | Deficits in active avoidance task | Swellings of neurons, glial reaction, and myelin debris in the hippocampus, laminal body and vacuoles formation (electron microscope) |

*B APP, B-amyloid precursor protein; GFAP, glial fibrillary acidic protein; AQP-4, aquaporin-4; MnSOD or SOD2, manganese superoxide-dismutase I; UCH-L1, ubiquitin C-terminal hydrolase; vWF, von Willebrand factor; NA, not applicable; NSE, neuronspecific enolase; Mwm, Morris water maze; CK-BB, brain-specific creatine kinase; MAP, mean arterial pressure; H&E, hematoxylin and eosin; 4-HNE, 4-hydroxynonenal; 3-NT, 3-nitrotyrosine; TNT, 2,4,6-trinitrotoluene; RDX, oxyhydrogen; ms, milliseconds; MMP8, matrix metalloproteinase 8; BOP, blast over pressure; NF-H, neurofilament-heavy chain.*

evoked by BOP are different than those described following acute models of brain injury (i.e., acceleration–deceleration injury or direct impact) (10, 55–58) highlighting at the complex pathways involved. Elegant work with experimental data by Cernak et al. has shown that primary closed non-impact blast injury-induced neurotrauma involves the interaction of cerebral, local, and systemic responses (31, 32, 45, 48). These experimental data seem to highlight the fact that blood vessels vasculature (venous as well as arterial) may be acting as a conduit for blast energy transfer to the brain contributing to blast pressure-induced fiber degeneration.

In non-blast brain injury, the primary injury occurs as a consequence of mechanical force due to direct contusion of the brain against skull's rough interior or due to shearing and stretching forces against the brain tissue (31, 59). This may also involve vascular injury including subdural hematoma from ruptured blood, brain edema from elevated permeability of cerebral vasculature along with reduced blood flow due to intra-cranial pressure or infarction (59). Taken together, these complications represent the secondary and tertiary phases of blast injury.

Cernak et al. assessed the contribution of body-central nervous system (CNS) cross talk involved in blast-induced trauma related to the activation of autonomous nervous system and the neuroendocrine–immune system which contributes significantly to the mechanism of blast injury. Inflammation has been proposed to play an important role in the pathogenesis of long-term neurological deficits due to blast (31). Experiments using rigid body- or head-protection in animals subjected to blast showed that head protection failed to prevent inflammation in the brain while body protection was able to alleviate blast-induced brain functional impairments highlighting the role of body-CNS interaction (31).

Cernak et al. studies have demonstrated that blast exposure (mild-to-moderate) induces the activation of autonomous nervous system in rabbit exposed to BOP. Distinct pathological components in the brain including impaired energy metabolism, and increase in the sodium–potassium ATPase measured in the brainstem and erythrocyte membranes were coupled with edema formation (48, 60). In addition, to link systemic alteration and cerebral inflammation to long-term neurological deficits caused by blast, migration, and accumulation of polymorphonuclear leukocytes as key inflammatory markers of host response were assessed after helium-driven shock tube delivering mild blast injury (103 kPa). *In vivo* real time imaging of myeloperoxidase (MPO) inflammatory enzyme activity of activated phagocytes was conducted on three groups of rats: (1) whole-body blast; (2) blast with "body armor," (chest and abdomen) with the head exposed; or (3) blast with "helmet" as head protection (neck and skull) while the rest of the body exposed. One day post-blast exposure, MPO activity was observed in the gastrointestinal tract and the diaphragmal mediastinal parts of the lungs (61).

In the brain, this activity was observed at 7, 14, and 30 days post-blast injury. Of interest, MPO increase in the brain was independent of head protection at 14 and 30 days post-injury suggesting chronic inflammation and highlighting the role of systemic origin of the inflammatory activation mediating brain injury which highly reflects on the role of the vagal afferent neurons mediating gut–brain communication. Taken together, the results of this study clearly demonstrate the importance of the indirect, i.e., blast–body interaction as well as the decisive role of autonomous nervous–neuroendocrine–immune systems interaction in the pathogenesis of blast-induced brain trauma (31).

Similarly, Chavko et al. assessed the theory of the indirect effect of kinetic energy transfer via the blood vessels and the surrounding cerebrospinal fluid (CSF) to the CNS (62). In their work, they evaluated the contribution of direct versus indirect transfer and its correlation to the head orientation and the surface area exposed.

Brain biomechanical responses involving pressure inside the brains were assessed in rats exposed to low blast exposure (35 kPa) and positioned in three different orientations with respect to primary blast wave. These positions included: frontal exposure (i.e., head facing blast) right side exposed and head positioned away from blast. Frontal exposures showed higher traces of pressure amplitude and longer duration, suggestive of dynamic pressure transfer (62). On the other hand, the pressure wave inside the brain in the head facing away was similar to hydrodynamic pressure within the brain. It has become more evident that the primary pressure wave can induce functional, biochemical, and morphological alterations in different ways than those observed in other types of traumatic injuries (penetrating head injury).

## MILD TBI AND NEUROPSYCHIATRIC IMPAIRMENTS IN BLAST INJURY AND PTSD COMORBIDITY

Another significant aspect of blast injury is psychological health which is highly affected. Many injured troops returning from war zones are afflicted with blast-induced BI experiencing post concussive symptoms (PCS), characterized by memory and cognitive disruption, irritability, anxiety, and fatigue (63). Among these with mTBI, PCS can persist long after exposure leading to major functional impairments (64). Unlike casualties suffered from moderate to severe TBI patients diagnosed with mTBI present with no apparent structural injury and are conscious with typical symptoms including headache, confusion, dizziness, memory impairment, and behavioral changes.

The nomenclature of mTBI has been a challenge for both civilian and military settings as described by Rosenfeld et al. (65). mTBI, according to the DoD, involves head trauma with loss of consciousness for <30 min or exhibiting post-traumatic amnesia for <24 h (66). Patients with mTBI have a Glasgow coma score of 13–15 usually experiencing poor unspecific diagnostic symptoms involving headaches, cognitive dysfunction, etc. independent whether mTBI is blast related or not. It is of high interest to deliver accurate diagnosis for such condition due to the overlapping symptoms mistaken with neuropsychiatric disorders. This in contrary to the moderate and severe blast-related TBI which have 9–12 and 3–8 Glasgow coma score respectively and require special treatment as they exhibit intra-cranial hemorrhage and brain edema (2, 67, 68). Patients with blast-related severe TBI are characterized with delayed vasospasm, and pseudoaneurysm formation requiring early intervention (2, 67). Severe blast-related TBI cases are usually due to the primary and secondary (penetrating injury) phases of blast and would require strict clinical guidelines that are similar to those in non-blast-related severe TBI cases (65).

Mild traumatic brain injury is the most frequent form of brain trauma among deployed military populations (69). It has been shown that repeated exposure to multiple low levels of blast injury account for the majority of mTBIs cases. These victims remain conscious and often are redeployed without proper diagnosis and treatment while they undergo severe mental stress (70, 71). The heterogeneous presentation of BOB injuries among mTBI patients depends on several factors (similar to what is observed in experimental blast injury studies) including: device composition, environment (e.g., presence of intervening protective barriers), distance from blast, and the use of protective shields, etc. (11, 72).

Primary blast component of blast injury is among the main contributors in developing neuropsychiatric impairments associated with the primary phase profile (30, 73). There had been an urgent quest to for future research examining the impact of blast concussion (particularly recurrent concussion) on neuropsychological performance. Neuropsychological evaluation of cognitive status post-blast exposure can be challenging for a variety of reasons. In particular, clinicians may have difficulty assessing: true concussion severity due to limited knowledge of the blast events which may be reflective of self-report months or years post the event(s) occurrence. In addition, the lack of several features of the blast environment may complicate the accuracy of the "blast self-report" involving distance from the blast, concussion severity which these are often unavailable from primary records (74). Thus, the lack of reliable information pertaining to injury characteristics makes it challenging to determine the course of cognitive recovery and rehabilitation. Usually, concussion severity is usually determined based on current PCS on screening instruments which are not necessarily specific to concussion and can be shared with depression or PTSD or even these PCS may be reflective of PTSD itself as elegantly discussed by Nelson et al. (74). Of interest, Hoge et al. reported that more than 40% of soldiers who experienced symptoms associated with mTBI (loss of consciousness) met the criteria for PTSD (1). This same study suggested that increased rates of health problems reported by soldiers exposed to mTBI are mediated mainly via neuropsychiatric disorders such as PTSD or depression, rather than mTBI (1).

Post-traumatic stress disorder, a psychiatric condition that arises after exposure to a life threatening experience such as conditions experienced in combat war zone with or without blast exposure as a form of mTBI (75). This, by itself, poses a challenge in the clinical diagnosis in veterans who are exposed to mTBI since the symptoms may overlap between these conditions exacerbated by other comorbid conditions such as drug abuse or other neuropsychiatric complications (75, 76). A Rand Corporation study indicated that ∼20% of returning service personnel (∼300,000) have had a TBI and that there was substantial overlap of TBI with the occurrence of PTSD (77).

Psychological stress resulting from exposure to blast wave leads to an altered psychological health status which contribute significantly to the development of PTSD (52, 70). However, a major recurring question arises-due to the similarity of blast injury clinical symptoms and those of PTSD, is how do we clinically differentiate between these two conditions and other neuropsychiatric conditions?

Post-traumatic stress disorder is deemed an effect of psychological and emotional determinants/trauma (i.e., event associated with threat of harm or loss of life to which the individual responds with extreme fear or horror), while mild bTBI is a result of destructive biomechanical forces acting on the brain (78). There is substantial overlap in symptom profile associated with these two conditions (1). For instance, impaired concentration, increased irritability, insomnia, and lack of interest are among the symptoms shared in the diagnosis for mTBI and PTSD (79). Additionally, blast TBI is a well-documented risk factor for the development of PTSD (80–82). The association between the two conditions is further supported by structural and functional neuroimaging studies

showing similar abnormalities in patients with blast-related mTBI as well as in those with PTSD (83–86).Such overlap and link determines and contributes to several ambiguities emphasizing the urgent need for finding reliable objective test to make an accurate diagnosis and to improve the understanding of the nature of the interaction and pathophysiology of PTSD and mild bTBI.

Clinical evaluation of a blast-exposed personnel can be challenging as symptoms may range from neurologic problems, psychiatric, or emotional difficulties which may be attributed to blast or due to other psychiatric disorder where in several instances the occurrence of TBI and PTSD may be suggested (81, 87). For neurological assessment in TBI, similar criterion-based methodology to that in PTSD has been used rendering a specific diagnosis to either condition or even to those with both conditions (PTSD or TBI-exposed) uncertain (87–89). Thus, in many cases, clinical diagnosis may result in high rate of inaccurate PTSD diagnosis in persons exposed to TBI (87).

Based on the above, it is of high interest that an accurate detailed knowledge of blast injury biophysics and injury threshold may assist clinicians in better diagnosis (87). This includes expanded neuropsychological studies of blast injury (both experimental and clinical) to identify accurate, specific and sensitive anatomic, pathophysiologic, and behavioral responses to blast injury as discussed by Bass et al. (87). This is complicated by the complex nature of blast injury involving several combinations of primary or other phases of blast injury (secondary, tertiary, and/or quaternary blast).

## ANIMAL MODELS OF BLAST INJURY

Over the last several decades, a number of experimental animal models have been implemented to study the mechanisms of blast wave impact which included rats, mice, ferrets, rabbits, and larger animals involving sheep and swine (33, 90–97). These experimental models exhibited heterogeneous outcomes and even contradictory findings which have been attributed to several factors. A summary of the recent and major blast injury studies (2001, 2009–2013) is summarized in **Table 1**. In addition, there is a lack in the reproducibility of blast injury models and a need to develop blast injury generators that precisely control blast injury parameters similar to other well-defined acute brain injury models such as (controlled cortical impact (CCI) and the fluid percussion (FP) which have been well characterized with predictable neurological, histological, physiological, and behavioral outcomes. Thus, the need of establishing well characterized reproducible models (animal and blast framework) is vital to identify relevant pathogenic pathways involved that can assist in the development of effective diagnostic, prognostic blast specific-biomarkers (panel of biomarkers) (98). Several blast injury instrumentations are available which include: compressed gas-driven shock tubes which are driven by air, helium, or nitrogen gas which may result in unrealistic duration of the overpressure wave leading to an inappropriate scaling between species (humans and animal models; **Table 1**) (99).

## CHALLENGES IN ANIMAL MODELS OF BLAST INJURY

There are limited available basic and translational studies relevant to the mechanisms of primary blast-induced brain injury. A better understanding of injury mechanisms is required for the development of protection and treatment options and biomarker identification for prognosis.

Several animal models have been proposed at translating intracranial biophysics and pathophysiology experienced in human blast exposure (87). These models have a number of limitations including: neuronal tissue biomechanical properties, anatomical differences as well as physiological differences (87). In addition, other factors that are challenging for proper scaling between experimental and human blast injury are associated with neuroanatomy and physiology involving: size of different brain structures, neural mass (brain size, head, body, position, and architecture), as well as body fluid composition (thickness, volume, and components) (87). Other key factors that need to be considered are the potential for exposure scaling, consistency in experimental protocols, frequency of exposure, and overpressure levels, which should be mimicking real life exposure or at least translate equally to human exposure (**Figure 1**). Other external factors include: distance from the blast, the use of protective shields and the presence or absence of noise stressors, etc. (12) (**Figure 1**). In real life situation, soldiers are often deployed several times and exposed to numerous psychological stressors such as blast noise with or without blast injury (87). Such conditions can induce adverse physiological changes leading to post-traumatic symptoms without sustaining any prior physical injury (discussed previously). Taken together, these challenging factors contribute to the difficulty of truly modeling blast injury in animals resulting in an in-appropriate neuropathological and neurobehavioral assessment.

## BLOOD–BRAIN BARRIER AND SECONDARY INJURY IN BLAST OVERPRESSURE

Traumatic brain injury leads to progressive pathophysiological changes resulting in a reduction in cerebral blood flow and a decrease in tissue oxygen levels leading to ischemia, BBB disruption with brain edema (100). Death of resident cells of the CNS has traditionally been accepted to take place in two phases: an early necrotic and an on-going long-term apoptotic phase. Secondary brain injury develops in minutes to months following the original insult, progressively contributing to the worsened neurological impairment. This complex phenomenon is defined by the activation of various neurochemical cascades and the systemic physiological responses which manifest following the traumatic event (101).

At the cellular level, the biphasic nature of secondary injury is mediated by numerous disturbed pathways which include: (a) excitotoxicity caused by an excess of the neurotransmitter glutamate; (b) free radical generation by mitochondrial dysfunction, causing damage to proteins and phospholipid membranes of neurons and glia; and (c) the neuroinflammatory response which takes place due to both CNS and systemic immunoactivation. Thus, diffuse brain injury mediated immune responses, BBB alterations, and neuroinflammation seem to play an important role in the pathology of BOP. The increase in BBB permeability was shown to recover by the third day after the blast exposure (44, 102). Following blast injury, loosening of the vasculature and perivascular unit is mediated by the activation of matrix metalloproteinases and

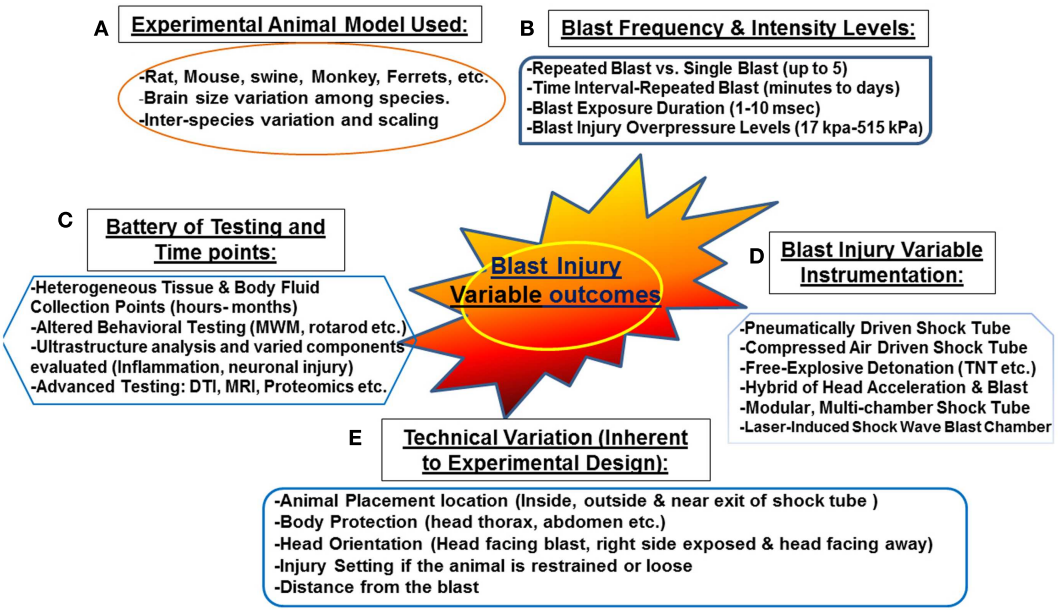


**FIGURE 1 | Challenges associated with "experimental blast injury" modeling real life blast exposure**. Several factors contribute to the heterogeneous behavioral, neuropathological, and systemic profile observed in the several experimental blast injury models. Even with models using the same injury parameters (animal model, blast shock tube, and intensity levels, etc.); reproducing the same results is rather challenging (refer to **Table 1**). These challenging variables are summarized in the following: **(A)** various animal models and interspecies variation, **(B)** blast injury frequency and intensity levels ranging from single blast up to five blast with some overpressure intensities reaching 515 kPa **(C)** the heterogeneous selection of biochemical/behavioral testing conducted and the several time points selected (hours to few months) **(D)** the non-standardized blast and not well characterized blast injury instrumentation **(E)** technical variation inherent to experimental design related to animal setting, body armor, head protection, and the distance from the blast. These factors contribute to the variable outcome observed in published work in blast injury field.

water channel aquaporin-4, promoting edema, enhanced leakiness of the BBB, and progression of neuroinflammation and neuronal degeneration (102). Although many studies demonstrate a similar pathophysiologic progression as the conventional TBI, a recent study reported that cerebrovascular injury due to primary blast is distinct from it; suggesting that BBB disruption in blast injury was an acute one, not resulting from a delayed inflammation as it is in the conventional ones (103).

Recent work from our laboratory has shown that blast injury leads to oxidative stress and autonomic dysfunction (104). Generation of free radicals and hypoxia leads to the failure of the $Na^+$, $K^+$-ATPase, a membrane-bound enzyme required for cellular transport. Dysfunction of this pump is a common feature in CNS pathologies related to ischemic conditions and TBI. The activity of $Na^+$, $K^+$-ATPase pump is very sensitive to free radical reactions and lipid peroxidation. Reductions in this activity can indicate membrane damage indirectly. Thus, $Na^+$, $K^+$-ATPase is clearly down regulated under low $O_2$ conditions which in turn triggers brain edema, enhances the loosening of tight junctions and causes BBB breakdown. MPO activity, an index for neutrophil infiltration, also increases as an evidence of inflammation (105). In summary, failure of pumps, cerebral edema, BBB permeability, neuroinflammation, and oxidative damage are among the major mechanisms that play important roles in the development of secondary brain injury following TBI.

## TRAUMATIC BRAIN INJURY AND AUTONOMIC DYSFUNCTION

One deleterious consequence of brain injury is autonomic nervous system dysregulation and/or dysautonomia. Autonomic nervous system dysfunction has been documented after TBI but is not well understood. Ninety percent of TBI patients demonstrate signs of autonomic dysfunction during the first week after injury, with about one third of the patients developing longer lasting autonomic dysfunction. Autonomic dysregulation is characterized by distinct changes in cardiovascular hyperactivity, sleep function, and specific biomarkers of neural damage. System dysregulation might lead to a range of comorbidities such as hypertension, endothelial dysfunction, and end-organ perfusion abnormalities. Specifically, TBI disruption of autonomic function most often results in sustained sympatho-activation. This sympathetic hyperactivity after TBI remains poorly understood, although sympathetic hyperactivity likely contributes to the high morbidity and mortality associated with TBI. Sympathetic hyperactivity contributes to systemic stress, including neuroinflammation and oxidative stress in the autonomic nervous system. Eventually these disturbances lead to cardiovascular dysfunction (31, 32, 106) and sleep complications (107). Systemic stress is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis (108) and the hypothalamic sympatho-adrenal medullary axis (109). It is known that TBI activates the HPA, however little is known regarding the TBI-induced activation of the sympatho-adrenal

medullary axis, and there are limited therapeutic options to treat this sympatho-activation.

We recently demonstrated selective biochemical markers of autonomic function and oxidative stress in male Sprague Dawley rats subjected to head-directed overpressure insult (104). There were increased levels of tyrosine hydroxylase (TH), dopamine-β hydroxylase (DβH), Neuropeptide Y (NPY) along with plasma norepinephrine (NE). In addition, blast-induced injury significantly elevated TH in the nucleus tractus solitarius (NTS) of the brain stem while AT1 receptor expression and NADPH oxidase activity, a marker of oxidative stress, was elevated in the hypothalamus suggesting that single BOP exposure results in increased sympatho-excitation. The mechanism may involve the elevated AT1 receptor expression and NADPH oxidase levels in the hypothalamus. Taken together, such effects may be important factors contributing to pathology of brain injury and autonomic dysfunction associated with the clinical profile of patients following BOP exposure (104).

## BLAST BRAIN INJURY AND OXIDATIVE STRESS

The primary effects of BOP have been generally attributed to its external physical impact on the body, causing internal mechanical damage. The pathophysiological effects on hollow organs have been extensively studied, but little attention has been given to the biochemical manifestations and molecular mechanism(s) of injury occurring in the brain after BOP exposure. Due to the biochemical nature of BOP compared to physical nature of TBI (impact or penetrating injury), subtle molecular changes such as free radical-mediated oxidative stress occur and contribute to the manifestation of BOP-induced brain injury (40, 44, 110). Previous studies have demonstrated that reactive oxygen species such as the superoxide radicals and nitric oxide can form peroxynitrite, a powerful oxidant that impairs cerebral vascular function following blast-induced brain injury (46, 111). Cernak et al. reported that bilateral vagotomy successfully mitigated bradycardia, hypotension, and apnea caused by blast; prevented extreme metabolic alterations and brain edema; but failed to eliminate oxidative stress in the brain due to blast (48). More recently, it was reported that the induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mTBI induced by primary blast (102). Brain-specific oxidatively modified protein markers that are indicative of biochemical/proteomic and functional changes occurring post-BOP need to be considered. Insufficient published data are available to describe the long-term effects of TBI on central noradrenergic systems, particularly on neuroplastic adaptations within numerous targets of central noradrenergic projections. In addition, understanding the etiology of these changes may shed new light on the molecular mechanism(s) of injury, potentially offering new strategies for treatment.

## BLAST INJURY BIOMARKERS IDENTIFICATION AND LIMITATIONS

The widespread recognition of the brain vulnerability to blast exposure and inadequate approaches to diagnose blast-related TBI led to design an mTBI Diagnostics Workshop (66) and the foundation of the Demographics and Clinical Assessment Working Group of the International and Interagency Initiative (112) to assess the current diagnostics technologies that can be used to detect brain injury following mTBI and BOP. One of the major recommendations was the use of biomarkers to supplement functional and imaging-based assessments for significant improvements in the diagnosis and characterization of the effects of blast exposure on brain and for distinguishing bTBI from other neuropsychiatric disorders including PTSD.

Current available imaging modalities, such as computed tomography (CT) and magnetic resonance imaging (MRI), primarily detect major structural changes in the brain (113); however, their utility has not been fully optimized following blast-related mTBI. More advanced neuroimaging techniques such as DTI, while have shown abnormalities post-blast-related TBI (114), have not been able to show consistent relationship to mild bTBI diagnosis (115). Additionally, there is no consensus on the ideal scan method or timing. Therefore, multiple studies have been conducted to identify ideal sensitive, inexpensive, non-invasive biochemical markers that can offer diagnostic and prognostic information, and reflect bTBI pathogenic mechanisms and pathology (116, 117).

To date, several biomarkers such as GFAP (118), UCH-L1 (119), and S-100ß(120) have been identified as potential excellent "candidates" of blast TBI. However, a limited number of studies did specifically evaluate biochemical brain damage markers in the setting of blast-induced brain injury (43, 121). In one study by Svetlov et al. they assessed temporal pattern of serum putative biomarkers that have been characterized in acute TBI including GFAP, NSE, and UCH-L1 in brain tissue, CSF, and blood. Serum biomarkers levels distinctively increased 24 h post-blast, followed by a decline thereafter, indicating a potential use to assess blast-induced brain damage acutely after injury (33). Supporting these observations, Gyorgy and colleagues, using reverse phase protein microarray (RPPM) technology to determine serum protein levels, showed a rise in S-100B, MBP, NF-H, and NSE protein levels in serum after injury in a large-animal model of bTBI. Remarkably, serum NF-H was reported to increase in an overpressure dose-dependent manner reflecting the extent of the damage caused by bTBI (122).

More recently, Balakathiresan et al. proposed microRNAs as novel serum diagnostic biomarkers of bTBI. They investigated microRNA signatures in CSF and serum of rats exposed to BOP injury. Specifically, microRNA let-7i was elevated in both CSF and serum post-blast wave exposure and was considered as an ideal candidate biomarker of brain injury (123). Importantly, microRNAs can be considered the third generation molecular signature after proteomics and genomics studies (123). Elevated concentrations of serum vascular endothelial growth factor, associated with neuroinflammation and vascular pathology in blast-related TBI have also been reported (124).

Studies investigating biomarkers of mTBI in humans continue to be limited as illustrated in one study by Ingebrigtsen and Romner (125). In their research paper, MEDLINE database was surveyed for biochemical serum markers specific to mild head injuries. Three serum markers including creatine kinase isoenzyme BB (CKBB), NSE, and S-100B were evaluated. Of these markers, S-100B protein was proposed as the most promising marker for mTBI while the other two lacked specificity, sensitivity, or injury correlation (125). In an another study by Blennow

et al. military personnel exposed to explosions or repeated firing of heavy weapons did not show any evidence of brain damage as assessed by CSF biomarkers. (126). Conversely, the New Zealand Breacher Study demonstrated a degree of brain perturbation as assessed by serum biomarker levels, neurocognitive performance, and self-reported symptoms in members of the New Zealand Defense Force exposed to repeated low-level blast (127). Taken the controversial results of these different studies, these findings, in fact, stimulate the need for further research to evaluate the usefulness of biochemical markers after repeated exposure of different blast levels.

Interestingly, recent experimental and human studies are suggesting a link between blast exposure and chronic traumatic encephalopathy (CTE), a tau protein-linked neurodegenerative disease (128–131). To date, no biofluid marker has been shown to assist with diagnosis of CTE. However, future studies to identify biomarkers tracking chronic processes and on-going degeneration and able to predict the development of neurodegenerative diseases of bTBI are of a critical need.

## FUTURE RECOMMENDATIONS

For long, TBI has been considered one of the "signature injuries" of current conflicts in Iraq and Afghanistan which attracted concern from the DoD, Department of Veteran Affairs, and National Institutes of Health, encouraging combined efforts to understand brain injury pathophysiology and identify therapeutics and assess different approaches for rehabilitation platforms as well as deciphering novel blast specific biomarkers (7, 11). Better understanding of the biophysics of blast shock injury and its body propagation to the neural tissue may enhance the development body armor protection. Given the complexity of blast TBI pathobiology, the development of an objective, specific, and quantifiable panel of biomarkers is highly needed for the purpose of providing better monitoring of the real time injury mechanism and progression post-blast exposure (121, 122, 132, 133). An important consideration is that a panel combining different biomarkers be assembled that can establish the nature and severity of the head injury and reflect the contributing pathogenic mechanism(s) of the acute phase as well as the neurodegeneration and recovery (rehabilitative stages). Additionally, the integration of such bTBI diagnostic markers into routine clinical care will require a thorough validation and extensive standardization protocols coupled with well-defined recommendations for immunoassay and different measurement technologies.

A non-trivial and urgent issue in biomarker-panel design will be determining an appropriate instrument platform that is suited to measure these biomarker changes. At present, biomarkers are analyzed in clinical laboratories using closed, high throughput immunoassay analyzers allowing for high performance in terms of accuracy and precision which are suitable for major hospitals. Future recommendation is to focus research on the development of a miniaturized point-of-care (POC) system, which can be transported in the "field" (military and civilian) providing accurate measurements at a reasonable cost with short turnaround time (116).

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# Repetitive traumatic brain injury and development of chronic traumatic encephalopathy: a potential role for biomarkers in diagnosis, prognosis, and treatment?

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The diagnosis of chronic traumatic encephalopathy (CTE) upon autopsy in a growing number of athletes and soldiers alike has resulted in increased awareness, by both the scientific/medical and lay communities, of the potential for lasting effects of repetitive traumatic brain injury. While the scientific community has come to better understand the clinical presentation and underlying pathophysiology of CTE, the diagnosis of CTE remains autopsy-based, which prevents adequate monitoring and tracking of the disease. The lack of established biomarkers or imaging modalities for diagnostic and prognostic purposes also prevents the development and implementation of therapeutic protocols. In this work the clinical history and pathologic findings associated with CTE are reviewed, as well as imaging modalities that have demonstrated some promise for future use in the diagnosis and/or tracking of CTE or repetitive brain injury. Biomarkers under investigation are also discussed with particular attention to the timing of release and potential utility in situations of repetitive traumatic brain injury. Further investigation into imaging modalities and biomarker elucidation for the diagnosis of CTE is clearly both needed and warranted.

**Keywords: chronic traumatic encephalopathy, CTE, TBI, biomarkers, imaging**

## INTRODUCTION

Increasing awareness by both medical professionals and the lay community concerning the potential long-term effects of repetitive traumatic brain injury, such as chronic traumatic encephalopathy (CTE) and cognitive impairment (Guskiewicz et al., 2005; Gavett et al., 2010; Daneshvar et al., 2011), has led to the identification of a need for improved diagnostic and prognostic tests. Investigators have focused primarily on the use of various imaging modalities and development of blood- or CSF-based biomarkers. In the following sections we attempt to briefly review findings associated with CTE diagnosis, proposed disease pathophysiology, and how these findings may potentially relate to imaging and/or biomarker discovery.

### EPIDEMIOLOGY AND CLINICAL PRESENTATION

Exposure to repetitive mild traumatic brain injury (mTBI) is a common occurrence in athletes on the playing field and soldiers on the battlefield. In fact, playing American football at higher levels results in documented exposure of up to 1400 impacts per season for select positions such as linemen with some players involved in both offense and defense sustaining nearly 2000 impacts (Stern et al., 2011). Similarly, mTBI has been identified as the most common combat-related injury in soldiers returning from present-day conflicts in Iraq and Afghanistan and consequently, has been described as the "signature injury of war" (Shenton et al., 2012). The diagnosis of mTBI remains particularly challenging due to the usual lack of abnormal findings on conventional CT and MR imaging (Shenton et al., 2012). Lack of a diagnostic test for mTBI is problematic considering the potential for both enduring cerebral effects (cognitive, neurophysiological, and clinical) and for identification of those at risk for development of CTE later in life.

Chronic traumatic encephalopathy represents a progressive neurodegenerative disease currently diagnosed only upon autopsy and subsequent neuropathological examination (Saulle and Greenwald, 2012). Despite the lack of specific diagnostic criteria required for pre-mortem clinical diagnosis, patients afflicted with CTE diagnosed post-mortem are often described as suffering behavioral, cognitive, and emotional changes or impairments prior to death (Gavett et al., 2011b; Omalu et al., 2011b; Saulle and Greenwald, 2012). Notably, symptom development occurs following a prolonged latency in most cases, although exceptions do exist (Gavett et al., 2011a; Omalu et al., 2011b). The tendency for a latent period creates a clear distinction between initial symptoms associated with traumatic brain injury (TBI) and the persistent, long-term degeneration, much like other neurodegenerative diseases such as Alzheimer's disease (AD).

Chronic traumatic encephalopathy has been diagnosed in a broad spectrum of individuals with a history of head trauma, although the number of and severity of impacts is often unclear, ranging from athletes playing American football, soccer, hockey, boxers, and wrestlers to soldiers who have received battlefield injuries (Omalu et al., 2005, 2006, 2010a,b,c, 2011b; McKee et al., 2009; Baugh et al., 2012; Goldstein et al., 2012; Lakhan and Kirchgessner, 2012). Due to the variety of individuals afflicted by CTE, emerging evidence indicates that CTE is likely more common than previously thought (Stern et al., 2011; Baugh et al., 2012). Based on the experience of one group of investigators, a conservative estimate of lifetime prevalence of CTE in American football players is at least 3.7% (Saulle and Greenwald, 2012). Estimates of CTE prevalence in retired professional boxers have been as high as 20% (Lakhan and Kirchgessner, 2012). Considering the number of individuals actively engaged in contact sports such as football or exposed to explosive devices on the battlefield, it is clear that CTE represents a clear public-health risk. Much of the early work involving CTE diagnosis focused on concussion history but recent studies have documented a potential role of repetitive subconcussive blows as well (Omalu et al., 2010b; Baugh et al., 2012).

## NEUROPATHOLOGIC FINDINGS OF CTE

### *Gross*

Gross neuropathologic examination of the brain in individuals afflicted with CTE may produce a range of findings. The brain may appear grossly normal or may show minimal lobar cortical atrophy for age without remote cortical contusions or lacerations (Omalu et al., 2005). There may be other non-specific gross pathologic changes like fenestrations of the septi pellucidi, communicating ventriculomegaly, subcortical ganglionic atrophy, cerebellar folial atrophy, and pallor of the substantia nigra (Gavett et al., 2011b). In general, however, the CTE brain of non-boxers is grossly normal without evidence of focal traumatic brain injury.

The frequent lack of gross neuroanatomical changes observed in CTE is in striking contrast to dementia pugilistica, believed to represent a more severe form of CTE observed in boxers (Millspaugh, 1937; Corsellis et al., 1973; Adams and Bruton, 1989; Casson et al., 2006). Dementia pugilistica, described originally as "punch drunk" by Martland (1928), was characterized neuropathologically by Corsellis et al. (1973) based on a tetrad of findings: (1) abnormalities of the septum pellucidum; (2) cerebellar and other scarring of the brain; (3) degeneration of the substantia nigra; and (4) the presence of neurofibrillary tangles (NFTs) in a regional manner. While some controversy exists concerning identification of CTE, the work of Corsellis and colleagues is notable in that at no point is encephalopathy defined by the presence of a fenestrated or cavum septum pellucidum and equally important, the complete tetrad was not observed in a third of the cases characterized as dementia pugilistica (Casson et al., 2006).

### *Microscopic*

Microscopic investigation of CTE has focused primarily on several factors: the presence of tau, amyloid, and presence of TAR DNA-binding protein 43 proteinopathy as well as low grade diffuse white matter rarefaction, microglial activation, and presence of reactive astrocytes (Gavett et al., 2011b). The presence of a tauopathy, whether it be in the form of neurofibrillary tangles (NFTs), neuropil threads (NTs), or glial tangles (GTs), is a defining feature of CTE. While other neurodegenerative diseases such as AD are also frequently defined and/or described by the presence of tau, CTE is clearly unique based on the topographic distribution of tauopathy (Gavett et al., 2011b). AD is characterized by a relatively uniform distribution of tau NFTs in layers containing large projection neurons, such as layers III and V (Gavett et al., 2011b). In contrast, CTE is exemplified by an irregular distribution of tau in more superficial cortical layers such as II and III. Similarly, the progressive topographic involvement of regions of the brain as seen in CTE differs from what is seen in other neurodegenerative diseases like AD. While neuritic amyloid plaques are seen in AD, neuritic amyloid plaques are not defining features of CTE, and are less frequently seen in CTE (Gavett et al., 2011b).

Another delineating factor between AD and CTE is the presence of neuritic beta amyloid (Aβ) plaques. Found extensively throughout the brains of those afflicted with AD, neuritic amyloid plaques are found in a minority of CTE sufferers. Diffuse and neuritic amyloid plaques are found in less than 40–45% of individuals with CTE (Blaylock and Maroon, 2011; Gavett et al., 2011b). Additionally, when found in CTE, amyloid plaques are more likely to be diffuse plaques and not the typical neuritic plaques that are diagnostic of AD (Gavett et al., 2011b). The role Aβ plays in CTE pathophysiology, and why it is present in some brains but not others, remains to be elucidated. Interestingly, amyloid precursor protein (APP), which can undergo cleavage to form Aβ, accumulates following axonal injury, and likely plays a role in plaque formation (Gavett et al., 2011b).

Chronic traumatic encephalopathy has been recently associated, in greater than 80% of cases, with accumulation of yet another phosphorylated protein aggregate, TDP-43 (Gavett et al., 2011b). In some cases, TDP-43 has been found extending into the anterior horns of the spinal cord, particularly in patients exhibiting motor neuron disease symptoms similar to those of amyotrophic lateral sclerosis (Gavett et al., 2011b; Stern et al., 2011). While TDP-43 proteinopathy occurs as a primary or secondary proteinopathy in a variety of neurodegenerative diseases, the significance of TDP-43 proteinopathy in CTE is presently not clear. The presence of another phosphorylated protein in aggregate form may indicate a shared process resulting in neurodegeneration following repetitive brain trauma (Gavett et al., 2011b). Being that TDP-43 has been suggested to mediate the response of the neuronal cytoskeleton following injury, brain trauma, and subsequent axonal injury may trigger a TDP-43-mediated process involved in neurodegeneration (Costanza et al., 2011).

The diversity of pathological findings associated with CTE, as represented by the varied findings presented above, has begun to be described with four distinct phenotypes emerging (**Table 1**; Omalu et al., 2011a). The significance of these phenotypes with regards to clinical correlate remains to be elucidated but it is becoming increasingly clear that CTE represents a diverse spectrum of disease, a finding consistent with the heterogeneous nature of injury history, genetic predisposition, and a variety of other factors.

## CTE PATHOGENESIS AND PATHOPHYSIOLOGY

The pathogenesis and pathophysiology of CTE remain unclear, as with many neurodegenerative diseases, but is believed to be

**Table 1 | The diversity of pathological findings in CTE has lead to the emergence of four distinct phenotypes.**

| Phenotype | Cerebral cortex | Subcortical nuclei/basal ganglia | Brainstem | Cerebellum |
|---|---|---|---|---|
| #1 | +Sparse to frequent NFTs and NTs<br>+No diffuse amyloid plaques | +With or without NFTs and NTs | +Sparse to frequent NFTs and NTs | +No NFTs and NTs |
| #2 | +Sparse to frequent NFTs and NTs<br>+Sparse to frequent diffuse amyloid plaques | +With or without NFTs and NTs | +Sparse to frequent NFTs and NTs | +No NFTs and NTs |
| #3 | +None to sparse NFTs and NTs<br>+No diffuse amyloid plaques | +None to sparse NFTs and NTs | +Moderate to frequent NFTs and NTs | +No NFTs and NTs |
| #4 | +None to sparse NFTs and NTs<br>+No diffuse amyloid plaques | +None to sparse NFTs and NTs | +None to sparse NFTs and NTs | +No NFTs and NTs |

*How each phenotype correlates to the clinical condition remains unclear but the varied disease findings are consistent with the diversity of clinical exposure and disease-related factors.*

a multifactorial process initiated by brain trauma. The development of CTE begins with subconcussive or concussive injury. The damage is progressive and often accelerated by the number of brain injuries that occur in an individual. Initially, mTBI causes diffuse axonal injury (DAI), which results in disruption of axonal transport and subsequent axonal swelling. The swelling causes a disconnection of the axons and later Wallerian degeneration (Johnson et al., 2012). This degenerative process, a portion of which is referred to in the literature as immunoexcitotoxicity, may lead to the development of CTE (Blaylock and Maroon, 2011). It is interesting to note that the abnormal tau and amyloid accumulations, which are seen in CTE are peptide derivatives of both membrane and cytoskeletal proteins, which are involved in traumatic axonal injury following concussive and subconcussive injury. This process is still relatively poorly understood clinically with post-mortem studies on young adults revealing that repetitive head injury is associated with the formation of NFTs and tau-based pathology surrounding vascular elements within the cortex (Geddes et al., 1999). Consequently, it is perhaps likely that microvascular damage plays a role in formation of the classical neuropathology associated with CTE. This is consistent with findings from classical literature exploring dementia pugilistica in which a large percentage of ex-boxers experienced perivascular hemorrhages with evidence of meningeal and/or subpial siderosis (Adams and Bruton, 1989). In addition to repetitive brain injury, there may be other identified factors that may contribute to or alter disease development, such as presence of certain genotypes (Omalu et al., 2010b). Notably, anabolic steroid use had been previously suggested as a potential contributing factor to CTE development but the use of exogenous anabolic steroids has been shown experimentally to not worsen mTBI (Mills et al., 2012).

While the precise pathway or mechanism via which repetitive brain trauma predisposes to CTE development is poorly elucidated, Blaylock and Maroon (2011) posit a logical process via which immunoexcitotoxicity mediates the transition. As part of this process, microglia are primed by initial impacts and with sustained trauma as well as aging, undergo phenotypic conversion from a non-destructive to destructive mode (Blaylock and Maroon, 2011; Saulle and Greenwald, 2012). Once phenotype switching occurs, this pro-inflammatory state can be maintained for prolonged periods, consistent with neurodegenerative processes and emergence of hyperphosphorylated tau. Similarly, mild injury has been demonstrated to damage axons due to degenerative processes, resulting in a progressive deterioration, rather than rapidly occurring axonal shearing. This furthers the notion of CTE as a chronic neurodegenerative process, clearly distinct from the immediate sequelae often associated with TBI (Blaylock and Maroon, 2011).

## IMAGING MODALITIES FOR REPETITIVE BRAIN INJURY AND CHRONIC TRAUMATIC ENCEPHALOPATHY

A major concern for clinicians and researchers is how to detect small but important brain changes prior to the development of CTE symptoms so that preventative measures can be taken or treatments implemented, once available (Baugh et al., 2012; Saulle and Greenwald, 2012). The obvious short term deficits seen in concussive injuries such as loss of consciousness, post-traumatic amnesia, and altered mental status have been historically hard to quantify using traditional imaging modalities such as computed tomography (CT) and magnetic resonance imaging (MRI; Difiori and Giza, 2010; McCrory, 2011; Prichep et al., 2012). The difficulties and shortcomings of more commonly utilized imaging techniques have led researchers and clinicians to define concussion as a biomechanically induced brain injury with no gross anatomic lesions (Signoretti et al., 2011). Despite the lack of gross lesions, it is still necessary to look at diffuse and microscopic alterations in the brain. Until recently, imaging techniques were not sophisticated enough to detect the subtle changes that are the hallmark of DAI. Clinicians have previously been forced to rely on post-mortem tissue analysis to discover mechanisms of CTE pathology (Lakhan and Kirchgessner, 2012). With the invention and application of new imaging modalities, it is now possible to identify and investigate more of the pathological changes that are a consequence of mTBI. The following sections will highlight several of the new imaging modalities that are being used successfully to study various phenomena associated with mTBI and also promising modalities for further investigation of CTE. These modalities

and potential strengths, as well as challenges, associated with each are summarized in **Table 2**.

