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## **Biomarkers Of Proteolytic Damage Following Traumatic Brain Injury**

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## Introduction

Brain injury resulting from traumatic, ischemic and/or chemical etiology is a significant international health concern, representing a potentially catastrophic debilitating medical emergency with poor prognosis for long-term disability. It represents a major problem to military care, accounting for 25% of all combat casualties and is the leading cause of death (approaching 50% incidence) among wounded soldiers reaching Echelon I medical treatment [8]. In civilian life, the incidence of brain injury and resultant long-term disabilities caused by traumatic insults (automobile accidents, gunshots, sports, etc.) and ischemic events (strokes, cerebral hemorrhage, cardiac arrest, etc.) are several orders of magnitude greater. There are more than 1 million traumatic brain injury (TBI) cases that are treated and released from an emergency department annually in the United States resulting in more than 230,000 hospitalizations, 50,000 deaths and 80,000 disabilities. Among all age groups, the top three causes of TBI are motor vehicle accidents, falls and violence [1]. Despite modern automobile design and injury prevention campaigns, important causes of TBI in children such as ejections from cars during traffic accidents, have increased in recent years [111]. The current estimation is 5.3 million Americans live with TBI-related disability. TBI is the greatest cause of death and disability in young people less than 24 years old [12].

With the exception of supportive measures, there are currently no approved drug treatments for TBI [77]. There have been a large number of clinical trials studying potential therapies for traumatic brain injury (TBI) that have resulted in negative findings with a cost of over \$200 million [15, 30]. Many investigators have pointed out that the absence of biochemical markers of injury could have contributed to these failures [77, 104]. Unlike other organ-based diseases where rapid diagnosis employing biomarkers (usually involving blood tests) prove invaluable to guide treatment of the disease, no such rapid, definitive diagnostic tests exist for

TBI to provide physicians with quantifiable neurochemical markers to help determine the seriousness of the injury, the anatomical and cellular pathology of the injury, and to guide implementation of appropriate triage and medical management.

### **Criteria For Biochemical/Surrogate Markers**

In the course of research on biomarkers, our laboratories have developed criteria for biomarker development. Useful biomarkers should employ readily accessible biological material such as CSF or blood (CSF is routinely accessible in severely injured TBI patients), predict the magnitude of injury and resulting functional deficits and possess high sensitivity and specificity, have a rapid appearance in blood and be released in a time-locked sequence after injury. Ideally, biomarkers should employ biological substrates unique to the CNS and provide information on injury mechanisms, a criterion often used to distinguish biochemical markers from surrogate markers of injury, which usually do not provide information on injury mechanisms. Potential gender and age related differences on biomarker profiles are also important and should be taken into account when developing useful biochemical markers [43].

### **Uses Of Biomarkers**

Biomarkers would have important applications in diagnosis, prognosis and clinical research of brain injuries. Simple, rapid diagnostic tools will immensely facilitate allocation of the major medical resources required to treat TBI and other brain injuries. Accurate diagnosis in acute care environments can significantly enhance decisions about patient management including decisions whether to admit or discharge or administer other time consuming and expensive tests including computer tomography (CT) and magnetic resonance imaging (MRI) scans. Biomarkers

could have important prognostic functions especially in patients suffering mild TBI, which make up an estimated 80% of the 2.5 to 6.5 million individuals who suffer from lifelong impairment as a result of TBI [2, 83]. Accurate identification of these patients could facilitate development of guidelines for return to duty, work or sports activities and also provide opportunities for counseling of patients suffering from these deficits. Biomarkers could provide major opportunities for the conduct of clinical research including confirmation of injury mechanism(s) and drug target identification. The temporal profile of changes in biomarkers could guide timing of treatment and assist in monitoring the response to therapy and intervention. Finally, biomarkers could provide a clinical trial outcome measure obtainable much more cheaply and readily than conventional neurological assessments, thereby significantly reducing the risks and costs of human clinical trials. Relevant, easily available biomarkers are needed in order to maximize chances of success in developing long awaited effective drugs for traumatic brain injury [77].

### **Current Status of Research on Markers of Traumatic Brain Injury**

Analysis of specific biochemical markers has provided useful information on the mechanism and diagnosis specific organ dysfunction in humans [112]. However, although analysis of cerebrospinal fluid, cerebral microdialysis samples, and brain tissue specimens has provided insight into the mechanisms of brain injury [61, 63], there are no biomarkers of proven clinical utility for TBI.