### DIFFUSION TENSOR IMAGING

Diffusion tensor imaging (DTI) represents one of the more thoroughly investigated techniques for detecting axonal injury clinically (Mac Donald et al., 2007; Smits et al., 2011; Shah et al., 2012; Shenton et al., 2012) and has even been shown to correlate with long-term outcome in preclinical rodents models of TBI (Maller et al., 2010). Perhaps most notably, preliminary studies in retired professional athletes subjected to repeat head trauma demonstrate a correlation between history of concussion and DTI measures, particularly in the callosal white matter (Gavett et al., 2011a). How DTI-based findings are altered with age and whether these findings correlate with neurodegenerative and behavioral changes associated with CTE remains to be seen (Tremblay et al., 2012). Regardless, DTI appears to be a promising technique for detecting and tracking structural correlates of repetitive brain injury (Chappell et al., 2008; Bazarian et al., 2012; Bennett et al., 2012; Hutchinson et al., 2012; Li et al., 2012), including those believed to be specific to CTE.

### FUNCTIONAL MAGNETIC RESONANCE IMAGING

Functional magnetic resonance imaging (fMRI) is unique amongst the various imaging modalities discussed within this work due to the ability to provide insight into functional alterations, particularly working memory, and anatomic or structural changes simultaneously. The capability to measure brain-behavior relationships has proved useful in other neurodegenerative diseases and appears promising for further investigation of CTE-related changes (Gavett et al., 2011a). Based on blood oxygen level dependent (BOLD) signal, fMRI is advantageous in that serial scans can be administered without exposure to harmful ionizing radiation (Sanchez-Carrion et al., 2008). This is particularly useful for fMRI-based studies as baseline measurements are required for comparison due to inter-individual variability (Rosenbaum and Lipton, 2012). Following exposure to head trauma, deficits on fMRI are apparent in both concussive and subconcussive injury groups with concussive injury producing a more severe deficit (Talavage et al., 2010; McDonald et al., 2012). Importantly, studies utilizing both fMRI and ImPACT for neuropsychological testing (NPT) have revealed deficits in working memory, even in the group sustaining only subconcussive impacts, and these deficits appear

**Table 2 | Numerous imaging modalities may provide insight into the development or tracking of CTE.**

| Imaging modality | Potential strengths | Potential weaknesses | References of interest |
|---|---|---|---|
| Diffusion tensor imaging (DTI) | +Radiologic and clinical deficits may correlate well<br>+Correlation between concussion history and DTI measures | +May be time-intensive if tractography required | Henry et al. (2011a), Gavett et al. (2011a), and Rutgers et al. (2008) |
| Functional magnetic resonance imaging (fMRI) | +Real-time assessment of brain activity and function<br>+Shown deficits in subconcussive injury | +Likely requires baseline scan for comparison | Gavett et al. (2011a), Talavage et al. (2010), McDonald et al. (2012), Breedlove et al. (2012), and Henninger et al. (2007) |
| Magnetic resonance spectroscopy (MRS) | +Insight into pathophysiology of CTE<br>+Shown persistent changes in professional athletes<br>+Metabolites may correlate with pathology and function | +Difficulty distinguishing between natural changes with aging and those of injury | Henry et al. (2010), Kleindienst et al. (2005), Lin et al. (2010, 2012); Gavett et al. (2011a), and Tremblay et al. (2012) |
| Positron emission tomography (PET) | +Identification of brain regions exhibiting decreased metabolism<br>+May measure tau deposition in CTE | +Exposure to radioactive isotopes may limit repeat scans | Venneti et al. (2007), Provenzano et al. (2010), Wagner (2012), and Small et al. (2006) |
| Single photon emission computer tomography (SPECT) | +Abnormalities in perfusion visualized in boxers with repeat trauma exposure | +SPECT fails to predict neuropsychological deficits | Kemp et al. (1995) and Lin et al. (2012) |
| Susceptibility weighted imaging (SWI) | +Can map blood-brain barrier disruption and tau deposition<br>+Shown to predict outcome in pediatric patients post-injury<br>+Can detect microhemorrhages with DTI | +Predictive ability limited in adults | Baugh et al. (2012), Kou et al. (2009), Ashwal et al. (2006), and Gavett et al. (2011a) |
| Electroencephalography (EEG) | +Components of P300 such as P3a/P3b altered chronically post-concussion<br>+Low cost | +Minimal investigation outside of boxing<br>+Analysis difficult | Costanza et al. (2011) and Gavett et al. (2011a) |

*Each modality exhibits potential strengths and weaknesses, presented here, affecting its viability for further CTE-related research.*

to be related to the number of impacts rather than the magnitude of any one impact (Breedlove et al., 2012). Furthermore, in a preclinical model of TBI, fMRI response was diminished acutely and correlated with functional deficits based on the forepaw placement test (Henninger et al., 2007). The use of fMRI for assessing the more subtle deficits produced by repetitive subconcussive impacts warrants further investigation, and in particular, longitudinal studies investigating the persistence of deficits on fMRI and the likelihood of further development for detecting, diagnosing, and tracking CTE.

#### MAGNETIC RESONANCE SPECTROSCOPY

Magnetic resonance spectroscopy (MRS) is a non-invasive method that measures brain chemistry associated with DAI and neuronal injury (Gavett et al., 2011a; Shenton et al., 2012). It is sensitive for changes occurring with age, mTBI, and mild cognitive impairment (MCI; Tshibanda et al., 2009). As an imaging modality, $^{1}$H MRS is used to detect changes in *N*-acetylaspartate (NAA), myoinositol (mI), choline, lactate, and adenosine triphosphate (ATP) production in specific ROI. NAA is a neuronal marker and is reported as a NAA:creatinine ratio. Other markers that may prove useful in neural injury include mI, a glial marker (Signoretti et al., 2010), choline-based compounds, indicative of membrane integrity, and lactate, an indirect marker for ischemia (Signoretti et al., 2010). The metabolite levels in an injured brain are compared to levels in a healthy brain for analysis (Vagnozzi et al., 2010). A decrease in NAA and ATP is indicative of reversible neuronal and mitochondrial dysfunction (Henry et al., 2011b). An elevation of the NAA:creatinine ratio indicates persistent damage following mTBI (Signoretti et al., 2011). $H_2O$ is often measured as an internal control in the patient during imaging (Tremblay et al., 2012). By monitoring the metabolites and $H_2O$, the progress of a patient can be tracked longitudinally (Bigler and Maxwell, 2012). It is even possible to detect changes in the brain prior to onset of symptoms such as in subconcussive injuries (Henry et al., 2010). Furthermore, metabolite levels remain altered long after symptoms subside, which gives credence to the idea of long-term degeneration occurring and presence of a latent period (Kleindienst et al., 2005). As MRS utilizes current clinical MR scanners and does not administer ionizing radiation to the patient, MRS may represent an ideal imaging modality for long-term studies requiring repeated measurements. Furthermore, a preliminary study conducted by Lin and colleagues demonstrated persistent changes in Cho and Glx in 5 professional athletes, aged 31–54 years. Specifically, both Cho and Glx were significantly increased as compared to controls (Lin et al., 2010, 2012; Gavett et al., 2011a). Additionally, studies have demonstrated changes in mI consistent with MCI literature that correlate with NFT count in AD brains upon pathological examination (Tremblay et al., 2012). Consequently, understanding the effect of repetitive brain injuries on mI levels over time may be of interest for future investigation due to the strong correlation with episodic memory deficits observed in formerly concussed athletes (Tremblay et al., 2012). Based on this evidence, MRS appears promising for not only elucidation of CTE pathophysiology but also tracking the effects of repetitive brain trauma longitudinally. In this manner, MRS may also be useful for the diagnosis of CTE in effected individuals.

#### POSITRON EMISSION TOMOGRAPHY

Positron emission tomography (PET) can be used to detect metabolic rates in a variety of tissues of interest, including the brain (Price and Jones, 1995). While PET has traditionally been used in cases of more severe injury, recent evidence indicates a potential role in mTBI (Mase et al., 2004; Venneti et al., 2007). In boxers, hypometabolism was observed in regions likely affected by impacts to the side of the head such as the portion of the frontal lobe anterior to Broca's area (Provenzano et al., 2010). Similarly, hypometabolism was observed in the posterior cingulate cortex and posterior parietal lobes (Provenzano et al., 2010). In other patients afflicted with TBI, hypometabolism may be seen commonly in the anterior temoporal lobe and orbitofrontal lobe due to rapid acceleration-deceleration mechanisms (Provenzano et al., 2010). Other promising developments in PET imaging include the development of ligands specific for disease states such as AD. For example, Pittsburgh compound B selectively binds Aβ whereas others under development non-selectively bind both Aβ and tau (Gavett et al., 2011a). While Aβ is not diagnostic for CTE, this technology appears potentially promising for the imaging of CTE should tau-specific compounds be identified. Furthermore, recent studies using $F^{18}$ DDNP glucose-PET, conducted by Dr. Gary Small at UCLA, show promise for identifying tau protein deposition in the form of NFT's in subjects with potential CTE (Wagner, 2012). $F^{18}$ DDNP glucose-PET allows for labeling of both plaques and tangles, as has been shown in cases of MCI as well as AD, making this technique exceptionally promising in identifying characteristic pathological features of CTE as they develop pre-mortem (Small et al., 2006). This effort is significant in that it could potentially allow, for the first-time ever, the diagnosis of CTE pre-mortem (Small, 2012). Similarly, identification of NFT's following brain injury would provide substantial insight into the timing of development and pathophysiology of CTE.

#### SINGLE PHOTON EMISSION COMPUTER TOMOGRAPHY

Single photon emission computer tomography (SPECT), similar to PET in the use of radionuclides, can be used for measurement of cerebral perfusion and has been shown to detect abnormalities in 41% of amateur boxers in comparison to 14% of controls. Furthermore, abnormalities were generally singular in the control group yet multiple in the group of boxers subjected to repetitive brain trauma (Kemp et al., 1995). While SPECT has not been utilized in studies of CTE, CTE is associated with amyloid protein deposition in a significant number of cases and amyloid is notably largely perivascular in deposition, particularly in regions of abnormal vasculature, which could account for abnormal findings on SPECT (Kemp et al., 1995). It has also been correlated with functional deficits revealed on NPT. Specifically, NPT strongly predicts diminished perfusion on SPECT but the inverse is not true (Lin et al., 2012). The reasons for this mismatch are currently unknown but may be due to SPECT being obtained at a resting state while NPT requires neural activity and processing (Lin et al., 2012). In spite of this lack of correlation and lack of specificity when considering other disease conditions, SPECT has clear value in recovery prognostication based on previously reported findings (Lin et al., 2012).

### SUSCEPTIBILITY WEIGHTED IMAGING

Susceptibility weighted imaging (SWI) is used to detect microhemorrhages, the intact structure of the venous system, and oxygen saturation following neurotrauma (Shen et al., 2007). Microhemorrhages, venous thrombosis, and ischemia are common secondary injuries following mTBI (Aiken and Gean, 2010). Furthermore, the extent of blood-brain barrier (BBB) disruption and perivascular tau deposition can be mapped with SWI (Baugh et al., 2012). Tau accumulates in the brain during the progression of CTE (Stern et al., 2011). The imaging technique works by taking phase data from a *T*2* weighted MRI and creating a second mapped image. This image allows both blood oxygen levels and the amount of deoxyhemoglobin to be measured (McCrea et al., 2008). It also localizes concentrations of paramagnetic iron and detects areas of iron translocation (Cheng et al., 2010). Iron deposition may play a role in CTE symptom manifestation (Zhang et al., 2010). The images produced with SWI are highly functional, velocity-compensated, and gradient echoed (Meoded et al., 2012). When used with DTI, SWI can accurately show which areas of white matter are damaged by microhemorrhages (Kou et al., 2009). This modality offers a unique tool to detect areas of injury early, and provides clinicians with information on how to manage the care of the patient, particularly in children in which SWI has been shown to predict future outcomes following injury (Ashwal et al., 2006; Gavett et al., 2011a). Unfortunately, the predictive ability is more limited in adults at present but necessitates further study (Gavett et al., 2011a).

### ELECTROENCEPHALOGRAPHY

Electroencephalography (EEG) has been used to demonstrate mental processing impairment in 12 professional concussed boxers based on increased P300 latency and lessened amplitude. P300 has been identified as a cognitive event-related potential (ERP) with a novel amplitude and latency, making it a potentially suitable measure of brain processing ability. Similarly, investigators have demonstrated altered components of P300, such as P3a/P3b, decades after concussion in older athletes with a history of past concussion compared to controls (Costanza et al., 2011; Gavett et al., 2011a). Based on these findings, EEG may have utility in assessing long-term cognitive effects associated with concussion and therefore, may be useful in identifying those predisposed to CTE development.

### FUTURE DIRECTIONS

Advances in imaging technology and improved access provide nearly limitless opportunities for both understanding the pathophysiology and diagnosis of CTE pre-mortem. As such, it may be possible to initiate both treatments and preventive measures prior to emergence of CTE. Many challenges remain such as clarifying the role of subconcussive injury in CTE development and how to most readily identify suspected subconcussive injuries on the athletic or battlefield. By utilizing techniques such as MRS and fMRI, pathology can be correlated with functional deficits and hopefully lead to more appropriate identification and monitoring of those with suspected injuries, regardless of severity. Other challenges such as the need for baseline measurements remain to be overcome as well but clearly warrant additional investigation.

## BIOMARKERS FOR CTE: A NEW FRONTIER

As discussed in previous sections modern imaging techniques are one way by which diagnosing and tracking the progression of CTE may be feasible in the near future. Unfortunately, access and economic issues associated with imaging techniques may somewhat limit their ultimate usefulness in the diagnosis of CTE, especially during the acute phases of injury. With CTE potentially being a large public-health issue, a simple and cost effective manner to diagnose and possibly track progression of the disease is crucial (Baugh et al., 2012).

Biomarkers represent one method by which CTE may one day be diagnosed. Additionally, it may be possible to track disease severity and progression through the use of a biomarker(s). Ideally, a potential biomarker for CTE would be minimally invasive, have diagnostic potential and would correlate to disease severity, allowing care providers to track progression of the disease. It should be sensitive enough to detect the disease and when used in conjunction with clinical evidence of CTE symptoms and a past history of repeated head injuries, allow for a diagnosis. As described above, there is currently no accepted method of diagnosing CTE until post-mortem pathological analysis has been conducted (Baugh et al., 2012). A readily available biomarker with the aforementioned traits would give practitioners a useful tool for the diagnosis and tracking of patients suffering from CTE.

Currently there is a paucity of studies aimed at determining a biomarker(s) specifically for CTE. Many studies aimed at elucidating biomarkers for TBI and other types of neurotrauma have been conducted. One difficult aspect of searching for a CTE biomarker is that CTE symptomology can be similar to a variety of other neurologic disorders. This may add a layer of complexity to conducting human clinical studies in an attempt to discover suitable biomarkers. The neuropathology of CTE, however, is not distinctly different, as hyperphosphorylated tau and TDP-43 deposition are found in a variety of neurodegenerative diseases outside CTE (McKee et al., 2010; Baugh et al., 2012; Saulle and Greenwald, 2012). Additionally, recent reports indicate that CTE may be associated with symptomology similar to ALS, further complicating the likelihood of clinical diagnosis without improved diagnostic tests (McKee et al., 2010). These reports emphasize the need for a biomarker with high specificity that can delineate CTE from other neurologic disorders.

There are two primary body fluids where readily attainable biomarkers of CTE may be located. These include the cerebral spinal fluid (CSF) and the blood. Blood or plasma samples have the distinct advantage of being minimally invasive and easy to obtain. CSF samples, however, are in more direct contact with the CNS and may confer an advantage in this regard to the determination of suitable biomarkers for CTE. Routine use of CSF in the diagnosis or tracking of CTE presents several obvious problems associated with obtaining CSF samples. Therefore, ideally a biomarker aimed at detecting CTE in the periphery would be found in serum or plasma samples obtained via venipuncture. As discussed above, currently there is a lack of primary studies aimed at the elucidation of biomarkers in either one of these body fluids for CTE. However, several studies have been conducted in the realm of TBI and mTBI and these will be discussed herein.

It may be possible that biomarkers in development for TBI may be useful during the acute injury phase of patients at risk for CTE. The repeated concussive and subconcussive blows that result in CTE may alter levels of particular biomarkers studied for TBI immediately following these events. The tracking of these acute changes, although not specific to CTE, may give healthcare professionals insight into those patients at the greatest risk for developing CTE later in life. Equally important though, is the potential utility for biomarker surveillance in patients with known or suspected post-concussion syndrome.

Many studies that have been conducted aimed at determining a biomarker for TBI or mTBI in the plasma or serum. A study conducted by Mondello et al. has determined that glial neuronal ratio (GNR), characterized as the ratio between glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) in the serum of TBI patients is characteristic of the type of TBI injury, focal vs. diffuse (Mondello et al., 2012). It was noted by Mondello et al. (2012) that their studies may have implications for diagnosis in TBI patients in the early acute phases of injury, however whether these results would have implications for CTE patients has yet to be determined. It is possible that after multiple concussive or subconcussive blows to the head that the GNR may be altered and may offer insight into the extent of injury and potential for later development of CTE.

Several other studies aimed at elucidating valuable biomarkers for TBI in the serum have been conducted and may offer insight into the production of a biomarker(s) specific to CTE. It has been reported that plasma bilirubin levels in TBI patients are elevated on days subsequent to injury (Dohi et al., 2005). Unfortunately, this study only determined bilirubin levels up to 4 days post-injury. The determination as to whether the increases in plasma bilirubin remain elevated for a significant amount of time post-injury or whether it is specific to the acute injury phase has yet to be made. Additionally, the determination as to whether bilirubin concentrations in the plasma would be elevated in patients at risk for CTE has also yet to be made.

Another intriguing potential serum biomarker for CTE may be the protein neuron-specific enolase. Zetterberg et al. (2009) have shown that this protein is increased in the serum of boxers as compared to healthy controls after the boxers abstained from boxing for a period of 2 months. Interestingly, elevated levels of neuron-specific enolase were detected 2 months after a non-participatory period of boxing, however other biomarker candidates for CTE such as S-100B, brain-derived neurotrophic factor (BDNF), and heart-type fatty acid binding protein (H-FABP) were found to be unchanged (Zetterberg et al., 2009). These results indicate that neuron-specific enolase may be one protein biomarker that has the potential for use as a diagnostic tool. It is possible that neuron-specific enolase remains elevated for an extended time period after injury and may be a useful tool for diagnostic purposes in athletes and patients who have suffered multiple concussive and subconcussive blows to the head.

An area of biomarker research that has received much attention is the field of microRNA. It is possible that potential biomarkers for CTE may not only be protein-based in nature. A recent report by Balakathiresan et al. (2012) has shown that levels of microRNA let-7i are elevated post-blast-induced TBI in both the CSF and the serum of rats exposed to a model of this type of TBI. Additionally, this group showed that the expression of five microRNA's were altered post-blast TBI in the serum of these animals (Balakathiresan et al., 2012). These included let-7i, miR-122, miR-200b, miR-340-5p, and miR-874 (Balakathiresan et al., 2012). Further study is needed in both animal models, as well as humans, to validate the potential for using microRNA's as potential biomarkers for TBI and possibly CTE. Although this study was conducted using a blast TBI animal model, a recent report has shown that this model produces pathology similar to that seen in CTE and models similar to this may be viable animal models for the study of CTE (Goldstein et al., 2012). The production of validated animal models could greatly aid in the discovery of biomarkers aimed at detecting CTE and the production of potential clinical therapies for treating CTE. Other studies have also successfully identified RNA-based biomarkers for mTBI based on both micro- and snoRNA in peripheral blood mononuclear cells (PBMCs). Using a panel of three markers, veterans that experienced mTBI were detected with 89% accuracy, 82% selectivity, and 78% specificity (Pasinetti et al., 2012). Future studies utilizing both animal models as well as clinical samples may reveal additional RNA-based molecules that may be viable biomarkers for detecting and possibly tracking CTE.

One particularly interesting report recently published, studied a variety of biomarkers aimed at detecting TBI and mTBI in Olympic boxers with an extended history of many bouts (Neselius et al., 2012). This study determined extent of increases in neurofilament light protein (NFL), total tau (T-tau), tau phosphorylated at threonine 181 (P-tau$_{181}$), H-FABP, GFAP, S-100B, and the 42 amino acid form of amyloid β (Aβ1–42) in Olympic boxers both in the acute phase after a bout and an extended phase of 2 weeks post-bout and compared them to non-boxing controls. It was found that both NFL and GFAP levels within the CSF were significantly different from controls in both the acute and extended phase of testing (Neselius et al., 2012). The determination as to whether either of these correlated to injury severity was unable to be conclusively made in this study (Neselius et al., 2012).

However, a previous study has shown that there may be a potential correlation between NFL levels in the CSF and injury severity (Zetterberg et al., 2006). Zetterberg et al. (2006) showed that NFL, T-tau, and GFAP were increased in the CSF of boxers and the levels appear to be correlated to injury severity, as boxers who received more hits in number or severity had increased levels of these markers. Only NFL in these studies however, was increased for an extended time period (tested at 3 months) as compared to controls (Zetterberg et al., 2006). The extended increase in NFL levels within the CSF post-injury may make it a viable candidate for a biomarker for mTBI and CTE however whether levels of NFL within the CSF correlate to later disease severity in the case of CTE or mTBI has yet to be conclusively made. NFL levels however, have been shown to be altered in other types of neurologic trauma and disorders so this may ultimately decrease its viability as a tool for detecting, diagnosing, and tracking CTE disease progression (Avsar et al., 2012; Tortelli et al., 2012).

Although levels of proteins such as NFL and tau may not be specific with regards to CTE, a battery of various biomarker proteins combined with patient history and imaging studies (discussed

in previous sections) may result in the ability to make a pre-mortem diagnosis of CTE via a reference fractional laboratory index using a high throughput analytical system. In this respect, a combined battery of proteins such as tau, GFAP, NFL, S100-B, and bilirubin may offer more accurate quantitative data with regards to a patients risk for developing CTE and when combined with other modalities of relevant clinical data, may be used as even more accurate diagnostic indicators at some point in the future.

## LINKING PATHOPHYSIOLOGY TO IMAGING AND BIOMARKER DISCOVERY

Perhaps the greatest challenge concerning development of biomarkers or imaging modalities for prevention, diagnosis, and prognosis of CTE is the current lack of well-elucidated disease pathophysiology. Consequently, few clearly identifiable and specific disease markers have been identified. Without first understanding disease development more clearly, likely via both clinical research and utilization of animal models of repetitive brain

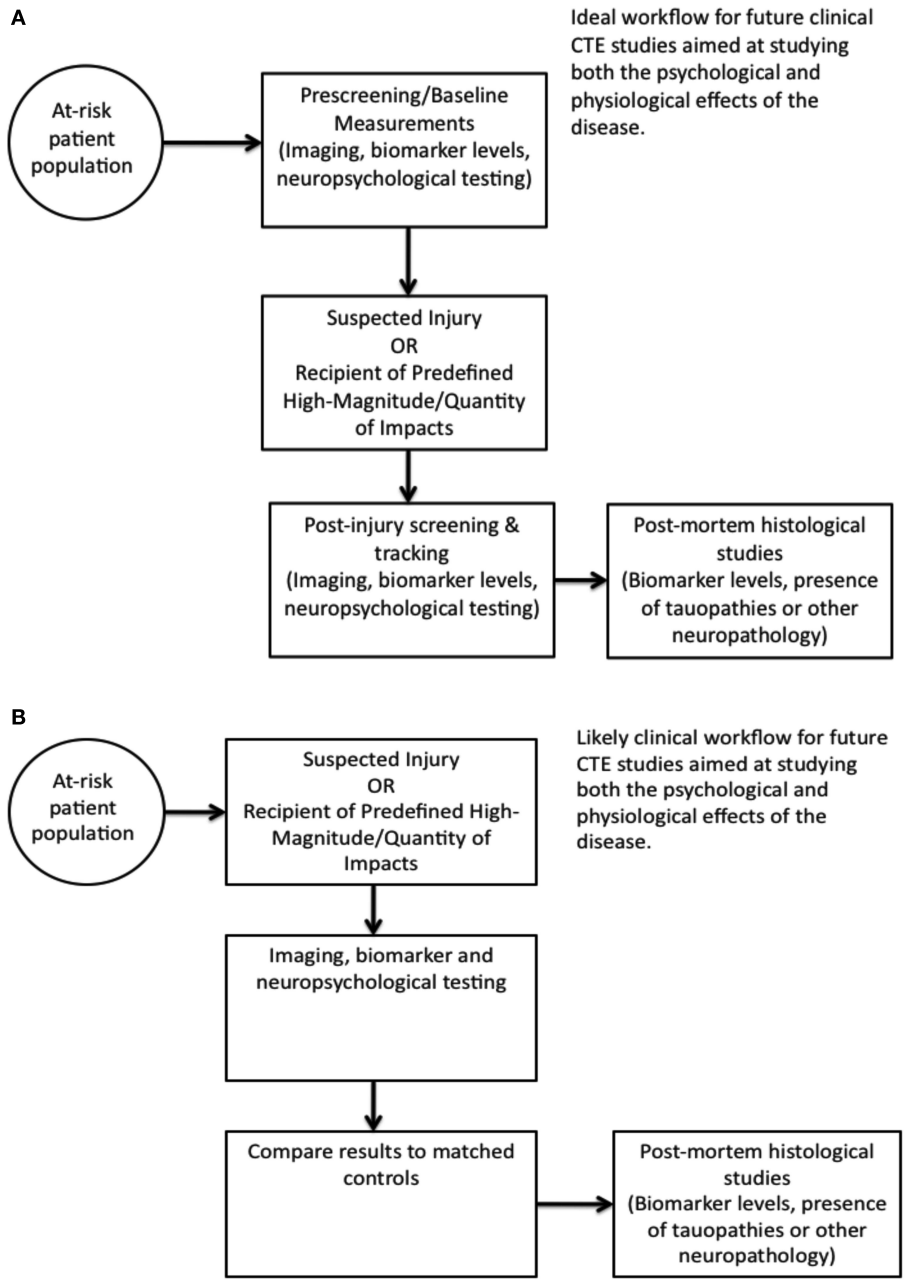


**FIGURE 1 | Imaging and biomarker studies may be useful in improving understanding of the role of repetitive concussive and subconcussive injury in disease development.** Two experimental paradigms are presented representing a potentially more ideal, but costly, scenario **(A)** and the more cost-conscious approach **(B)**.

trauma, logical discovery of CTE-associated markers appears unlikely. Recent work has demonstrated the presence of CTE-like neuropathology in wild-type mice following single-blast exposure and increased formation of NFTs in a transgenic mouse model following repetitive injury (Yoshiyama et al., 2005; Goldstein et al., 2012). In a single-injury experiment, again using transgenic mice utilized in AD models, tau and amyloid-β accumulation was accelerated (Tran et al., 2011). Similarly, studies have shown the presence of phosphorylated tau and amyloid-β months after initial fluid percussion injury in the genetically unmodified rat (Hoshino et al., 1998).

Despite the present lack of clear elucidation of disease pathophysiology, certain imaging techniques such as MRS, fMRI, and tau-specific labeling compounds in PET, have begun and will likely continue to provide insight into CTE development via longitudinal changes in neurologic function and health via a variety of markers. Similarly, work in other disease conditions that allows for imaging of activated microglia, consistent with the notion of immunoexcitotoxicity, may provide further insight into disease development and progression (Venneti et al., 2007).

In addition to assisting in the diagnosis and prognosis of patients suffering from CTE, continued improvements in imaging and biochemical assays associated with CTE identification may promote risk factor identification and provide insight into periods of increased risk. For example, the role of age at time of impact and associated brain trauma in disease development is unclear. Understanding this issue warrants further investigation as neuropathologic changes associated with CTE have been found in an asymptomatic 18-year-old high school football player and consequently, age at time of first head injury may contribute to CTE incidence (Saulle and Greenwald, 2012).

## CONCLUSION

Chronic traumatic encephalopathy is an emerging public-health concern due to disease prevalence likely higher than previously recognized and continued popularity of contact sports as well as involvement in military combat that places soldiers at risk for explosive blasts and subsequent TBI. As CTE represents a devastating deterioration of neurologic function, a clear need for improved diagnostic and prognostic tests exists. To accommodate this need, as well as more clearly elucidate disease pathophysiology, imaging, and/or biomarker-based tests are required. We propose a potential work-flow for implementation of both imaging and biomarker-based studies for improving the understanding of the role concussive and subconcussive impacts play in long-term disease development (**Figure 1**). While the specifics remain open to interpretation, obtaining pre-injury exposure studies as well as after a suspected or confirmed injury parameters is likely to provide further insight into the effects of TBI. It is only by more accurately identifying CTE pre-mortem and studying disease progression and mechanisms that we can establish improved therapeutic approaches.

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# The diagnosis of traumatic brain injury on the battlefield

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The conflicts in Iraq and Afghanistan have placed an increased awareness on traumatic brain injury (TBI). Various publications have estimated the incidence of TBI for our deployed servicemen, however all have been based on extrapolations of data sets or subjective evaluations due to our current method of diagnosing a TBI. Therefore it has been difficult to get an accurate rate and severity of deployment related TBIs, or the incidence of multiple TBIs our service members are experiencing. As such, there is a critical need to develop a rapid objective method to diagnose TBI on the battlefield. Because of the austere environment of the combat theater the ideal diagnostic platform faces numerous logistical constraints not encountered in civilian trauma centers. Consequently, a simple blood test to diagnosis TBI represents a viable option for the military. This perspective will provide information on some of the current options for TBI biomarkers, detail concerning battlefield constraints, and a possible acquisition strategy for the military. The end result is a non-invasive TBI diagnostic platform capable of providing much needed advances in objective triage capabilities and improved clinical management of in-Theater TBI.

**Keywords: TBI, military, biomarkers, diagnosis, concussion**

## THE TBI PROBLEM

In the early years of the wars in Afghanistan (Operation Enduring Freedom, OEF) and Iraq (Operation Iraqi Freedom, OIF), there appeared to be an increase in the numbers of causalities sustaining a traumatic brain injury (TBI). By early 2005 TBI was being called the "signature wound of war in Iraq" as cases of soldiers suffering from TBI were appearing in National newspapers, such as USA Today (Okie, 2005; Zoroya, 2005). The increased awareness and emphasis of TBI in the military population spurned a movement to identify and collect data on the incidence of TBI in our deployed forces.

The incidence of TBI in the deployed forces varies depending upon the type of information collected. One initial study examined a cohort of casualties that were wounded in OEF/OIF from 2001 to 2005. The study reported that approximately 30% of wounds were to the head and neck area with 8% of the total attributed as head wounds (Owens et al., 2008). However, this study was based on casualties treated for wounds, and excluded those that were returned to duty within 72 h, thus potentially missing TBI cases that were mild and did not accompany an open wound. The RAND Corporation's "Invisible Wounds of War" collected data from April 2007 to January 2008, part of which included a telephone survey of 1,965 previously deployed persons. From this survey they reported that 19.5% of the previously deployed persons suffered from a "probable TBI." They further estimated that of the 1.64 million deployed service members (at that time), 19.5% of them, or 320,000 have suffered a TBI (Tanielian and Jaycox, 2008). However, data collected from the Armed Forces Health Surveillance Center show that by the end of 2008 there had only been approximately 130,000 clinically confirmed cases of TBI. In addition, the number of TBI diagnosis rose sharply between the years of 2005 thru 2009, specifically in the cases of mild TBI (mTBI; http://www.health.mil/Research/TBI_Numbers.aspx). Is this rise in TBI a real rise, or an increase in TBI awareness and improved vigilance for detecting TBI? Clearly there appears to be a real challenge on getting an accurate estimate of the actual incidence of TBI experienced by our deployed Forces.

## DIAGNOSING TBI

One of the issues creating this challenge lies in the current limitations in the diagnosis of TBI, specifically for the military. In general, the diagnosis of TBI relies on a clinician to accurately interpret a patient's signs and symptoms of injury, often through some type of self-report from the patient and possibly corroborated by a witness. In the case of a penetrating brain injury, the signs and symptoms are more straightforward. However, with closed-head injuries, especially mTBIs/concussions, the symptoms are often not as straightforward or clear. Typically the initial evaluation of a possible TBI captures the patients Glasgow Coma Scale (GCS), the length of loss of consciousness, alteration of consciousness/mental state, and/or post-traumatic amnesia (Harrington et al., 1993; Meyer et al., 2010). If certain symptoms are present the clinician can further evaluate the injury by image analysis of the brain via a computed tomography (CT) scan or even magnetic resonance imaging (MRI), both of which have their own limitations in the ability to detect TBI, especially mTBI (Chastain et al., 2009; Mondello et al., 2011a; Prabhu, 2011). Besides the image analysis, most of the tools to aid in the diagnosis of TBI are subjective and as stated above involve self-reporting. Hence, there are no true objective measures available to determine that the brain indeed has been injured, thus the ability to diagnose closed-head TBI, especially those of the mild-moderate severity, is at best limited by

available methods. However, the ability to diagnosis a TBI can also be limited by the setting.

The civilian environment and the military (deployed) environment are vastly different. In a civilian setting, if someone experiences a head injury from a motor vehicle accident, fall, or a sporting event, the event is often witnessed; they are quickly transported to the local medical facility and promptly evaluated by a medical professional, sometimes with access to the latest imaging technology. In the military setting, there is often some type of battle taking place either before, during, or after, when someone experiences a head injury. By its very nature the event is dangerous, and often chaotic and loud, occurring in remote locations and possibly lasting hours before casualties can be evaluated by a clinician. In addition, the military casualty can and often will experience multiple serious injuries (Kelly et al., 2008; Owens et al., 2008) and the evaluation of a potential head injury can take a back seat to life saving interventions, possibly missing the treatment window for the TBI. Further, evacuation priorities and logistics may impact the ability to evaluate all suspected mTBI cases to the same extent as a civilian setting as the ability to image the brain is only located at specific locations in the theater of operations. Clearly the military setting creates another layer of difficulty and complexity in the ability to diagnose TBI.

## ON THE BATTLEFIELD

Due to the complexities of war and of TBI, it has been extremely difficult to get a handle on the rate of TBIs experienced during a deployment, not to mention the severity of TBIs or the incidence of multiple TBIs our service members are actually experiencing, or not experiencing. One of the major reasons is the lack of a method to objectively determine if the brain has been injured. In 2006 the Military Acute Concussion Evaluation (MACE) was add to the list of tools to screen casualties (Meyer et al., 2010). While the MACE has been well integrated into the military medical evaluation, it still relies on subjective recall of the events, may be affected by fatigue as other neuropsychological tests and has shown low sensitivity when administered greater than 12 h (Coldren et al., 2010). Thus there remains a gap for the ability to objectively measure brain injury with a method that is not impacted by other factors such as extra-cranial injury (i.e., polytrauma), stress, fatigue, or battlefield conditions.

Due to the logistic constraints faced on a battle field, any method involving a piece of equipment, must meet additional requirements sometimes not faced in civilian medical care. The battlefield is often a very austere environment. The most restrictive environments are often isolated locations where power supply is lacking or being provided by a generator which must sustain all of the electrical needs for the deployed force. Since units often need to remain quickly mobile, the footprint of the unit is limited, so space, and weight of equipment is a priority concern. In addition, if medical refrigeration exists, it is typically small and in high demand and there is little hope of having specialized reagents such as deionized water. Further consideration is given to the shelf-life of a piece of equipment and its ability to sustain high altitude, large temperature, and/or humidity fluctuations and a considerably dusty and dirty environment. Taking all of these issues into consideration, the ideal method for the diagnosis of TBI would be quick, simple, easy to obtain, not rely on self-report of symptoms, and be portable. In addition, the results of the test should be able to differentiate the severity of injury and in a most ideal world, be predictive of some level of clinical outcome.

## OBJECTIVE DIAGNOSTIC TEST FOR TBI

In recent years there have been a number of technologies under development to objectively aid in the diagnosis of TBI (Marion et al., 2011). Some of these include advances in MRI (Kumar et al., 2010; Prabhu, 2011), quantitative electroencephalogram (EEG; Nuwer et al., 2005), visual tracking (Maruta et al., 2010), and serum based biomarkers of brain injury (Dash et al., 2010). Using the logistical constraints mentioned above and the need for the test to not be confounded by conditions of deployment (sleep deprivation, stress, fatigue, etc.), one of the more promising options is the development of a simple blood test to detect brain specific proteins after a TBI. Blood based tests have been successful in the diagnosis of other disease conditions such as cardiac disease and cancer, so it is possible biomarkers could be identified for TBI as well.

The TBI community has been actively engaged in the discovery of biomarkers for TBI in the last decade. A number of review articles have captured the pros and cons of various potential markers (Dash et al., 2010; Mondello et al., 2011a). Some of the more promising candidates have been tested in human clinical trials of TBI patients, most of them in severe TBI (Hergenroeder et al., 2010; Liliang et al., 2010; Mondello et al., 2010, 2011b, 2012a,b; Vos et al., 2010; Brophy et al., 2011; Stein et al., 2011, 2012; Gong et al., 2012). A number of proteins of interest are markers that are not solely found in the brain (i.e., cytokines, growth factors, interleukins; Hergenroeder et al., 2010; Stein et al., 2011; Gong et al., 2012). While these may provide clinical utility in situations of head trauma with no confounding injuries, this is typically not the case in the military combat causality. However, a number of markers of interest are more brain specific, to include S100B, glial fibrillary acidic protein (GFAP), Ubiquitin C-terminal hydrolase L1 (UCH-L1), Neuron Specific Enolase (NSE), spectrin breakdown products (SBDP), and Tau (Siman et al., 2009; Liliang et al., 2010; Mondello et al., 2010, 2011b, 2012a,b; Vos et al., 2010; Brophy et al., 2011; Stein et al., 2012).

Most of the clinical trials have been conducted with severe TBI patients, but a few have also included moderate and mTBI patients as well and show promise (Honda et al., 2010; Papa et al., 2012a,b; Topolovec-Vranic et al., 2011; Egea-Guerrero et al., 2012). In an early study of S100B in mTBI subjects, S100B serum levels were significantly different between mTBI patients and uninjured controls (Nygren De Boussard et al., 2004). However, S100B is also increased in other extra-cranial injuries, making less than ideal in the military environment (Savola et al., 2004). Promising recent studies include a study on moderate-mTBI (GCS $\leq$ 12), that demonstrated that the levels of GFAP in patient serum were able to significantly differentiate not only between TBI patients and uninjured controls, but also between TBI patients and trauma controls (trauma injury without head injury). Further, when TBI groups were dichotomized into traditional groups of mTBI (GCS 13–15) and moderate TBI (GCS 9–12), the level of serum GFAP were significantly different between groups (Papa et al., 2012a). Similar results were demonstrated with serum levels of UCH-L1 differentiating between mTBI, moderate TBI, normal, and trauma controls (Papa et al., 2012b). Another study recently characterized

the differences in areas under the curve (AUC) for sensitivity and specificity of GFAP, S100B, and NSE in TBI patients (GCS range 5–14) determined by positive CT findings. GFAP performed the best with sensitivity set to 100% and the corresponding specificity was 88.9% on day one compared to S100B with a specificity of 27.8% and NSE with a specificity of 22.2% on day one (Honda et al., 2010).

## TBI BIOMARKER PLATFORMS FOR THE FIELD

Although recent trials have indicated that it is possible to detect TBI biomarkers in the serum of injured patients, all of the studies have taken place in civilian centers and were able to use a research laboratory platform for analysis. Typically this platform is a standard enzyme-linked immunosorbent assay (ELISA) which has a number of limitations for use in a combat environment. First, the typical assay requires multiple pieces of equipment (reader, incubator, and automated washing machine), refrigeration of reagents, and deionized water. Second, the assay can be lengthy to run, taking as little as 4 h and as many as 24 h. Third, the standard assay plate consists of 96 wells, which could equate to wasting two-thirds of a plate if there is only one sample to assay. However, when we consider the needs for our military applications the assay in an ELISA platform is indeed the most mature in development and testing of clinical samples. Similarly this platform could potentially have greater sensitivity (lower limits of detection) and serve as a benchtop in a reference laboratory. In addition, porting the standard ELISA assay onto an automated benchtop platform could cut down on some of the size and weight of the system.

On the other end of the spectrum would be the development of a hand-held device that performs similar to a TBI "pregnancy test." Such hand-held systems would be lightweight, portable, and require little logistical support. The assay time and waste would likely be reduced as well. However, these devices do not exist today for the TBI biomarkers that have been examined in clinical studies; therefore they are the least mature in development and Food and Drug Administration (FDA) approval timeline. In addition, it is possible that this platform could suffer in the limit of detection.

As mentioned above, the development of a device platform for the military environment presents unique challenges with different restrictions upon instrumentation at each level of care. The initial level of combat casualty care is located close to the point of injury such as a Battalion Aid Station (BAS). This level of care is the most remote and has the highest logistical constraints concerning power, refrigeration, and footprint. In addition, this level of care does not have much capacity, if any, to hold patients for treatment and evaluation. The most robust level of care on the battlefield is located at a combat support hospital (CSH) or a field hospital. It includes specialist diagnostic resources, specialist surgical and medical capabilities, and operational stress management teams. The holding capacity is sufficient to allow diagnosis, treatment, and holding of those patients who can receive total treatment and be returned to duty. However, it is still a deployed environment faced with the same challenges of reduced and restricted footprint, especially for power and refrigeration. For the highest level of definitive care, patients are evacuated back to fixed facility hospitals such as Landstuhl Regional Medical Center or Walter Reed National Military Medical Center. These hospitals operate as any civilian medical facility with few, if any, logistical constraints.

## MILITARY ACQUISITION APPROACH

Given (1) the importance of providing an objective test to aid in the diagnose of TBI, (2) the level of maturity of platforms to evaluate TBI biomarkers, and (3) the unique abilities and constraints on each level of possible medical care, the best approach to solve this problem may be an phased acquisition approach (Mondello et al., 2011a; **Figure 1**). The first phase of development/fielding should focus on the technology that is the most mature, and potentially fieldable, not only in terms of platform logistics but also in clinical testing and familiarity with the FDA. Therefore, Phase I could focus on an automated *benchtop* system suitable for use in a fixed medical facility. Although this system is the most complex and logistically intensive, it is the most similar to the standard research ELISA that have been employed in previous testing of clinical samples. In addition, it could possibly have the highest level of sensitivity for detection of biomarkers and ultimately serve as a reference platform for confirmatory testing (higher diagnostic specificity). Phase II could be a small single assay *point-of-care system*, such as a physician's office or a field hospital. Phase II systems would ideally have the ability to screen for the presence of mild-moderate TBI and differentiate the level of severity for all TBIs. Ideally Phase II systems would be more portable than Phase I systems, have the availability of battery operation and require less refrigeration of reagents. Phase III systems could be a miniaturized *hand-held system*, most suitable for use by emergency medical personnel or personnel deployed in the remote and more austere locations on the battlefield, such as a BAS. These systems would ideally use whole blood from a finger stick to screen for the presence of TBI. They would use little power supply and require no refrigeration. Currently, Phase III systems are the least mature in development and testing for TBI biomarkers, but would provide the most use for screening of casualties (highest diagnostic sensitivity).

If all three Phases of systems were developed and deployed, one could image a scenario that starts with screening casualties for the presence of TBI biomarkers immediately after an event. Screening tests can typically have higher diagnostic sensitivity than diagnostic specificity (Ruan et al., 2009; i.e., more false positives than false negatives). Using a screening test designed in this manner will ensure you do not miss a casualty with a possible TBI, however you will have more people identified as having a TBI that could later be ruled out. The casualty could be evacuated back to the next higher level of care that can provide a more robust confirmatory test for the TBI biomarkers. At the highest level of care, the test could be more confirmatory and have a higher diagnostic specificity than sensitivity (lower number of false positive) and essentially rule in those with TBI and determine which casualties were false positives for the screening test. In addition, TBI biomarker assays at the highest level of care could also be used to possibly determine what type of injury has occurred and monitor if the injury is getting worse over time or better with treatment (Mondello et al., 2011a). This scenario also fits well with the current military levels of medical care on the battlefield. The biomarker assay screen (Phase I/II) could be completed during the initial assessment after

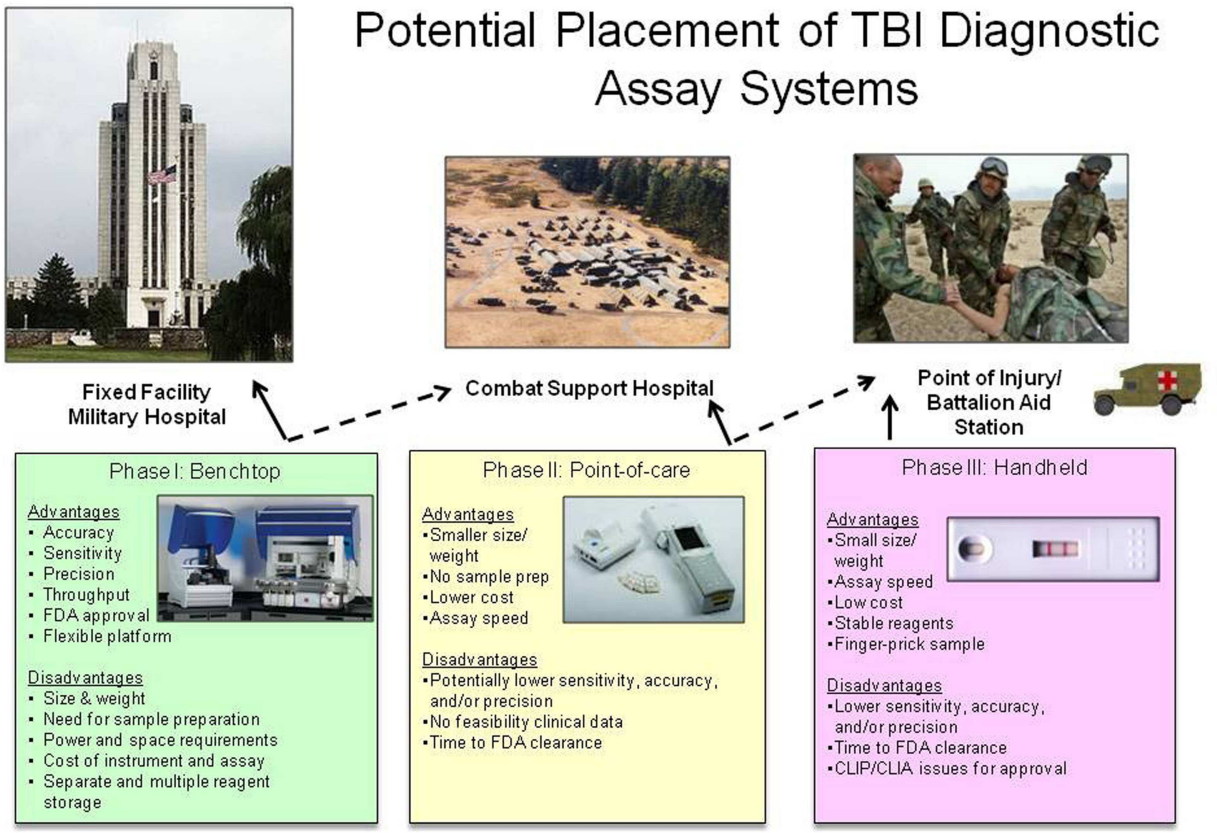


**FIGURE 1 | Potential placement of TBI diagnostic assay systems.** Each potential device has advantages and disadvantages that constrain its use in the military medical system. Solid lines represent ideal placement of each system, dashed lines represent possible placement of each system depending on logistical support. All device pictures are representative examples of commercially available devices, but do not indicate the use of TBI biomarkers on each device. Pictured benchtop device is Dynex Technologies DS2™and DSX™(http://www.dynextechnologies.com), pictured point-of-care device is Abbott Laboratories ISTAT® (http://www.abbottpointofcare.com).

injury, where the other screening test, the MACE is performed. The biomarker assay confirmatory test (Phase III) could be performed at a field hospital where other more extensive diagnostic tools are available, such as a CT.

## SUMMARY

Overall the current methods to diagnosis a TBI could be improved with the development and addition of a non-invasive, objective test for the presence of TBI. Current research and development in the field of biomarkers give hope the development of such an objective diagnostic test. However, military conditions contain logistical constraints which may require different platforms for different levels of care. Development of this non-invasive TBI diagnostic platform applicable to all levels of military care would provide much needed advances in objective triage capabilities and improved clinical management of in-Theater TBI. The ability to objectively determine the occurrence of an initial mTBI/concussion and the incidence of multiple mTBIs on our front lines of defense is critical to the success of our military operations, and to the long term health of our warfighters. Likewise, improved TBI diagnosis will also significantly advance the management of civilian patients.

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# A military-centered approach to neuroprotection for traumatic brain injury

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Studies in animals show that many compounds and therapeutics have the potential to greatly reduce the morbidity and post-injury clinical sequela for soldiers experiencing TBI. However, to date there are no FDA approved drugs for the treatment of TBI. In fact, expert opinion suggests that combination therapies will be necessary to treat any stage of TBI recovery. Our approach to this research effort is to conduct comprehensive pre-clinical neuroprotection studies in military-relevant animal models of TBI using the most promising neuroprotective agents. In addition, emerging efforts incorporating novel treatment strategies such as stem cell based therapies and alternative therapeutic approaches will be discussed. The development of a non-surgical, non-invasive brain injury therapeutic clearly addresses a major, unresolved medical problem for the Combat Casualty Care Research Program. Since drug discovery is too expensive to be pursued by DOD in the TBI arena, this effort capitalizes on partnerships with the Private Sector (Pharmaceutical Companies) and academic collaborations (Operation Brain Trauma Therapy Consortium) to study therapies already under advanced development. Candidate therapies selected for research include drugs that are aimed at reducing the acute and delayed effects of the traumatic incident, stem cell therapies aimed at brain repair, and selective brain cooling to stabilize cerebral metabolism. Each of these efforts can also focus on combination therapies targeting multiple mechanisms of neuronal injury.

**Keywords: TBI biomarkers, combination drug therapy, isobolographic, pre-clinical, neuroprotective agents**

## BACKGROUND

Historically, combat-related traumatic brain injury (TBI) has been one of the leading causes of military casualties, responsible for 20–25% of battle-incurred injuries in previous conflicts and accounting for upwards of 42% of combat-related deaths that occur "after" reaching a surgical ward (Arnold and Cutting, 1978; Leedham et al., 1993; Salazar et al., 1995; Jevtic et al., 1996; Owens et al., 2008). More recent epidemiological data generated from the current conflicts in Iraq and Afghanistan indicates that up to 30% of combat-related trauma occurs in the head and neck region and that the vast majority (over 80%) of these casualties result from blast explosion (Owens et al., 2008). Explosive devices (i.e., improvised explosive devices (IEDs), propelled grenades, mortars, mines, bombs etc) accounted for 76% of U.S. fatalities in Iraq from June 2006–December 2006 alone, demonstrating a 20% increase over blast fatalities in 2004. In large part, this is due to the fact that enemy use of IEDs has become increasingly more deadly with larger fire balls and more explosive power causing increased fragmentation (Schreiber et al., 2008).

While advances in body armor, helmets, and clinical advanced trauma life support measures have lead to a significant decrease in mortality on the battlefield (Young and Andrews, 2008) an increasing number of these patients are facing a lifetime of cognitive and physical disabilities. In 2003, over 40% of TBI survivors had a TBI related disability one year after injury (Corrigan et al., 2010). Not only do people with TBI face disability, TBIs have also been shown to increase long-term mortality and reduce life expectancy. Further, TBI is associated with the increased incidences of seizures, sleep disorders, neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, and epilepsy), neuroendocrine dysregulation, and psychiatric diseases (Masel and Dewitt, 2010; Smith et al., 2013).

Further analysis into the mechanisms of combat-related moderate/severe TBI indicates that *over 70% of blast-induced moderate to severe TBI are confounded by a penetrating injury to the brain* (Bell et al., 2009). These data come from a 5-year retrospective study (2003–2008) conducted at the National Naval Medical Center and Walter Reed Army Medical Center which reported that over half (229/408) of neurosurgical casualties evacuated from Theater had sustained a TBI from blast events and that 71% of these blast TBI victims also suffered penetrating TBIs (PTBI). From the total population, 40% (163/408) presented with a blast/PTBI whereas only 16% presented with a blast/closed-head TBI (66/408). Gunshot inflicted-PTBI accounts for an additional 13% of this patient segment. Overall, these data indicate that combat blast encounters resulting in moderate-severe TBI are more likely to have a penetrating rather than closed-head injury (Masel et al., 2012).

Although severe TBI represents the most significant life-threatening trauma, the vast majority of non-fatal TBIs (>80%) have been classified as "mild" (mTBI) typically caused by

closed-head concussion[1]. It has been estimated that up to 28% of U.S. military personnel sustained at least one concussive mTBI event while deployed in Iraq and Afghanistan (Warden, 2006). In fact, the extremely high incidence of which concussive mTBI has occurred in our soldiers has defined this combat wound as the "signature injury" of these wars (Elder and Cristian, 2009). Further, combat troops may experience increased risk of exposure to more than one concussion or mTBI in a short timeframe, the cumulative effects of which can produce long-lasting neuropsychological disorders including physical, mental, emotional, and cognitive impairments and may place our returning soldiers at increased risk for PTSD and/or neurodegenerative disorders including chronic traumatic encephalothapy (CTE) (MacGregor et al., 2011; Goldstein et al., 2012; McKee et al., 2013). Critically, TBI in military personnel is not limited to combat situations (MacGregor et al., 2012). The most recent epidemiological data from the Defense and Veterans Brain Injury Center (see text footnote 1) and the Armed Forces Health Surveillance Center (AFHSC, 2013) estimates that over 80% of military-related TBI occurs in non-deployed environments. Therefore, even in times of peace, TBI will remain a significant medical concern for the military and poses an even greater economic concern for the military as service members retire and face potential long-term consequences from brain injury.

Listed in the Guideline for Management of Severe TBI (Brain Trauma Foundation et al., 2007) are at least 14 emergency room (ER) approaches for managing severe TBI in the neuro-intensive care unit. These include, but are not limited to, hyperventilation, monitoring intracranial pressure, anti-seizure prophylaxis, infection prophylaxis, and sedation. The primary goal of these ER managements is to achieve stabilization of all vital systems and allow further assessment and treatment, particularly neuroprotective therapies that can improve neurological, motor, and cognitive functions. Presently, no drug therapy is approved as standard of care for the treatment of TBI.

Our primary mission under the directive of the Combat Casualty Care Research Program (CCCRP) is to conduct pre-clinical studies of neuroprotection therapies aimed at mitigating TBI. During the past decade and under the directive of the CCCRP, our research team established a rodent model of penetrating ballistic-like brain injury (PBBI) which was designed to model the permanent injury tract created by the path of a ballistic and the large temporary cavity generated by the ballistic energy dissipated from the penetrating object (Williams et al., 2005, 2006a,b). The PBBI model can be adjusted to represent any penetrating projectile that carries either a low (9 mm and/or fragments) or high (7.62 round = AK-47, M-16, etc.) velocity capable of producing a leading pressure or shock wave to the brain.

The unilateral frontal PBBI model has been extensively characterized and captures the acute neuropathological events associated with penetrating TBI, including lacerated brain damage, intracerebral hemorrhage, increased intracranial pressure, axonal degeneration, up-regulation of pro-inflammatory cytokines, and electrocortical disturbances (Williams et al., 2005, 2006a,b). It also produces reliable and enduring motor and cognitive deficits (Shear et al., 2010, 2011; Mountney et al., 2013) and electrophysiological insults (Lu et al., 2011, 2013), and has proven useful for assessing neuroprotective effects of promising therapeutic interventions (Lu et al., 2009b; Shear et al., 2009; Wei et al., 2009; Deng-Bryant et al., 2012). Specifically, to date we have reported evidence indicating that DM, a potent NMDA antagonist and sigma-1 receptor ligand, and NNZ-2566, a glypromate analog, and novel neuroprotective compound (Neuren Pharmaceutical Inc.) are effective in promoting functional recovery following PBBI (Lu et al., 2009a; Shear et al., 2009). We have also demonstrated that NNZ-2566 protects against PBBI-induced up-regulation of pro-inflammatory cytokines (Wei et al., 2009). Our pre-clinical NNZ-2566 data from the PBBI model has directly contributed to the recent clinical advancement of this compound into a multi-center Phase II trial for moderate-severe TBI.

More recently our research team took on the task of developing a rodent model of closed-head concussive mTBI. Our approach to this model was to produce molecular changes in the brain and alterations in behavior that would be indicative of an mTBI without making any surgical incisions and without producing any gross morphological damage like skull fracture or intracerebral hemorrhage. We recently reported the proof-of-concept development of a projectile concussive impact (PCI) injury model that produces a true closed-head concussive event resulting in significant cellular, molecular, and sensorimotor changes with no evidence of gross contusional injury (Chen et al., 2012). Studies currently underway include longitudinal and multi-modal designs to fully characterize the neuromotor, cognitive, emotional, and neuropathological evidence of concussive brain injury using the PCI model. The overall goal is to develop a more thorough understanding of the changes taking place at a cellular level following a single or multiple concussive events, for the purpose of evaluating putative therapeutic interventions.

## DRUG DISCOVERY AND DEVELOPMENT

Our approach to drug discovery and development consists of our Cooperative Research and Development Agreement (CRADA) partnerships with major pharmaceutical companies and our ongoing collaborative effort with the Operation Brain Trauma Therapy (OBTT) Consortium (Kochanek et al., 2011). Novel drug discovery and development in partnership with private pharmaceutical companies represents a critical component of our TBI/Neuroprotection Research Program. Our CRADA partnerships give us access to lead neuroprotective drug candidates keeping us at the drug discovery forefront. Importantly, our Program has a long history of successful collaborations with drug companies and our efforts have directly resulted in two clinical trials: Phase I clinical trial on MLN 519 for stroke (terminated after successful Phase I), and the Phase II clinical trial on NNZ-2566 for moderate and severe TBI (*INTREPID-2566*, ongoing). We currently have CRADAs with several private pharmaceutical companies to conduct studies assessing novel compounds in our PBBI model that target a number of different TBI mechanisms. The basic premise of this work is to first establish proof-of-principle therapeutic efficacy for a novel CRADA-sponsored drug in the PBBI model and evaluate the full dose-response monotherapy

[1]http://www.dvbic.org/dod-worldwide-numbers-tbi

profile of the most promising drugs for potential consideration as a candidate for advanced combination drug therapy studies. For the combination therapy studies, we focus primarily on the most promising neuroprotective drugs described in the TBI literature that either have already been approved by the FDA for other clinical indications, or are in the process of being advanced into clinical trials.

The OBTT is a multi-center consortium developed with the primary purpose of rapidly screening potential TBI therapies and TBI biomarkers and translating them ultimately to combat casualty care (Kochanek et al., 2011). The inception of the OBTT Consortium was predicated on the observation that the mechanistic-based approach to TBI research, which dominated the field over the past two decades, has hindered the rapid advancement of new therapies to the clinic. The primary purpose of the OBTT Consortium was to address this issue by screening drugs of high interest across three TBI rodent models with the idea the best drug(s) would be subjected to advanced testing in a TBI pig model with the ultimate goal to facilitate the rapid translation of the most promising therapies to the clinic (Kochanek et al., 2011).

## ALTERNATIVE THERAPEUTIC APPROACHES FOR TBI

### NEURAL STEM CELL TRANSPLANTATION

We have previously demonstrated that human amnion-derived progenitor (AMP) cell transplantation protects against injury-induced neuropathology and motor deficits in the PBBI model (Chen et al., 2009, 2011). However, the functional recovery observed in those studies occurred too rapidly (within 1 week post-injury) to be attributed to any host-graft functional connectivity. This suggested the transplanted cells may be mediating functional recovery through a variety of mechanisms associated with inducing neuroplasticity, including the sustained secretion of cytokines/growth factors which are abundant in amnion-derived cellular cytokine solution (ACCS).

Amnion-derived cellular cytokine solution contains physiological concentrations of dozens of factors, many of which are involved in the wound healing cascade, including the growth factors TGF-B2 and PDGF and the metalloproteinase inhibitors Timp-1, Timp-2 (Steed et al., 2008). Accordingly, ACCS has been shown to have a significant effect in a variety of burn and incisional wound healing models (Franz et al., 2008; Uberti et al., 2009; Payne et al., 2010). Our most recent work has demonstrated that chronic intracerebroventricular infusion of ACCS promoted significant protection against PBBI-induced neuropathology and motor abnormalities (Deng-Bryant et al., 2012). However, in that study ACCS was not effective in reducing cognitive deficits, nor was it effective when delivered intravenously, indicating that blood brain barrier (BBB) permeability may be a mitigating factor.

### SELECTIVE BRAIN COOLING

Research focused on elucidating the effects of mild-to-moderate therapeutic hypothermia on severe TBI has consistently demonstrated therapeutic benefits in pre-clinical studies. However, the majority of these studies have utilized whole-body cooling techniques, which may pose an increased clinical risk of adverse side effects including coagulopathy, hypotension, and infectious pneumonia in TBI patients (Shiozaki et al., 2001; Bernard et al., 2002; Milhaud et al., 2005; Hemmen and Lyden, 2007; Sydenham et al., 2009). Clinically, these adverse effects have raised serious concerns for the application of therapeutic hypothermia, particularly when treating patients with severe hemorrhage (Romlin et al., 2007). In order to maximize the potential benefits of hypothermia while minimizing the potential for adverse effects, we developed a novel method of selective brain cooling (SBC) using bilateral common carotid artery (CCA) cooling cuffs that can achieve rapid and sustained reductions in core brain temperature while maintaining normal (37 °C) body temperature (Wei et al., 2008). We recently published results demonstrating the therapeutic efficacy of SBC in the PBBI model including significant reductions in acute post-injury measures of intracranial pressure, brain edema, BBB permeability, and lesion volume (Wei et al., 2011).

## COMBINATION DRUG THERAPY DEVELOPMENT FOR TBI

Research in the TBI field has generated a plethora of data demonstrating significant pre-clinical therapeutic efficacy from over 130 drugs, which in turn has resulted in over 20 Phase II/III clinical trials over the past two decades. However, this approach has yet to succeed in producing a single therapy which has demonstrated clinically significant neuroprotective efficacy in TBI (Margulies et al., 2009). One major reason cited for these disappointing outcomes is that monotherapy approaches, that target single or limited mechanisms, are simply not adequate to address the complex and dynamic milieu of the injured brain. In recognition of the limitations of the monotherapy approach to treating TBI, increased attention is now being directed toward developing combination therapeutic strategies. This issue was addressed by a panel of TBI experts and called for a revisiting of the most promising neuroprotective agents and challenged the TBI research community to develop step-by-step strategies for pre-clinical and clinical research on combination drug therapy development (Margulies et al., 2009).

A more recent focus of our military-focused research program was to address the challenge of combination drug therapy development. Our approach to this problem was to apply the *isobolographic method* of combination drug therapy development to our TBI neuroprotection studies. The isobolographic method represents the industry "gold standard" pharmacological approach for detecting drug–drug interactions (Tallarida, 2012). This step-by-step statistical method was originally introduced in 1953 (Loewe, 1953) and has since been developed and extended by Tallarida (2012) and others, and applied to numerous pre-clinical and clinical analyses of combination data. For example, Dr. Tallarida has published >80 peer-reviewed papers and several textbooks on this subject matter and his isobolographic analysis guided the pre-clinical and clinical studies that led to a patent (U.S. 5,336,691) for the analgesic combination of tramadol and acetaminophen (Tallarida and Raffa, 1996) and to the subsequent development of the product Ultracet ©that is a synergistic combination of the two drugs.

Overall, the key criteria for a successful pre-clinical combination therapy is to (1) improve the therapeutic effects achieved via monotherapy through the *synergistic* interaction of two or more drugs administered in combination and (2) to effectively lower the risk of adverse effects by using sub-threshold doses of

the individual drugs in combination (Tallarida, 2012). Thus, the strength in the isobolic approach to combination therapy development lies in its ability to distinguish between additive and synergistic effect of drug-pairs. Of equal importance is that the isobolic approach provides a well-established statistical framework for identifying sub-additive or potentially antagonistic effects of drug-pairs that could be indicative of contraindication.

## PROGNOSTIC AND THERAGNOSTIC VALUE OF TBI BIOMARKERS

In addition to treatment, of paramount concern to the military is the lack of a rapid, objective test, or criteria for clinical diagnosis of mTBI/concussion and/or a means of tracking the chronic evolution of the TBI across all levels of injury severity. Mild cases of TBI are often under-diagnosed and under-reported, and often escape detection by brain imaging. In contrast, moderate and severe cases of TBI may be easier to detect, accurate prognostic indications and long-term therapeutic management remains a challenge.

Overall, numerous efforts across the TBI field are attempting to solve this problem and much of these efforts are reviewed in this special edition of Frontiers. TBI-specific biomarkers that have been established in experimental models of TBI and implicated in human clinical TBI studies include include S100B, glial fibrillary acidic protein (GFAP), Ubiquitin C-terminal hydrolase L1 (UCH-L1), Neuron Specific Enolase (NSE), Alpha-II spectrin breakdown products (SBDP), and Tau (Brophy et al., 2011; Mondello et al., 2011, 2012a,b). Of these, S100B has been shown to upregulate in response to other trauma in the absence of TBI and thus its diagnostic value to the military may be limited (Bloomfield et al., 2007). In contrast, serum GFAP levels have been reported to show both good specificity and sensitivity to TBI (Mondello et al., 2011, 2012a; Papa et al., 2012a) and serum levels of GFAP breakdown products have been correlated with brain imaging studies of mild and moderate TBI suggesting that GFAP may serve as a marker for mTBI (Brophy et al., 2011). Research has also shown UCH-L1 (a marker of neuronal damage) is significantly increased in the CSF of TBI patients during the acute post-injury phase and has been correlated with negative outcome (Brophy et al., 2011; Papa et al., 2012b). Alpha-II spectrin is located primarily in axons and presynaptic terminals of neurons (Riederer et al., 1986) and is cleaved by calpain and caspase 3 (Nath et al., 1996; Wang et al., 1998) representing both necrotic and apoptotic mechanisms. SBDPs have been detected in animals in both brain and CSF after moderate CCI injury (Ringger et al., 2004) and brain tissue following mild FPI (McGinn et al., 2009).

However, there still remains a critical need for research on TBI-specific biomarkers that are sensitive to the chronic evolution of TBI neuropathology and that can reliably measure the therapeutic efficacy of a particular drug. Collectively, as regards our gaps in treatment and diagnosis, there is an increased demand for preclinical TBI research addressing these concerns, particularly across animal models of mild, moderate and severe TBI.

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# Biomarkers of hypoxic-ischemic encephalopathy in newborns

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As neonatal intensive care has evolved, the focus has shifted from improving mortality alone to an effort to improve both mortality and morbidity. The most frequent source of neonatal brain injury occurs as a result of hypoxic-ischemic injury. Hypoxic-ischemic injury occurs in about 2 of 1,000 full-term infants and severe injured infants will have lifetime disabilities and neurodevelopmental delays. Most recently, remarkable efforts toward neuroprotection have been started with the advent of therapeutic hypothermia and a key step in the evolution of neonatal neuroprotection is the discovery of biomarkers that enable the clinician-scientist to screen infants for brain injury, monitor progression of disease, identify injured brain regions, and assess efficacy of neuroprotective clinical trials. Lastly, biomarkers offer great hope identifying when an injury occurred shedding light on the potential pathophysiology and the most effective therapy. In this article, we will review biomarkers of HIE including S100B, neuron specific enolase, umbilical cord IL-6, CK-BB, GFAP, myelin basic protein, UCHL-1, and pNF-H. We hope to contribute to the awareness, validation, and clinical use of established as well as novel neonatal brain injury biomarkers.

**Keywords: biomarkers, hypoxic-ischemic encephalopathy, brain injury**

## INTRODUCTION

Biomarkers are molecules released by or specific to a particular organ, can give a glimpse into the physiologic or pathologic status of that specific organ (Ling and Sylvester, 2011). Biomarkers can be obtained from the blood, urine, cerebrospinal fluid (CSF), or any other bodily fluid. In neonates with brain injury, biomarkers may be able to predict the degree and location of injury shortly after the injury occurs. The discovery of neonatal brain injury biomarkers is a key step in neonatal neuroprotection. Biomarkers may enable the clinician-scientist to screen infants for brain injury, monitor the progression of disease, identify injured brain regions, and assess the efficacy of neuroprotective strategies procedures in clinical trials. In addition, large-scale validation of the potential biomarkers is required, because the potential confounders (especially for biomarkers that are non-organ specific such as inflammatory mediators). Currently, clinicians do not routinely use biomarkers to care for neonates with brain injuries. This review will examine potential biomarkers the bedside clinician-scientist may use to hone the treatment of neonates with hypoxic-ischemic encephalopathy.

## HYPOXIC-ISCHEMIC ENCEPHALOPATHY

Systemic asphyxia manifests in the brain as hypoxic-ischemic encephalopathy (HIE; Vannucci, 1997). Systemic asphyxia occurs in about 2% of full-term infants and in nearly 60% of very low birth weight (premature) newborns (Mulligan et al., 1980; Giffard et al., 1990; Low et al., 1997). Twenty to fifty percent of asphyxiated babies who exhibit severe HIE die during the newborn period (MacDonald et al., 1980). Of the survivors of severe HIE, up to 25% have permanent neuropsychological handicaps in the form of learning disabilities, epilepsy, cerebral palsy, with or without associated mental retardation, learning disabilities, or epilepsy (Finer et al., 1981; Robertson et al., 1989). Systemic asphyxia that causes HIE may occur prior to delivery (e.g., placental abruption, toxemia, maternal collagen vascular disease), during delivery (e.g., prolonged labor, difficult delivery, abnormal presentation), or after delivery (e.g., sepsis, shock, respiratory distress). Currently, hypoxic-ischemic injury is diagnosed based on clinical criteria. This review will use the term HIE although recently medical experts have proposed use the term neonatal encephalopathy instead of HIE.

A clinician's ability to predict the outcome of neonates with HIE is not straightforward. The Sarnat grading system (Sarnat and Sarnat, 1976) stages HIE based on clinical criteria. This scoring system divides neonates into mild, moderate, or severe categories, and measures the progression of the neurologic insult to predict a neonate's prognosis (Finer et al., 1981). Nevertheless, the Sarnat score system is subjective and changes over time. A new bedside tool, amplitude integrated electroencephalogram (aEEG), may help stage the severity of injury and predict prognosis (Hellstrom-Westas et al., 1995). Unfortunately, the Sarnat score and aEEG are not as effective in predicting outcomes in neonates during hypothermia (Thoresen et al., 2010) and do not provide information about the timing of the injury. Brain MRI can help determine

**Abbreviations:** aEEG, amplitude integrated electroencephalogram; BDNF, brain derived neurotrophic factor; CPK-BB, brain type creatine phosphokinase; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; HIE, hypoxic-ischemic encephalopathy; IL-6, interleukin-6; NSE, neuron specific enolase; UCHL-1, ubiquitin carboxyl-terminal hydrolase L1.

when the injury occurred, but obtaining an MRI is not possible in unstable patients.

Recently, therapeutic hypothermia has evolved into standard of care for neonates with moderate to severe HIE. Prior to this therapy, neonates were treated with systemic supportive care with no specific therapy aimed at preventing or ameliorating ongoing brain injury. Large randomized multicenter trials demonstrated that hypothermia in neonates with moderate to severe HIE is safe, improves outcomes, and has a combined number needed to treat of one in nine (Gluckman et al., 2005; Azzopardi et al., 2009). The next step in brain neuroprotection is the identification of biomarkers that can facilitate clinical decisions. Biomarkers will help clinicians identify neonates that will respond to hypothermia and those that will need other new neuroprotective interventions. If clinicians are able to stratify patients using biomarkers, neonates will be protected from exposure to unnecessary, ineffective therapies. Furthermore, these same infants may benefit from other specific therapies more tailored to their biological profile. Biomarkers will be a key feature of future neuroprotective trials and will help gage the intervention's short- and long-term efficacy.

## BIOMARKERS OF HYPOXIC-ISCHEMIC ENCEPHALOPATHY

To date, potential biomarkers have been identified in neonates with HIE. These biomarkers were obtained from CSF, serum, and urine and include S100B, neuron specific enolase (NSE), umbilical cord Interleukin-6 (IL-6), CPK-BB, glial fibrillary acidic protein (GFAP), myelin basic protein, Ubiquitin carboxyl-terminal hydrolase L1 (UCHL-1), and pNF-H (see **Table 1**).

As discussed above, a primary goal of biomarkers is to identify injury and predict long-term outcomes. The best sources for biomarkers in critically ill neonates are those fluids obtained the least invasively. Therefore, an ideal biomarker would come from the urine or saliva. Ideally, biomarkers could be collected shortly after birth and help to determine the time at which the hypoxic-ischemic injury occurred and predict the neonate's outcome. Counter-intuitively, biomarkers that do not originate from brain could be good predictors of outcomes such as death and long-term neurodevelopmental handicaps. For example, IL-6 is an inflammatory cytokine produced by T-cells and macrophages, and was found by Chiesa et al. (2003) to be 376-fold higher in 50 infants without infection who developed HIE compared to 113 normal infants. The IL-6 concentrations was 5.5-fold higher in the HIE infants than the asphyxiated newborns without HIE. In addition IL-6 concentrations were significantly related to the severity of HIE and the neurodevelopmental outcome at 2 years of age. Maternal serum IL-6 concentration did not correlate with the risk of neonatal HIE.

**S-100** is a calcium binding protein and is a major component of the cytosol in various cell types. In particular, glial cells have a high concentration of S100B. S100B immunoassay kits are commercially available and can detect S100B in many biological fluids (urine, blood, CSF, amniotic fluid, saliva, and milk; Gazzolo and Michetti, 2010; Gazzolo et al., 2010). Furthermore, reference ranges are available for newborns and children through age three (Bouvier et al., 2011) and urine S100B reference ranges for preterm and term healthy newborns (Gazzolo et al., 2007). Serum S100B concentrations in healthy children are higher than concentrations reported in adults. These serum concentrations decrease over time, especially during the first 6 months after birth. Similarly, urinary S100B protein concentrations are higher in premature infants than in term newborns and steadily decrease with advancing GA.

Gazzolo et al. (2004) demonstrated that S100B concentrations in the first urine after birth were significantly higher in HIE patients than in controls. S100B has been investigated in cord blood samples and has been linked to HIE. Cord blood of 40 neonates with HIE had elevated S100B protein concentrations when compared with controls (Qian et al., 2009). In the same study, concentrations of S100B greater than 2.02 μg/L had a sensitivity of 86.7% and a specificity of 88% for predicting the development of moderate or severe HIE.

Gazzolo et al. (2009) also demonstrated that an S100B concentration cut-off of 0.41 mcg/L had a sensitivity of 91.3% and

**Table 1 | Summary of biomarkers characteristics.**

| Biomarker | Description | Cell specificity | Pathophysiology of high plasma concentrations |
|---|---|---|---|
| *BDNF* | Neurotrophic factor | Secreted by *neural progenitor stem cells, astrocytes, and neurons.* There are trace amounts in platelets | Released after brain injury (neuronal and astrocyte cell death) but concentration can be altered by exercise, depression, and autoimmune disease |
| *S100*β | It is a protein that binds calcium and is a major component of the cytosol in various cell types | *Astroglial* cells have a high concentration of S100B. Other cells can release S100B | Released predominantly after astrocyte death but can be released from other tissue damage |
| *GFAP* | It is a cytoskeletal intermediate filament protein found in the astrocytes | Specific marker of *differentiated astrocytes* | Released after astrocyte death |
| *NSE* or neuron specific enolase or enolase 2 | Glycolytic isoenzyme (γγ) | High concentrations of NSE are found in mature central and peripheral *neurons.* Although there are trace amounts of similar isoenzyme (αγ) in platelets | Released after neuronal death |

*Summarizes main biomarkers for hypoxic-ischemic encephalopathy including its description, cell specificity, and pathophysiology of high plasma concentrations.*

a specificity of 94.6% for predicting the development of HIE. The sensitivity and specificity increased to 100 and 98.8%, respectively, when urine samples were collected at 4–72 h after birth. In another study of 132 term infants, urinary S100B concentrations were higher in infants who suffered perinatal asphyxia or died and urine S100B above 1 mcg/L predicted neonatal death with a sensitivity and specificity of 100%. A study by the same group demonstrated that urinary S100B concentrations were not affected by renal failure (Risso et al., 2011).

Glial fibrillary acidic protein is a cytoskeleton intermediate filament protein of the astrocytes and is only released into the blood upon astrocyte death. GFAP have been correlated with poor outcomes in adult patients after stroke, cardiac arrest, or traumatic brain injury (Pelinka et al., 2004a). GFAP has been used as a predictor of mortality or poor neurological outcomes in children requiring extracorporeal membrane oxygenation (Pelinka et al., 2004b; Vos et al., 2004; Lumpkins et al., 2008; Kaneko et al., 2009; Bembea et al., 2010). A recently published pilot study compared 23 HIE neonates who met the criteria for hypothermia with 23 NICU patients without neurologic injury (Ennen et al., 2011). The patients with HIE had significantly elevated GFAP concentrations when compared with controls. In addition, a GFAP equal to or greater than 0.15 ng/mL upon NICU admission was predictive of an abnormal brain MRI.

Other serum biomarkers have been explored to predict long-term neurologic deficits after neonatal asphyxia. In a recent meta-analysis, Ramaswamy et al. (2009) pooled data from published studies of neonatal HIE biomarkers that followed patients beyond 12 months of age. Serum and CSF concentrations of IL-1b, IL-6, and serum NSE were predictive of abnormal outcomes. In addition, high GFAP concentrations in CSF were predictive of death.

Neuron specific enolase belongs to the family of enolases, enzymes present in all tissues and organisms capable of glycolysis. Enolases have three subunits (α, β, and γ) each one encoded by separate genes. The subunits can combine to form five different isoenzymes: αα, αβ, αγ, ββ, and γγ. Enolase 1 (αα) is found in liver, kidney, spleen, and adipose tissue. Enolase 3 (ββ) is muscle specific enolase. Enolase 2 (γγ) is NSE found in central and peripheral neurons and neuroendocrine cells. The mature neurons and glia can be distinguished by the content of enolase: neurons only have NSE and glia express enolase 1 (Marangos et al., 1980a). Minimal quantities of enolase can be found in platelets (0.045% of the total soluble protein of platelets); nevertheless most of the enolase found in platelets is αγ subunits (Marangos et al., 1980b). High levels of NSE in CSF and serum are correlated with poor outcome in patients with cardiac arrest (Roine et al., 1989; Rundgren et al., 2009), in patients with cerebrovascular accident (Hay et al., 1984) and pediatric patients with traumatic brain injury (Berger et al., 2005). Detection of NSE in peripheral serum is only expected to occur after both, neuronal death and disruption of the blood brain barrier. Animal models (Costine et al., 2012) have demonstrated a correlation between the volume of cortical injury and levels of NSE following a traumatic brain injury. Elevated serum NSE concentrations in neonates undergoing cardiac surgery correlate with poor prognosis even when parallel samples of CSF do not reveal elevated NSE levels (Schmitt et al., 1998).

Celtik et al. (2004) explored serum neuron specific enolase as a predictor of HIE severity. According to ROC curves, serum NSE above 40 mcg/L obtained between 4 and 48 h could distinguish infants with no or mild HIE from infants with moderate or severe HIE. Additionally, serum NSE concentrations with a cut-off point of 45.4 mcg/L could distinguish infants with poor outcomes from infants with normal outcomes.

Analyses of brain MRIs in patients with HIE have identified the most common patterns of brain injury: basal ganglia injury, diffuse or focal cortical injury, and injury to watershed areas of the cortex. Two studies have attempted to correlate biomarkers of HIE with various MRI patterns of brain injury. Ennen et al. (2011) found that high serum GFAP concentrations in the first 2 days of life in neonates undergoing whole body hypothermia correlate with abnormal brain. Douglas-Escobar et al. (2010) measured serum **UCHL-1** (found in neuronal cell bodies) and **pNF-H1** (found in white matter brain regions) in patients with severe HIE and controls. Correlations were found between the serum levels and the MRI patterns of injury. Both studies were pilot studies with very low patient numbers therefore need further validation. The ability to predict the outcomes of HIE patients may be improved when biomarkers are used in combination with brain MRI. For example, combining trajectory of biomarkers such as NSE with MRI, improved the long-term prognostic prediction (Berger et al., 2010).

A final interesting category of potential biomarkers is the neurotrophins. Brain derived neurotrophic factor (BDNF) is a neurotrophin that binds to the TrkB and $p75^{NTR}$ receptors. BDNF supports the survival of existing neurons and encourages the growth and differentiation of new neurons and synapses. Imam et al. (2009) described higher cord plasma BDNF levels in newborns with HIE when compared with control neonates. These elevated BDNF levels predicted poor neurologic outcomes. Our laboratory has found evidence that brain BDNF concentrations are increased after rodent model of neonatal HI, similar to reports by others in the post-stroke milieu (Bejot et al., 2011). Researchers have postulated that BDNF increases the migration of stem cells (Borghesani et al., 2002). We can speculate from animal models, the high plasma concentrations of BDNF are reflection of high brain BDNF concentrations released by neural progenitor cells and astroglia cell in an attempt to foster brain cell recovery.

## FUTURE DIRECTIONS

Hypothermia is the most promising of the neuroprotective therapies that have emerged over the past decade and is rapidly becoming the baseline therapy upon which future neuroprotective agents will be added. However, only one in eight neonates treated with hypothermia respond to the treatment. Biomarkers may help the bedside clinician identify neonates that will responders and non-responders to hypothermia. Non-responder patients could to be selected to add new neuroprotective strategies. Biomarkers may help to determine the time that the injury occurred. This is important, because hypoxic-ischemic injury often begins *in utero and if* too much time has elapsed from the brain injury, neonates would not benefit from treatment with hypothermia. This may explain why some neonates with HIE do not respond to hypothermia. The timing of injury also has major medico-legal ramifications for the obstetric and neonatal team taking care of the infant.

**Table 2 | Biomarkers and their potential use in neonatal brain injury.**

| Biomarker | Category | Fluids locations | Associations | Usefulness |
|---|---|---|---|---|
| S100β | Brain-specific protein | Cord blood, urine, saliva, milk blood, CSF | Pregnancy complicated with growth restriction and trisomy 21<br>Neonates with asphyxia and HIE<br>Mortality in term newborns | ++ |
| Interleukin 6 | Inflammatory maker | Cord blood and blood | Neonates with HIE | + |
| GFAP | Brain-specific protein | CSF | Neonates with HIE | + |
| Neuron specific enolase | Brain-specific protein | Blood and CSF | Neonates with HIE, mortality | +++ |

*Summarizes potential biomarkers of asphyxia and hypoxic-ischemic encephalopathy (HIE). These biomarkers have been detected in blood, urine, saliva, milk, cerebrospinal fluid (CSF), and brain tissue. Usefulness of the biomarkers: (+) limited use because CSF samples are required, (++) very useful but can be altered by other factors such as gestational age and intrauterine growth restriction, (+++) very useful because it is more specific to brain injury and detected in serum.*

Using a panel of biomarkers for neonatal brain injury also holds the promise of allowing for more individualized care of neonates (see **Table 2**). For example, certain neonates may have more of an inflammatory component than others. Once identified, these patients could be treated with agents that minimize the inflammatory cascade. Serum levels of biomarkers could also be utilized to monitor the neonate's response to pharmacologic agents. A decrease in plasma biomarker concentrations could potentially indicate a preservation of endogenous tissue.

Biomarkers may also be able to identify specific brain regions that undergo injury following HIE. These regions may respond better to a specific treatment. Therefore, in the future panel of biomarkers may be utilized to identify injury to particular brain regions. To date, none of the examined biomarker trials have predicted the aforementioned due to small patient numbers.