TBI is difficult to assess and clinical examinations are of restricted value during the first hours and days after injury. Conventional diagnoses of TBI are based on neuroimaging techniques such as CT scanning, MRI and single-photon emission CT scanning [46, 58, 72]. CT scanning has low sensitivity to diffuse brain damage, and the availability of MRI is limited [60,

64]. Single-photon emission CT scanning detects regional blood-flow abnormalities not necessarily related to structural damage.

A recent review of biomarkers of TBI highlighted the need for biomarker development [43]. The most studied potential biochemical markers for TBI include creatine kinase (CK), glial fibrillary acidic protein (GFAP), lactate dehydrogenase (LDH), myelin basic protein (MBP), neuron-specific enolase (NSE) and S-100 proteins. The bulk of research in TBI has focused on NSE and S-100 $\beta$ . The specificity of NSE for brain is high [49], sex- and age-related variability is low [28, 51, 74, 86, 98, 120, 121, 133], and NSE is rapidly detectible in serum after TBI [129]. However, studies relating NSE serum levels to admission GCS in patients with severe TBI show conflicting results. Similar data have been reported concerning relationships with CT scan findings, ICP and long-term outcomes. In mild TBI, NSE failed to separate patients from controls [43, 44, 108, 131]. Thus, NSE is predominantly used as a marker for tumors [22]. NSE is also released in the blood by hemolysis, which could be a major source of error [22].

The S-100 protein family now consists of 19 members, of which S-100B is the one viewed as a marker of brain damage [49, 65], although it is present in other tissues such as adipocytes and chondrocytes [40]. Investigators have reported S-100 $\beta$  serum levels correlate to both GCS scores, neuroradiologic findings at admission and long-term outcomes [100, 99, 128]. However, investigators have recently raised questions about the utility of S-100 $\beta$  reporting that high serum levels of S-100 $\beta$  are detectible in trauma patients not having head injuries, a factor not adequately controlled for in earlier studies [3]. In addition, serum levels of S-100 $\beta$  following mild TBI do not show strong correlations with neuropsychological outcome [107]. Research in this area continues and recent reports have indicated the potential utility of measures of blood GFAP [71], spinal fluid Interleukin-6 [115] and cleaved tau protein in serum [45, 114] and spinal fluid [135] following brain injury.

Investigators have also generally recognized the need for more objective assessments of outcome following stroke, including biochemical markers [27, 68]. The approval of tPA as a treatment for acute stroke has additionally highlighted the potential utility of biochemical markers. Use of tPA may be hindered by diagnostic concerns because neurological deficits accompanying stroke can mimic those seen during transient ischemic attacks, complex migraine, space-occupying lesions and post-ictal paralysis. A reliable biochemical marker might give assurance to physicians considering administering thrombolytic agents for acute stroke [41, 50].

Previously reported biomarkers of cerebral ischemia include NSE, brain specific creatine kinase enzyme (CPK-BB), S-100 $\beta$  and inflammatory cytokines such as IL-6 [76, 89]. NSE and S-100 $\beta$  have been the most studied. After cardiac arrest, NSE elevations in serum and CSF have been correlated with neurological recovery [26, 67, 106]. Serum and CSF NSE values were reported to be elevated in rodent models of focal ischemia in proportion to the eventual infarct volume [24, 25, 42]. In clinical trials, peak serum NSE values also predicted infarct volumes as shown by CT. Correlating serum NSE values with functional outcome was less successful [24, 25, 70], possibly because functional neurological deficit is influenced as much by location of brain injury as by infarct size [70]. S-100 $\beta$  protein has been studied most extensively for characterization of ischemic injuries after cardiac surgery, and several reports have documented post-operative serum elevations [29, 113, 127]. However, many of these reports do not include careful studies of neurological outcome, and several investigators have recently criticized the diagnostic utility of S-100 $\beta$  during cardiac surgery. [3].

### **Proteolytic Damage and the Pathobiology of Traumatic Brain Injury**

After TBI, brain cells can deteriorate following more than one pathway, and many genes and proteins may be involved. Programmed cell death is an evolutionarily conserved form of cell

suicide that occurs widely throughout development [13]. This type of cell death often has the morphological appearance of apoptosis [119]. Apoptosis occurs following TBI in animals [20, 59, 130] and humans [16, 17]. Studies of apoptosis pose special challenges since there are multiple apoptotic pathways, and apoptosis is extremely sensitive to a number of variables including injury type and magnitude [10, 14, 101], cell type [38, 57] and stimulation/antagonism of specific receptors [14, 19, 23, 38, 39, 48].