*In summary, more studies are needed to correlate and validate the clinical use of possible biomarkers of hypoxic-ischemic brain injury. In the future, more sensitive and precise instruments for brain imaging (such as brain MRI), brain functioning (such as NIRS, aEEG), and long-term neuro-assessment should be incorporated to validation of biomarkers of neonatal brain injury.*

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# Biomarkers of brain injury in the premature infant

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The term "encephalopathy of prematurity" encompasses not only the acute brain injury [such as intraventricular hemorrhage (IVH)] but also complex disturbance on the infant's subsequent brain development. In premature infants, the most frequent recognized source of brain injury is IVH and periventricular leukomalacia (PVL). Furthermore 20–25% infants with birth weigh less than 1,500 g will have IVH and that proportion increases to 45% if the birth weight is less than 500–750 g. In addition, nearly 60% of very low birth weight newborns will have hypoxic-ischemic injury. Therefore permanent lifetime neurodevelopmental disabilities are frequent in premature infants. Innovative approach to prevent or decrease brain injury in preterm infants requires discovery of biomarkers able to discriminate infants at risk for injury, monitor the progression of the injury, and assess efficacy of neuroprotective clinical trials. In this article, we will review biomarkers studied in premature infants with IVH, Post-hemorrhagic ventricular dilation (PHVD), and PVL including: S100b, Activin A, erythropoietin, chemokine CCL 18, GFAP, and NFL will also be examined. Some of the most promising biomarkers for IVH are S100β and Activin. The concentrations of TGF-β1, MMP-9, and PAI-1 in cerebrospinal fluid could be used to discriminate patients that will require shunt after PHVD. Neonatal brain injury is frequent in premature infants admitted to the neonatal intensive care and we hope to contribute to the awareness and interest in clinical validation of established as well as novel neonatal brain injury biomarkers.

**Keywords: biomarkers, intraventricular hemorrhage, post-hemorrhagic ventricular dilation, periventricular leukomalacia, brain injury**

## INTRODUCTION

Increasing rates of survival of extremely premature infants has produced a shift of paradigms from "survival" to "prevention of morbidity" including brain injury. The discovery and validation of neonatal biomarkers of brain injury is a key step in the evolution of neonatal neuroprotection. These markers may enable the clinicians to screen infants for brain injury, monitor the progression of disease, identify injured brain regions, and assess the efficacy of neuroprotective strategies procedures in clinical trials. Currently, clinicians do not use biomarkers to care for neonates with brain injuries. This review will examine potential biomarkers for the most common brain injuries in premature infants, such as intraventricular hemorrhage (IVH), post-hemorrhagic hydrocephalus, and periventricular leukomalacia (PVL).

## BIOMARKERS OF INTRAVENTRICULAR HEMORRHAGE

One major source of long-term neurologic deficits in premature neonates is the injury to the germinal matrix and the subventricular zone (Volpe, 2009). These injuries produce IVH. Approximately 20–25% of premature infants weighing less than 1,500 g will have an IVH (Volpe, 2008). The risk of IVH is inversely related to gestational age and birth weight. Forty-five percent of infants weighing 500–750 g develop IVH (Wilson-Costello et al., 2005). Immature blood vessels in the germinal matrix, a highly vascular region of the brain, combined with poor tissue vascular support, predispose premature infants to hemorrhage (Volpe, 2009). Clinical presentation of IVH can range from an acute newborn deterioration of the newborn (with apnea, pallor, acidosis, hypotension, bulging fontanel, seizures, and decreased muscle tone) to a "clinically silent syndrome" (no symptoms). Biomarkers that hold promise in predicting which neonates may suffer an IVH and long-term deficits will be reviewed.

*The protein S100 family* encompasses many calcium sensor proteins that modulate biological activity via calcium binding (Ikura, 1996). In particular, *S100*β (a homodimer of the subunit beta) protein is primarily synthesized in the brain by astrocytes and is quickly released from the brain into the blood when the blood-brain-barrier is disrupted (Kapural et al., 2002; Marchi et al., 2003). In the central nervous system S100β protein is predominantly concentrated in the astroglial cell population (Heizmann, 1999). However, reports of extra cranial sources of S100β, especially from adipose and muscle tissue, may confound its interpretation in the clinical setting (Otto et al., 2000; Bloomfield et al., 2007). S100β has a dual function depending on its concentrations. At nanomolar physiological concentrations, S100β is neurotrophic (Haglid et al., 1997). However, when S100β is overexpressed (in micromolar concentrations), it enhances neuroinflammation and

**Abbreviations:** aEEG, amplitude integrated electroencephalogram; CPK-BB, arain-type creatine phosphokinase; CSF, cerebrospinal fluid; CCL18, chemokine ligand 18; CRP, C-reactive protein; EPO, erythropoietin; GA, gestational age; IVH, intraventricular hemorrhage; MMP-9, matrix metalloproteinase-9; PVL, periventricular leukomalacia; PAI-1, plasminogen activator inhibitor 1; TGF-β1, transforming growth factor beta 1; TGF-β2, transforming growth factor beta-2; UA, uric acid.

neuronal apoptosis (Van Eldik and Wainwright, 2003). Recently an excess of S100β and amyloid precursor protein has been linked to impaired neurogenesis (due to the gliocentric shift of neural progenitor cells) in Down syndrome (Bouvier et al., 2011; Lu et al., 2011).

S100B has been well studied in the pediatric population. Immunoassay kits are commercially available and can detect S100β in many biological fluids such as urine, blood, CSF, amniotic fluid, saliva, and milk (Gazzolo and Michetti, 2010; Gazzolo et al., 2010). Furthermore, reference ranges are available for the pediatric population including preterm and term healthy newborns (Gazzolo et al., 2007; Bouvier et al., 2011). In general, healthy children have higher serum S100β concentrations than adults and the concentrations decline over time, especially during the first 6 months of life (Bouvier et al., 2011). Similarly, urinary S100β concentrations are higher in premature infants than in term newborns and steadily decrease with advancing gestational age (Gazzolo et al., 2007).

Gazzolo et al. (2006) reported that maternal blood concentrations of S100β >0.72 mcg/L were able to predict neonatal IVH with 100% sensitivity, 99% specificity, and 0.999 area under the ROC curve. However, measurements of serum S100β during pregnancy could be affected by multiple factors, such as gestational age, intrauterine growth, prenatal steroids use, twin gestation, and trisomy 21 (Gazzolo et al., 2003a,b; Sannia et al., 2010).

Premature infants with high concentrations of S100β in urine have higher mortality than matched controls for gestational age and weight with a positive predictive value of 78% and a negative predictive value of 100% (Gazzolo et al., 2005). S100β also plays a role in predicting IVH in neonates. Newborns that developed IVH have elevated S100β concentrations in blood and urine (Gazzolo et al., 1999, 2001). In addition, the urine S100β level correlates with the degree of IVH (Gazzolo et al., 2001). Taken together, these publications support the hypothesis that early brain injury may be responsible for a continuous release of S100β protein from the CNS into the systemic circulation and urine. Because IVH is more frequent in very small infants with birth weight 500–750 g, biomarkers that do not require blood samples are more clinical relevant. Therefore, one major advantage of S100β is that it can be measured in urine. In addition, there are commercially available kits to measure S100β. We believe that the next step in the validation of S100β as biomarker of IVH would be studies that incorporate its association with brain injury assessed by brain MRI and long-term functional outcomes.

*Activin* is another proposed biomarker for IVH. Activin, a member of the transforming growth factor-β superfamily, is a trophic factor that regulates differentiation and proliferation of neurons and a wide variety of cells (Florio et al., 2007). Activin receptors are highly expressed in neuronal cells and neuronal activity up-regulates activin mRNA expression (He et al., 2012). In animal models, Activin is neuroprotective during excitotoxic brain injury (Mukerji et al., 2007). In transgenic mice, activin regulates spine formation, behavioral activity, anxiety, adult neurogenesis, late-phase long-term potentiation, and the maintenance of long-term memory (Ageta et al., 2008; Zheng et al., 2009). Florio et al. (2006) found that premature newborns that developed IVH had high concentrations of Activin A in blood samples drawn during their first hour of life. In his cohort of 53 infants <32 weeks gestational age, 21% developed IVH detected by serial head ultrasound (HUS). Activin above 0.8 mcg/L predicted IVH with 100% sensitivity of 100%, and 93% specificity (with positive predictive value of 79%). Activin A is also increased in term newborns with moderate or severe asphyxia suggesting that activin is released after neuronal (Florio et al., 2004). Activin A should be validated in larger cohort of premature infants and correlated not only with IVH diagnosed by HUS but also with term corrected brain MRI (a more sensitive and specific brain injury detection).

*Erythropoietin* (EPO) is also a potential biomarker of IVH. EPO and its receptor are expressed in astrocytes, neurons, and endothelial brain cells (Marti, 2004). Bhandari published a prospective pilot cohort study of cord blood concentration of EPO in 116 infants less than 34 weeks GA (Bhandari et al., 2011). In this study, 25% infants had IVH diagnosed by serial HUS. Elevated cord blood EPO levels were predictors of IVH even after correction for gestational age. In the same cohort, inflammatory markers (such cord blood IL-6, pH, and early onset of neonatal sepsis) were not associated with IVH. These results suggest that elevated cord blood EPO may predict neonatal risk for IVH, independent of fetal inflammatory status. EPO production is increased in response to fetal hypoxia (Davis et al., 2003; Teramo and Widness, 2009). Thus, elevated EPO in cord blood may indicate fetal hypoxic conditions that lead to injury of the germinal matrix resulting in IVH. EPO is attractive as a biomarker because it can be measured at birth and the results are available the same day. Nevertheless it is important to establish if high levels Epo correlate with functional outcomes.

*Chemokine ligand 18 (CCL18)* belongs to the CC-chemokine family, is encoded in chromosome 17q11.2 and participates in the lymphocytes homing and the primary immune response (Zlotnik et al., 2006). As a result, inflammatory conditions may increase levels of CCL18 (Schutyser et al., 2005). Preterm infants who developed cerebral palsy have lower cord blood levels of CCL18 (Kaukola et al., 2004). Patients with traumatic brain injuries have elevated CCL18 in biopsies of brain tissue (Chang et al., 2010). In a prospective cohort study of 163 premature infants (less than 32 weeks of gestation), Kallankari et al. (2010) analyzed 107 cord blood immunoproteins, 12 cytokines from the peripheral blood, serial HUS in all newborns, and brain immunohistochemistry of chemokine receptors from the autopsies of 14 patients. Cord chemokine CCL18 robustly predicted the risk of IVH grades II-IV and was not associated with chorioamnionitis or funisitis. CCL18 receptor was detectable in the choroid plexus, periventricular capillary endothelium, ependymal cells, and the germinal matrix which may explain that high cord levels of CCL18 could be protective against IVH and brain injury by blocking the action of agonistic ligands on CCR3, thereby inhibiting leukocyte degranulation and inflammatory activation. Because CCL18 is an inflammatory mediator, it may be a very sensitive but not specific biomarker for IVH.

*Uric acid* (UA) is the end product of purine metabolism. Because UA has poor solubility, continuous renal excretion is necessary to avoid its toxic accumulation. High serum UA concentrations are expected when there are increase in production or decrease in its excretion. During hypoxic-ischemic events, hypoxanthine, a purine intermediate metabolite, accumulates. During the reperfusion states, hypoxanthine is then converted to UA (Perlman and Risser, 1998). Perlman and Risser (1998) reported elevated concentrations of UA (during first 24 h) in premature

infants that developed IVH and PVL. In support of these findings, Aliefendioglu et al. (2006) found that high UA concentrations in CSF were associated with a higher risk of IVH. However, other study of low birth weight infants did not find association between elevated UA in serum and IVH (Sysyn and Rozycki, 2003). UA has conflicting results as brain injury biomarker.

*Brain-type creatine phosphokinase (CPK-BB)* is an enzyme expressed in various cell types and catalyzes the conversion of creatine to phosphocreatine (energy reservoir for cells that consume ATP rapidly). Van de Bor et al. (1988) found that higher serum CPK-BB during the first day of life was associated with IVH detected by serial HUS. Nevertheless, the small number of newborns with severe IVH limited the interpretation of the results of this pilot study. In a later study, Amato et al. (1989) found that only the CPK-BB values obtained during the first 6 h of life, but not later, were associated with IVH. This early elevations of CPK-BB concentration suggest association with prenatal events. One plausible explanation is that during hypoxic-ischemic events, brain cells deplete their ATP and increase CPK-BB concentrations to obtain more phosphocreatine (other source of cell energy). In our opinion, this biomarker shows limited predictability for brain injury because it is not specific to brain injury and is only increased for few hours.

*IL-6 and C-reactive protein (CRP)* are inflammatory markers, therefore non-specific markers of brain injury. Sorokin et al. followed a cohort of 475 asymptomatic pregnant women at risk for preterm birth. He found that high maternal serum concentrations of IL-6 and CRP were associated with increase risk of IVH in their premature infants even after adjusting for gestational age (Sorokin et al., 2010). Notably, 25 out of 30 neonates had grade I IVH. It is possible maternal inflammatory markers reflect the fetal environment and therefore could be associated with brain pathology but they are not specific for brain injury.

## BIOMARKERS FOR POST-HEMORRHAGIC VENTRICULAR DILATION

Following a large IVH, blood clots throughout the ventricular system may block the channels for the reabsorption of cerebrospinal fluid and the lateral ventricles enlarge producing Post-hemorrhagic ventricular dilation (PHVD; Whitelaw and Aquilina, 2012). Overtime, the chronic inflammation, free iron, free radicals, and the excessive intracranial pressure produce not only ventricle enlargement but also progressive periventricular white matter injury. Patients with PHVD have worse outcomes: 40% develop cerebral palsy and 25% have multiple impairments (Ventriculomegaly trial group, 1994; Kennedy et al., 2001). Patients with large amounts of blood clots in the ventricles have a higher risk for shunt placement (Whitelaw, 2001; Whitelaw and Aquilina, 2012). Deciding when to intervene in patients with PHVD could be a challenge in part due to the small size of the patient (often between 1 and 2 kg) and complications related to drains. There is no precise test that could help to determine when is the best time to place a drain (external drain or shunt) to avoid secondary periventricular brain damage (Davies et al., 2000; Whitelaw and Aquilina, 2012). The following biomarkers are associated with PHVD and need for shunt:

*Transforming growth factor beta 1 (TGF-β1)* is released into CSF after intraventricular bleeding and up-regulates the genes to increase production of extracellular matrix (ECM) proteins such as collagen and fibronectin (Whitelaw and Aquilina, 2012). Excessive production of ECM could lead to blockage of CSF reabsorption. Therefore, TGF β1 could serve as a biomarker of PVHD. Whitelaw et al. (1999) found higher concentrations of TGF β1 in CSF of preterm infants with PHVD than preterm controls. Among the PHVD patients, those with highest TGF β1 concentrations had higher rate of shunt placement. TGFβ1 > 6.5 ng/mL in CSF was 80% sensitive and 78% specific to discriminate which infants with PHVD would require a shunt. By contrast, Heep et al. (2004) found that increased concentrations of TGFβ-1 in CSF did not correlate with PHVD but correlated with white matter injury. Lipina et al. (2010) reported that patients with PVHD with TGF β-1 > 2,396 pg/mL had a sensitivity of 79% and specificity of 80% to predict which patients would not benefit of endoscopic third ventriculostomy (surgery that allow CSF to leave the ventricular system) and need shunt placement. Elevated TGF β-1 is associated with worse course of PHVD either because patient has higher risk of shunt placement or white matter injury. Unfortunately, this biomarker is only measured in CSF and need validation in larger studies.

*Transforming growth factor beta-2 (TGF-β2)* is associated with a decreased proliferation of neuronal precursors and induction of cell death of oligodendrocytes. Chow et al. (2005) found that in patients with PHVD, TGF β-2 in CSF was 20 times greater if patients required shunt and it was associated with worse neurodevelopment outcome at 15 months. It is possible that high concentrations of TGF-β2 in CSF correlate with worse prognosis due to its effects with neurons and oligodendrocytes. This potential biomarker needs validation in larger cohort of patients.

*Proteins of the matrix metallo-proteinases (MMPs)* family are involved in the breakdown of ECM proteins (Rosell et al., 2008). Okamoto et al. (2008) found that *MMP-9* in CSF was higher in patients with PHVD than controls. Higher MMP-9 concentrations possible reflect ongoing brain tissue remodeling in patients with PHVD. Patients with PHVD that required a shunt had higher MMP-9 levels than controls but lower MMP-9 concentrations than PHVD patients without need for a shunt. A plausible explanation for this finding is that patients with lower MMP-9 concentrations could not degrade the amount of extracellular proteins produced and end with CSF outflow obstruction requiring shunt placement. Validation studies are necessary for this pilot report.

*Plasminogen activator inhibitor 1 (PAI-1)* is one of the main inhibitors of fibrinolysis (physiological breakdown of blood clots; Booth et al., 1988). Hansen et al. showed that CSF concentration of PAI-1 was highest in patients with PHVD compared with neonates with IVH without PHVD (Hansen et al., 1997, 2000). It is possible that neonates who develop IVH and have impaired blood breakdown due to high concentrations of PAI-1 would develop PHVD. This is promising biomarkers but needs more validation studies.

Whitelaw et al. (2001) found that *median neurofilament (NFL) and glial fibrillary acidic protein (GFAP)* concentrations in infants with PHVD were 20–200 times higher than controls. In the same study, patients with PHVD had four times higher S100 protein in CSF than control patients and GFAP concentrations correlated with death or disability. We think that NFL and GFAP are plausible biomarkers of PHVD but they required CSF samples. Again, this

**Table 1 | Summary of biomarkers characteristics.**

| Biomarker | Description | Cell specificity | Pathophysiology |
|---|---|---|---|
| S100β | Protein that binds calcium and is a major component of the cytosol in various cell types (Ikura, 1996) | *Astroglial* cells have a high concentration of S100β (Heizmann, 1999). Other cells can release S100β (Bloomfield et al., 2007) | Increased concentrations of S100β occur predominantly after astrocyte death (Van Eldik and Wainwright, 2003) |
| Activin A | Trophic factor, member of the transforming growth factor-β superfamily (Florio et al., 2007) | Activin receptors are highly expressed in *neuronal* cells (Florio et al., 2007) | Increased concentrations of Activin A occur predominantly after neuronal injury (Florio et al., 2006, 2007) |
| Erythropoietin | Trophic factor and is synergistic with other growth factors (Marti, 2004) | Produced mainly by interstitial fibroblasts in the kidneys and placenta and hepatocytes in the fetus (Davis et al., 2003). EPO and its receptor are expressed throughout the brain in *glial cells, neurons*, and *endothelial cells* (Marti, 2004) | Increased concentrations of EPO occur after hypoxic conditions (endogenous mechanism neuronal protection; Marti, 2004; Teramo and Widness, 2009) |
| Chemokine CCL18 | Member of the CC-chemokine family (Zlotnik et al., 2006) | Monocytes and dendritic cells secrete CCL18. CCL18 receptor is detectable in the choroid plexus, periventricular capillary endothelium, ependymal cells, and the *germinal matrix* (Kallankari et al., 2010) | High concentrations of CCL18 blocks the action of agonistic ligands on CCR3 (decreasing inflammatory response) and could be protective factor for IVH (Chang et al., 2010; Kallankari et al., 2010) |
| TGF-b1 | Member of the transforming growth factor-β superfamily (Pal et al., 2012) | Main sources of TGF-β1 in the injured brain are *astrocytes* and *microglia* but *neurons* can produce it as well (Heinemann et al., 2012; Pal et al., 2012). TGF-β1 released into CSF after IVH, up-regulate the genes for *extracellular matrix* (Whitelaw and Aquilina, 2012) | High concentrations of TGF- (β1 may trigger excessive production of ECM leading to blockage of CSF reabsorption, therefore could serve as biomarker of PVHD (Whitelaw et al., 1999) |
| MMP-9 | Member of the proteins of the matrix metallo-proteinases (MMPs) family (Rosell et al., 2008; Pal et al., 2012) | *All cell types of the CNS* are potential sources of MMPs. MMP-9 is involved in the breakdown of *extracellular matrix* proteins (Okamoto et al., 2008; Rosell et al., 2008) | Higher concentrations of MMP-9 are needed to degrade the extracellular proteins after IVH. Lower MMP-9 concentrations in CSF of patients with PVHD could predict patients that will need shunt (Okamoto et al., 2008) |
| PAI-1 | Main inhibitor fibrinolysis (Booth et al., 1988) | PAI is mainly produced by *vascular endothelial cells*, but also secreted by many other tissues (hepatic, adipose, etc.). This protein that *inhibit* tissue plasminogen activator and urokinase, the *activators of plasminogen* (Booth et al., 1988) | High concentrations of PAI-1 in CSF could impaired blood removal (fibrinolysis) after IVH, leading to PVHD (Hansen et al., 1997, 2000) |
| GFAP and NFL | Cytoskeletal intermediate and median filament protein found in the astrocytes (Mayer et al., 1989; Eng and Ghirnikar, 1994; Middeldorp and Hol, 2011) | Specific marker of *differentiated astrocytes* (Middeldorp and Hol, 2011) | Higher concentrations of GFAP and NFL in CSF are expected after astrocyte death (Whitelaw et al., 2001) |

*Summary of potential biomarkers of brain injury including intraventricular hemorrhage (IVH), and post-hemorrhagic ventricular dilation (PHVD).*

is promising pilot data but needs confirmation with large number of patients.

## BIOMARKERS OF PERIVENTRICULAR LEUKOMALACIA

Periventricular leukomalacia is a cerebral white matter injury that occurs to some degree in 50% of neonates with birth weights less than 1,500 g (Volpe et al., 2011). PVL is associated with a decrease in the volume of the cortex, thalamus, and basal ganglia (Volpe et al., 2011). This injury likely account for 90% of the neurologic deficits, including cerebral palsy, cognitive, behavioral, and attention deficits, that occurs in surviving premature neonates. In addition, up to 50% of neonates with

**Table 2 | Most promising biomarkers and usefulness in neonatal brain injury.**

| Biomarker | Fluids locations | Change | Associations (reference) | Usefulness |
|---|---|---|---|---|
| S100β | Urine, Blood, CSF | ↑ | IVH (Gazzolo et al., 1999, 2007, 2010) | ++ |
| | | | Asphyxia and HIE (Gazzolo et al., 2001, 2005) | |
| Activin A | Blood | ↑ | IVH (Florio et al., 2006, 2007) | ++ |
| | | | Asphyxia (Florio et al., 2004) | |
| Epo | Blood | ↑ | IVH (Bhandari et al., 2011) | ++ |
| CCL18 | Blood | ↓ | Lower concentrations in neonates that developed IVH (Kallankari et al., 2010) | ++ |
| TGF-β1 | CSF | ↑ | PHVD (Whitelaw et al., 1999, 2001; Lipina et al., 2010) | + |
| | | ↑ | PHVD patients that that required shunt (Whitelaw et al., 1999) | +++ |
| TGF-β2 | CSF | ↑ | PHVD (Chow et al., 2005) | + |
| | | | PHVD patients that develop white mater injury and worse neurodevelopmental outcomes at 15 months (Chow et al., 2005) | + |
| MMP-9 | CSF | ↑ | PHVD (Okamoto et al., 2008) | + |
| | | ↓ | Lower concentration in neonates with PHVD that required shunt (Okamoto et al., 2008) | +++ |
| PAI-1 | CSF | ↑ | PHVD (Hansen et al., 2000) | + |
| | | | Highest concentration observed in neonates that required shunt (Hansen et al., 2000) | ++ |
| GFAP and NFL | CSF | ↑ | PHVD (Whitelaw et al., 2001) | + |

***Table 1*** *provides a summary of potential biomarkers. These biomarkers have been detected in blood, urine, and cerebrospinal fluid (CSF). Usefulness of the biomarkers: (+) limited use because CSF samples are required, (++) very useful but can be altered by other factors such as gestational age and intrauterine growth restriction, (+++) useful because it may foster different therapy discussion (such shunt placement in patients with PHVD) although required CSF sample.*

congenital heart disease requiring surgery acquire PVL (Galli et al., 2004).

Diagnosis of PVL has been limited to later stages of PVL where radiological changes are visible in ultrasound or brain MRI. There is a paucity of publications on PVL serum biomarkers. Recent research done on autopsy of premature newborns with PVL has lead to the discovery of immunomarkers of early stages of PVL and could change our understanding of its physiopathology and prevalence. Some of the new described tissue biomarkers of PVL are:

*Human beta-amyloid precursor protein (β-APP)* is marker of wide diffuse axonal damage for early stages of PVL (Arai et al., 1995; Hirayama et al., 2001) and *Fractin* that is an apoptotic marker (Haynes et al., 2008). β-APP and fractin could improve understand the pathogenesis of diffuse axonal damage in PVL, including whether or not this damage results in irreversible axonal loss and impaired neuronal function. There is a paucity of publications on biomarkers of PVL, an area on increasing interest due to its high frequency in extremely premature neonates and infants with congenital heart disease.

## SUMMARY

We found that some of the most promising biomarkers for IVH are S100β and Activin. PHVD biomarkers like TGF-β1, MMP-9, and PAI-1, could be used to discriminate patients that will require shunt. We summarized the characteristic of the biomarkers and its potential usefulness in **Tables 1** and **2**. There is a paucity of publications that validated the potential biomarkers of brain injury with more accurate brain damage assessment, such as brain MRI. In addition potential biomarkers should explore their correlation with functional brain outcomes such as amplitude integrated EEG, full EEG, functional brain MRI, and long term neurodevelopmental follow-up. The available data indicate that such studies are not only justified but also urgently needed to care for newborns, especially those with extreme prematurity.

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# Can S100B predict cerebral vasospasms in patients suffering from subarachnoid hemorrhage?

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**Background:** Protein S100B has proven to be a useful biomarker for cerebral damages. Increased levels of serum and cerebrospinal fluid (CSF) S100B have been shown in patients suffering subarachnoid hemorrhage (SAH), severe head injury and stroke. In patients with SAH, the course of S100B levels has been correlated with neurological deficits and outcome. Cerebral vasospasm is a major contributor to morbidity and mortality. The primary aim of this study was to investigate the potential of S100B protein as a predictor of cerebral vasospasm in patients with severe SAH.

**Materials and Methods:** Patients with SAH, Fisher grade 3 and 4, were included in the study. Five samples of CSF and serum S100B were collected from each patient. The first sample (baseline sample) was drawn within the first 3 days following ictus and the following four samples, once a day on days 5–8, with day of ictus defined as day 1. Clinical suspicion of cerebral vasospasm confirmed by computed tomography angiography was used to diagnose cerebral vasospasm.

**Results:** A total of 18 patients were included. Five patients (28%) developed cerebral vasospasm, two (11%) developed ventriculitis. There were no significant differences between S100B for those with and without vasospasm. Serum S100B levels in patients with vasospasm were slightly lower within the first 5 days following ictus, compared to patients without vasospasm. Two out of five patients had elevated and increasing serum S100B prior to vasospasm. Only one showed a peak level of S100B 1 day before vasospasm could be diagnosed. Due to the low number of patients in the study, statistical significance could not be reached.

**Conclusion:** Neither serum nor CSF S100B can be used as predictor of cerebral vasospasm in patients suffering from SAH.

**Keywords: protein S100B, subarachnoid hemorrhage, cerebral vasospasm, CT angiography, cerebrospinal fluid, serum**

## INTRODUCTION

Subarachnoid hemorrhage (SAH) accounts for approximately 6–8% of all strokes, and the leading cause is rupture of intracerebral aneurysms in 85% of the cases. The remaining causes are due to arteriovenous malformation (AVM) bleeding, vertebral artery dissection (about 5% together), or due to more undefined causes or the perimesencephalic SAH, which account for about 10% of the cases (van Gijn and Rinkel, 2001). The overall incidence of SAH is approximately 9 per 100,000 persons/year, slightly higher in the Scandinavian countries, an highest in Finland and Japan with 19.7 and 22.7 per 100,000/year, respectively (de Rooij et al., 2007). Mortality and morbidity is high, accounting for up to 50% in patients suffering from aneurysmal SAH (aSAH). About 25% never reach medical attention (Diringer, 2009).

Cerebral vasospasm is an important cause of morbidity and death after SAH (Rowland et al., 2012), for those who survive to receive medical treatment. It is defined as clinical neurological symptoms of ischemia (confusion, decreased level of consciousness, focal neurological deficits), with narrowing of cerebral vessels, visualized by computed tomography angiography (CTA). Cerebral vasospasm occur in approximately one third of aSAH patients (Frontera et al., 2009), and the risk of vasospasm is related to the thickness and amount of blood in the subarachnoid space and/or the presence of intraventricular blood assessed on computed tomography (CT), the Fisher grade (Fisher et al., 1980; Jung et al., 2012). The risk of developing cerebral vasospasm is highest during dag 6–8 following ictus (Weir et al., 1978). SAH patients are clinically monitored with daily neurological examinations, but the means of diagnosing cerebral vasospasm varies. The techniques in use for diagnosing vasospasm are by means of clinical evaluation, transcranial doppler sonography (TCD), CTA, digital subtraction angiography (DSA), or by computed tomography perfusion (CTP) (Yoon et al., 2006; Washington and Zipfel, 2011; Kunze et al., 2012), although the most relevant technique is still not defined (Frontera et al., 2009).

A biomarker for detection of cerebral vasospasm in patients with SAH, could ideally allow for early detection to prevent delayed ischemic neurological deficits. Protein S100B is a calcium binding protein, predominant in nervous tissue, and mainly expressed in astroglial cells (Donato, 2001). It is also found in extracerebral tissues, such as long bones, fat, melanocytes, heart, and kidneys, though in lesser extent (Anderson et al., 2001; Unden et al., 2005). S100B is increased in serum and in cerebrospinal fluid (CSF) after brain injury, mainly as a result of the opening of the blood brain barrier (Marchi et al., 2003). In recent years, studies have shown that S100B is useful as a predictive marker for outcome after cerebral infarction (Herrmann and Ehrenreich, 2003; Ahmad et al., 2012), anoxic brain injury (Shinozaki et al., 2009), and SAH (Wiesmann et al., 1997; Stranjalis et al., 2007; Sanchez-Pena et al., 2008).

The aim of our study was to investigate the potential of S100B protein as a predictor of cerebral vasospasm in patients suffering from severe SAH.

## MATERIALS AND METHODS

### PATIENTS

We prospectively studied patients with SAH, admitted to Copenhagen University Hospital, neurointensive care unit (NICU) between September 2012 and January 2013. The inclusion criteria were: patients with SAH confirmed by CT, Fisher grade 3 and 4, age 18 years and above, admission and external ventricular drain (EVD) within 3 days of ictus, no other major injuries. Patients who had their EVD removed within the first 8 days after ictus were excluded from the study.

Glasgow Coma Scale (GCS) score (Teasdale and Jennett, 1974) and WFNS grading scale (Teasdale et al., 1988) was used to assess the neurological status on admission. Location and thickness of the hemorrhage on CT scan was determined by the Fisher grading scale (Fisher et al., 1980).

### SAMPLES

A total of five blood samples and five CSF samples were obtained from each patient. The day of ictus was defined as day 1. The first blood and CSF samples (Baseline sample) were obtained between day 1 and 3, depending on when the patient was transferred to the NICU and had received an EVD. The following four samples were obtained on day 5–8 following ictus. Each blood sample consisted of 4 ml of blood obtained in a SST-tube with separating gel without additives, and the CSF samples consisted of minimum 1 ml of CSF obtained from the EVD. About 4 ml of blood and 1 ml of CSF was taken as waste prior to sampling, as a precaution to contamination and dilution effect. Following collection all samples were centrifuged at 3500 rpm for 7 min at room temperature. The separated serum and CSF samples were stored at −80 °C, and thawed prior to analysis. Samples were analyzed with the method of electrochemiluminescence immunoassay, and the equipment used was Elecsys 2010, Modular Analytics E170, Roche Diagnostics (Mannheim, Germany). The lower detection limit was 0.005 and the upper limit 39 μg/L.

### VASOSPASM

Daily neurological status was assessed to determine clinical signs of worsening with cerebral vasospasm as the primary cause. Cerebral vasospasm was confirmed by CTA.

### STATISTICS

Statistical analysis was performed in IBM SPSS Statistics ver. 19.0.0. Figures and tables were computed in Microsoft Excel 2007. Student *t*-test was performed for comparing mean S100B levels between patients with and without cerebral vasospasm. Significance level was set to 0.05.

### ETHICAL CONSIDERATIONS

The study and the collection of S100B in both serum and CSF had been approved by the local ethics committee.

## RESULTS

A total of 18 patients with severe SAH were included, 16 had an aSAH, one had SAH due to a small AVM bleeding, and one due to dissection of the vertebral artery. Mean age was 60 years (range 42–84 years), there were 11 females and 7 males. The mean GCS score on admission was 8, the mean score of the WFNS grading scale was 3.5, and the mean Fisher grade was 3.6. Patients with aSAH were treated with endovascular coiling (81%) or surgical clipping (19%). The two patients without aSAH were treated conservatively.

The baseline samples were obtained on day 1 from one patient, on day 2 from four patients and on day 3 from 13 patients. Five patients developed cerebral vasospasm during the first week. None developed cerebral vasospasm later than on day 8. Three patients died, of which one developed cerebral vasospasm during the trial period. Two patients developed bacterial ventriculitis during the trial period.

When comparing patients who developed cerebral vasospasm with those who did not, there were no significant differences in GCS score at admission, Fisher grade or WFNS grade. Mean age was slightly, but not significantly, higher in patients that developed cerebral vasospasm (**Table 1**). The mean serum S100B level in

**Table 1 | Characteristics of patients with cerebral vasospasm compared to patients without.**

| | Cerebral vasospasm *n* = 5 | No cerebral vasospasm *n* = 13 |
|---|---|---|
| Gender (M:F) | 2:3 | 5:8 |
| Mean age (years) | 67 | 58 |
| Mean GCS at admission | 8 | 9 |
| Mean WFNS grading score at admission | 3.6 | 3.5 |
| Mean Fisher grading score | 3.6 | 3.5 |
| Mean body temperature ( °C) | 38.0 | 38.0 |
| Intracranial infection | 0 | 2 |
| Death | 1 | 2 |
| Sample collection (*n*) | | |
| Baseline | | |
| Day 1 | 1 | 0 |
| Day 2 | 1 | 3 |
| Day 3 | 3 | 10 |
| Days 5–8 | 5 | 13 |

*GCS, glasgow coma scale; WFNS, World federation of neurological societies grading scale.*

patients who developed cerebral vasospasm compared to those who did not, was lower within the first 5 days after ictus. Following day 5, serum S100B levels in the group with cerebral vasospasm, was increased and exceeded the mean level of serum S100B in patients who did not developed vasospasm, though not statistically significant (**Table 2**). The mean CSF S100B levels in patients who developed cerebral vasospasm were lower compared to patients without vasospasm, but not statistically significant. The peak and mean body temperatures were generally high, but did not differ between those who developed vasospasm and those who did not.

Among the five patients who developed cerebral vasospasm, baseline samples of serum and CSF S100B were drawn on day 1 in one patient, at day 2 in one patient, and at day 3 in three. Two patients had increasing levels of serum S100B compared to the other three, which had an overall decreasing tendency and normal levels of serum S100B when vasospasm was diagnosed. Only one patient reached a peak level of serum S100B the day before vasospasm was confirmed (**Figure 1**, patient B), while the other patient reached peak S100B 1 day after angiographic confirmation of cerebral vasospasm and ongoing cerebral infarction (**Figure 1**, patient E). Both patients had a WFNS score of 5 and a GCS of 3 at admission, and both developed pneumonia during the trial period. The other three patients with cerebral vasospasm had slightly lower WFNS and slightly higher GCS scores at admission, and none had pneumonia or other infections diagnosed during the trial period.

Among patients who did not developed cerebral vasospasm, the baseline samples were drawn on day 2 in three patients and on day 3 in 13 patients. Two developed bacterial ventriculitis, with a sudden 10-fold increase in serum S100B levels. One reached peak level of serum S100B the day before developing ventriculitis, while peak levels for the other patient occurred on the same day as the

**Table 2 | Mean serum and CSF S100B levels in patients with cerebral vasospasm compared to patients without*.**

| Samples | Mean serum S100B (μg/L) | | Mean CSF S100B (μg/L) | |
|---|---|---|---|---|
| | Cerebral vasospasm $N = 5$ | No cerebral vasospasm $N = 13$ | Cerebral vasospasm $N = 5$ | No cerebral vasospasm $N = 13$ |
| Baseline | 0.10 | 0.19 | 23.5 | 29.6 |
| Day 5 | 0.15 | 0.37 | 13.8 | 21.8 |
| Day 6 | 0.21 | 0.15 | 10.1 | 16.3 |
| Day 7 | 0.27 | 0.11 | 12.7 | 15.7 |
| Day 8 | 0.24 | 0.25 | 12.0 | 12.3 |

**There are no statistical differences between the two groups (vasospasm vs. no vasospasm).*

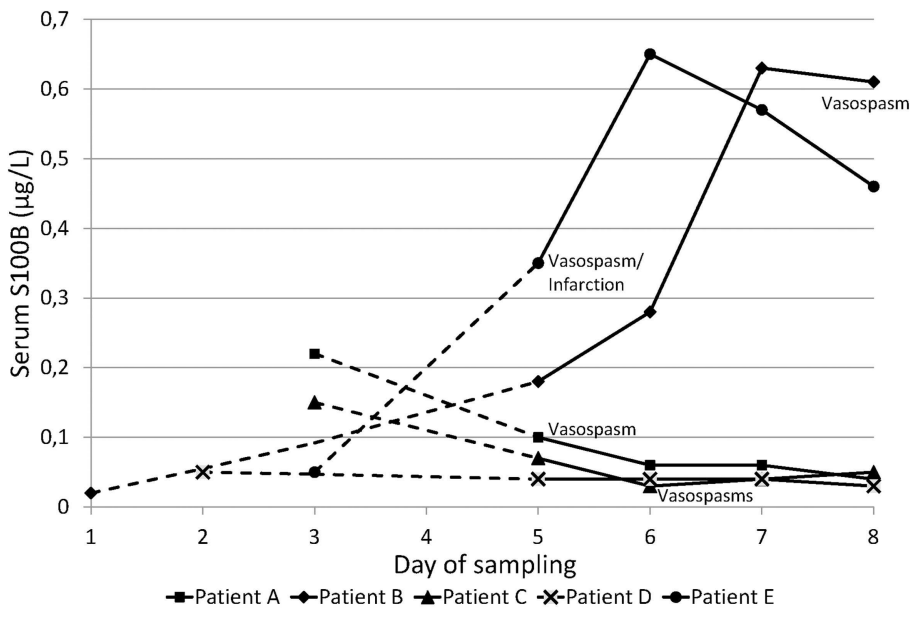


**FIGURE 1 | Serum S100B levels for the five patients with cerebral vasospasm**. Day 1 = day of ictus. Baseline sampling was made between day 1 and 3. The dotted lines are extrapolations from baseline sample to the second sample drawn on day 5. The day on which cerebral vasospasm was confirmed is indicated for each patient.

infection was diagnosed. No such relation was observed for CSF S100B.

## DISCUSSION

We studied a total of 18 SAH patients by measuring S100B in serum and CSF within the first 8 days following ictus. Five patients developed cerebral vasospasm, detected by neurological deterioration and confirmed with CTA. No significant differences for S100B levels were observed when comparing patients who developed cerebral vasospasm to patients who did not, and S100B failed predict the development of cerebral vasospasm.

Only two patients with cerebral vasospasm developed increased levels of serum S100B during the trial period. Both patients were in worse neurological condition (GCS 3, WFNS 5 at admission) compared to the other patients who developed cerebral vasospasm (Stranjalis et al., 2007). In addition, these two patients developed pneumonia during the trial period. Two patients in the group without cerebral vasospasm developed ventriculitis. High levels of serum S100B were found on the day before or on the same day as the clinical diagnosis of bacterial ventriculitis was set. The results indicate an association between increased serum S100B and intracerebral infections as shown in a previous study (Unden et al., 2004).

Measurements of S100B in our patients were done once daily with baseline sample obtained within the first 3 days of ictus and the rest of the four samples on days 5–8 following ictus. The baseline samples were obtained within the first 3 days of ictus, as cerebral vasospasm usually do not occur before day 4 following ictus. As the highest risk for developing vasospasm is between 6 and 8 days following the subarachnoid bleeding (Weir et al., 1978), the subsequent samples were drawn on day 5–8. None of the patients developed either clinical or angiographic vasospasm after day 8.

In two patients the bleeding source was not an aneurysm, but a small deep AVM in one patient, and a vertebral artery dissection in the other. The risk of developing cerebral vasospasm increases with increasing volume of subarachnoid blood visualized by CT (Fisher et al., 1980; Adams et al., 1987). Both of these patients had high amount of subarachnoid blood visualized on CT (Fisher grade 3 and 4), and thus high risk of developing cerebral vasospasm.

Currently, neurosurgical centers use daily assessment of neurological status, TCD sonography and CTA to determine the development of cerebral vasospasm. Most patients suffering from severe SAH, have already, prior to transport to the NICU, been sedated and intubated, making the assessment of the neurological status in these patients challenging. In our study, clinical suspicion of vasospasm in addition with CTA, rather than TCD, was used to diagnose cerebral vasospasm. CTA has been found to correlate well (overall agreement of 95%) with DSA (Yoon et al., 2006), which has been considered as the gold standard in detecting cerebral vasospasm. Furthermore, Kunze et al. (2012) have shown that the accuracy of neurological examination and CTP is higher in detecting cerebral vasospasm than TCD, and in a systematic review by Lysakowski and colleagues, they concluded that although TCD has a high specificity (0.99) and positive predictive value (0.97) for detecting vasospasm, this accounts only for the middle cerebral artery. For all the other arteries, there is no evidence for the usefulness of TCD as a diagnostic tool for vasospasm (Lysakowski et al., 2001).

Our study also show a tendency toward lower serum S100B levels during the first 5 days following SAH in patients who developed cerebral vasospasm compared to those who did not. These results were not statistically significant, thus not supporting useful value of serum S100B in predicting vasospasm. Similar results have been found by Oertel et al. (2006) who showed lower levels of serum S100B within the first 3 days after SAH in patients who developed cerebral vasospasm compared to those who did not. Cerebral vasospasm was determined by neurological deterioration and increasing flow velocity on TCD.

Identifying a specific biomarker for prediction of cerebral vasospasm in this group of patients is of high value, since earlier detection and hence earlier treatment of vasospasm could lower the morbidity and mortality in this group of patients. The statistical analysis of our end results are limited by the small number of cases enrolled in the study. We can, however, conclude that, although S100B is a promising prognostic biomarker of secondary brain damage and outcome in patients with SAH (Stranjalis et al., 2007; Sanchez-Pena et al., 2008), the potential of S100B as a predictor of cerebral vasospasm is very limited. This is in accordance with two previous studies (Moritz et al., 2010; Jung et al., 2013). The need of a marker in predicting cerebral vasospasm still remains, and other biomarkers such as myeloperoxidase (Lim et al., 2012), amino acids in addition to microdialysis (Jung et al., 2013), endothelin-1, interleukin-6, and indicators of thrombin activity might be of greater utility (Lad et al., 2012).