The molecular events occurring after TBI are just beginning to be understood. Elevated neuronal calcium levels activate a number of calcium-dependent enzymes such as phospholipases [84], kinases [132], phosphatases [75], and proteases [5, 81], all of which can modulate post-TBI cytoskeletal protein loss. Caspase-3 is a member of the caspase family of cysteine proteases. Activated caspase-3 has many cellular targets that, when severed and/or activated, produce the morphologic features of apoptosis [18]. Calpains are calcium-activated, neutral cysteine proteases with relative selectivity for proteolysis of a subset of cellular proteins. Calpain activation has been implicated in different models of apoptosis and in different cell types, including neurons [93]. Understanding of the contributions of calpains and caspases to cell injury/death following TBI may have important diagnostic and therapeutic implications.

### **Contributions of Caspase-3 and Calpain to Cell Death Following Traumatic Brain Injury**

Numerous studies from our own [6, 91, 93] and other laboratories [31, 35, 73] have provided evidence that the caspase family of cysteine proteases is an important intracellular effector of apoptosis in various cell lines and apoptotic models. Caspase 3-like proteases have been shown to cleave a variety of cytoplasmic, nuclear and cytoskeletal proteins during apoptosis including  $\alpha$ II-spectrin [69, 78, 79, 122], poly(ADP-ribose) polymerase (PARP: 51) and others (52-57). *In vitro* studies in our laboratories using a model of stretch injury have demonstrated

caspase-3 processing of  $\alpha$ -spectrin to the apoptotic-linked 120-kDa fragment 24 hours after moderate, but not mild or severe injury [6]. *In vivo* studies have provided evidence of caspase-3 activation following TBI. First, Clark et al. demonstrated cleavage of caspase-3 to its p18 and p12 subunits in humans [17]. Yakovlev et al. reported that TBI increased caspase-3, but not caspase-1, activity [130]. Caspase-3 inhibition reduced DNA fragmentation and TUNEL staining and improved behavioral outcome. We have also concurrently examined caspase-3 and calpain activation after TBI. Distinct regional and temporal patterns of calpain/caspase-3 processing of  $\alpha$ II-spectrin in brain regions ipsilateral to the site of injury after TBI have been observed. Caspase-3-mediated break down products (BDP's) to  $\alpha$ II-spectrin were absent in the cortex but showed significant increases in hippocampus and striatum early (hours) after TBI [92]. Immunohistological examinations revealed increased expression of the proteolytically active subunit of caspase-3, p18, in neurons, astrocytes, and oligodendrocytes from 6 to 72 hours following controlled cortical impact injury. Moreover, concurrent assessment of nuclear histopathology using hematoxylin identified p18-immunopositive cells exhibiting apoptotic-like morphological profiles in the cortex ipsilateral to the injury site [6].

Calpains are  $\text{Ca}^{2+}$  activated cysteine proteases that have been implicated in a variety of neuropathological conditions [55, 126]. Intracellular substrates of activated calpain include cytoskeletal proteins, calmodulin-binding proteins, enzymes involved in signal transduction, membrane proteins and transcription factors [110, 118, 125]. While calpain activation has historically been associated with necrotic cell death [82], calpain activation has also been implicated in different models of apoptosis and in different cell types, including neurons [7, 52, 78, 117, 123]. Research in our own and other laboratories have documented calpain activation following TBI *in vivo* [55]. TBI results in altered  $\text{Ca}^{2+}$  homeostasis [134] and activates several  $\text{Ca}^{2+}$ -dependent enzymes including the calpains. Overactivation of calpains occurs in many neurodegenerative diseases and injuries to the CNS [4, 55, 126]. Increased calpain activity

following TBI has been inferred by a variety of techniques [54, 81, 94, 97], including protection by calpain inhibitors [95, 109].

Pathological calpain activation is believed to occur when intracellular free calcium levels surpass a certain threshold. Importantly, increases in free calcium via voltage and receptor gated calcium channels have been reported in CNS trauma *in vivo* [19, 48, 56]. Calpains are located throughout the neuron, in somatodendritic regions and in axons [57]. Calpain may also be a constituent of myelin [116]. Therefore, pathological calpain activity and subsequent substrate proteolysis can have profound effects on neuronal structure and function.