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# Increased seizure susceptibility in mice 30 days after fluid percussion injury

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Traumatic brain injury (TBI) has been reported to increase seizure susceptibility and also contribute to the development of epilepsy. However, the mechanistic basis of the development of increased seizure susceptibility and epilepsy is not clear. Though there is substantial work done using rats, data are lacking regarding the use of mice in the fluid percussion injury (FPI) model. It is unclear if mice, like rats, will experience increased seizure susceptibility following FPI. The availability of a mouse model of increased seizure susceptibility after FPI would provide a basis for the use of genetically modified mice to study mechanism(s) of the development of post-traumatic epilepsy. Therefore, this study was designed to test the hypothesis that, mice subjected to a FPI develop increased seizure susceptibility to a subconvulsive dose of the chemoconvulsant, pentylenetetrazole (PTZ). Three groups of mice were used: FPI, sham, and naïve controls. On day 30 after FPI, mice from the three groups were injected with PTZ. The results showed that FPI mice exhibited an increased severity, frequency, and duration of seizures in response to PTZ injection compared with the sham and naïve control groups. Histopathological assessment was used to characterize the injury at 1, 3, 7, and 30 days after FPI. The results show that mice subjected to the FPI had a pronounced lesion and glial response that was centered at the FPI focus and peaked at 3 days. By 30 days, only minimal evidence of a lesion is observed, although there is evidence of a chronic glial response. These data are the first to demonstrate an early increase in seizure susceptibility following FPI in mice. Therefore, future studies can incorporate transgenic mice into this model to further elucidate mechanisms of TBI-induced increases in seizure susceptibility.

**Keywords: traumatic brain injury, post-traumatic epilepsy, pentylenetetrazole, mouse models**

## INTRODUCTION

An estimated 1.7 million people in the U.S. experience a traumatic brain injury (TBI) each year, 80,000 of which develop long-term disabilities and 50,000 of which are fatal (Faul, 2010). Approximately 3.2 million individuals are living with such disabilities in the U.S., resulting in a large economic burden, primarily through loss of work and medical expenses (Finkelstein et al., 2006). TBI causes several neuropathological manifestations, including cognitive, emotional, physiological, and psychological deficits (Rosenthal et al., 1998; Junqué, 1999; Vakil, 2005; Nampiaparampil, 2008; Bales et al., 2009). In addition, to these deficits, another pathology often associated with TBI is increased seizure susceptibility and the development of epilepsy (D'Ambrosio and Perucca, 2004). TBI is responsible for the development of 10–20% of symptomatic epilepsy in the general population (Pitkänen and Bolkvadze, 2012) and has also been reported to increase seizure susceptibility (Kharatishvili and Pitkänen, 2010). Why TBI results in the development of epilepsy and increased seizure susceptibility remains largely unknown, although several candidate mechanisms have been postulated, including: neurodegeneration, neuroplasticity, neuroinflammation, and connective tissue formation. The use of animal models that mimic these effects will aid in the understanding of the mechanisms of TBI and may provide help in the development of better treatment strategies.

Previous studies in rats using the fluid percussion injury (FPI) model have demonstrated an increase in seizure susceptibility, as measured by a second-hit chemoconvulsant challenge, as well as the development of spontaneous epileptiform discharges, the hallmark of epilepsy (Silva et al., 2011; Kharatishvili et al., 2006). One of the main benefits of this model in rats is the high reproducibility. However, because this model has not been extended to mice, it lacks the benefits of using different genetic models. Fundamental studies are needed to enable mechanistic studies using transgenic mice in the FPI model. Therefore, this study examined second-hit seizure susceptibility in mice, using Pentylenetetrazol (PTZ) at 30 days after FPI. To further characterize this mouse model of FPI, histopathological and glial response data are also provided.

## MATERIALS AND METHODS

### STRAIN AND SURGERIES

All experimental protocols were carried out as previously approved by the Institutional Animal Care Committee (IACUC) of Texas

A&M University Health Science Center and Scott &White hospital. Male C57Bl6 mice from Charles River were used in these studies. All the mice from FPI and sham groups underwent surgery. Mice were initially anesthetized with 4% isoflurane and oxygen for anesthesia induction and later to 2% isoflurane for maintenance. Once under anesthesia, the heads of the animals were shaved. Strict sterile technique was maintained during surgical procedures. Animals were placed in a stereotaxic instrument with an attachment for mouse surgery (Stoelting, Inc., IL, USA). A 2-mm hole was drilled, with dura intact, in the skull over the left parietal cortex (antero-posterior: +1.5 mm; medio-lateral: −1.2 mm). A female luer-lock (PlasticOne) was connected to the hole in the skull. Animals in the FPI group received a pressure pulse of 1.5–1.7 atm from the FPI apparatus through the luer-lock for 12–16 ms. Sham animals received identical treatment except no pressure pulse was delivered. Naïve animals were not surgically manipulated. Animals were housed singly after FPI with a 12-h light–dark cycle (light on 6:00 and light off 18:00). All animals had continuous access to food and water.

### HISTOPATHOLOGY

Forty C57Bl6 male mice were used for histological examination. Animals were randomly assigned to experimental ($N = 16$), sham ($N = 16$), and naïve control ($N = 8$) groups. In order to define the injury and subsequent inflammatory response, separate groups of mice were sacrificed at 1, 3, 7, and 30 days after FPI ($N = 4$ sham, 4 FPI, and 2 Naïve mice per time point). Mice were euthanized via a transcardiac perfusion of saline followed by paraformaldehyde (PFA) as previously described (Arisi et al., 2011). Briefly, animals were given an overdose of i.p. Euthasol, followed by an incision in the right atrium while simultaneously pumping 0.9% sterile saline through the left ventricle. After the blood ran clear (∼50 ml), 4.0% PFA was pumped through the left ventricle. Brains were allowed to post-fix in the skull for 24 h following perfusion, after which they were removed, and post-fixed for another 24 h in PFA. Brains were subsequently hemi-sectioned and cut at 50 μm for analysis. Gross examination of the impact lesion was performed upon extraction from the skull, prior to cutting, in addition to histological and immunocytochemical analysis.

#### *Cresyl violet*

Sections were mounted onto gelatin-coated slides and allowed to dry overnight. Slides were then dehydrated and defatted in 70, 95, and 100% ETOH, followed by rehydration and staining in the cresyl violet solution (Sigma, St Louis, MO, USA). Slides were rinsed in de-ionized $H_2O$, again dehydrated, cleared with xylenes, and coverslips were applied using permount. Sections were then visualized using a Leica SCN 400 (Leica Corp., Wetzlar, Germany) slide scanner.

#### *Fluoro-Jade C histology for damaged cells*

Sections were mounted onto gelatin-coated slides and Fluoro-Jade C staining took place according to the packaging instructions (AG325, Millipore Inc., Billerica, MA, USA). Once these slides were dry, they were immersed in xylenes and then cover slips were applied using DPX mounting media. Sections were then visualized on a Olympus IX81 (Olympus Inc., Center Valley, PA, USA) inverted microscope equipped to visualize FITC.

#### *GFAP and Iba1 immunocytochemistry for astrocytes and microglia*

Sections were reacted free-floating as previously described (Shapiro et al., 2008, 2009). Briefly, fluorescent labeling of both antibodies was performed in order to provide a qualitative temporal description of the inflammatory response in the ipsi and contralateral cortex following FPI. For GFAP-labeling, a fluorescent-tagged primary GFAP antibody (1:2000; Sigma #C9205) was used for analysis. For Iba1, a rabbit polyclonal antibody (1:500; Wako labs # 019-19741) was used, followed by fluorescent-conjugated goat anti-rabbit IgG (Alexa-fluor 555; Invitrogen Inc.). Sections were then visualized on a Olympus IX81 (Olympus Inc.) inverted, laser-scanning confocal microscope. In addition, we performed a peroxidase reaction using DAB for GFAP (Rabbit polyclonal 1:1000; Sigma#G9269) and these slides were visualized on the Leica SCN 400 slide scanner (Leica Corp.).

### PTZ SECOND-HIT SEIZURE CHALLENGE

Twenty three male C57Bl/6 mice (23–28 g) were used in this part of the study. The 30-day post-FPI timepoint was selected because previous studies using other models of epileptogenesis have examined the 30-day timepoint for increased seizure susceptibility (Blanco et al., 2009; Wilhelm et al., 2012). Animals were randomly assigned to experimental ($N = 9$), sham ($N = 9$), and naïve control ($N = 5$) groups. To test for seizure susceptibility, 30 days after the surgery, all the animals from FPI, sham, and naïve control groups were injected i.p. with a subconvulsive dose (Jain et al., 2011) of PTZ (30 mg/kg; Sigma). Immediately following the single injection of PTZ, mice were monitored and videotaped, and seizure scores were calculated for 20 min by reviewers blind to the condition of the animal. Seizures were scored as per a modified Racine Scale (Shapiro et al., 2005). Briefly, stage 1 seizures were classified by movement of mouth and facial muscles; stage 2 seizures were classified as head-bobs and rocking; stage 3 seizures were classified by forelimb clonus; stage 4 seizures were classified as forelimb and hindlimb clonus; stage 5 seizures were classified as tonic clonic activity and loss of balance. The seizure parameters that were examined in this study were: severity, frequency, and duration of seizures. Data was analyzed by contingency table analysis with Chi-Square, using SPSS 9.0.

## RESULTS

### GROSS EXAMINATION

Gross examination of the brain following removal revealed a substantial lesion surrounded by blood in mice that received the FPI. The lesion was present both at the 1- and 3-day timepoints (data not shown). This was not observed in the sham animals. The blood surrounding the lesion site appeared to be more prevalent at 3 days relative to the 1-day timepoint. By 7 days, the lesion was still present, but the blood surrounding the lesion was no longer evident. At 30 days, there was no gross evidence of a lesion.

### HISTOPATHOLOGY

In general, the FPI results in a consistent lesion that is focused around the center of the impact area and emanates deep to, lateral and medial to the impact area. Examination of Cresyl Violet stained tissue sections revealed that at 1 day after FPI, the lesion size spanned from Anterior/Posterior (AP) +0.3 to −2.54 mm from

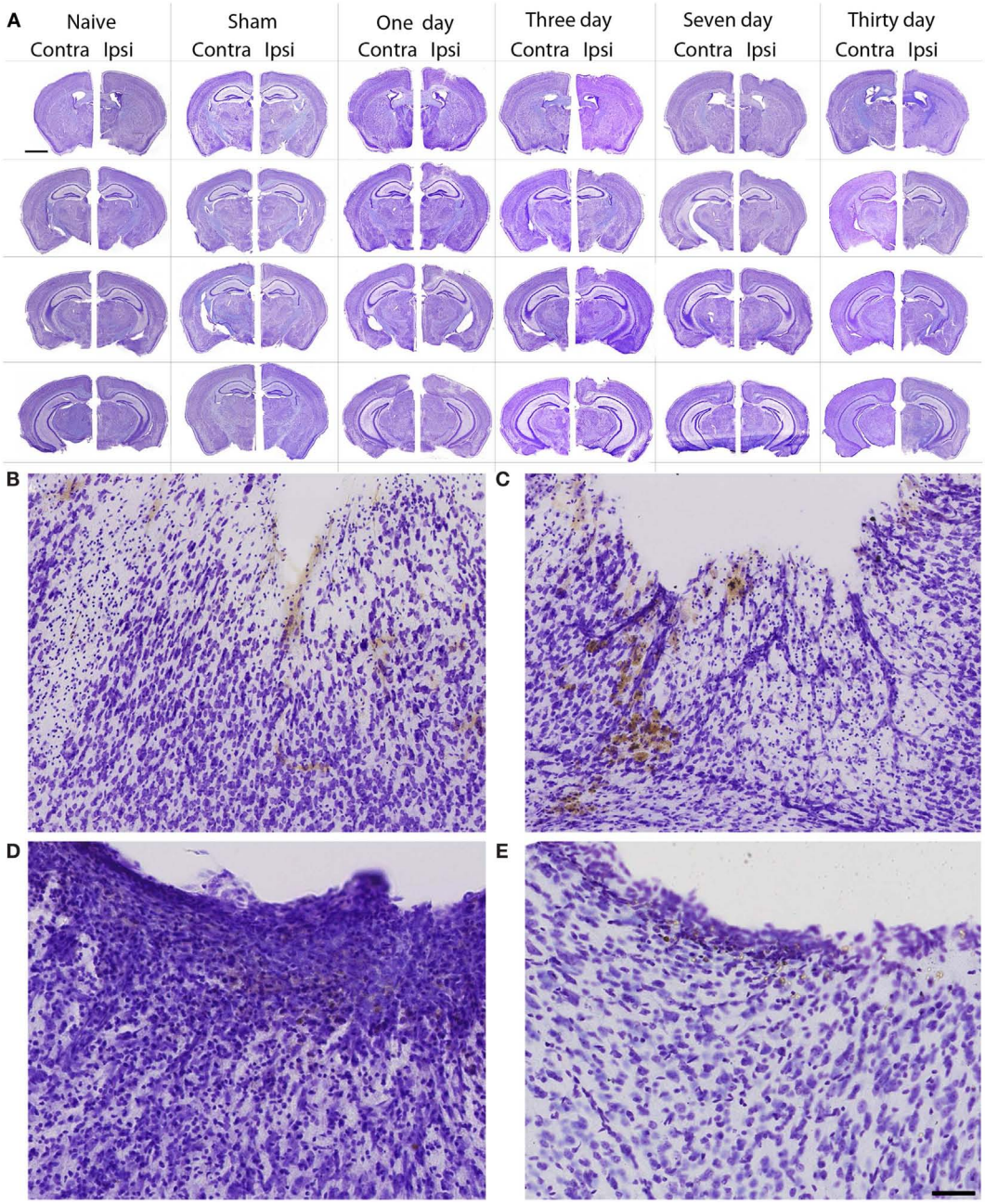


**FIGURE 1 | Cresyl violet stained tissue from FPI mice, sham mice, and naïve mice.** In **(A)**, low magnification images are shown to illustrate the location and span of the lesion resulting from the FPI. Note that the sham column depicts each of the timepoints (1–30 days in descending order) in the central hippocampus. Note that the dural surface is intact in the sham animals. In **(B–E)**, high magnification images are shown from the FPI mice. In **(B)**, the lesion area is enlarged from the 1-day post-FPI timepoint. Tissue damage is clearly evident. The brown reaction product is likely a reaction to the iron associated with hemoglobin of erythrocytes. Since blood degradation products are ingested by macrophages, the cells containing the brown staining are quite possibly macrophages. Note that the cortical layers subjacent to the lesion appear relatively intact at this timepoint. In **(C)**, the boxed area from the 3-day FPI mice is shown in enlargement. In **(D)**, the dark blue staining is likely indicative of pial repair. Note that the typical layering and columnar appearance of the cortex is altered. At 30 days post-FPI **(E)**, the pial surface appears intact, but remnants of blood in the peri-lesion area remain. Note that the typical layering of the cortex remains altered at this timepoint. Scale bar in **(A)** = 2 mm for all low magnification images and scale bar in in **(E)** = 80 μm for **(B–E)**.

bregma. The average medial to lateral (ML) span was 1.82 mm (±0.42 mm) at its widest margin, which was located around the epicenter of the impact (AP −1.2 mm, ML +1.5 mm from bregma). The median ML length was 1.78 mm. The range of the dorsal/ventral (DV) damage was between 0.18 and 1.09 mm from the pial surface (**Figure 1**). Leukocyte infiltration was observed in

the region surrounding the lesion (**Figure 1**). Fluoro-Jade staining was performed and damaged cells were seen immediately lateral, medial, and subjacent to the impact zone (**Figure 2**) at the 1 day post-FPI timepoint.

At 3 days post-FPI, the lesion spanned from AP +0.26 to −2.60 mm from bregma. The average ML span was 1.44 mm (±0.47 mm) at its widest margin, which was located around the epicenter of the impact (AP −1.2 mm, ML +1.5 mm from bregma). The median ML length was 1.31 mm (**Figure 3**). The range of the DV damage was between 0.15 and 0.86 mm from the pial surface. Leukocyte infiltration was observed in the region surrounding the lesion (**Figure 1**). Fluoro-Jade staining was performed and damaged cells were seen extending deep into the tissue, reaching as far ventral as the corpus callosum (**Figure 2**) at the 3-day post-FPI timepoint.

At 7 days post-FPI, the lesion size was noticeably smaller relative to the 1- and 3-day timepoints. In two of the four FPI animals examined, the pial surface had healed such that no breach was evident. In the other two mice, only a small disruption of the pial surface was evident. At this timepoint, the lesion size spanned from AP +0.26 to −2.54 mm from bregma. The average ML span was 1.08 mm (±0.28 mm) at its widest margin, which was located around the epicenter of the impact (AP −1.2 mm, ML +1.5 mm from bregma). The median ML length was 1.28 mm (**Figure 1**). The range of the DV damage was between 0.43 and 0.65 mm from the pial surface. Fluoro-Jade staining detected only a small number of Fluoro-Jade cells at the 7-day post-FPI timepoint. These cells were located in the immediate surrounding area of the impact zone (**Figure 2**).

At 30 days after FPI, the pial surface appeared to be fully restored and intact in all of the mice examined. Although only minimal superficial evidence of the lesion was evident, the cortical layers within and surrounding the region where the lesion occurred no longer exhibit a clear I–VI pattern (**Figure 1**). No Fluoro-Jade labeling was found at this timepoint.

## GLIAL RESPONSE TO FPI

### *GFAP*

In order to examine the glial response following FPI in mice, we performed immunohistochemistry for astrocytes using anti-GFAP and microglial cells using anti-Iba1. The general pattern of the glia was such that at 1-day post-FPI, there were astrocytes with processes extending toward the lesion (**Figure 3**), indicative of astrocytes migrating to the injury site. However, in the peri-lesion area, only minimal GFAP-labeling is observed (**Figure 3**). Minimal GFAP-labeling was also observed throughout ipsi and contralateral cortex (**Figures 4** and **5**). By 3 days after FPI, there was an intense astrocyte activation located in the peri-lesion region (**Figure 3**) that was considerably more pronounced and widespread compared to 1 day post-FPI (**Figure 3**). In addition to the peri-lesion area, activated astrocytes were robustly observed throughout the entire ipsilateral cortex in the FPI mice (**Figure 4**), although there was only minimal GFAP-labeling in the contralateral hemisphere (**Figure 4**). At 7 days post-FPI, the number of GFAP-positive astrocytes was decreased throughout the ipsilateral cortex relative to the 3-day timepoint (**Figures 3** and **4**). In the peri-lesion area, a robust number of hypertrophied astrocytes are still observed (**Figure 3**), although considerably less astrocytes are seen relative to the 3-day timepoint. In the ipsilateral cortex, GFAP-labeling is observed, but not in the contralateral cortex (**Figure 4**). Overall, at 7 days post-FPI, there appears to be less GFAP-labeled astrocytes relative to the 3-day timepoint (**Figures 3–6**).

### *Iba1*

At the 1-day timepoint, activated microglial cells are only sparsely observed in the peri-lesion area (**Figure 6**). No changes were apparent in the contralateral hemisphere. Microglial activation was most robust at 3 days post-FPI (**Figure 6**). The majority of activated microglial cells are observed in the peri-lesion zone (**Figure 6**), but to a lesser extent, were also observed throughout the ipsilateral cortex (**Figure 6**). At 1 and 3 days after FPI, some of the microglial cells exhibited a rod shaped morphology (**Figure 7**). Some of these rod shaped cells were in pairs or small trains of cells, similar to that observed by Ziebell et al. (2012). By 7 days post-FPI, activated microglial cells were only observed in the immediate peri-lesion region (**Figure 6**) and were relatively less abundant than the 1 and 3 day post-FPI timepoints. At this time point and beyond, very few if any of the microglial cells exhibited a rod shaped appearance (data not shown). Activated microglial cells were relatively sparse at the 30-day timepoint in both the ipsilateral and contralateral hemispheres (**Figure 6**).

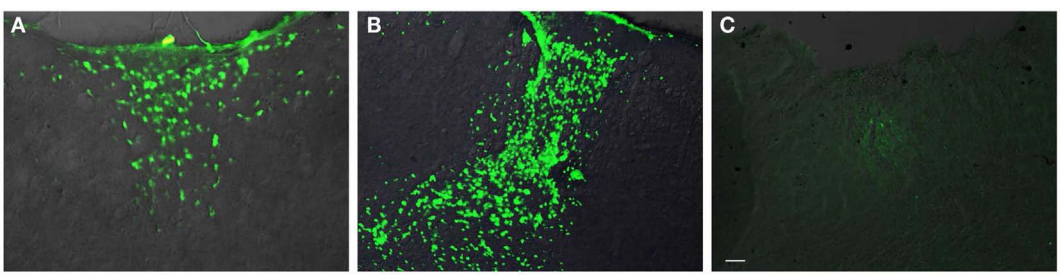


**FIGURE 2 | Fluoro-Jade labeling in peri-lesion area following FPI.** In **(A)**, degenerating cells are observed in the immediate peri-lesion region at 1-day post-FPI. In **(B)**, degeneration is considerably more robust at the 3-day post-FPI compared to the 1-day post-FPI timepoint. By 7 days post-FPI **(C)**, the number of degenerating cells is considerably less (and was non-existent in most of the sections examined) than the 1- or 3-day post-FPI timepoints. Scale bar in **(C)** = 50 μm for all images.

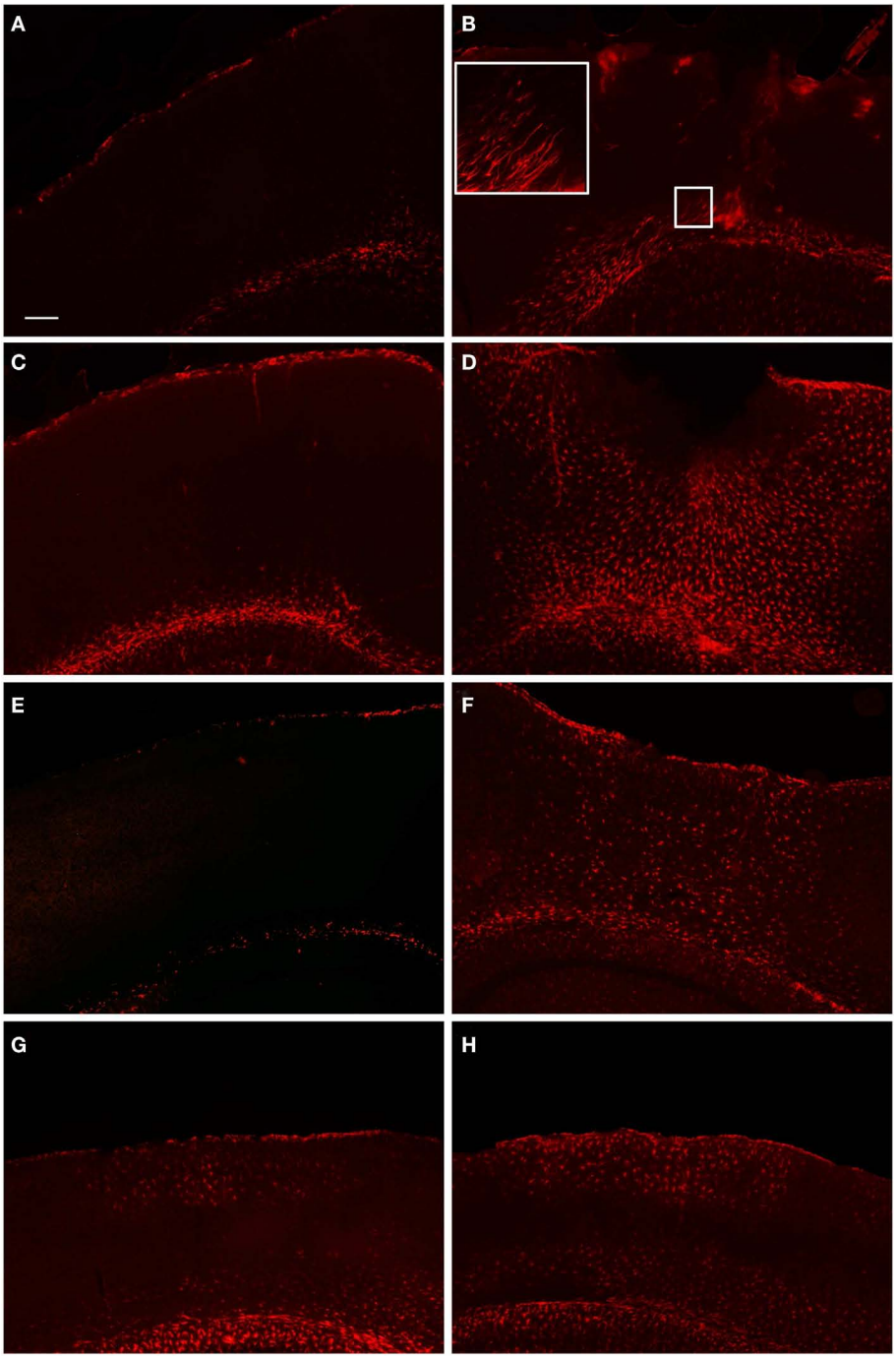


**FIGURE 3 | Fluorescent microscopy of GFAP-labeling in the peri-lesion area and corresponding contralateral hemisphere.** At 1 day after FPI **(A,B)**, there is only a minimal astrocyte reaction in the peri-lesion area in the ipsilateral cortex **(B)**. Note in the inset image, the elongated appearance of these astrocytes oriented toward the lesion emanating from the area of the corpus callosum, or possibly the underlying lateral ventricle. This morphology, coupled with the minimal astrocytic staining in the peri-lesion area, is indicative of the early stages of astrocyte activation. In the corresponding contralateral hemisphere, minimal GFAP-labeling is observed. At 3 days post-FPI **(C,D)**, a robust number of GFAP-labeled cells with an activated appearance are observed in the peri-lesion area **(D)**. Only minimal GFAP-labeling is observed in the contralateral hemisphere at this timepoint **(C)**. At 7 days post-FPI **(E,F)**, the appearance of GFAP-labeled astrocytes with an activated appearance is decreased relative to the 3-day timepoint. In the corresponding contralateral hemisphere at 7 days post-FPI, there appears to be diminished GFAP-labeling relative to 1 and 3 days, as well as sham and naïve mice (data not shown). At 30 days post-FPI **(G,H)**, a sizable population of GFAP-labeled astrocytes is observed in both the contralateral **(G)** and ipsilateral **(H)** hemispheres. In both hemispheres, the labeling is quite robust in layers I–IV and VI, but conspicuously absent in layer V. Scale bar in **(A)** = 50 μm for all images.

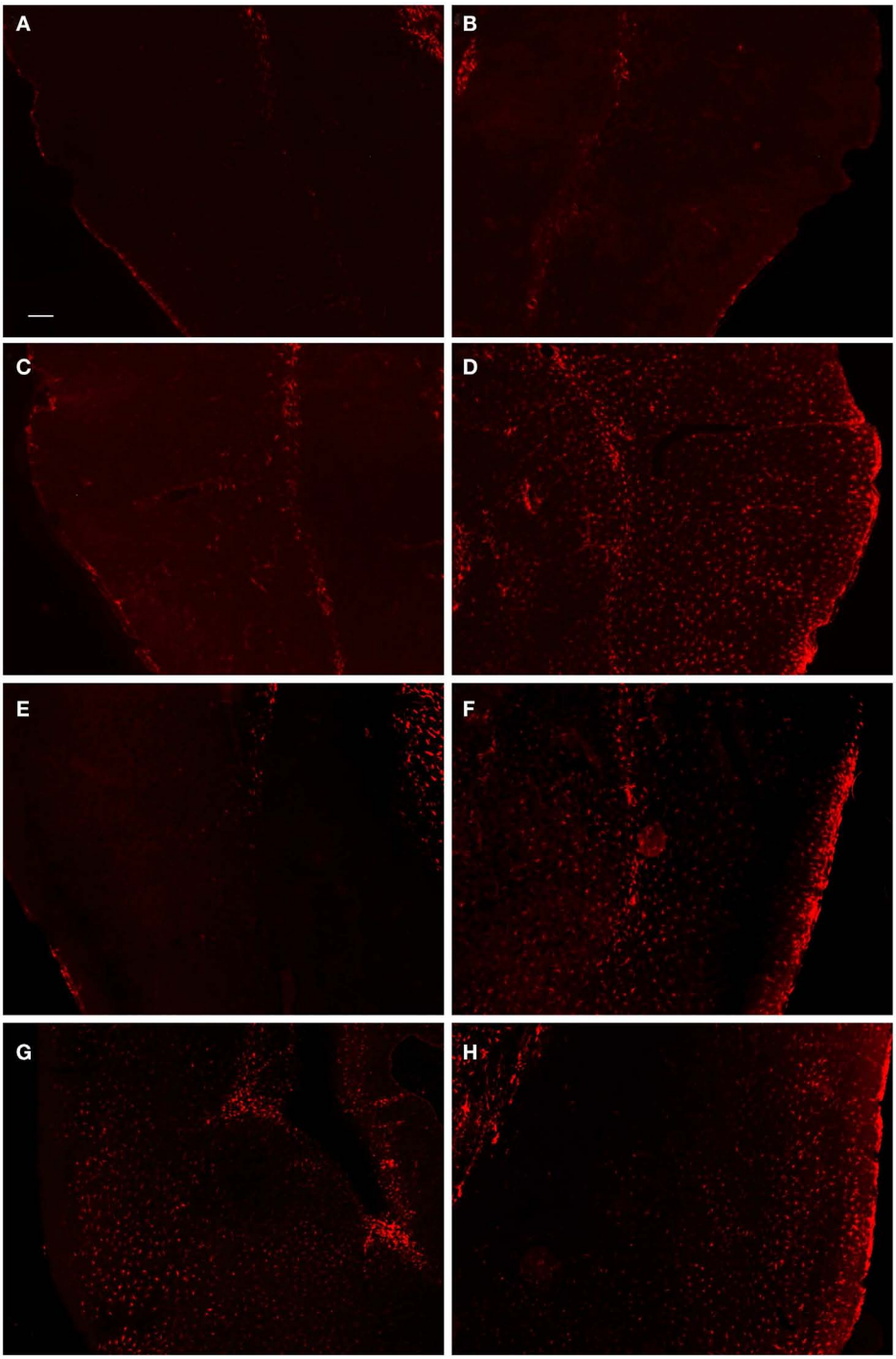


**FIGURE 4 | Fluorescent microscopy of GFAP-labeling in the ipsilateral and contralateral hemispheres.** At 1-day post-FPI, only minimal GFAP-labeling is observed in the contralateral **(A)** and ipsilateral **(B)** hemispheres. At 3 days post-FPI, only minimal GFAP-labeling is observed in the contralateral hemisphere **(C)**, but a robust number of GFAP-labeled astrocytes are observed in the ipsilateral hemisphere **(D)**. This pattern of labeling is also evident at 7 days post-FPI **(E,F)**. It is pertinent to note that in the contralateral hemisphere **(E)**, an overall depletion of GFAP-labeling is observed. This is similar to the observation in **Figure 3 (E,F)**, in which the contralateral hemisphere corresponding to the lesion site also appeared depleted of GFAP-labeling. At 30 days post-FPI **(G,H)**, GFAP-labeled astrocytes are widely distributed throughout both, ipsilateral **(G)** and contralateral **(H)** hemispheres. Note, that we have also provided light microscopic images of DAB-reacted tissue (**Figure 5**) demonstrating these same observations. Scale bar in **(A)** = 50 $\mu$m for all images.

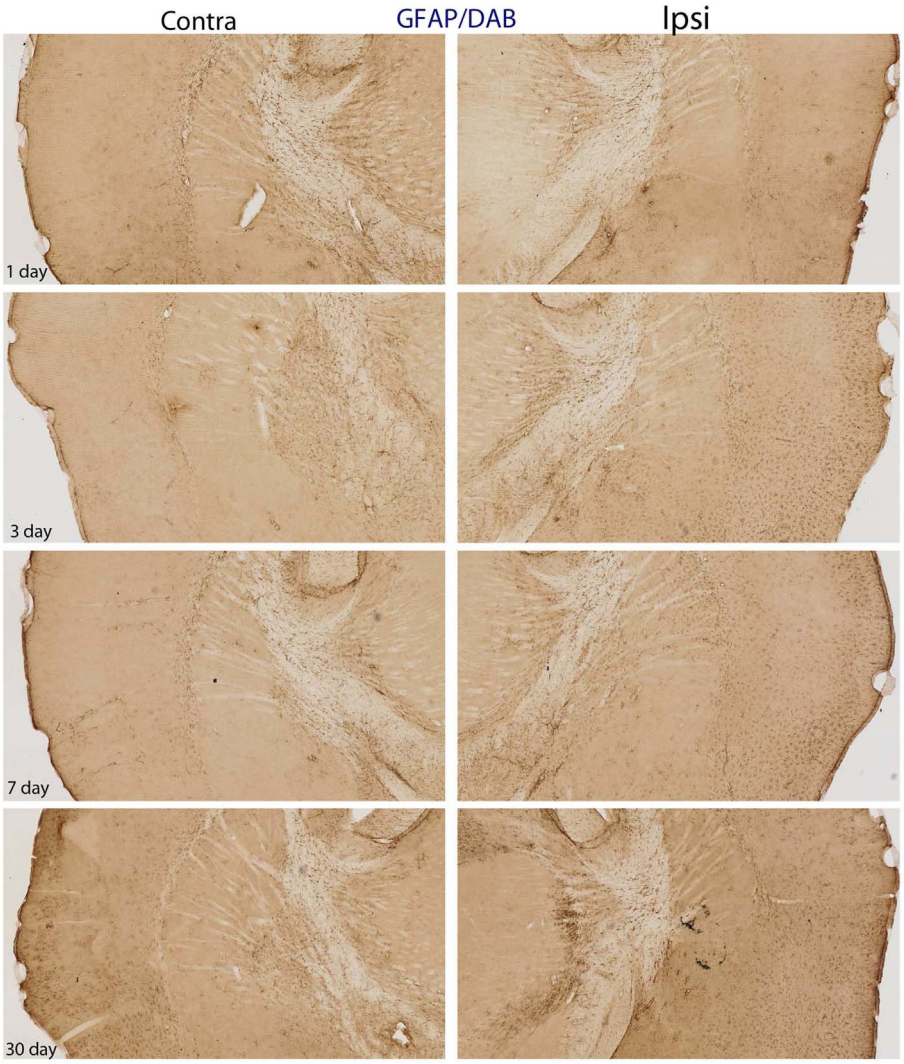


**FIGURE 5 | DAB-reacted GFAP immunohistochemistry.** This figure depicts the same pattern of GFAP staining as that shown in **Figure 4**. The advantage of the DAB-reacted tissue is that it allows for the reader to appreciate the coordinates of the tissue, as well as the relative staining in the ipsi and contra lateral hemispheres at lower magnification.

### SECOND-HIT PTZ SEIZURES

In response to the PTZ challenge, eight out of the nine mice from the FPI group exhibited stage IV/V seizures compare to one mouse in the sham group and no mice in the naïve control group (**Table 1**). Contingency table analysis was performed and the Chi-Square results revealed a significant increase in the development of stage IV/V seizures between the FPI group, compared to the sham group ($\chi^2 = 10.888$; $p < 0.001$), and the Naïve group ($\chi^2 = 10.37$; $p < 0.001$). There was no difference observed between sham and naïve control groups ($\chi^2 = 0.598$; $p = 0.439$, NS).

The frequency of seizures was also significantly different between the three groups such that there was an increase in the FPI mice when compared with mice from the sham ($\chi^2 = 11.455$; $p < 0.001$) and naïve ($\chi^2 = 11.455$; $p < 0.001$) groups (**Table 2**). There was no difference observed between sham and naïve control groups ($\chi^2 = 0.498$; $p = 0.455$, NS). In addition, FPI altered the total duration of seizures between the three groups ($F = 4.90$; $p < 0.033$), such that the FPI mice had a significantly greater total duration of seizures relative to naïve ($p < 0.049$) but not relative to sham ($p = 0.35$) groups (data not shown).

## DISCUSSION

The results from this study demonstrate increased seizure susceptibility in mice at 30 days after FPI. This finding is entirely novel and has not been reported in mice at the 30-day timepoint after TBI. It is pertinent to note that a recent study did demonstrate increased seizure susceptibility in mice after a FPI, but that study looked at the 6-month post-FPI timepoint (Bolkvadze and Pitkänen, 2012). The two studies also contain several other important differences in methodology. First, the present study used a lower subconvulsive PTZ dose of 30 mg/kg, compared with 50 mg/kg used in the aforementioned study (Bolkvadze and Pitkänen, 2012). Second,

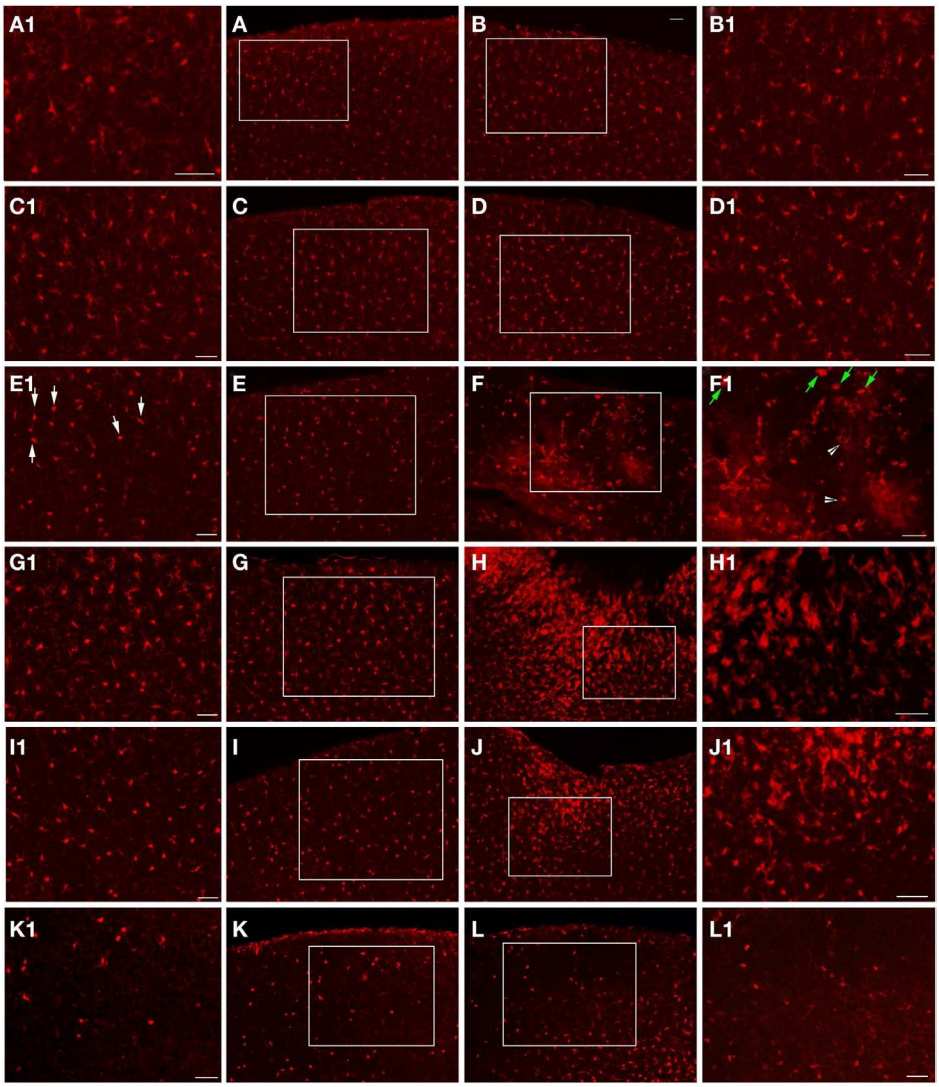


**FIGURE 6 | Epi-fluorescent microscopy of Iba1-labeling following FPI.** In **(A,B)**, images are from a naïve control mouse. In **(C,D)**, images are from a sham mouse. Note the typical distribution of Iba1-labeled microglial cells in both of these animals. Enlargements **(A1–D1)** are provided in order to illustrate the normal appearance of resting microglial cells in the region corresponding to the FPI-induced lesion **(B,D)** and the region corresponding to this area in the contralateral hemisphere **(A,C)**. Note that microglial cells in normal conditions show minimal overlap, with each cell occupying a specific domain within the parenchyma. At 1-day post-FPI **(E,F)**, some of the Iba1-labeled microglial cells in the contralateral hemisphere **(E1)** exhibit the early stages of microglial activation (white arrows). In the peri-lesion region **(F1)**, robust Iba1-labeling is apparent. Many of these labeled cells at this timepoint exhibit a relatively simple morphology (green arrows) and to a lesser extent more complex microglial cells (white arrowheads) are also evident (Shapiro et al., 2008). At 3 days post-FPI **(G,H)**, microglial activation is increased in the contralateral hemisphere **(G)** relative to 1-day post-FPI. In the peri-lesion region **(H)**, a robust Iba1-labeling is observed. At 7 days post-FPI **(I,J)**, the distribution and appearance of Iba1-labeled microglial cells in the contralateral hemisphere **(I)** is similar to 1-day post-FPI. In the peri-lesion area **(J)**, the microglial response is decreased relative to the 3-day timepoint, although there is still a substantial appearance of activated microglial cells. At 30 days post-FPI **(K,L)**, the Iba1-labeled microglial cells in the contralateral hemisphere **(K)** appear to be depleted. Of the few remaining Iba1-labeled microglial cells, many continue to exhibit an activated morphology. Similarly in the peri-lesion region **(L)**, there is a noticeable lack of Iba1-labeled microglial cells in layers I–IV in the region subjacent to where the lesion occurred. In layers V and VI, there are considerably more Iba1-labeled microglial cells, some of which exhibit varying degrees of activation. Scale bar in **(B)** = 50 μm for images **(A–L)**. Scale bar = 50 μm in images **(A1–L1)**.

the present study delivered a more moderate trauma (1.5–1.7 atm for 12–16 ms) compared with ∼2.9 atm for 21–23 ms (Bolkvadze and Pitkänen, 2012). It should be noted that previous studies in rat have shown that increasing the pressure level of the FPI, increases the extent of tissue damage, acute impairment, and the probability of post-traumatic epilepsy (Curia et al., 2011). However, since very few studies have examined FPI in mice, it is unclear what pressure level is required to produce epileptogenic effect. The data presented in the current study show that a moderate FPI is capable of increasing seizure susceptibility, a hallmark of the epileptogenic

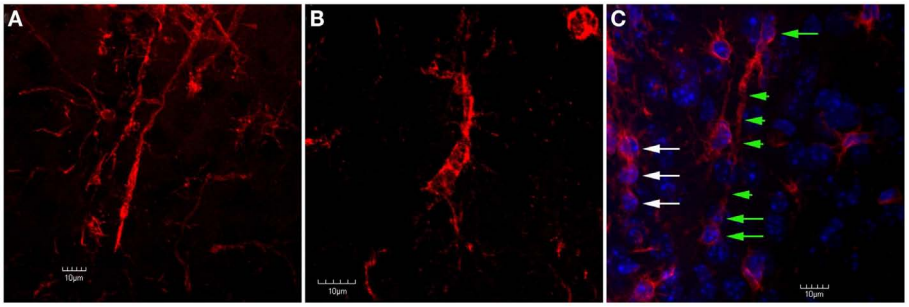


**FIGURE 7 | Confocal images of trains of Iba1-labeled microglial cells in the peri-lesion area.** In **(A)**, two separate trains of microglial cells are shown. In these trains, the microglial cells exhibit a rod-like appearance. Ziebell et al. (2012) have described a similar appearance of trains of rod-like cells in the somatosensory cortex following fluid percussion injury in rats. Similar to Ziebell et al. (2012), the rod-cells observed in the present study (both single and in trains) appeared perpendicular to the pial surface. In **(B)**, despite the lack of an elongated, rod-like appearance, the cells within this train appear to have their apical and basal processes connected. In **(C)**, Iba1-labeled cells are shown in tissue that has been counterstained using DAPI. Note that there are at least three rod-cells (green arrows) within this train that contain relatively long apical and basal processes (green arrowheads). Another train of cells that does not exhibit this rod shape (white arrows) is seen at the left edge of the image. Although these cells do not exhibit a rod shape, they still appear to be connected by their apical and basal processes. Scale bars in all images = 10 μm.

**Table 1 | Chi-Square results from contingency table analysis of stage IV/V seizures.**

| | Stage IV/V seizure | No stage IV/V seizure |
|---|---|---|
| FPI | 8* | 1 |
| Sham control | 1 | 8 |
| Naïve control | 0 | 5 |

*Note that there is no significant differences between sham control and naïve control groups. *Denotes significant difference from sham control and naïve control group ($p < 0.005$).*

**Table 2 | Chi-square results from contingency table analysis of median seizure frequency.**

| Treatment groups | Seizure frequency (median) |
|---|---|
| FPI | 7* |
| Sham control | 0 |
| Naïve control | 0 |

*Naïve control was not different from sham control. *Denotes significant difference from sham control and naïve control group ($p < 0.005$).*

progression. Third, the coordinates and size of the burr hole used in the two studies is different. In the present study, the burr hole diameter is 2 mm, compared to Bolkvadze and Pitkänen (2012) in which a 3-mm burr hole was drilled. Moreover, the present study used the coordinates (antero-posterior: +1.5 mm; medio-lateral: −1.2 mm), whereas the previous study did not indicate specific coordinates, rather that study indicated that it was the area over the left parietotemporal cortex between bregma and lambda. These latter coordinates are considerably more lateral compared to the current study. Moreover, a shift in the location of the craniotomy is associated with a alteration to the resulting lesion, such that more lateral coordinates are associated with an increased ipsilateral tissue damage in rats (Vink et al., 2001). Nevertheless, previous studies in the rat have shown that coordinates analogous to the ones used in the current study result in a robust pro-epileptogenic response (D'Ambrosio and Perucca, 2004; D'Ambrosio et al., 2004).

Histopathological examination of the tissue at several timepoints post-FPI revealed a stereotypical lesion, followed by scarring and a robust glial response. The glial response has clearly been initiated by 1 day post-FPI and peaks at 3 days post-FPI (**Figures 2–6**), as observed by astrocyte and microglial cell staining. By 7 days after FPI, a moderate level of healing has occurred such that the integrity of pial surface has been mostly restored. There are fewer activated astrocytes and microglial cells, and Fluoro-Jade labeling is considerably diminished relative to the 1- and 3-day timepoints. One cannot rule out the possibility that the decreased Fluoro-Jade labeling at 7 days post-FPI is as much a result of cells that have died, as it is cell that have been rescued. Consistent with this notion, a persistent area of necrotic tissue is evident in the region immediately surrounding the impact zone. It is pertinent to note that stereological cell counts for neurons was beyond the scope of this study, but it is entirely possible, if not likely, that neuronal loss and remodeling persists following FPI. By 30 days after the FPI, superficial examination of the tissue reveals only minor necrosis and the pial surface appears to have been repaired.

Although this study does not directly assess infiltrating components of neuroinflammation, the fact that there is blood brain barrier breakdown following FPI, as well as a robust astroglial and microglial response, is typically indicative of acute neuroinflammation (Streit et al., 2004). Moreover, we have previously performed analogous studies in the rat using the FPI paradigm and showed a robust inflammatory response in the cortex by 24 h after injury (Mukherjee et al., 2011). Previous studies examining molecular correlates of inflammation following traumatic brain injuries have indicated that the drilling of the Burr hole alone is sufficient to cause inflammation and mild levels of neuropathology (Cole et al., 2011). Therefore, the present study incorporated the use of both, a sham group that received identical treatment to the FPI group, minus the actual FPI delivery, as well as a naïve control which had

no experimental manipulations performed. In the sham groups, there was no noticeable lesion at any of the timepoints examined, nor was there appreciable astrocyte or microglial activation. Despite this lack of a lesion, or glial activation, it is still possible that neuroinflammatory proteins are altered in response to the craniotomy in sham mice. The fact that one animal in the sham group did exhibit stage IV/V seizures following the PTZ second-hit challenge supports the idea that the sham surgery has the potential to not only cause an inflammatory response (Cole et al., 2011), but may also increase seizure susceptibility (Galic et al., 2012). This idea is further supported by the fact that previous studies have shown that some inflammatory proteins are pro-convulsive (Kramer et al., 2012; Vezzani and Granata, 2005). Such findings underscore the need to incorporate both, a sham and a naïve control group when performing studies that pertain to inflammation and/or seizure effects of TBI.

In conclusion, this study demonstrates increased seizure susceptibility to a sub-threshold dose of PTZ at 30 days following a FPI. Taken together with the study from Bolkvadze and Pitkänen (2012), future studies can be carried out using transgenic mouse strains and the FPI method to further elucidate mechanisms of TBI.

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# Controlled cortical impact and craniotomy induce strikingly similar profiles of inflammatory gene expression, but with distinct kinetics

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An immediate consequence of traumatic brain injury (TBI) is the induction of an inflammatory response. Mounting data suggest that inflammation is a major contributor to TBI-induced brain damage. However, much remains unknown regarding the induction and regulation of the inflammatory response to TBI. In this study we compared the TBI-induced inflammatory response to severe parenchymal injury (controlled cortical impact) vs. mild brain injury (craniotomy) over a 21-day period. Our data show that both severe and mild brain injury induce a qualitatively similar inflammatory response, involving highly overlapping sets of effector molecules. However, kinetic analysis revealed that the inflammatory response to mild brain injury is of much shorter duration than the response to severe TBI. Specifically, the inflammatory response to severe brain injury persists for at least 21 days, whereas the response to mild brain injury returns to near baseline values within 10 days post-injury. Our data therefore imply that the development of accurate diagnostic tests of TBI severity that are based on imaging or biomarker analysis of the inflammatory response may require repeated measures over at least a 10-day period, post-injury.

**Keywords: traumatic brain injury, inflammation, genomics, glia, response to injury, mouse models, cytokines, diagnostics**

## INTRODUCTION

A major component of the biological response to traumatic brain injury (TBI) is induction of inflammation. TBI, like other forms of tissue injury, induces an immune response which is a form of sterile inflammation. This type of immune response is driven by the release of intracellular antigens which are normally hidden from leukocytes (Medzhitov, 2008; Chen and Nunez, 2010). A subset of these hidden antigens is highly immunogenic, driving immune activation by many of the same mechanisms employed in responses to pathogenic organisms. Specifically, certain autologous hidden antigens bind to and activate the same pattern recognition receptors [PRRs; e.g., toll-like receptors (TLRs)] that are the receptors for specific pathogen products. For example, TLR4 serves as the receptor for both bacterial lipopolysaccharide (LPS) and a number of hidden self-antigens, including HMGB1, hyaluronan, and specific proteins of the S100 family (Medzhitov, 2008; Chen and Nunez, 2010).

Toll-like receptors and other PRRs are found on many cell types throughout the body. However, hidden self-antigen mediated activation of such receptors on specific sentinel cells, particularly macrophages, triggers the initial release of pro-inflammatory cytokines, such as IL-1β. This cytokine response results in rapid activation of local blood vessel endothelial cells, resulting in recruitment of leukocytes from the blood stream, particularly neutrophils and monocytes. These phagocytic cells remove dead cells and other debris in the injured tissue, as well as performing a variety of other functions that promote tissue repair (Chen and Nunez, 2010).

In the case of brain injury, substantial evidence suggests that the PRR-expressing sentinel cells that initially trigger the injury-associated inflammatory response are brain tissue-resident macrophages (microglia) and astrocytes. The pro-inflammatory cytokines produced by microglia and astrocytes then initiate endothelial cell activation and recruitment of blood leukocytes (Fitch and Silver, 2008; Whitney et al., 2009), analogous to the inflammatory cascade that occurs in other tissues throughout the body. Although post-TBI inflammation plays an essential role in the healing response, there is also much evidence that the inflammatory response contributes to the death of bystander cells in the brain tissue that were not directly damaged by TBI (Loane and Byrnes, 2010). Furthermore, aspects of the inflammatory response may favor the formation of scar tissue (i.e., the glial scar), while disfavoring neuroregeneration (Fitch and Silver, 2008; Whitney et al., 2009; Griffiths et al., 2010; Neher et al., 2011). Thus, a detailed understanding of the inflammatory response to TBI is a necessary component in the effort to formulate successful strategies to diminish bystander cell injury and to promote neuroregeneration.

One of the gaps in the understanding of the biological response to TBI is how the magnitude of the injury influences the qualitative, quantitative, and kinetic characteristics of the inflammatory

response. A recently published study using a rat model of TBI (in which members of our team participated) included the interesting finding that specific inflammatory mediators were produced in significant amounts in response to craniotomy. This study assessed a small collection of cytokine protein levels at days+1 and +7 post-injury, comparing tissues from craniotomy vs. naïve animals (Cole et al., 2011). However, as there was no comparison to a more severe brain injury in this study, it was not possible to determine how the observed cytokine levels induced by craniotomy compare to the cytokine response to more severe injury of the parenchyma.

Therefore, to extend the findings of this previous report, we initiated a study in mice to determine to what degree the magnitude and kinetics of the inflammatory response to severe and mild brain injury differ (note that the inflammatory response to TBI in mice and rats is highly similar Natale et al., 2003). To address this question, we performed histological, behavior, protein, and global gene expression analyses comparing a model of severe parenchymal injury [controlled cortical impact (CCI); Lighthall, 1988] to mild brain injury (craniotomy). Our data show that both severe and mild brain injury induce a qualitatively similar inflammatory response, involving highly overlapping sets of genes. However, kinetic analysis revealed that the inflammatory response to mild brain injury is of much shorter duration than the response to severe TBI, allowing severe- and mild-TBI to be readily discriminated at day +10 post-injury and beyond. These data therefore have implications for the diagnosis of TBI severity. Specifically, the development of accurate diagnostic tests of TBI severity that are based on imaging or biomarker analysis of the inflammatory response may require repeated measures over at least a 10-day period, post-injury.

## MATERIALS AND METHODS

### ANIMALS AND SURGICAL PROCEDURES

Ten- to twelve-week old C57BL/6 male mice were subjected to TBI using an Impact One™Stereotaxic Impactor (myNeuroLab, St. Louis, MO, USA). Briefly, mice were anesthetized with 2% isoflurane in 98% oxygen, and were then positioned in the stereotaxic frame. Craniotomy was performed by a single skilled surgeon using a hand-held 5 mm trephine over the motor cortex (1.8 mm medial-lateral, 2 mm from Bregma). Mice were then subjected to CCI using a 3 mm flat-tip with a velocity of 5 m/s, a depth of 2.0 mm, and a duration of 200 ms. After trauma, the craniotomy was closed with the previously removed bone and bone wax, and the incision was closed with sutures. Craniotomy animals underwent the same procedure as the CCI group, except that the stereotaxic impactor was not used. Craniotomies were performed with great care, in order to avoid disruption of the dura. A few mice in the CCI group displayed slight hemorrhage, primarily on days +1 and +3 post-CCI. When hemorrhage was present, the wound was cleaned prior to tissue harvest. There was no visible hemorrhage in any craniotomy-only animal. Naïve mice were anesthetized with 2% isoflurane in 98% oxygen, monitored until recovery from anesthesia, and transferred to fresh cages. The Institutional Animal Care and Use Committee at Uniformed Services University (USU) approved all animal procedures.

### HISTOLOGY

For hematoxylin and eosin (H&E) staining, brains were harvested from CCI, craniotomy, and naïve animals at day +7. Brains were perfused with 1 × PBS then fixed and stored in 4% paraformaldehyde. Tissue embedding, processing, and H&E staining were performed by Histoserv Inc. Note that the dura was lost from some regions of craniotomy and naïve brains during sectioning, although it was intact at the time of tissue harvest. Histology slides were viewed and scanned using the Nanozoomer Digital Pathology version 2.0-RS (Hamamatsu Photonics, Japan). Nanozoomer data were analyzed using NDP viewer software (Hamamatsu Photonics, Japan).

For immunofluorescence microscopy analysis, mice were perfused with a cold solution of 4% paraformaldehyde in 1 × PBS, followed by immediate brain harvest and 8–10 h cryopreservation in 30% sucrose. Brains were then frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA) and stored at −80°C. Coronal cryosections (20 μm) were collected and stored at −80°C until immunostaining with the anti-GFAP antibody.

### TISSUE HARVESTING AND RNA EXTRACTION

Animals were sacrificed on days +1, +2, +3, +7, +10, and +21. Mice were perfused with 1 × PBS and brains were collected. Two brain regions were harvested from naïve, craniotomy and CCI mice: a 5 mm diameter punch biopsy encompassing the exact injury site on the left hemisphere and another 5 mm biopsy recovering the equivalent non-injured (contralateral) site on the right hemisphere. The depth of the punch was approximately 5 mm, penetrating the base of the brain. RNA was extracted from the biopsy tissue using guanidinium isothiocyanate-phenol extraction (Chomczynski and Sacchi, 1987).

### REAL-TIME PCR ANALYSES

The above RNA samples (2 μg) were reverse transcribed to cDNA, using random hexamers and Superscript II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA), in a 1-h reaction at 42°C. Real-time PCR analysis of cDNA was performed using an RT-PCR master mix for TaqMan assays (SydLabs, Inc., Malden, MA, USA) and an iQ5 instrument (Bio-Rad, Hercules, CA, USA) in 96-well format with 20 μl reaction volume per well. Primers for TaqMan assays were designed using Primer Express 3.0 software (Life Technologies, Carlsbad, CA, USA). PCR primers and FAM-ZEN double-quenched probes were purchased from IDT (Coralville, IA, USA). Primer sequences are listed in **Table 1**. GAPDH was used as a normalization control for all probe sets. Samples were collected from both the ipsilateral and contralateral sites. Three or four mice were used for each experimental group at each time point.

The delta Ct ($\Delta C_t$) method was used for PCR array data analysis. The normalized $\Delta C_t$ for each gene of interest (GOI) was calculated by deducting the $C_t$ of the housekeeping gene (HKG: GAPDH) from the $C_t$ of each GOI: $\Delta C_t = \left(C_t^{GOI} - C_t^{HKG}\right)$. The ΔΔCt for each GOI was calculated by deducting the average $\Delta C_t$ of GOI in the naïve or craniotomy group from the $\Delta C_t$ of each GOI in the CCI group: $\Delta\Delta C_t = \Delta C_t$ (CCI group) – average $\Delta C_t$ (naïve or craniotomy group). The fold-change of each

**Table 1 | Real-time PCR primers.**

| | |
|---|---|
| **CCL2** | |
| Sense | GGCTCAGCCAGATGCAGTTAA |
| Anti-sense | CCTACTCATTGGGATCATCTTGCT |
| Probe | CCCCACTCACCTGCTGCTACTCATTCA |
| **IL-1β** | |
| Sense | GAGCACCTTCTTTTCCTTCATCTT |
| Anti-sense | CACACACCAGCAGGTTATCATCA |
| Probe | AGAAGAGCCCATCCTCTGTGACTCATGG |
| **TNF-α** | |
| Sense | GGTCCCCAAAGGGATGAGAA |
| Anti-sense | TGAGGGTCTGGGCCATAGAA |
| Probe | TTCCCAAATGGCCTCCCTCTCATCA |
| **AQP4** | |
| Sense | GGTTGGAGGATTGGGAGTCA |
| Anti-sense | GTGAACACCAACTGGAAAGTGATT |
| Probe | CACGGTTCATGGAAACCTCACCGC |
| **VIMENTIN** | |
| Sense | GGAGATGCTCCAGAGAGAGGAA |
| Anti-sense | GTGCCAGAGAAGCATTGTCAAC |
| Probe | CGAAAGCACCCTGCAGTCATTCAGACA |
| **MMP3** | |
| Sense | TGATGAACGATGGACAGAGGAT |
| Anti-sense | AGCCTTGGCTGAGTGGTAGAGT |
| Probe | TTGCTGCTCATGAACTTGGCCACTCC |
| **SAA3** | |
| Sense | CGCAGCACGAGCAGGAT |
| Anti-sense | GCTGTCAACTCCCAGGATCAA |
| Probe | AGCCTTCCATTGCCATCATTCTTTGCA |
| **C3** | |
| Sense | GCCAAGGACTGCAGACTGAAC |
| Anti-sense | CACTTCCGAAGACCCTTGTCA |
| Probe | CGCCGCCGTCGCTCAGTACAGT |
| **GAPDH** | |
| Sense | TGTGTCCGTCGTGGATCTGA |
| Anti-sense | CCTGCTTCACCACCTTCTTGA |
| Probe | CCGCCTGGAGAAACCTGCCAAGTATG |

*Sequences of primers (5′ to 3′) used for TaqMan real-time PCR are listed. All hydrolysis probes were FAM-labeled and included an internal ZEN quencher. Each PCR amplicon crosses a splice junction.*

GOI compared to the naïve or craniotomy group was calculated as: Fold-change $= 2^{(-\Delta\Delta Ct)}$.

### ELECTROCHEMILUMINESCENT IMMUNOASSAY ANALYSIS OF CYTOKINES IN BRAIN HOMOGENATES

Brain homogenates were prepared from punch biopsies (5 mm diameter cannula) from the injury and contralateral sites. Tissue was weighed and homogenized in 10 volumes per weight of T-Per extraction buffer (Pierce Biotechnology, Rockford, IL, USA) with Halt protease Inhibitor (Pierce Biotechnology, Rockford, IL, USA) utilizing a Bioruptor UCD-200 ultrasonic disruptor (Diagenode, Sparta, NJ, USA), as previously described (Cole et al., 2011). Total protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Analyte levels of cytokines were measured using the Mouse Pro-inflammatory 7-Plex Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, MD, USA). During the protocol, plates were washed using the BioTek ELx405 Select automated liquid handling platform. Imaging of the plates was performed using a Sector 6000 Imager (Meso Scale Discovery, Gaithersburg, MD, USA). A standard curve for each analyte was curve-fitted, allowing determination of the concentration in pg cytokine/mL sample volume in each well, which was normalized to total protein input, yielding analyte amount, expressed as pg cytokine/mg total protein.

### MICROARRAY ANALYSES

MouseRef-8 v2.0 Expression BeadChips (Illumina Inc., San Diego, CA, USA) were used to measure relative levels of mRNA expression for over 19,000 unique genes. Preparation of cDNA, probe hybridization, and data collection were carried out at the Cleveland Clinic. Background subtracted, quantile normalized data were analyzed using GenomeStudio (Illumina Inc., San Diego, CA, USA) and GSEA (Broad Institute, Cambridge, MA, USA) software packages.

### BEHAVIOR STUDIES

All behavior tests were performed on days −1, +1, +3, +7, +10, +14, and +21, relative to surgical procedures. Rotarod testing was performed as previously described (Vitali and Clarke, 2004). Briefly, mice were acclimated to the rotarod apparatus (Ugo Basile, Collegeville, PA, USA) for 60 s at a fixed speed of 5 rpm. After the adaptation phase, animals were placed on the rotarod and the acceleration was increased from 5 to 60 rpm within 180 s. Latency to fall from the accelerating rotarod and the reached speed were recorded for each mouse. Three trials were performed for each animal and the average was reported.

For balance beam testing, mice were placed on a narrow beam (0.5 cm) and trained to cross the beam for three consecutive days before the first test. On the testing day, the mice were placed on the beam and the time spent to cross and the number of foot slips occurring during the beam cross were recorded. Three trials were performed for each animal and the average was calculated.

### STATISTICAL ANALYSES

Behavior data and protein expression data were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons with *Tukey's post hoc test*. A two-tailed Student's *t*-test was used for comparison between two groups. Real-time PCR data were analyzed using a Mann–Whitney test. A $p$ value $< 0.05$ was considered statistically significant.

## RESULTS

### HISTOLOGICAL ANALYSIS OF BRAIN INJURIES

Hematoxylin and eosin staining of coronal sections of brains from CCI, craniotomy, and naïve animals was performed to assess tissue damage. Low-magnification images revealed severe damage to the parenchyma of CCI brains, but no obvious tissue disruption in craniotomy animals (**Figure 1A**). Higher magnification H&E images showed immune cell infiltration and/or microglia activation and expansion around the site of injury, in response to the severe CCI injury (**Figure 1B**). Moreover, at the injury site in craniotomy animals, H&E staining suggested immune cell infiltration

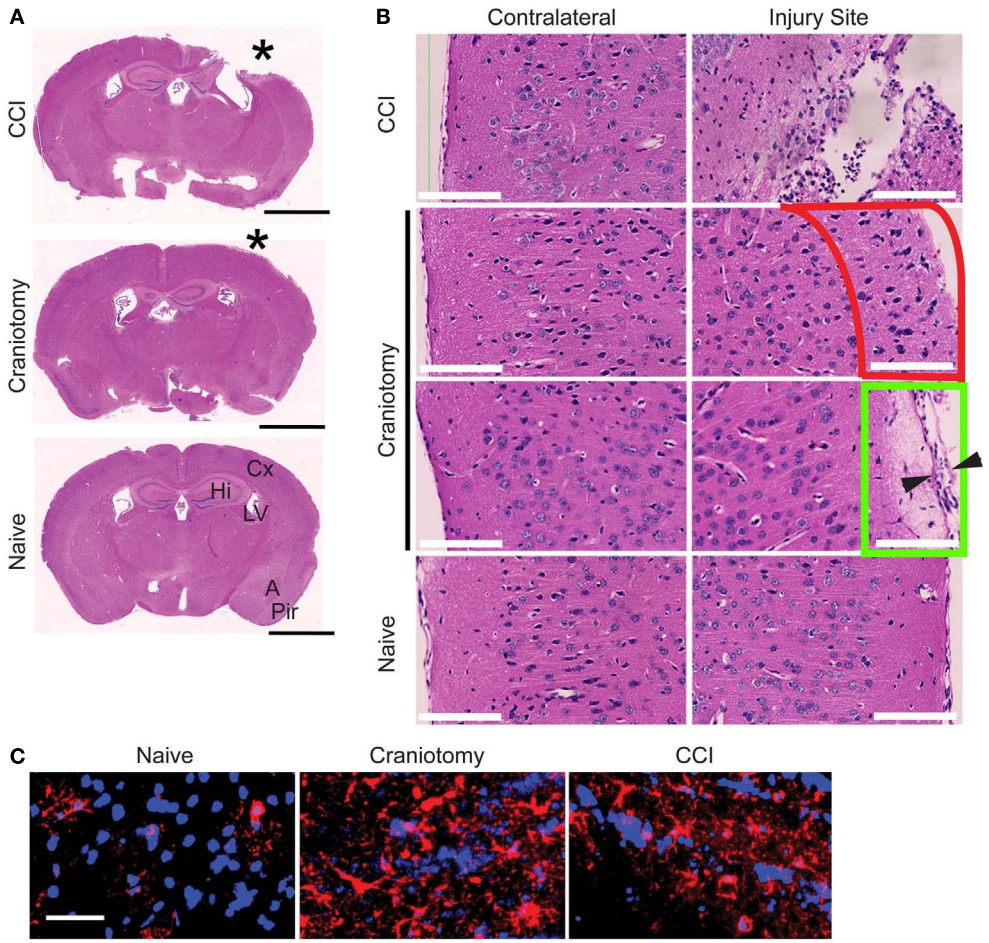


**FIGURE 1 | Both severe and mild brain injury is accompanied by a cellular inflammatory response. (A)** H&E staining of coronal sections prepared from brains of CCI, craniotomy, or naïve animals at day +7. Images were collected on a Nanozoomer instrument and are displayed at a 2 × magnification. *, injury site. Bar, 2 mm. Cx, cortex; Hi, hippocampus; LV, lateral ventricle; A, amygdala; Pir, piriform cortex. **(B)** Tissue sections from **(A)** are shown at a 40 × magnification at the injury site or the equivalent contralateral location, as indicated. Red outline indicates cortical layer 1, and Green outline indicates region of altered dura (arrowheads) and underlying tissue at the injury site of the craniotomy samples. Bar, 100 μm. **(C)** Anti-GFAP immunofluorescence microscopy analysis of frozen brain sections from CCI, craniotomy, and naïve animals. Regions imaged were proximal to the injury site. CCI and craniotomy brains were harvested at day +3. Data are representative of three mice from each experimental group (naïve, craniotomy, and CCI). Bar, 40 μm.

and/or expansion of activated microglia in cortical layer 1 (outlined in red), with disruption of the normal architecture of the dura and underlying cortical cells (outlined in green. Compare to contralateral sections; **Figure 1B**). Additionally, immunofluorescence microscopy using an anti-GFAP antibody showed evidence of astrogliosis near the site of injury in both CCI and craniotomy animals. Specifically, CCI and craniotomy animals had regions of high astrocyte density and enlarged astrocyte bodies, relative to naïve controls (**Figure 1C**). Together, the data in **Figure 1** provide evidence that CCI and craniotomy-induced severe injury and mild injury to the brain, respectively. Additionally, this histological analysis provided evidence of a cellular inflammatory response following both CCI and craniotomy.

## BEHAVIORAL ANALYSIS OF CCI AND CRANIOTOMY ANIMALS

CCI injury and craniotomy were performed directly over the motor cortex. Post-injury motor function was assessed via rotarod and balance beam assays ($n = 12$). In the rotarod task, CCI mice showed a significant deficit in performance on day +1, compared to pre-injury performance on day −1 (**Table 2**). Moreover, **Figures 2A,B** shows that both the maximum speed attained and the latency to fall from an accelerating rotarod decreased significantly among the CCI mice compared to the craniotomy mice at days +1 and +3. Although not statistically significant after day +3, there was a clear and consistent difference between the CCI and craniotomy animals, which persisted for at least 3 weeks post-injury (study end). Indeed, craniotomy mice showed no deficit following surgery, but rather continued to improve their performance throughout the first 2 weeks following injury [naïve mice show a very similar learning-based improvement over the same interval (data not shown)]. By day +7, the performance of CCI mice returned to the baseline level, and this improvement was statistically significant when comparing day +1 to day +14 (**Table 2**).

**Table 2 | Statistically significant behavior data.**

| Assay | *p* Values |
|---|---|
| **ROTAROD, ATTAINED SPEED** | |
| CCI day −1 vs. CCI day +1 | $p < 0.05$ |
| CCI day +1 vs. CCI day +14 | $p < 0.05$ |
| **ROTAROD, LATENCY TO FALL** | |
| CCI day −1 vs. CCI day +1 | $p < 0.05$ |
| CCI day +1 vs. CCI day +14 | $p < 0.05$ |
| **BALANCE BEAM, CROSSING TIME** | |
| Craniotomy day +1 vs. craniotomy day +3 | $p < 0.05$ |
| Craniotomy day +1 vs. craniotomy day +7 | $p < 0.01$ |
| Craniotomy day +1 vs. craniotomy day +10 | $p < 0.05$ |
| CCI day −1 vs. CCI day +1 | $p < 0.001$ |
| CCI day +1 vs. CCI day +3 | $p < 0.001$ |
| CCI day +1 vs. CCI day +7 | $p < 0.001$ |
| CCI day +1 vs. CCI day +10 | $p < 0.001$ |
| CCI day +1 vs. CCI day +14 | $p < 0.001$ |
| CCI day +1 vs. CCI day +21 | $p < 0.001$ |
| **BALANCE BEAM, FOOT SLIPS** | |
| Craniotomy day −1 vs. craniotomy day +1 | $p < 0.001$ |
| Craniotomy day +1 vs. craniotomy day +3 | $p < 0.01$ |
| Craniotomy day +1 vs. craniotomy day +7 | $p < 0.001$ |
| Craniotomy day +1 vs. craniotomy day +10 | $p < 0.01$ |
| CCI day −1 vs. CCI day +1 | $p < 0.05$ |
| CCI day −1 vs. CCI day +3 | $p < 0.001$ |
| CCI day +1 vs. CCI day +3 | $p < 0.01$ |
| CCI day +3 vs. CCI day +7 | $p < 0.001$ |
| CCI day +3 vs. CCI day +10 | $p < 0.001$ |
| CCI day +3 vs. CCI day +14 | $p < 0.01$ |
| CCI day +3 vs. CCI day +21 | $p < 0.01$ |

*Behavior data in **Figure 2** were analyzed by ANOVA with Tukey's multiple comparison test. Significant differences between groups ($p < 0.05$) are shown. All comparisons not shown in this table were not significant, with the exception of significant CCI vs. craniotomy t-test data (included in **Figure 2**).*

In the balance beam task, both CCI and craniotomy mice were significantly affected during the first week post-injury (**Figures 2C,D**). The crossing time was significantly increased for CCI animals, comparing day −1 to day +1, and the number of foot slips was significantly increased on days +1 and +3 (**Table 2**). For the craniotomy animals, only the number of foot slips was significantly increased between day −1 and +1 (**Table 2**). Notably, during the first week post-injury, motor performance was more severely impaired by CCI than by craniotomy. These data were significant for the beam crossing time on day +7 and for foot slips on days +3 and +7 (**Figures 2C,D**). By day +10, both craniotomy and CCI mice showed a significant improvement in their performance on the balance beam, vs. the post-injury day of most severe impairment (**Figures 2C,D**; **Table 2**).

Together, the data in **Figure 2** show that the effect of brain injury on motor function was most pronounced during the first week post-TBI, with some deficits in function observed for both CCI and craniotomy animals. The significant differences observed in the performance of CCI vs. craniotomy animals during the first week post-injury is consistent with the severe parenchymal damage induced by CCI vs. the more subtle brain injury induced by craniotomy (**Figure 1**).

### INFLAMMATORY CYTOKINE PROTEIN RESPONSE TO TRAUMATIC BRAIN INJURY

Previous studies have established that moderate to severe TBI is accompanied by inflammation (Ciallella et al., 2002; Harting et al., 2008; Rhodes et al., 2009; Dalgard et al., 2012). A recent study by members of our team suggested that there is also significant induction of several inflammatory mediators in response to mild brain injury (craniotomy) in the rat model system (Cole et al., 2011). To determine whether mice also show similar inflammatory responses to both CCI and craniotomy, we profiled the protein expression of a subset of cytokines in brain tissue using a multiplexed ELISA detection platform. For this analysis, we employed a 5 mm punch biopsy to recover the tissue at the site of CCI and craniotomy, and from the equivalent site at the non-injured (contralateral) hemispheres. Tissues were harvested from injured animals at days +1, +3, and +7, and from naïve mice.

Of the seven cytokines measured, six were significantly increased following CCI, as compared to naïve controls (**Figure 3**; **Table 3**). Of these six cytokines, peak expression was observed at day +1 for three cytokines (CXCL1, IL-1β, and IL-6), while the other three cytokines exhibited peak expression at day +3 (IL-12p70, IFN-γ, and IL-10).

In comparing naïve animals to CCI animals at the injury site, CXCL1 and IL-6 were significantly increased at days +1 and +3, while IL-1β was significantly increased only at day +1. The craniotomy tissue demonstrated a similar significant increase in CXCL1 and IL-6 protein expression at day +1, in comparison to naïve tissue. The contralateral site did not exhibit significant increases in production of these cytokines in CCI or craniotomy subjects. The exception was CXCL1, which was significantly increased in craniotomy subjects at day +1, as compared to controls.

In contrast to CXCL1, IL-1β, and IL-6, the changes in peak expression for IL-12p70, IFN-γ, and IL-10 after injury were delayed and modest. Significant increases were detected only at the injury site. When comparing injured to naïve tissue, IL-12p70, IFN-γ, and IL-10 were all significantly elevated at day +3, while only IL-10 was significantly elevated at day +1. After day +3, expression of these cytokines declined to non-significant levels, vs. naïve. Interestingly, the kinetics of expression of these cytokines in craniotomy tissue was slower than in CCI tissue, with significant increases of IL-12p70, IFN-γ, and IL-10 not observed until day +7.

Together, the data in **Figure 3** and **Table 3** illustrate that expression of six different cytokines was significantly increased in response to severe brain injury (CCI). Importantly, expression of all but one of these cytokines was also significantly increased at the injury site in response to mild brain injury (craniotomy). Additionally, the peak levels of cytokine production were similar (within a factor of three), when comparing CCI vs. craniotomy at the injury site. Indeed, when comparing each day for CCI to the corresponding day for craniotomy, the only significant difference was IL-6 at day +1 (**Figure 3**; **Table 3**). Thus, severe brain injury

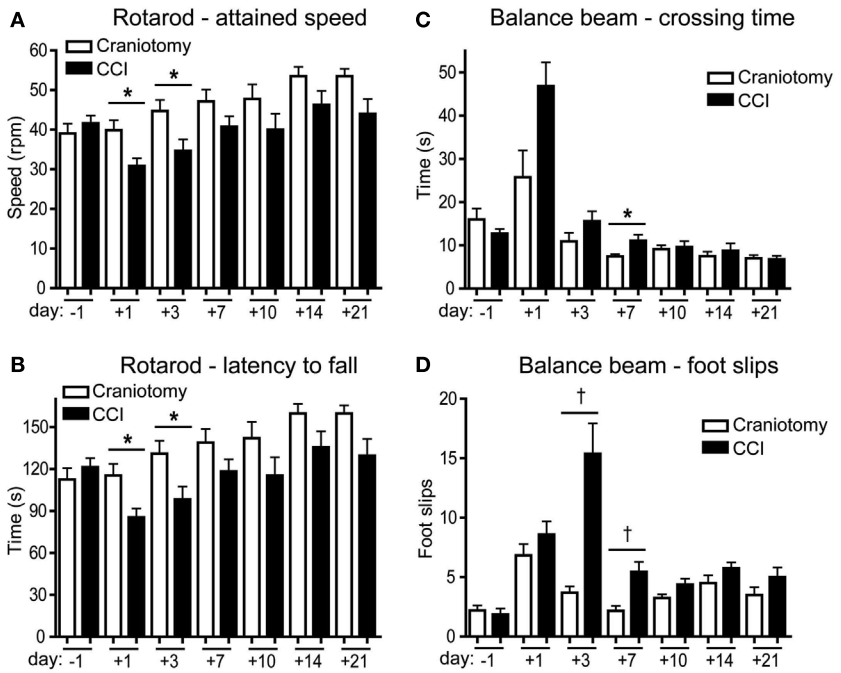


**FIGURE 2 | Both severe and mild injury induce behavioral deficits.** **(A,B)** Rotarod assay ($n = 12$). Mice were assessed to determine the maximum speed attained **(A)** and the latency to fall **(B)** during execution of the accelerating rotarod task **(B)**. **(C,D)** Balance beam assay. Mice were assessed for latency to cross the beam **(C)** and the number of foot slips during the cross **(D)**. All tests were performed at day −1, and at multiple time points post-injury over a 21-day period. Error bars are SEM. The Student's *t*-test was used to determine significance when comparing CCI to craniotomy samples; ($^* = p < 0.05$, $^\dagger = p < 0.005$). Significant differences within the CCI and craniotomy groups were determined using ANOVA with Tukey's multiple comparison test (**Table 2**).

and mild brain injury induce a quantitatively similar inflammatory cytokine response during the first 7 days post-injury.

### MICROARRAY ANALYSIS OF INFLAMMATORY GENE EXPRESSION

To assess whether the above expression data for selected inflammatory proteins could be generalized to the global inflammatory response, we performed a genome-wide microarray analysis. Brain tissue biopsies were collected from CCI, craniotomy, and naive animals, as described for the cytokine protein analysis in **Figure 3**. mRNA was harvested from three to four animals per time point per condition, and samples from individual animals were pooled prior to cDNA synthesis. Pooled cDNAs were analyzed via Illumina bead-chip microarrays. We examined selected markers of inflammation to assess general trends in inflammatory gene expression. We chose genes from four diverse functional sets: inflammatory cytokines, astrocyte activation markers, markers of antigen presenting cell (APC)/microglia activation, and effectors of opsonization and phagocytosis. Although a number of different kinetic patterns were noted, a consistent observation was that the genes induced by CCI were also induced by craniotomy (**Figure 4**). Interestingly, the general gene expression kinetic trends were quite similar in the CCI and craniotomy groups, with a day +1 or +3 expression peak frequently observed in both groups. For both CCI and craniotomy groups, induction of inflammatory gene expression in the contralateral sample was either not detected or less than that observed in the CCI tissue. However, a general difference between the CCI and craniotomy groups was that values generally returned to baseline in the craniotomy group by day +21. In contrast, the CCI tissues generally remained above the naïve baseline at day +21. Thus, these data show that inflammation-related gene expression is highly similar between severe parenchymal injury (CCI) and mild brain injury (craniotomy), with regard to the intensity of gene expression and the kinetic pattern of gene expression. The major notable difference was the persistence of inflammation in response to severe injury (CCI) at day +21, in apparent contrast to the mild injury (craniotomy) group.

### GENE SET ENRICHMENT ANALYSIS OF MICROARRAY DATA

We also analyzed the microarray data by Gene Set Enrichment Analysis (GSEA; Subramanian et al., 2005) to determine whether the patterns of inflammatory gene expression among genes sampled in **Figure 4** were representative of the global inflammatory response to CCI and craniotomy. For this analysis, we chose CCL3 as a representative phenotype among the gene expression profiles shown in **Figure 4**. GSEA software identified gene expression profiles with similarity to CCL3 and clustered these profiles into functionally related gene sets. Over 200 gene sets were scored as enriched, and approximately 100 of these sets were scored as statistically significant.

Among the sets with highest statistical significance were the Immune System Process set and the Regulation of IκB Kinase/NF-κB Cascade set, both of which reflect components of the inflammatory response (**Figure 5**). For these two sets, the false discovery rate (FDR) *q*-values were 0.082 and 0.115, respectively (values < 0.25

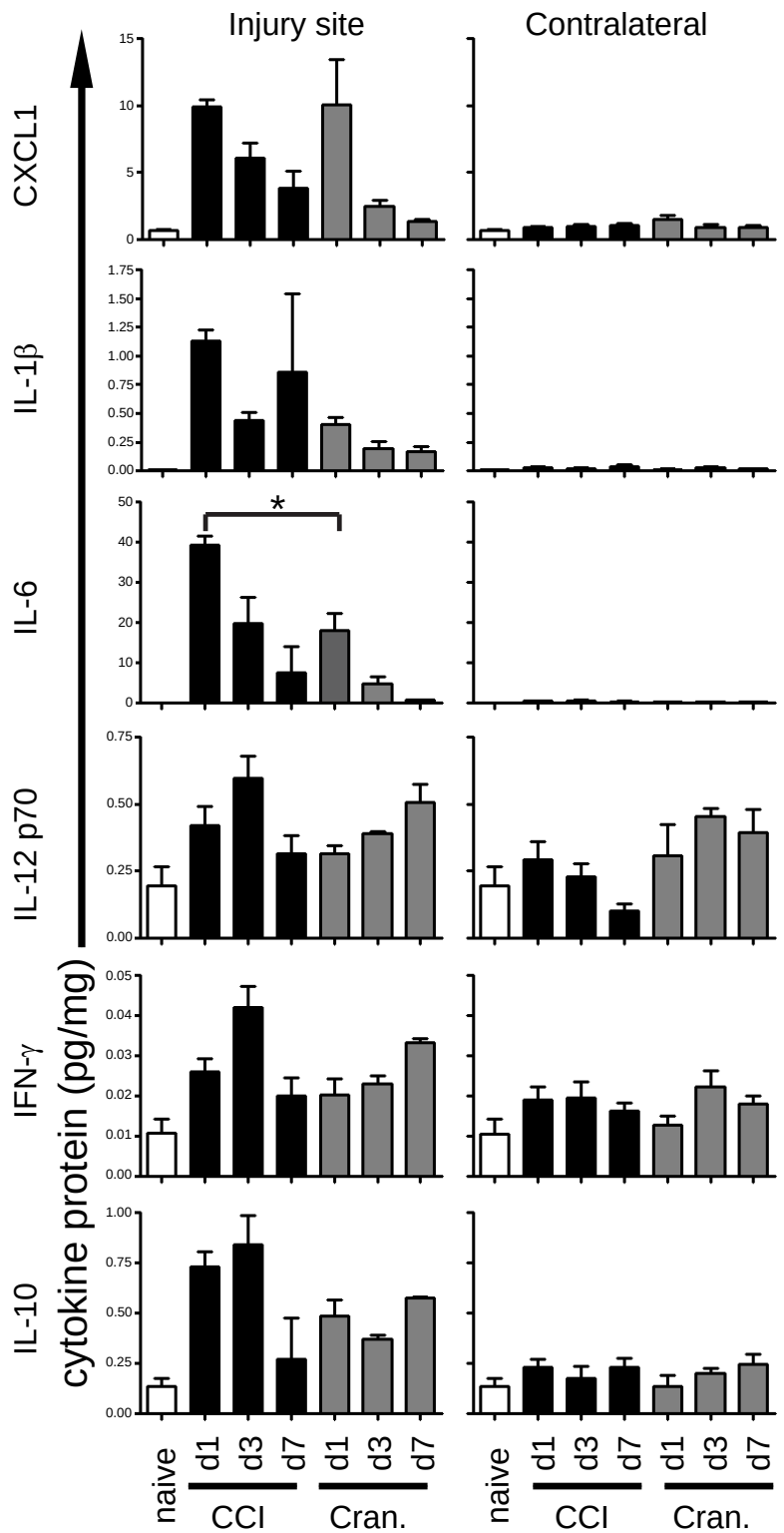


**FIGURE 3 | Inflammatory cytokines are induced to similar levels by both severe and mild brain injury.** Brain tissue biopsies from the injured or contralateral sites were harvested from CCI and craniotomy (Cran.) animals on the indicated days post-injury. Tissues were also harvested from naïve animals to establish the baseline for each assay. Cytokine proteins in brain tissue homogenates were quantified by electrochemiluminescent immunoassay. Three mice from each experimental group (naïve, craniotomy, and CCI) were used for this analysis. Error bars are SEM. Statistically significant differences between groups are indicated in **Table 3**. Additionally, the single finding of a statistically significant difference between CCI and craniotomy on equivalent days is indicated by an asterisk (*).

**Table 3 | Statistically significant changes in cytokine protein levels.**

| Cytokine measurement | *p* Values |
|---|---|
| **CXCL1 – INJURY SITE** | |
| Naive vs. CCI day +1 | $p < 0.001$ |
| Naive vs. CCI day +3 | $p < 0.05$ |
| Naive vs. craniotomy day +1 | $p < 0.001$ |
| Craniotomy day +1 vs. craniotomy day +3 | $p < 0.01$ |
| Craniotomy day +1 vs. craniotomy day +7 | $p < 0.01$ |
| **CXCL1 – CONTRALATERAL SITE** | |
| Naive vs. craniotomy day +1 | $p < 0.01$ |
| **IL-6 – INJURY SITE** | |
| Naive vs. CCI day +1 | $p < 0.001$ |
| Naive vs. CCI day +3 | $p < 0.01$ |
| Naive vs. craniotomy day +1 | $p < 0.05$ |
| CCI day +1 vs. CCI day +3 | $p < 0.05$ |
| CCI day +1 vs. CCI day +7 | $p < 0.001$ |
| CCI day +1 vs. craniotomy day +1 | $p < 0.05$ |
| **IL-1β – INJURY SITE** | |
| Naive vs. CCI day +1 | $p < 0.05$ |
| **IL-10 – INJURY SITE** | |
| Naive vs. CCI day +1 | $p < 0.01$ |
| Naive vs. CCI day +3 | $p < 0.001$ |
| Naive vs. craniotomy day +7 | $p < 0.05$ |
| CCI day+3 vs. CCI day +7 | $p < 0.05$ |
| **IL-12 p70 – INJURY SITE** | |
| Naive vs. CCI day +3 | $p < 0.01$ |
| Naive vs craniotomy day +7 | $p < 0.05$ |
| **IFNγ – INJURY SITE** | |
| Naive vs. CCI day +3 | $p < 0.001$ |
| Naive vs. craniotomy day +7 | $p < 0.01$ |
| CCI day +3 vs. CCI day +7 | $p < 0.05$ |

*Cytokine data in **Figure 3** were analyzed by ANOVA with Tukey's multiple comparison test. Significant differences between groups ($p < 0.05$) are shown. All comparisons not shown were not significant.*

are considered significant Subramanian et al., 2005). For both sets, similar trends in gene activation were observed between CCI and craniotomy animals, with a day +3 activation peak being most prominent for both CCI (large arrow) and craniotomy (small arrow). Activation was strong at the injury site, but not detected or weak at the contralateral site, consistent with the analysis of selected genes in **Figure 4**. However, such differences in magnitude of activation appeared greatest at day +21, at which point the CCI-induced RNAs generally remained elevated relative to naïve; whereas the craniotomy-induced RNAs had generally returned to the naïve baseline value.

### REAL-TIME PCR ANALYSIS OF INFLAMMATORY GENE EXPRESSION KINETICS

The above microarray data suggested that CCI and craniotomy induce a highly similar inflammatory gene expression program in the brain. Moreover, these data suggested that the magnitude of inflammation-associated gene expression was similar at all time points through day +7. However, the data also suggested that the inflammatory gene expression in the CCI and craniotomy groups diverge by day +21, with robust inflammation maintained by the CCI animals, but not the craniotomy animals. To confirm these observations with a more sensitive technique, we used TaqMan real-time PCR to quantify relative levels of transcription for an assortment of eight inflammation-associated genes (**Figure 6**; **Table 4**), which included cytokines (CCL2, IL-1β, TNF-α), markers of astrocyte activation [Aquaporin-4 (AQP4), Vimentin (VIM), Matrix metalloproteinase-3 (MMP3)], and RNAs encoding proteins of the macrophage acute phase response [serum amyloid A3 (SAA3), complement C3]. In addition to the days +1, +3, +7, and +21 time points analyzed in the microarray data, we also included days +2 and +10. This real-time PCR analysis was performed on samples from individual mice (3–4 mice/condition/time point), allowing quantification of biological variability.

Data were quantified as fold-difference in expression for three ratios: CCI/Naïve, Craniotomy/Naïve, and CCI/Craniotomy (**Figure 6**). This analysis generally provided a confirmation of microarray data presented in **Figure 4**. For example, in the case of IL-1β, the peak mRNA levels at the injury site were at day +1 for both CCI and craniotomy. Also, the IL-1β mRNA levels at the injury site for the CCI samples at day +1 was approximately 2 × higher by microarray vs. 4 × higher by real-time PCR, as compared to the craniotomy samples. For certain transcripts, such as CCL2, the real-time PCR provided much higher signal-to-noise than the microarray analysis, revealing a strong induction of CCL2 transcription by craniotomy that was not evident in the microarray data. Also, the inclusion of additional time points in the real-time PCR analysis revealed important details of the kinetics for the expression of certain genes. For example, day +10 represents the peak AQP4 and C3 mRNA expression (among included time points) at the injury site for CCI animals. Day +10 also represents a secondary peak of transcription of CCL2 and IL-1β genes, in response to CCI.

Importantly, the real-time PCR analysis confirmed the overall trend suggested by the microarray analyses (**Figures 4** and **5**). Specifically, at the site of injury, whereas inflammatory gene expression persisted beyond day +7 (and in some cases continued to intensify) in the CCI animals, inflammatory gene expression in the craniotomy animals generally reached or approached baseline values beyond day +7. This trend was particularly apparent when assessing the CCI/craniotomy ratios for the injury site data, in which the greatest difference between CCI and craniotomy was at day +10 or +21 for all eight mRNAs. Moreover, whereas the majority of transcripts exhibited less than a 10-fold difference in the CCI/craniotomy ratio between days +1 and +7, three genes (MMP3, SAA3, and C3) showed a greater than 50-fold difference between mRNA abundance in CCI and craniotomy animals at day +10 or +21 (note, however, that the MMP3 data were not significant, due to high variability at these late time points).

Notably, for all transcripts except for MMP3, statistically significant differences were observed between CCI and craniotomy tissues (at the injury site) for day +10 and/or +21. Although significant differences between these same groups were also seen at day +1 for five transcripts (IL-1β, TNFα, VIM, MMP3, and C3), it is important to note that the fold-differences were greater at day +10 and/or +21. Together, these data reinforce the conclusions suggested by **Figures 3** and **5**. Specifically,

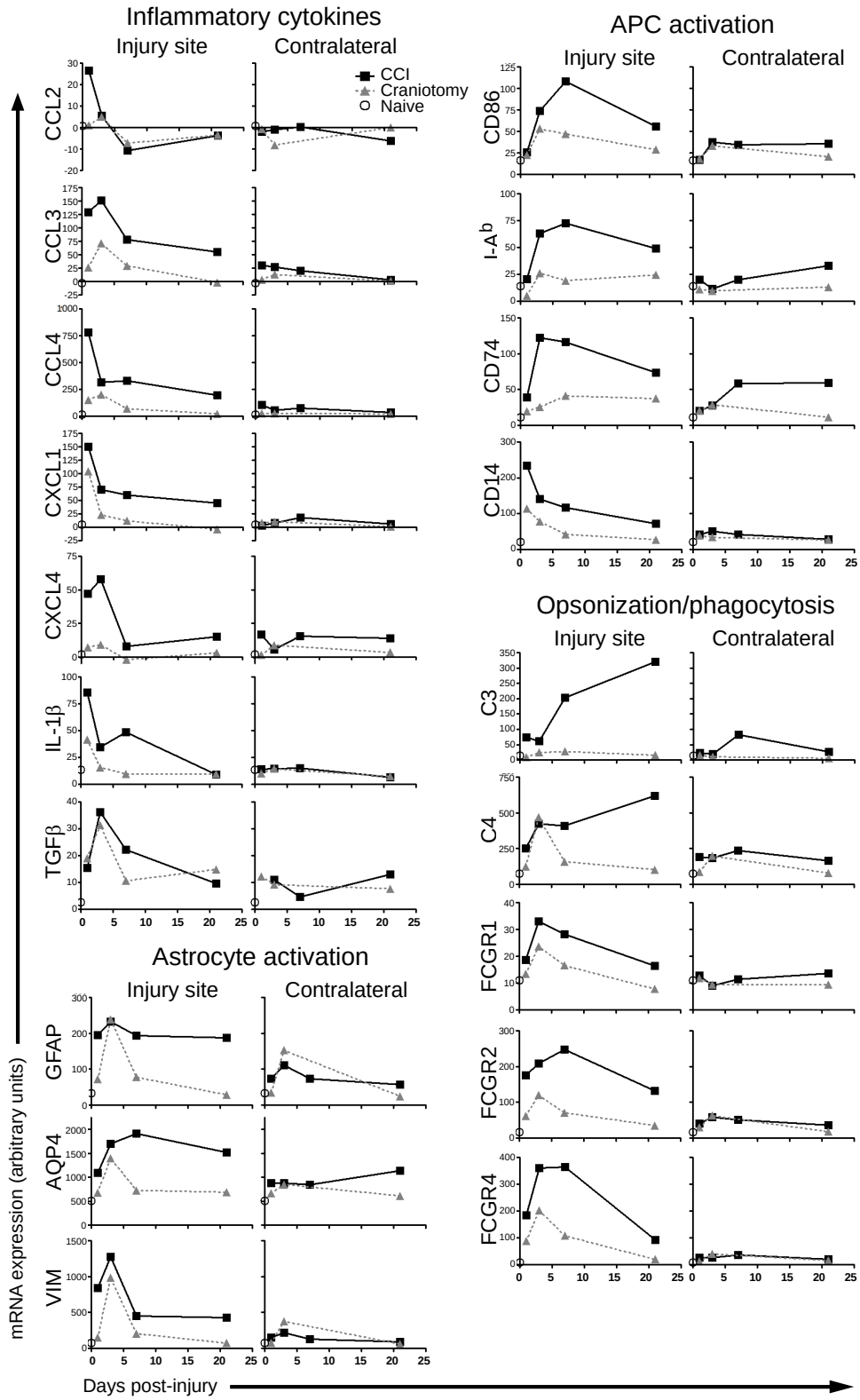


**FIGURE 4 | Microarray analysis shows severe and mild brain injury induce expression of multiple inflammation-related transcripts with similar kinetics.** Injury site and contralateral site biopsies were harvested from CCI and craniotomy mice at the indicated times post-injury. Brain tissue was also harvested from naïve animals. Total RNA samples from individual animals from each experimental group (3–4 mice/group) were pooled, and cDNA was synthesized and analyzed by microarray. Relative expression levels are shown for selected genes in the indicated functional groups.

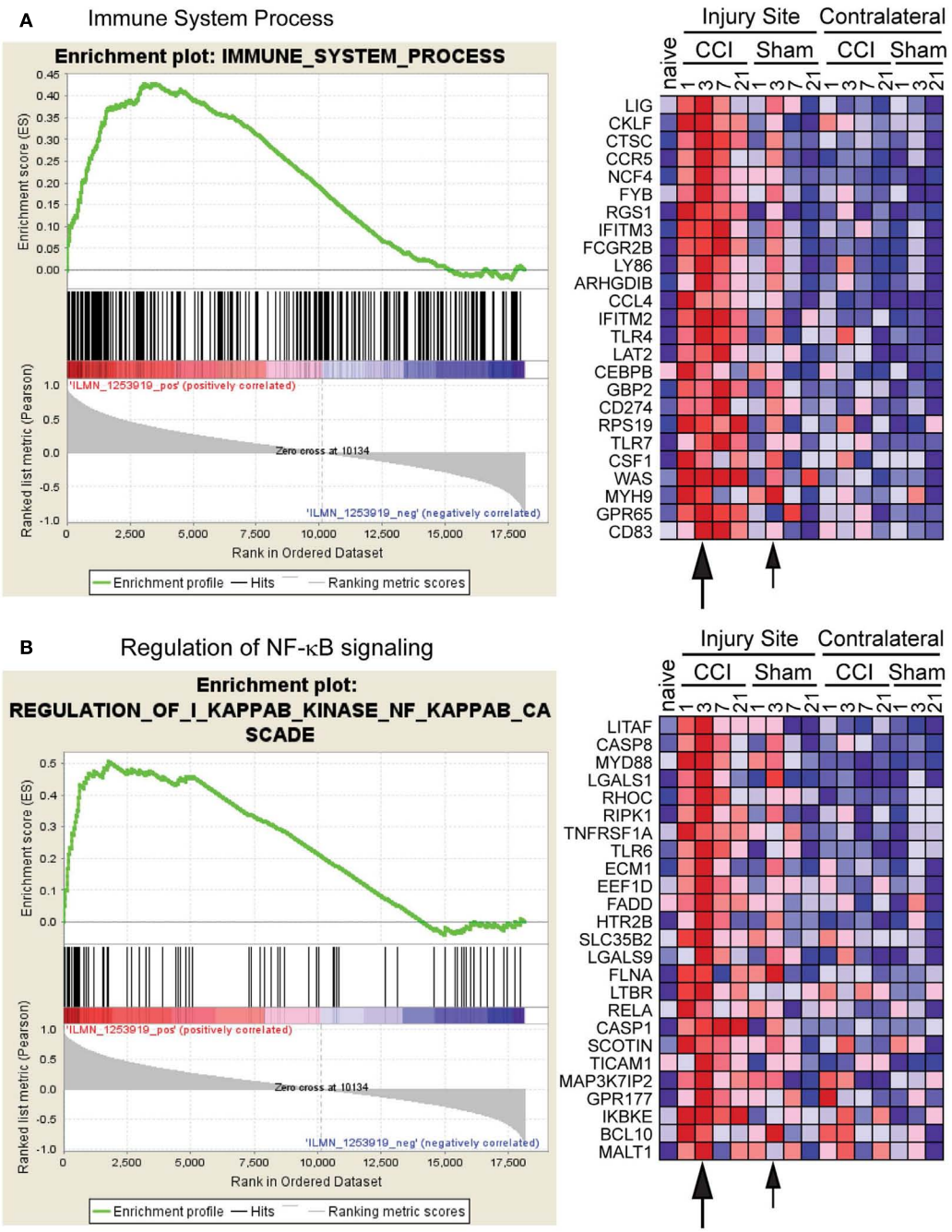


**FIGURE 5 | Gene set enrichment analysis (GSEA) confirms induction of a broad inflammatory response by both severe and mild injury.** Microarray data were analyzed using GSEA software to identify functionally related groups of genes (gene sets) with statistically significant enrichment, using CCL3 as the gene expression phenotype. The figure shows the enrichment plot and the top 25 enriched genes for **(A)** the Immune System Process set and **(B)** the Regulation of NF-κB Signaling (Regulation of IκB kinase/NF-κB Cascade) set. The plot on the left shows the distribution of genes in the set that are positively and negatively correlated with the CCL3 phenotype. The plot on the right shows the relative gene expression (red = high, blue = low) for each gene for the indicated samples. Note that the overall kinetic profiles are similar for the CCI and craniotomy (Cran) samples, with a prominent gene expression peak at day +3 (large and small arrows indicate the day +3 peak for CCI and craniotomy, respectively). However, the craniotomy samples generally show a lower intensity of gene expression, particularly at day +21.

severe brain injury and mild brain injury induce very similar inflammatory responses through approximately day +7. Following day +7 (during the period of days +10 through +21 in our analysis), the inflammatory response to severe injury persists, whereas the response to mild injury returns to baseline for most genes.

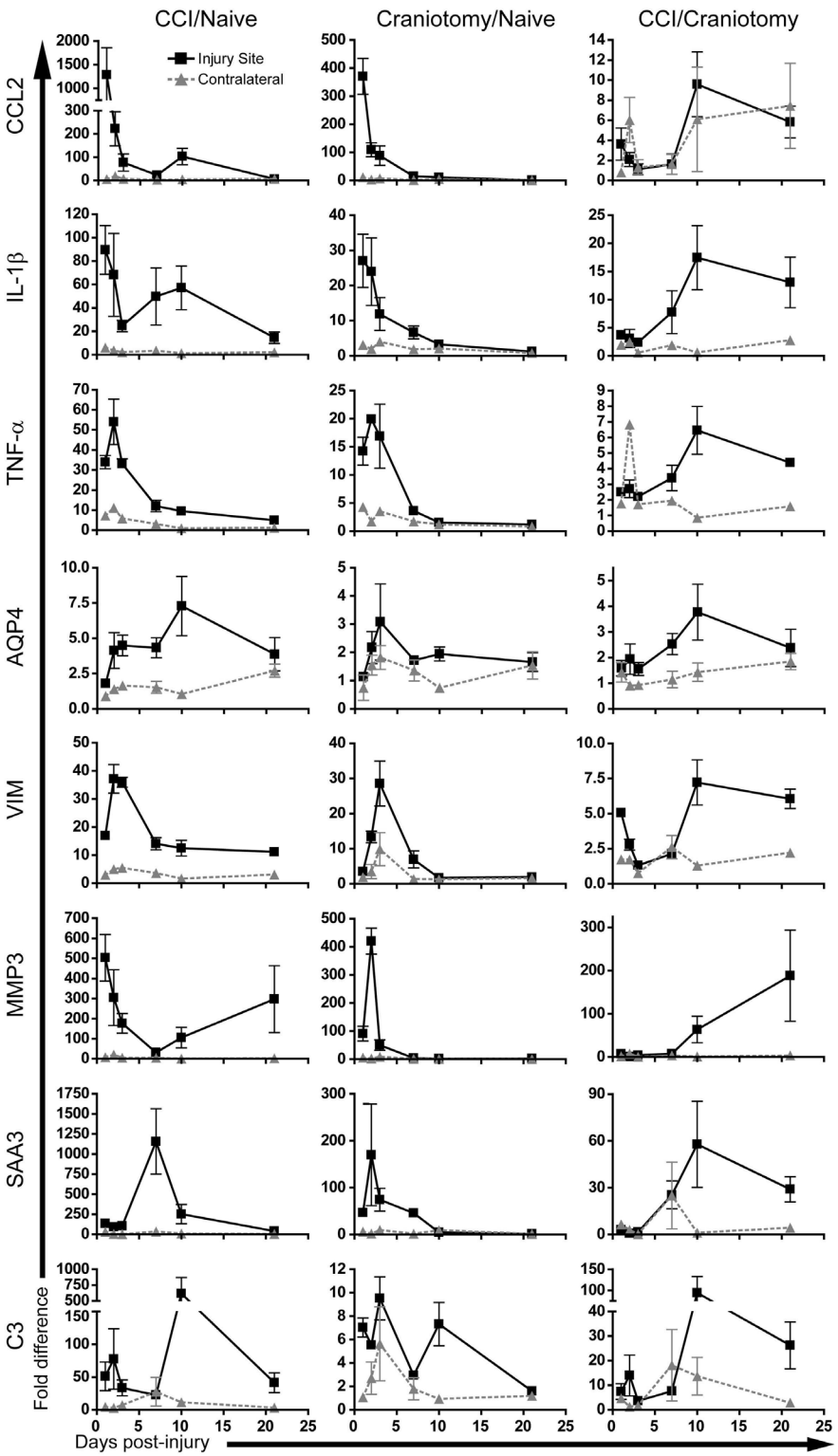


**FIGURE 6 | The inflammatory response to severe brain injury persists for several weeks, whereas the response to mild injury declines rapidly after day +7.** Real-time PCR analysis of mRNA levels for the indicated genes at days +1, +2, +3, +7, +10, and +21. Samples from individual animals from both ipsilateral and contralateral sites were tested for the expression of the specified genes. Three or four mice were analyzed for each time point in each experimental group. Data are expressed as ratios of CCI/Naïve, Craniotomy/Naïve, and CCI/Craniotomy, as indicated, with the *y*-axis indicating fold-difference in gene expression. Note that the CCI/Craniotomy fold-difference at the injury site is greatest after day +7 for all analyzed genes. Significant differences in mRNA levels in the CCI vs. Craniotomy groups are listed in **Table 4**. Error bars are SEM.

**Table 4 | Statistically significant changes in gene expression.**

| Assay | *p* Values |
|---|---|
| **CCL2 – INJURY SITE** | |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |
| **IL-1β – INJURY SITE** | |
| CCI vs. craniotomy – day+1 | $p < 0.05$ |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| **TNFα – INJURY SITE** | |
| CCI vs. craniotomy – day+1 | $p < 0.05$ |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |
| **AQP4 – INJURY SITE** | |
| CCI vs. craniotomy – day+7 | $p < 0.05$ |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |
| **VIM – INJURY SITE** | |
| CCI vs. craniotomy – day+1 | $p < 0.0001$ |
| CCI vs. craniotomy – day+2 | $p < 0.05$ |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |
| **MMP3 – INJURY SITE** | |
| CCI vs. craniotomy – day+1 | $p < 0.05$ |
| **SAA3 – INJURY SITE** | |
| CCI vs. craniotomy – day+10 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |
| **C3 – INJURY SITE** | |
| CCI vs. craniotomy – day+1 | $p < 0.05$ |
| CCI vs. craniotomy – day+7 | $p < 0.05$ |
| CCI vs. craniotomy – day+21 | $p < 0.05$ |

*Real-time PCR data in **Figure 6** were analyzed using a Mann–Whitney test. Significant differences between CCI and craniotomy groups ($p < 0.05$) are shown. All comparisons not shown were not significant.*

## DISCUSSION

In this study, we performed a 21-day kinetic analysis of the inflammatory response to severe and mild brain injury, examining both the injury site and the equivalent site on the contralateral hemisphere. The histological data in **Figure 1** support the severe nature of the CCI injury and the more subtle nature of injury-associated with craniotomy. Specifically, CCI-induced a substantial loss of brain tissue beneath the injury site, accompanied by considerable inflammatory cell infiltration and astrocyte activation. In contrast, the craniotomy animals showed no evidence of tissue loss. However, craniotomy was not innocuous, as demonstrated by histological changes within the injury site: H&E staining of coronal sections showed increased numbers of inflammatory cells and changes to the dura and underlying parenchyma. Anti-GFAP immunofluorescence suggested astrogliosis. Thus, although there was no apparent brain tissue loss due to craniotomy, there was clear evidence of an inflammatory response.

Behavior data (**Figure 2**) showed a clear difference in impairment of motor function in CCI vs. craniotomy animals. Between day −1 and day +1, only the CCI animals exhibited a significant impairment in performance in the rotarod assay, whereas both injuries significantly impaired performance on the balance beam. Thus, both severe and mild injury to the motor cortex cause at least a transient functional impairment. However, direct comparison of CCI vs. craniotomy animals revealed significant differences on days +1 and +3 for the rotarod, and on days +3 and +7 for the balance beam. Based on these data, we conclude that craniotomy induces a mild and transient functional impairment, while CCI more severely impairs function for at least a week post-injury. The behavior data are consistent with the histological data (**Figure 1**).

Quantification of protein levels for a limited selection of inflammatory cytokines also supported the histological data. In comparison to naïve controls, there was a significant elevation of each of the measured inflammatory cytokines at one or more time points at the injury site in both the CCI and craniotomy groups (with the exception of IL-1β, for which the measured increase did not reach significance in the craniotomy group). Although increases in cytokine expression were generally confined to the site of injury, it is notable that in one instance (CXCL1 at day +1), we did detect a significant increase at the contralateral site in the craniotomy group. This finding suggests that certain cytokines diffuse over considerable distances following brain injury, and/or that long-range diffusion of hidden self-antigens stimulates resident pro-inflammatory cells (e.g., microglia and astrocytes) far from the site of injury. The mRNA expression data in **Figures 4** and **6** are suggestive of the latter possibility.

Surprisingly, over the first week post-injury, the levels of inflammatory protein expression in the CCI and craniotomy groups were of similar magnitude, even though the extent of tissue damage was substantially different. Indeed, the only statistically significant difference between CCI and craniotomy was for IL-6 at day +1. These data suggest that major differences in the extent of brain tissue injury are reflected by modest differences in inflammatory cytokine production.

Because our cytokine protein analysis included a limited number of inflammatory mediators, we performed a genome-wide microarray analysis. The microarray data confirmed and extended the cytokine protein measurements. Specifically, these data showed that CCI and craniotomy induce the transcription of an identical or highly overlapping set of soluble and cell-associated regulators of inflammation. Furthermore, the kinetics and magnitude of induction of these genes was highly similar during the first week post-injury. Therefore, through day +7, there is little difference between the global inflammatory response induced by a severe brain lesion with substantial tissue destruction vs. a mild brain injury with minimal damage to the parenchyma.

Importantly, however, our data show that following day +7, the inflammatory responses to severe and mild brain injury become discordant. In general, inflammatory gene expression persisted thorough at least day +21 in the CCI group, while returning to naïve baseline levels in the craniotomy animals by day +10. We presume that this difference reflects both the time required for phagocytic cells to clear dead tissue and the ongoing cell death (and concomitant pro-inflammatory signaling by persistent release of hidden self-antigens) in the penumbral region surrounding the site of direct tissue damage (Fitch and Silver, 2008; Loane and Byrnes, 2010) in the severe injury (CCI) group. Not only did the

CCI animals exhibit persistent (≥21 days) expression of the great majority of measured inflammation-associated genes, but a subset of genes, including complement C3, reached their peak expression *after* day +7.

In general, the mRNA and protein expression data are consistent with the behavior data: the greatest behavioral deficit correlated with the peak of the inflammatory response. Such a finding is consistent with the phenomenon of cytokine-induced sickness behavior, in which pro-inflammatory cytokines interact with the brain, inducing broad behavioral changes (Dantzer, 2001; Capuron and Miller, 2011). Based on our behavior data (which showed no significant behavioral deficit beyond day +7), it is not clear whether the persistent inflammatory response in CCI animals is correlated with any functional deficits. As the CCI injury resulted in clear tissue destruction, it is also difficult to assess the degree to which cell loss vs. inflammation contributed to the observed phenotypes. To better assess the relationship between inflammation and functional deficits, it will thus be important to develop TBI models that yield persistent inflammation with minimal tissue destruction, for testing with a wide array of behavioral assays.

We are not aware of detailed kinetic assessments of the global inflammatory response following other types of TBI, although the limited existing data are in general agreement with our findings. Specifically, a recent study using a mouse model of blast injury (Cernak et al., 2011) included semi-quantitative PCR findings consistent with our data. Measurements of CCL2 in the hippocampus and brainstem and GFAP in the hippocampus showed significant elevation of transcription in response to moderate blast, persisting until at least day +30 (study end). Mild blast also caused increased transcription of these genes, with day +1 levels very similar to moderate blast. However, by day +30, mRNA levels in the mild blast animals returned to baseline. Thus, the relationship between the inflammatory response induced by mild blast vs. moderate blast may be analogous to the relationship between craniotomy and CCI.

Regarding closed-head concussive injury models (weight drop or impactor device), investigators have reported transient increases in transcription of inflammatory genes (Crack et al., 2009; Israelsson et al., 2009) and persistent activation of microglia (Venkatesan et al., 2010). Another study failed to detect significant elevations of inflammatory cytokine proteins (Semple et al., 2010), although there were trends toward elevation of inflammatory mediators at early times post-injury. Because of differences in injury delivery, time points assessed, and analytical methods, it is difficult to distil these data to a consensus finding regarding inflammation following closed-head concussive injury. In general, however, these studies do suggest the induction of a transient inflammatory response, with peak expression by day +1 or day +3.

Although we speculate that repeated closed-head injury will trigger a more persistent inflammatory state in the brain, we are not aware of published data addressing this prediction. Given the accumulating clinical data showing striking pathology resulting from repeated concussive injuries (Baugh et al., 2012), it will be important to determine whether persistent inflammation contributes to the neurodegenerative response associated with repeated closed-head concussive injury.

With the increasing focus on TBI resulting from military deployments, concussion-prone sports, and auto accidents, many investigators are attempting to develop minimally invasive strategies to assess the extent and/or severity of brain damage resulting from a known or suspected recent TBI (Kubal, 2012). As inflammation is a predictable response to brain injury, measurement of inflammation is being explored as a proxy for brain damage. Specific approaches include the use of probes to detect activated macrophages in the brain through magnetic resonance imaging (MRI) and positron emission tomography (PET; Stoll and Bendszus, 2009; Wunder et al., 2009; Sibson et al., 2011), and use of antibody-based assays to detect biomarkers of inflammation in the blood or cerebral-spinal fluid (Agoston et al., 2009; Korfias et al., 2009; Svetlov et al., 2009). However, our data illustrate that such strategies, when employed as single time point tests within the first week of injury, may be unable to accurately assess the severity of brain tissue injury. Based on our data, we predict that accurate quantification of TBI severity will require repeated measures of inflammation performed over a period of at least 10 days. We furthermore propose that those inflammation-associated genes which show peak expression after day +7 may represent ideal biomarkers of TBI severity. This idea will require further validation.

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# Cerebrospinal fluid biomarker candidates for Parkinsonian disorders

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The Parkinsonian disorders are a large group of neurodegenerative diseases including idiopathic Parkinson's disease (PD) and atypical Parkinsonian disorders (APD), such as multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration, and dementia with Lewy bodies. The etiology of these disorders is not known although it is considered to be a combination of genetic and environmental factors. One of the greatest obstacles for developing efficacious disease-modifying treatment strategies is the lack of biomarkers. Reliable biomarkers are needed for early and accurate diagnosis, to measure disease progression, and response to therapy. In this review several of the most promising cerebrospinal biomarker candidates are discussed. Alpha-synuclein seems to be intimately involved in the pathogenesis of synucleinopathies and its levels can be measured in the cerebrospinal fluid and in plasma. In a similar way, tau protein accumulation seems to be involved in the pathogenesis of tauopathies. Urate, a potent antioxidant, seems to be associated to the risk of developing PD and with its progression. Neurofilament light chain levels are increased in APD compared with PD and healthy controls. The new "omics" techniques are potent tools offering new insights in the patho-etiology of these disorders. Some of the difficulties encountered in developing biomarkers are discussed together with future perspectives.

**Keywords: Parkinson disease, Parkinsonian disorders, cerebrospinal fluid, biomarkers, proteomics**

## PARKINSONIAN DISORDERS

The Parkinsonian disorders have in common, to various degrees, the parkinsonism, defined as the presence of at least two of six movement abnormalities, of which either no. 1 or no. 2 are compulsory: (1) hypokinesia or diminished movement activity (also called bradykinesia, slowness of movement); (2) rest tremor; (3) rigidity (muscular stiffness); (4) loss of postural reflexes; (5) flexed posture; and (6) the freezing phenomenon (when the feet seem temporarily to be glued to the floor; Fahn, 2003). In addition to the motor abnormalities, specific combinations of non-motor symptoms such as autonomic and neuropsychiatric disorders, balance and ocular movement abnormalities, developing at various disease stages, characterize each particular Parkinsonian disorder, with major implications with regard to morbidity, treatment, and prognosis.

The Parkinsonian disorders (**Figure 1**) represent a large group of neurodegenerative diseases affecting a considerable number of patients, most of whom are elderly. Parkinson's disease (PD) dominates the group by far, as the most prevalent in the population, but also on scientific grounds, as a flagship for neurodegeneration in general, and due to the overwhelming impact which levodopa, its highly efficacious symptomatic treatment, has had on neurology. To the more uncommon atypical Parkinsonian disorders (APD) belong multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and dementia with Lewy bodies (DLB). Depending on the nature of the abnormal proteins which aggregate in the nervous tissue in these diseases, they can be subclassified as either synucleinopathies (PD, MSA, and DLB) with alpha-synuclein accumulation, or tauopathies (PSP and CBD) with tau protein accumulation. The oftentimes deceptively similar clinical pictures of these diseases can make the differential diagnosis difficult, especially in early stages; generally, the clinical diagnostic accuracy is lower for APD compared with PD (Hughes et al., 2002). Due to the global aging of the population, the number of patients affected by these, for now, incurable disorders will expand in the future (Dorsey et al., 2007), with considerable strains on the health care system and society at large, increasing the need for developing new, efficacious therapies.

## BIOMARKERS

### DEFINITION

The word "biomarker" is being used widely but not always correctly. The term was defined in 2001 by the Biomarkers Definitions Working Group as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Surrogate endpoints are a subgroup of biomarkers. They are a substitute for clinical endpoints which is what we really are interested in, reflecting how the patient is doing in reality. The requirements for a biomarker to serve as a surrogate endpoint are very strict and, at the present time, we do not have any surrogate endpoints in Parkinsonian disorders. However, any reliable biomarker, even if not strong enough to be a surrogate endpoint,

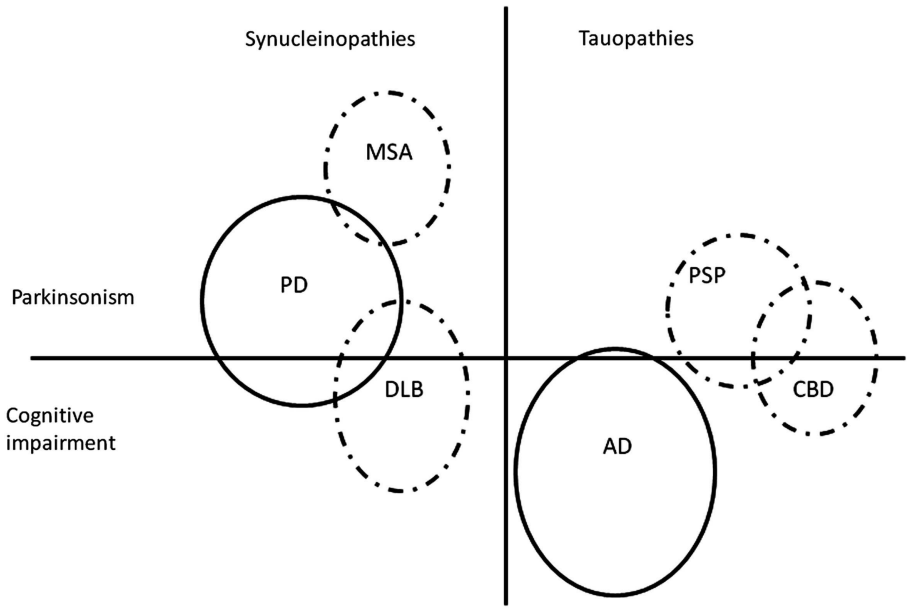


**FIGURE 1 | Simplified and non-exhaustive visual representation of two groups of protein accumulation disorders (synucleinopathies and tauopathies), two major groups of symptoms (Parkinsonism and cognitive impairment), and some but not all possible interactions in-between.** All of the depicted disorders are Parkinsonian disorders with the exception of Alzheimer's disease. The figure is not on scale. AD, Alzheimer's disease; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; PD, Parkinson's disease; PSP, progressive supranuclear palsy. Dashed line, atypical Parkinsonian disorders.

would be tremendously valuable. In order for a parameter to be considered a biomarker for a certain disease, it must fulfill several requirements: (1) Validity: there must be a correlation between the biomarker and the disease which it stands for; a treatment must affect the disease and not only the biomarker itself; (2) Performance: how good is the biomarker? How well does it differentiate between affected and non-effected? The biomarker assessment must be reliable and reproducible, both in the same patient at different points in time, and at different centers. It must be feasible in a clinical context and that implies safety, tolerability, simplicity, and low cost; (3) Generalizability: the performance in different patient subsets, based, e.g., on age, gender, disease stage, and medication, must be known (Brooks et al., 2003; Marek et al., 2008). It is easy to use the word "biomarker," but the implications of this word are profound, and despite all the efforts, we cannot say, for the time being, that we really have a biomarker for Parkinsonian disorders. What we do have in neurological sciences are: (1) biomarkers for certain disease-related processes, such as neurofilament light chain (NFL) as a biomarker of axonal degeneration, particularly damage to large-caliber, myelinated axons; and (2) different forms of protein inclusions, such as the 42 amino acid isoform of amyloid β (Aβ42) as a biomarker of Alzheimer-related senile plaque pathology.

### TYPES OF POTENTIAL BIOMARKERS FOR PARKINSONIAN DISORDERS

There are different types of potential biomarkers for neurodegenerative disorders: biochemical analysis of blood, cerebrospinal fluid (CSF), urine or brain tissues, genetics, and multiple imaging modalities (e.g., different MRI techniques, SPECT, PET, and ultrasound of substantia nigra). In addition, several clinical markers are used to measure different aspects of the diseases and to track their progression: motor analysis; assessments of olfaction, autonomic functions, cognition, sleep, speech and swallowing, neuropsychological, and psychiatric investigations (Marek et al., 2008). This overview is only concerned with biochemical markers, mostly in the CSF but to a lesser degree also in the blood.

According to the aims of the investigation and the technique utilized, there are two main approaches to assess body fluids and body/brain tissues for biomarkers:

(1) Targeted search to investigate one or several *a priori* defined compounds in patients and in healthy controls and looking for differences, patterns, and associations.
(2) Untargeted search to investigate broadly a large amount of components in a sample and compare patients with healthy controls. Nowadays, this is achieved by the "omics" techniques.

## THE "OMICS" TECHNIQUES

The relatively new "omics" techniques present both an enormous potential, through their capacity of screening wide and complementary areas of different biological materials, and a significant challenge, through the huge amount of data that are generated and need interpretation. In biologic materials, transcriptomics, proteomics, and metabolomics evaluate the transient, momentaneous, or "state" characteristics of a sample while genomics mirror its permanent or "trait" characteristics.

## GENOMICS

Genomic studies survey and compare genomes in patients and controls, looking for associations between gene alleles, genetic risk factors, and disease. The more restricted candidate gene approach investigates specific genes in the context of a certain disease, such as mutations in the alpha-synuclein gene (SNCA) causing a rare form of autosomal dominant PD. The genome-wide association studies, a more recent technique, investigate the whole genome. Genetic studies and metaanalyses have found more than 16 PARK loci associated with PD and 11 genes for PARK loci, and new insights are gained every year (International Parkinson's Disease Genomics Consortium and Wellcome Trust Case Control Consortium, 2011; Lill et al., 2012). Five of the identified genes induce a roughly typical PD presentation [a-synuclein, parkin, PTEN induced putative kinase 1, DJ-1, and leucine-rich repeat kinase 2 (LRRK2)] while mutations of ATP13A2 (PARK9) cause Kufor–Rakeb disease characterized by both Parkinsonism and many atypical features (Coppede, 2012). A genetic biomarker is unchangeable and indicates a trait, a predisposition to develop a disease. However, it does not indicate whether the disease has started or how advanced it is; it does not provide information about the state. Due to environmental factors, age, or reduced penetrance, the trait may or may not induce a state of disease during the lifetime of the bearer. The LRRK2 mutation is an example of a genetic trait for an autosomal dominant form of PD with variable penetrance probably due to non-genetic factors. Through genome-wide association studies, Simon-Sanchez et al. (2009) found a strong association in PD with the alpha-synuclein gene (SNCA) and, surprisingly for a synucleinopathy, also with the MAPT locus, related to tau protein.

An emerging research field is epigenetics which may bridge the gap between the apparently unchanging genome and the ever changing environment. There is evidence from both human but mostly from *in vitro* and animal models that DNA methylation, histone modifications, and small RNA-mediated mechanisms, could modify the expression of PD-related genes such as the alpha-synuclein gene, DJ-1, LRRK2, and parkin-gene, and thereby contributing to the development of the disease (Marques et al., 2011; Coppede, 2012).

## TRANSCRIPTOMICS

Transcriptomics investigates mRNA levels of expressed genes coding for proteins. Several studies have examined cells from substantia nigra in PD patients, controls, and PD animal models. Differences were found between controls and patients but the results in regard to particular genes were not similar between studies (Smith, 2009; Caudle et al., 2010). However, looking at patterns, findings became more consistent across studies and a pattern could be discerned showing that genes involved in oxidative stress, mitochondrial function, protein degradation, dopaminergic transmission, and axonal guiding were expressed differently in the different diagnostic groups (Smith, 2009; Caudle et al., 2010).

## PROTEOMICS

Proteomics characterizes the protein content – the proteome of a sample. Comparing the proteomes of patients and controls, differences may be found. The technology is based on three components: (1) separation of proteins; (2) analyzing proteins through mass spectrometry; and (3) quantifying and identifying the proteins through advanced data processing (Caudle et al., 2010). Using this technique, a comprehensive characterization of the proteome in substantia nigra was made by one group (Kitsou et al., 2008). Many of the proteins known to be involved in PD such as DJ-1 and UCHL-1 were identified. Using proteomics, the proteome of the CSF was characterized and over 1500 proteins were identified and grouped according to their functions, such as cell cycle, signal transduction, and cellular transport. In addition, a large number of proteins unique to PD, AD, and DLB were identified (Abdi et al., 2006). Seventy two of them were uniquely altered in PD compared with healthy controls. Apolipoprotein H (Apo H) and ceruloplasmin appeared to be able to segregate PD from healthy controls and from non-PD (AD and DLB). Using the same material, Zhang et al. (2008) validated a multianalyte CSF profile, identifying a panel of eight CSF proteins that were highly effective at recognizing PD. In a study in PD, MSA, CBD, PSP, and healthy controls, a panel of four proteins (ubiquitin, β2-microglobulin, and 2 secretogranin 1 [chromogranin B] fragments) was identified which could differentiate PD and healthy controls on one side from APD on the other side with an AUC of 0.8 (Constantinescu et al., 2010a).

Subcellular proteomics investigates the proteome at the subcellular level, in compartments of the cell. Such a compartment is neuromelanin, a granular pigment associated with lysosomes and present in cathecolaminergic neurons. It interacts with compounds in the cytoplasm such as iron, lipids, pesticides, neurotoxins, and it sequesters them, thus having a cytoprotective function. However, if it malfunctions, it could turn out to become cytotoxic and be involved in neurodegeneration. The proteins associated with neuromelanin were investigated using proteomics (Tribl et al., 2006). Several were associated with mitochondrial function and chaperons. Interestingly, antibodies against neuromelanin have been found in serum from PD patients (Double et al., 2009). Subcellular proteomics was also used for analyzing Lewy bodies. Several proteins thought to be involved in the pathogenesis of PD were found, associated with alpha-synuclein, such as chaperons, proteins involved in oxidative stress, and proteosomal degradation (Xia et al., 2008). Analyzing mitochondrial fractions, 119 proteins were found to differ in PD compared with controls. Especially interesting is mortalin, involved in mitochondrial function and oxidative stress reactions. Low levels of mortalin were found in substantia nigra from PD patients compared with controls (Jin et al., 2006).

A shortcoming of the proteomics technique is that it is often biased toward identification of abundant proteins. As albumin and immunoglobulins represent more than 70% of CSF proteins, a way to enhance the discovery of proteins present in small amounts is to exclude the abundant proteins from the sample through fractionation. Blood contamination with its high protein content can dramatically alter CSF proteomic pattern and it has been suggested to exclude from proteomic analyses CSF containing more than 10 erythrocytes per microliter (Caudle et al., 2010).

## METABOLOMICS

Metabolomics investigates end products of metabolic pathways. These are molecules with low molecular weights required for the maintenance, growth, and normal function of a cell (Beecher,

2003). Adequate sample collection and preparation prior to analysis is very important for accurate results. Metabolomic studies conducted by Bogdanov et al. have confirmed the inverse association between blood urate levels and the risk for PD. In addition, they found higher levels of glutathione and 8-hydroxydeoxyguanosine (8-OHdG) in PD compared with controls. These compounds are markers of oxidative processes and support the oxidative stress hypothesis in PD (Bogdanov et al., 2008). The same group could differentiate controls from idiopathic PD patients, patients with idiopathic PD from those with hereditary PD caused by the G2019S variant of the LRRK2 mutation, and also symptomatic LRRK2 mutation carriers from asymptomatic carriers, based on the metabolomic profile (Johansen et al., 2009).

### CONCLUSION

Ideally, findings from the four "omics" techniques applied on different materials (e.g., substantia nigra cells or the CSF) should be consistent. Thus, if genomics shows an altered gene in neuronal nuclei, then the mRNA (transcriptomics) should reflect that in the cytoplasm, and further, after translation, in proteins and through them metabolic products detected in the cell or in the CSF by proteomics and ultimately by metabolomics. Findings in the CSF should be replicated in substantia nigra cells. Unfortunately, this congruence of findings is not often to be seen. That may be due to the limitations of the techniques or experimental incongruences, along with the use of different techniques and the inherent complexities of living organisms (Caudle et al., 2010). Better equivalence is achieved when findings from different techniques are categorized within pathways such as oxidation, synaptic transmission, mitochondrial function, or protein degradation. Of these, the oxidative stress pathway is the most robust with similar results from both cellular and CSF analysis, from genomics, transcriptomics, proteomics, and metabolomics. Thus, oxidative stress appears to be the final common pathway in the neurodegenerative process in PD (Caudle et al., 2010). Better integration of these techniques should lead to a deeper understanding of the pathophysiology of PD as well as other neurodegenerative disorders, and open venues for developing new treatment strategies.

## CEREBROSPINAL FLUID

The first lumbar puncture (LP) was done in London 1889 and CSF studies have a long tradition in neurology, both in research and in clinical practice (Frederiks and Koehler, 1997). We know mainly from AD research that CSF studies in patients with neurodegenerative disorders are feasible with a low rate of post LP headache or other complications (Andreasen et al., 2001) and CSF analysis for assessing tau protein and beta-amyloid belongs now to the standard of care in the management of dementias. Brain-derived proteins do not usually appear in the blood due to the blood-brain barrier. In contrast, CSF is very close to the pathologic processes in the brain, and may better reflect changes in brain metabolism (Mollenhauer and Zhang, 2012). This may offer advantages when investigating neurodegenerative disorders. Even though protected by the blood-brain barrier, the CSF is dynamic. Proteins that diffuse in the CSF from plasma have a concentration gradient with a 2.5 times higher lumbar concentration than cranial. Proteins secreted in the CSF from the brain have about the same concentration in the CSF space, but some, including tau protein, may actually have a lower concentration distally, in the lumbar region. There are also diurnal variations, as the secretion of proteins into the CSF is higher at night. In addition, the protein concentration decreases between the first ml CSF tapped at the LP and the later portion which is the preferred one as it more accurately reflects the environment in the brain. All this makes imperative the standardization of the CSF sampling protocol (Kroksveen et al., 2011).

It has been suggested that CSF itself mediates humoral signaling which is distinct from synaptic neurotransmission. In one study, spherical nanometric-scale structures were identified in the CSF containing synaptic vesicles (Harrington et al., 2009). Cell-line studies have shown that CSF from PD patients affects dopaminergic cells differently than CSF from healthy controls, implying that there are differences in their composition (Le et al., 1999). Due to all this, CSF has been widely investigated in Parkinsonian disorders and it might be considered to offer the most promising insights in the disease processes (Lewitt, 2012).

There have been concerns regarding CSF sample handling and its impact on the acuity of CSF data as post-translational modifications, protein loss, and degradation can be caused by non-optimal CSF related procedures including sampling, freezing, thawing, and storage. Therefore it is important to have standard operating procedures in place (Lewczuk et al., 2006). A consensus protocol for the standardization of CSF collection and handling has been published in 2009 and is being followed by many European centers (Teunissen et al., 2009). In regard to analysis, for increasing the reliability of results, a study should ideally include a training subgroup and a validation subgroup, the latter preferably run by a different research group (Zetterberg et al., 2008; Mollenhauer and Trenkwalder, 2009).

## CSF BIOMARKER CANDIDATES FOR PARKINSONIAN DISORDERS

In a review by Mollenhauer et al. from 2008 of all then current publications regarding CSF biomarkers in PD, MSA, PSP, CBD, and DLB, no less than 67 tested compounds were identified, most of them in PD. However, several limitations were found in most of the studies: sensitivity and specificity were low; there was a lack of reproducibility of results by independent cohorts; and the analysis methods in use were still considered to be in their infancy (Mollenhauer and Trenkwalder, 2009). Thus, there is no scarcity of investigations on CSF compounds with biomarker potential in Parkinsonian disorders. What we barely have are mature CSF biomarker candidates and what we still lack is a real biomarker.

Historically, due to the prominence of the dopaminergic abnormalities in these disorders, the first compounds to be tested were dopamine and other monoamines and their metabolites. As these results were prone to be influenced by a multitude of other factors, the quest went further to compounds which were already known and tested in other diseases such as tau protein, beta-amyloid, and NFL. With advancing knowledge and technical capabilities, the search turned further toward specific targets following theoretical considerations in regard to patho-etiology, such as alpha-synuclein, or inflammatory markers. Later on, the newer and far-reaching possibilities offered by the "omics" techniques

led to broad searches surveying large, nod-discriminate entities like the genome or the proteome. The overview presented here has no claim on being exhaustive; instead it focuses on a number of compounds perceived to be more mature and/or promising for the future.

## SPECIFIC BIOMARKER CANDIDATES IN THE CSF AND BLOOD

A summary is presented in **Table 1**.

### *Alpha-synuclein*

***Background.*** Alpha-synuclein is the main component of intracytoplasmatic Lewy bodies and of Lewy neurites in neuronal processes. These structures are found in PD and in DLB in the remaining dopaminergic neurons in substantia nigra, and also in non-dopaminergic cortical and non-cortical neurons (Jellinger, 1990, 2003). In MSA, alpha-synuclein is a component of the characteristic glial intracytoplasmatic inclusions.

Mutations affecting the gene coding for alpha-synuclein cause rare hereditary forms of PD, such as in PARK1 (missense) and PARK4 (duplication, triplication; Polymeropoulos et al., 1997) but are also important for sporadic forms of PD (Farrer et al., 2001). In addition, in both PD and MSA, genome-wide association studies showed a strong association between disease risk and distinct single nucleotide polymorphisms (SNPs) in the α-synuclein encoding gene (Simon-Sanchez et al., 2009). There seems to be a dose-effect of alpha-synuclein as increased levels of synuclein caused by duplications and triplications of the gene cause PD (Fuchs et al., 2008; Simon-Sanchez et al., 2009).

***Alpha-synuclein's role in the pathogenesis of synucleinopathies.*** Although it is widely expressed in the brain, the precise function of alpha-synuclein is not known. It might play an important role in neurotransmission by regulating synaptic vesicle size and recycling. Mutant alpha-synuclein builds fibrils, aggregates, resists degradation, and ultimately interferes with vital cell functions such as transcription, the ubiquitin-proteasome system, lysosomes and mitochondria, disrupting protein metabolism, and energy production. Oxidation, pesticides, and mitochondrial dysfunction can damage alpha-synuclein and initiate its metamorphosis to toxic forms (Moore et al., 2005). It has been proposed that alpha-synuclein pathology and subsequent neurodegeneration could represent a common event for different forms of PD, with different etiologies. A recent theory proposes pathologic "seeding" throughout the nervous system of abnormal alpha-synuclein which, after finding its way in the body, might, through a prion-like induction, spread from cell to cell, causing the neurodegenerative process in PD (Angot et al., 2010; Jucker and Walker, 2011). Due to alpha-synuclein's prominence in the pathogenesis of these disorders, PD, MSA, and DLB are considered to be synucleinopathies.

***Previous findings in Parkinsonism.*** Cerebrospinal fluid alpha-synuclein levels in PD have been investigated using different techniques in over 10 studies. A majority of them showed decreased levels in PD (Tokuda et al., 2006; Mollenhauer et al., 2008; Hong et al., 2010; Mollenhauer et al., 2011) but not all (Borghi et al., 2000; Ohrfelt et al., 2009).

Four studies have investigated CSF alpha-synuclein levels in MSA. Three of them found decreased levels in MSA compared with controls but not with PD patients (Mollenhauer et al., 2011; Shi et al., 2011; Hall et al., 2012). In one of them levels were similar in MSA, PD, and controls (Tateno et al., 2012). In one study, PD and MSA could be differentiated by the CSF Flt3 ligand, not by alpha-synuclein (Shi et al., 2011).

In one study, CSF alpha-synuclein levels in PSP and CBD were not significantly different compared with controls. However, levels in PSP but not in CBD were higher than in PD (Hall et al., 2012).

Alpha-synuclein levels have also been investigated in plasma in PD and MSA but with conflicting results. Both higher (Lee et al., 2006) and similar (Li et al., 2002) levels compared with controls have been found and there was no correlation with PD severity. A major difficulty in measuring both alpha-synuclein and DJ-1 in plasma is the risk for contamination with erythrocytes or platelets as more than 95% of these compounds reside in erythrocytes and about 4% in platelets. However, even after controlling for that, there were no statistically significant differences between PD patients and controls in regard to these compounds although there was a trend for lower levels in PD. It does not seem that plasma alpha-synuclein can be used as a biomarker for PD for the time being (Shi et al., 2010).

Oligomeric forms of alpha-synuclein protein in plasma were higher in PD than in controls, in one study (El-Agnaf et al., 2006). However, in another study, phosphorylated alpha-synuclein, but

**Table 1 | Cerebrospinal fluid biomarker candidates in Parkinsonian disorders.**

| Compound | PD | MSA | PSP | CBD | Conclusion |
|---|---|---|---|---|---|
| Alpha-synuclein | ↓ ↔ | ↓ ↔ | ↔ ↑ | ↔ | Decreased in PD and MSA but not in PSP and CBD. Inconsistent data |
| NFL | ↔ | ↑ | ↑ | ↑ | NFL normal in PD but increased in MSA, PSP, and CBD, vs. controls |
| Total tau protein | ↓ (↑) ↔ | ↑ ↓ ↔ | ↔ | ↑ ↔ | Decreased in PD and increased in CBD. Inconsistent data |
| Aβ42 | ↓ ↔ | ↓ ↔ | ↓ ↔ | ↓ ↔ | Decreased in PDD and DLB. Inconsistent data in PD, MSA, PSP, and CBD |
| DJ-1 | ↑ ↓ | – | – | – | Data is not consistent |
| 8-OHdG | ↑ | – | – | – | Limited results. Probably increased in PD |
| Urate | [↓] | [↓] | | – | Lower urate levels are associated with a higher risk for developing PD and with a faster rate of disease progression in PD and MSA |

*Aβ42, amyloid-β; CBD, corticobasal degeneration; DLB, dementia with Lewy bodies; MSA, multiple system atrophy; NFL, neurofilament light chain; PD, Parkinson's disease; PDD, Parkinson's disease with dementia, PSP, progressive supranuclear palsy; vs., versus. 8-OHdG, 8-hydroxydeoxyguanosine.*

not total alpha-synuclein nor oligomers of alpha-synuclein, was higher in PD than in controls (Foulds et al., 2011). Interestingly, antibodies directed against monomeric alpha-synuclein were found in plasma of PD patients, with higher response in earlier disease phases (Yanamandra et al., 2011). Studies in animal models suggest that immunomodulatory interventions such as vaccination with alpha-synuclein (Masliah et al., 2005) or administration of alpha-synuclein antibodies (Masliah et al., 2011) may have a positive impact on the intraneuronal accumulation of alpha-synuclein, presumably reflected by reduced neuropathological and behavioral deficits. Intravenous immunoglobulin reduced alpha-synuclein oligomer neurotoxicity in human neuroblastoma cells (Smith et al., 2012). These results may motivate further research aiming to find whether immunomodulation might be a novel therapeutic approach in PD.

Alpha-synuclein was found not only in the brain and the blood but in other peripheral locations too. It was found in the colonic mucosa years before the emergence of PD symptoms and the question was raised whether it can be a biomarker for premotor PD stages (Shannon et al., 2012a,b). In saliva, alpha-synuclein was lower in PD patients than in controls and it inversely correlated with the UPDRS score (Devic et al., 2011).

Cerebrospinal fluid alpha-synuclein levels increase non-specifically in Creutzfeldt–Jakob's disease, presumably due to massive neuronal death (Mollenhauer et al., 2008). The same phenomenon but on a smaller scale occurs in AD, with increased CSF alpha-synuclein levels (Hall et al., 2012).

Although alpha-synuclein is a strong biomarker candidate due to its important role in the pathogenesis of synucleinopathies and to several promising results, currently it cannot be considered a mature biomarker. However, in a group of parkinsonian patients, low CSF alpha-synuclein levels could help with their stratification, due to its high positive predictive value for synucleinopathies. An additional marker (e.g., non-motor prodromal symptoms) would strengthen the stratification process and help to select a group of patients who may benefit from future synuclein-reducing therapies (Mollenhauer et al., 2011). Longitudinal studies and studies in early disease stages are needed in order to better understand the value of alpha-synuclein as potential biomarker in Parkinsonism.

### *Neurofilament light chain protein*

***Background.*** Neurofilaments (NF) are major neuronal structural elements, composing the intermediate filaments present in nerve fibers. They are mainly involved in maintaining the axonal caliber and the neuronal shape and size (Lasec, 1988) and are thereby critical for the morphological integrity of neurons and for the conduction of nerve impulses along the axons (Hoffman et al., 1987). The NF are composed of three subunits of different molecular weights: light chain NF (NFL), medium chain NF (NFM), and heavy chain NF (NFH). The NFL forms the backbone to which NFH and NFM chains copolymerize to form NF. Increased levels of CSF NF primarily reflect axonal degeneration of large myelinated axons, such as those present in the pyramidal tracts. NFL is a mainly non-phosphorylated protein, whereas NFH is substantiality phosphorylated (pNFH), and can be measured in that form. CSF NFL has been shown to be increased in a variety of acute and chronic neurological diseases (Rosengren et al., 1996; Rosengren et al., 1999; Zetterberg et al., 2006; for review, see Norgren et al., 2003).

***Previous findings in Parkinsonism.*** NFL has been investigated in Parkinsonian disorders in a relatively large number of studies (Holmberg et al., 1998; Holmberg et al., 2001; Abdo et al., 2007a; Abdo et al., 2007b). A review from 2009 concluded that NFL could differentiate between PD and controls on one side and MSA and PSP on the other side, although with overlap. NFL could not discriminate between MSA with predominant Parkinsonism and MSA with predominantly cerebellar symptoms, nor between MSA and PSP (Constantinescu et al., 2009). Consecutive analyses of CSF NFL did not show any significant changes over 1 year and no correlation with disease severity. CSF NFL levels were also increased in CBD (Constantinescu et al., 2010b). Several studies have been conducted since then with similar findings (see Combinations of CSF Compounds for the most recent results). Hall et al. (2012) found increased NFL in MSA, PSP, and CBD. In one study in advanced PD patients treated with deep brain stimulation of nucleus subthalamicus, CSF NFL levels increased sharply directly after surgery but normalized gradually and were normal at 1 year and later. Thus, using this method, no signs of accelerated neuronal death due to active DBS could be found (Constantinescu et al., 2011). To be able to ascertain that a therapy is not in itself deleterious for the disease being treated remains a key point, and even more as new therapeutic approaches to PD are envisioned that employ potentially harmful techniques (e.g., intracranial catheters for injection of neurotrophic factors, cell transplants, and genetical modifications using viral vectors). Thus, in the future there may arise the need to detect adverse events using a sensitive, albeit non-specific, marker for brain damage. In this context, CSF NFL with its high sensitivity for detecting more aggressive neuronal death than it occurs in PD, even if enfeebled by a low diagnostic specificity, might be of use.

### *Tau protein*

***Background.*** Tau protein is important for the function of axonal microtubules and thereby for the structural integrity of the neuron and for axonal transport. In hyperphosphorylated form it has reduced binding affinity for microtubules and leads to their malfunction. At the same time, it adopts an abnormal configuration favoring aggregation and inclusion formation (Kouri et al., 2011). Tau protein is the main structural element of neurofibrils in Alzheimer's disease (AD) but it has also been found in neurofibrillary tangles in PSP, in neuronal cytoplasmatic inclusions, and in ballooned neurons in CBD and PSP (Mori et al., 1994).

***Previous findings in Parkinsonism.*** Cerebrospinal fluid tau protein levels in Parkinsonism have been investigated in many studies in the past, with inconclusive results. In PD, most studies found normal values, but both higher and lower values were reported. In atypical Parkinsonism, tau levels tended to be higher in MSA than in PD, but not in PSP. The results for CBD are mixed, with both higher and lower levels than in controls being reported (for review of older literature, see Constantinescu et al., 2009).

Recently, in a large study on patients with dementia, total tau and phosphorylated tau levels were not significantly different in

PSP and CBD compared with controls (patients with subjective memory complaints; Schoonenboom et al., 2012). In four recent large studies, tau protein was investigated along with other CSF compounds (see Combinations of CSF Compounds).

### *Amyloid-β*

***Background.*** Aβ42, derived from the proteolytic processing of a larger protein, amyloid precursor protein, is a major component of neuritic plaques in AD. Due to its sequestration in plaques, the characteristic pattern in AD is low CSF Aβ42 levels. Low CSF concentrations have also been found in Creutzfeldt–Jakob's disease, in DLB, in frontotemporal and vascular dementias, and in PD with dementia.

***Previous findings in Parkinsonism.*** Previous studies in Parkinsonism were inconclusive, with both normal and decreased levels in the same disorder, and did not allow drawing any conclusions (Hall et al., 2012; for review, see Constantinescu et al., 2009). However, *in vitro* studies have shown that Aβ42 promotes accumulation of alpha-synuclein making it interesting in a PD context (Masliah et al., 2001).

More recent studies have found a correlation between Aβ42 and cognitive dysfunction in PD, with significantly lower CSF Aβ42 and higher total tau protein levels in Parkinson's disease with dementia (PDD) compared with PD (Mollenhauer et al., 2006). In addition, this pattern also distinguished AD from PD, DLB, and MSA, although CSF Aβ42 was lower in DLB compared with controls and PD (Zhang et al., 2008; Shi et al., 2011; Hall et al., 2012). In a study from Norway, non-demented PD patients with memory impairment had lower Aβ42 than those without memory impairment (Alves et al., 2010). Significant associations were found between cognitive performance and CSF levels of Aβ42 and Aβ42/total tau (Leverenz et al., 2011). Interestingly, in a rare occurrence, the ratio fractalkine/Aβ42 correlated with PD severity assessed by UPDRS-III (Shi et al., 2011).

### *DJ-1*

***Background.*** DJ-1 is a gene product associated with PD in both familial and sporadic forms. Its exact function is not known but it seems to play an important role in oxidative processes where it probably acts as a protease, chaperon, or antioxidant (Choi et al., 2006). Loss of DJ-1 function leads to neurodegeneration.

***Previous findings in Parkinson's disease.*** Previous studies have found both higher (Waragai et al., 2006) and lower (Hong et al., 2010) CSF DJ-1 levels in sporadic PD compared with non-PD controls. DJ-1 will be investigated in the ongoing Parkinson Progression Markers Initiative study aiming to identify markers for disease progression (Parkinson Progression Marker Initiative, 2011).

### *8-Hydroxydeoxyguanosine*

***Background.*** 8-Hydroxydeoxyguanosine (8-OHdG) is produced when reactive oxygen radicals react with guanine residues in DNA. When the oxidized DNA is repaired, 8-OHdG is excreted in the blood and eventually in urine, where it can be measured. As such, it has emerged as a marker of oxidation and mitochondrial dysfunction, not only in neurodegenerative disorders but also in cancer research.

***Previous findings in Parkinson's disease.*** Sato et al. (2005) found that the mean urinary 8-OHdG increased with the disease stage in PD patients and another group found an association between hallucinosis in PD and urinary 8-OHdG levels (Hirayama et al., 2011).

The CSF 8-OHdG levels were increased in non-demented PD compared with controls (Gmitterova et al., 2009). 8-OHdG is one of the parameters selected for assessment in the FS-ZONE study, investigating the effect of pioglitazone, a potential antioxidant, in early PD[1]. Increased 8-OHdG blood levels in PD were identified in metabolomic studies as previously discussed in the metabolomics section.

### *Urate*

***Background.*** In humans, uric acid is the major product of the catabolism of the purine nucleosides adenosine and guanosine. Purines are derived from dietary intake as well as from endogenous metabolic processes (synthesis and cell turnover). The enzyme uricase which breaks down urate is absent in humans and apes, due to mutations which occurred millions of years ago (Wu et al., 1989). As a result, along with an extensive reabsorption of filtered urate (>90%), humans have high serum urate levels (about 5 mg/dL in men), close to the maximum solubility. Levels above the saturation limit (7 mg/dL) can result in hyperuricemia which may be a cause of disease in humans. However, higher urate levels may account for the greater longevity of humans, e.g., due to lower cancer rates compared with shorted-lived mammals. During the evolution, urate has replaced ascorbate as the most potent antioxidant in humans.

***Previous findings in Parkinsonian disorders.*** There is a substantial amount of evidence showing a relationship between urate and PD. Higher serum urate levels and higher dietary urate intake are associated with lower risk for developing PD, and with slower disease progression, better cognitive performance, and reduced loss of striatal [123I] β-CIT uptake in patients already having PD (Davis et al., 1996; Annanmaki et al., 2007; Annanmaki et al., 2008; Ascherio et al., 2009). In a recent study, the ratio between the immediate precursor of urate, xanthine, and homovanillic acid, the major catabolite of dopamine, was different in PD patients compared with controls and correlated with disease severity (Lewitt et al., 2011). The odds for having parkinsonism but without signs of dopaminergic deficit on iodine-123-labeled 2-β-carboxymethoxy-3-β-(4-iodophenyl) tropane ([123I]b-CIT) scan were higher in subjects with higher urate levels (Schwarzschild et al., 2011). In one small study serum urate levels were higher in tauopathies compared with synucleinopathies (Constantinescu et al., 2012). In MSA, higher serum urate was associated with a lower rate of disease progression (Lee et al., 2011). In DLB, serum urate levels were lower than in controls (Maetzler et al., 2011). There are discrepancies in the reported data concerning the importance of gender in this context. Some studies have found the association with urate levels to be significant in men only, others in both genders.

The title of a recent article reflects the encouraging data centered on urate and its future perspectives: "Urate: a novel

[1] http://www.ninds.nih.gov/disorders/clinical_trials/-NCT01280123.htm

biomarker of PD risk, diagnosis, and prognosis" (Cipriani et al., 2010).

#### *Peroxisome proliferator-activated receptor gamma coactivator-1 alpha*

***Background.*** Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a key transcriptional co-regulator involved in mitochondrial respiration, oxidative stress defense, and adaptive thermogenesis (Puigserver and Spiegelman, 2003).

***Previous findings in Parkinson's disease.*** Reduced mRNA levels of PGC-1α leading to mitochondrial dysfunction and neurodegeneration were found in Huntington's disease models (Cui et al., 2006), opening up for new therapeutic targets (McGill and Beal, 2006). The same phenomenon seems to occur in PD (Keeney et al., 2009; Pacelli et al., 2011) and PGC-1α is under investigation in new PD studies such as Pioglitazone in Early PD[2] (FS-ZONE).

#### *Combinations of CSF compounds*

Hong et al. investigated PD patients, healthy controls, and AD patients, and found that both DJ-1 and alpha-synuclein were decreased in PD compared with the other groups. Alpha-synuclein discriminated PD from controls with a sensitivity of 92% and a specificity of 58%. For DJ-1 the sensitivity was 90% and the specificity 70%. There was no association with disease severity. Combining alpha-synuclein with DJ-1 did not enhance the performance of the test model. They emphasized that blood contamination must be an exclusion criterion for sample analysis as it influenced the results; likewise, age must be taken into consideration as both DJ-1 and alpha-synuclein increased with age (Hong et al., 2010).

Mollenhauer et al. investigated a large number of patients with both synucleinopathies (PD, MSA, and DLB) and tauopathies (PSP and AD) plus neurological controls, first in a training set and afterward in a validation set. They found that a CSF alpha-synuclein concentration of 1.6 pg/μL discriminated PD from non-synucleinopathies with a 70% sensitivity and a 53% specificity. At this cut-off, the positive predictive value for any synucleinopathy was 91%. In the training set, a combination of alpha-synuclein, tau protein, and age discriminated between synucleinopathies and neurological controls and AD with an area under the curve (AUC) of 0.908. In the validation cohort the AUC was 0.702 for discriminating between synucleinopathies and a mixture of PSP, normal pressure hydrocephalus, and neurological controls. Age, not diagnosis, was the strongest factor affecting total tau protein levels. Only mean alpha-synuclein levels and not total tau, or Aβ42 levels differentiated PD and MSA from neurological controls (Mollenhauer et al., 2011).

Hall et al. assessed patients with synucleinopathies (PD, MSA, DLB, and PDD), tauopathies (PSP, CBD, and AD) and healthy controls using a panel of compounds: alpha-synuclein, total tau protein, hyperphosphorylated tau, Aβ42, and NFL. Alpha-synuclein levels were decreased in synucleinopathies compared with controls, PSP, and AD. NFL levels were substantially increased in APD. A receiver operating characteristics (ROC) analysis conducted to determine the value of NFL to differentiate PD from APD resulted in an AUC of 0.93. Total tau protein was decreased in PD compared with controls, but increased in MSA and CBD compared with PD. No significant change was seen in PSP. Aβ42 did not differ significantly between controls and PD, MSA, PSP, and CBD (Hall et al., 2012).

Shi et al. examined patients with PD, MSA, AD, and healthy controls. The fractalkine/Aβ1–42 ratio correlated positively with PD severity (in cross-sectional studies) and with PD progression (in longitudinal studies). No other marker had shown this association before. Fractalkine is important for the proper function of microglia. In addition, the Flt3 ligand, a cytokine which acts as a neurotrophic and anti-apoptotic factor in CNS, could alone differentiate between PD and MSA with a sensitivity of 99% and a specificity of 95%. Aβ1–42 levels were lower in PD and MSA than in controls but higher than in AD. They could not differentiate between PD and MSA. Total tau levels were also lower in PD and MSA than in controls and AD. A combination of alpha-synuclein and phosphorylated tau/total tau could also differentiate PD from MSA with a sensitivity of 90% and a specificity of 71% but only when samples with blood contamination were excluded. Alpha-synuclein was decreased in both PD and especially in MSA compared with controls, presumably reflecting aggregation or metabolic abnormalities (Shi et al., 2011).

Bech et al. investigated a group of patients with Parkinsonian disorders (PD, MSA, PSP, CBD, DLB, and PDD). They could confirm previous results concerning NFL. Thus, a ROC analysis of NFL showed a sensitivity of 86% and a specificity of 81% with a cut-off value of 284.7 ng/L for differentiating PD from atypical parkinsonism. Aβ42 was low in DLB. Neither phosphorylated tau nor total tau differed between the diagnostic groups (Bech et al., 2012).

## WHY ARE BIOMARKERS FOR PARKINSONIAN DISORDERS NEEDED?

The ultimate reason for needing a biomarker is the fact that we still do not have any disease-modifying treatment in movement disorders. The lack of biomarkers is considered to be one of the greatest limitations for developing such a treatment (Olanow et al., 2008). Over years, there has been no shortage of therapeutic hypotheses or compounds to be tested; the list with failed compounds is very long. The real problem has been the lack of a reliable way to assess the underlying disease process and whether an intervention could influence it and alter the course of the disease (Ravina et al., 2003; Kieburtz and Ravina, 2007; Sherer, 2011).

It has been assessed that it takes 5 years of follow-up and 600 subjects participating in a randomized placebo-controlled trial in order to detect a 20% slowing of functional decline. A biomarker could dramatically reduce the resources needed for that (Hersch and Rosas, 2011).

Considering the very nature of Parkinsonian disorders and the limits it puts on the process of developing disease-modifying therapies, biomarkers could be useful for solving many limiting issues.

[2]http://clinicaltrials.gov/ct2/show/NCT01280123

### THE DIFFERENTIAL DIAGNOSTIC ISSUE

Differential diagnosis can be difficult during early phases of Parkinsonian disorders. What might look as PD in the beginning could turn out to be PSP, MSA, or even CBD. What was initially considered to be a synucleinopathy may end as a tauopathy. Ultimately, the gold standard for diagnosis remains neuropathology. Considering the substantial differences between these disorders, mixing together patients with different diagnoses may lead to negative or inconclusive results in any therapeutic trial, even when the therapy itself is efficient for one of these diagnoses. A biomarker pointing early toward the right diagnosis would increase the probability of success.

A diagnostic biomarker would decrease the cost, time, and effort it would take to secure a diagnosis. Currently, that is best achieved through an assessment done by a movement disorders specialist. A biomarker would simplify the diagnostic process.

Even when there is no doubt regarding diagnosis, an ideal biomarker could help stratify patients in subgroups which may show different responses to a given therapy. That would make possible a distinction between respondent and non-respondent diagnostic subgroups, preventing the dismissal of a therapy when it does not benefit the diagnostic group as a whole. Such a distinction would also permit, within a given diagnostic group, to differentiate and individualize treatment according to expected benefits or risks, and expected disease progression and complications (Marek et al., 2008). For example, young PD patients with an increased risk for developing dyskinesias, once levodopa therapy is instituted, might need a different treatment approach compared with patients with late disease onset and a low risk for dyskinesia but high for dementia.

### THE TIME OF DISEASE ONSET AND PROGRESSION ISSUE

To date, it is impossible to determine the exact date of onset in Parkinsonian disorders. Once started, the disease is asymptomatic for several years, followed by the emergence of non-specific, non-diagnostic symptoms. Our "early" diagnosis based on the emergence of motor symptoms probably describes an already advanced disease process.

Thus, in PD, it has been calculated that up to 50–70% of substantia nigra neurons are lost before symptomatic motor abnormalities develop (Fearnley and Lees, 1991) and the pre-motor period could be between 5 and 20 years long (Marek et al., 2008). In one positron emission tomography study in PD, a mean preclinical period of $5.6 \pm 3.2$ years was calculated (Hilker et al., 2005). Results from the Honolulu-Asia Aging Study do also place the onset of non-motor symptoms, such as bowel movement abnormalities, 10 years or more before the emergence of diagnostic motor symptoms (Abbott et al., 2001).

The fact that the disease onset predates with years the time when enough symptoms emerge for a diagnosis to be made, implies that even efficacious therapies may show themselves powerless if given when neurodegeneration has gone that far (Stern et al., 2012). An ideal biomarker could detect the disease in presymptomatic individuals or early in the disease course allowing an efficacious disease-modifying therapy to act and "cure" or at least delay the progression of disease.

For now, there is also no way of measuring disease progression. The tools we have been using are clinical scales of which UPDRS (Fahn et al., 1987) is the most widespread for PD and the Unified Multiple System Atrophy Rating Scale (UMSARS) for MSA. However, these scales are no biomarkers and they are subject to both investigator and patient bias and cannot be considered truly objective; they are not reliable as their score can vary from hour to hour due to medication, placebo, food intake, or a myriad of other causes; they measure a combination of dopaminergic and non-dopaminergic effects and not the disease process itself, nor the direct effects of treatment over this process. Biomarkers are needed to identify the development of disease, and monitor and measure its progression.

### THE EFFECTS OF THERAPY ISSUE

At the present time we do not have a way of assessing whether and to which degree a therapeutic intervention has an impact on the disease process: we cannot measure the effects of a therapy. The clinical scales which we use today are subject to error, as discussed before. In addition, as it was shown in the ELLDOPA study, clinical measures such as UPDRS, and a more objective assessment, radiotracer imaging, moved in different directions after the therapeutic intervention, levodopa treatment, leading to confusion in regard to interpretation (Fahn et al., 2004). A further problem is that radiotracer imaging, which, currently, is the best we have achieved in regard to a PD biomarker, does only assess the integrity of the dopaminergic pathways in the striatum and, maybe, although it is controversial, the impact of therapy on these dopaminergic pathways (Agarwal and Stoessl, 2012). However, PD and also the APD, are not only disorders of the dopaminergic system, but of several other neurotransmitter systems, which these radiotracers do not visualize.

In conclusion, biomarkers that can identify and monitor the biochemical effect of drugs, also called "theragnostic markers," would greatly benefit the search for disease-modifying therapies as well as could be employed usefully as surrogate markers in clinical trials.

### THE PATHO-ETIOLOGICAL ISSUE

The ultimate cause of Parkinsonian disorders remains unknown, despite an abundance of theories. Most research has been directed toward the elucidation of the etiology of PD. The vast majority of PD cases are sporadic but approximately 5–10% are genetic. A combination of both environmental and genetic factors is thought to underlie the pathological processes. Considerable evidence implicates oxidative stress in the degeneration of dopaminergic neurons, through deficiencies in the major antioxidant systems, and not only in the brain, but also in the periphery (Jenner, 1991; Kikuchi et al., 2002). Closely linked to oxidative stress is mitochondrial dysfunction (Lin and Beal, 2006). Several hereditary forms of Parkinsonism are caused by mutations in genes related to mitochondria, such as PINK1 and PARK2 (Mortiboys et al., 2008; Gegg et al., 2009). Environmental toxins such as rotenone and paraquat, which can disturb mitochondrial function, are positively associated with PD (Tanner et al., 2011). Alpha-synuclein, a major component of Lewy bodies, inhibits the mitochondrial complex I (Devi et al., 2008) and may cause impaired protein degradation

and accumulation of abnormal proteins by disturbing the two major systems which remove damaged proteins: (1) the ubiquitin–proteasome pathway; and (2) the autophagy–lysosome pathway. Transcription abnormalities caused by alpha-synuclein may disturb metabolic pathways (Desplats et al., 2012). Abnormal inflammation in the central nervous system, with activated microglia and massive astrogliosis with increased levels of proinflammatory cytokines (tumor necrosis factor – TNF-α, interleukins), has been found in the CSF in PD; these proinflammatory compounds may promote apoptosis and neuronal death (Hirsch et al., 2003, 2012) and have been suspected to contribute to the development of PD (Czlonkowska et al., 2002) and PSP (Litvan, 2003). Supporting this theory, it has been shown that use of non-steroidal anti-inflammatory drugs (NSAIDs), particularly ibuprofen, was associated with a lower risk for PD (Chen et al., 2003; Gao et al., 2011). It is not known whether the glial activation is secondary to neuronal death induced by other factors, or if it is the primary cause to neuronal death (Schapira and Jenner, 2011).

The cause of MSA, a synucleinopathy, is not known. As for PD, mitochondrial dysfunction and oxidative stress, genetic predisposition, microglial activation, pesticides, and other environmental toxins have been suggested as putative causes (Hanna et al., 1999; Stefanova et al., 2007; Ahmed et al., 2012). Alpha-synuclein accumulates in the oligodendrocytes but its source is not known, neither why it leads to neuronal death. Presumably, disturbances in the neurotrophic support offered by oligodendroglia to neurons result in their degeneration (Ubhi et al., 2011).

As for the synucleinopathies, the ultimate causes of PSP and CBD are not known. Again, a combination of environment and genetics may start the pathological process resulting in accumulation of hyperphosphorylated tau isoforms with four repeats, oxidative stress, and neurodegeneration. Inflammation may also be involved; using PET, microglia cell activation could be found in the same regions where the PSP pathology is usually located (Gerhard et al., 2006).

The bewildering complexity of the current etiological theories may just confirm that we still do not understand the etiology of Parkinsonism but it could also imply that treatment must also be complex and oriented toward several potential targets at the same time (Lang et al., 2012). The same may apply to biomarkers; it could be preposterous to expect to find a single biomarker covering such a complex disease. A biomarker reflecting the etiology of the disease might offer insights into the pathological mechanism itself, thereby opening the way for potentially successful interventions.

## CHALLENGES IN THE DEVELOPMENT OF BIOMARKERS FOR PARKINSONIAN DISORDERS

Although several promising candidates exist, we still lack a reliable biomarker for Parkinsonian disorders. Some of the obstacles on the road to developing biomarkers will be discussed here.

### DISEASE HETEROGENEITY

In Parkinsonian disorders in general and PD in particular, considering the heterogeneity of clinical presentations at onset, the variability in clinical progression, the multitude of genetic variants and of possible etiologies, it is conceivable that no single biomarker will ever be sufficient, but that several biomarkers will need to be developed, covering biochemical, imaging, pathological, and clinical aspects of the diseases (Marek et al., 2008).

### DIAGNOSTIC UNCERTAINTIES

In Parkinsonian disorders, the diagnosis still remains clinical. Even in the minority of PD cases which are identified through genetic testing, the time for phenoconversion cannot be assessed in a precise and objective way. Clinical diagnostic criteria are susceptible to subjective interpretation and may change over time, as it has happened to a certain degree with PSP. Ultimately, the diagnostic gold standard remains neuropathological examination that can only be ascertained post mortem. Obviously, this is a serious limitation for all research regarding Parkinsonism.

### SLOW RATE OF NEURODEGENERATION IN PD

The neurodegenerative process in PD develops insidiously over many years and the degree of degeneration with associated CSF alterations may be too low to be detected by the current laboratory methods. A consequence of that is the high susceptibility to blood contamination which can have profound influence on CSF analysis results.

### AGE IMPACT

As most cases of Parkinsonian disorders occur in people aged 55 years and older, there is a high probability for concomitant disorders including neurodegeneration related to other causes, e.g., AD or cerebrovascular pathology. The impact of high age *per se* and comorbidities associated with it has not been sufficiently investigated and more needs to be done in that respect.

### METHODOLOGICAL UNCERTAINTIES

It is not always clear which kind of measurement is most appropriate and which compounds are the best to explore, making comparisons between studies sometimes difficult. Several of the proteins associated with neurodegeneration are suspected to be aggregation-prone and may exist in different forms, e.g., phosphorylated or unphosphorylated or have different post-translational conformations. Should oligomers or polymers, the mother substance or its metabolites be investigated?

### BLOOD CONTAMINATION

While 80% of all proteins in the CSF derive from blood, only 20% are brain-derived (Reiber, 2001). The protein concentration in the blood is much higher than in the CSF, due to the brain-blood barrier which isolates the CSF space. Proteins such as alpha-synuclein are also present in the blood, in erythrocytes, and in thrombocytes. Even minor blood contamination may profoundly affect the results of CSF analysis. The integrity of the blood-brain barrier is crucial for ascertaining that what is found in the CSF reflects the brain environment and not a blood contamination and therefore results from blood-contaminated CSF should not be used. According to one American group, samples should not contain more than 10 erythrocytes per microliter CSF (Caudle et al., 2010), or 500 erythrocytes per microliter CSF according to an European recommendation (Teunissen et al., 2009).

## FUTURE PERSPECTIVES

Some of the approaches which may benefit the quest for biomarkers in Parkinsonian disorders are proposed here.

### STANDARDIZATION OF CSF RELATED PROCEDURES

Although there are some guidelines in place for the collection and analysis of CSF, from both Europe and the US (Teunissen et al., 2009; Caudle et al., 2010), there is no uniformly accepted protocol making possible the standardization of CSF related procedures. The creation of such a protocol would increase the quality, compatibility, and comparability of CSF related investigations.

### INVESTIGATIONS IN UNMEDICATED PATIENTS

The impact of dopaminergic medications on potential markers for Parkinsonian disorders is not sufficiently investigated and most of the patients studied so far had been treated with one or more antiparkinsonian medications at the time for LP. There is a need to investigate CSF from unmedicated patients. Some ongoing studies, such as the Parkinson Progression Marker Initiative will make that possible in PD (Parkinson Progression Marker Initiative, 2011) but similar studies are also needed in APD.

### INVESTIGATIONS IN EARLY ATYPICAL PARKINSONISM

Although early PD has been and is being studied, there is a lack of similar studies in early atypical Parkinsonism. This will have to be addressed as understanding the early disease stages probably holds the key to the development of useful biomarkers and efficacious disease-modifying therapies.

### INVESTIGATING PATTERNS OF POTENTIAL BIOMARKERS

Given the difficulties encountered when trying to identify single compounds as biomarkers in Parkinsonism, there may be more feasible to identify patterns of compounds serving as biomarkers. Some illustrations of this concept are presented previously in this review. The nature of these disorders may imply minute modifications in single CSF compounds, impossible to perceive, while patterns of several such modifications might be more prone to detection.

### LONGITUDINAL STUDIES

Most of the available data concerning CSF biomarkers comes from cross-sectional studies. Considering the chronic and insidious nature of Parkinsonian disorders, there is a need for longitudinal studies which alone could examine changes and patterns over longer time periods.

### GROUPING DIAGNOSES

Grouping diagnoses together, such as PD contra APD or synucleinopathies contra tauopathies, may facilitate developing biomarkers for these diagnostic groups. These biomarkers would be limited and not able to distinguish between single diagnostic entities, but they could be useful in particular circumstances.

### INCREASED GENERALIZABILITY

All biomarker studies come from highly selected patient populations recruited via movement disorder clinics. In the future it will be necessary to investigate a more heterogeneous Parkinsonian population.

## CONCLUSION: CLINICAL APPLICATIONS OF BIOMARKERS

Reliable biomarkers could be of great use in the development of disease-modifying therapies and in the management of Parkinsonian disorders, once a disease-modifying therapy is developed, by:

(1) Indicating promising therapeutic approaches derived from a patho-etiologic understanding of the disease;
(2) Translating results of drug tests in animals to human populations;
(3) Enriching study populations by identifying patients at risk for a disease;
(4) Determining disease onset at an early stage, hopefully even before the emergence of symptoms;
(5) Stratifying populations according to estimated disease progression, anticipated complications, expected therapy benefits, and potential risks;
(6) Measuring the effects of a therapy on the disease process and on disease progress;
(7) Determining when a therapeutic intervention can be discontinued;
(8) Simplifying the drug regulatory process.

Considering their high positive potential in the management of Parkinsonian disorders, the quest for biomarkers for these diseases must continue unabated.

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