Cytoskeletal alterations after experimental brain injury have pointed to the likelihood of calpain mediated proteolysis. Preferred substrates for calpains include the cytoskeletal protein spectrin [47, 66], microtubule associated protein-2 (MAP-2)[33], and neurofilament proteins [20, 21, 85, 105]. Increased degradation of MAP2 [32, 130], the neurofilament triplet proteins [53, 87] and spectrin [80] have been reported in cerebral ischemia. In addition, loss of MAP-2 [62], neurofilament 68 (NF 68) and neurofilament 200 (NF 200)[94-96] have been reported following traumatic brain injury (TBI) *in vivo*. Additional evidence that calpain is activated in neurons following experimental brain injury has been provided by the use of antibodies which bind specifically to calpain mediated BDP's of cytoskeletal proteins in models of TBI [81].

### **$\alpha$ II-Spectrin Degradation-A Prototype Biomarker**

Our research program to develop biomarkers for TBI has focused on  $\alpha$ -spectrin degradation as a prototypical biochemical marker [34, 103].  $\alpha$ II-spectrin (280 kDa) is the major structural component of the cortical membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals [36, 37]. Importantly,  $\alpha$ II-spectrin is a major substrate for both calpain and caspase-3 cysteine proteases [124]. Our laboratory has provided considerable

evidence that  $\alpha$ II-spectrin is processed by calpains and/or caspase-3 to signature cleavage products *in vivo* after TBI [6, 11, 81, 92] and *in vitro* models of mechanical stretch injury [91]. Calpain produces two major  $\alpha$ II-spectrin breakdown products of 150 kDa and 145 kDa (SBDP150 and SBDP145) in a sequential manner (Wang TINS 2002). On the other hand, caspase-3 initially produces a 150 kDa SBDP that is further cleaved into a 120 kDa fragment (SBDP120) (Wang TINS 2000). Immunoblots of  $\alpha$ II-spectrin degradation thus provide concurrent information on the activation of calpain and caspase-3, potentially important regulators of cell death following TBI. The calcium sensitivity and low basal levels of calpain optimize its utility as a marker of cell injury. Although not found in erythrocytes and thus robust to confounding by blood contamination,  $\alpha$ II-spectrin is not specific to the CNS [37]. Following injury, native  $\alpha$ II-spectrin protein was decreased in brain tissue and increased in CSF from 24 hrs to 72 hrs after injury. Calpain-specific breakdown products increased in both brain and CSF after injury. Caspase-3-specific breakdown products increased in some animals, but to a lesser degree [90]. Considerable laboratory data exists on the potential utility of  $\alpha$ II-spectrin degradation as a biomarker for TBI, including our ability to detect differences in severity of injury based on  $\alpha$ II-spectrin detection in spinal fluid of rodents after TBI (Figure 1). Preliminary human data are also promising (Figure 2). However, what is not known is whether BDP's and other biomarkers that discriminate injury magnitudes at the biochemical level (i.e., magnitude differences in CSF/serum levels of a biomarker) will be useful as predictors of clinically relevant measures of outcome. That is, two different injury magnitudes may produce significantly different biochemical responses in the brain that are discernable in CSF or serum, but these same injury magnitudes may not result in functionally different behavioral, pathological, or other clinical outcome measures. Thus, biomarkers that do not correlate with clinically relevant outcome measures will not be useful for assessment of functional ability, functional recovery, or for gauging effects of therapy on outcome. In addition, preliminary results from our laboratories

suggest that there is a spontaneous susceptibility of cytoskeletal protein degradation by calpain in aging rats [9]. These findings emphasize the importance of accounting for multiple clinical variables including but not limited to age when evaluating the clinical utility of biomarkers of brain injury.

### **Additional Cytoskeletal Proteins with Potential Utility as Biomarkers**

Initial research focused on proteolytic processing of cytoskeletal proteins such as lower molecular weight neurofilament 68 protein (NF-68) highlights their potential to provide useful information on activity of specific proteases such as  $\mu$ -calpain and m-calpain. Importantly, 2-D gel electrophoresis studies suggested dephosphorylation of NF-68 may be associated with NF protein loss following TBI, a post translational modification that could have significance for biomarker development [81, 97]. This important biomarker could provide important information on the pathophysiology of both dendritic and axonal damage after TBI [96]. Importantly, NF-68 has been used to quantify axonal injury in closed head injury models [102]. Since diffuse axonal injury (DAI) is presently considered one of the most common types of primary lesions in patients with severe closed head injury [88], a biomarker that provides information on axonal injury could potentially have clinical utility.

### **Future Directions**

The pathology of TBI is extremely complex. As our understanding of the numerous biochemical cascades involved continues to evolve, sophisticated diagnostic tools such as biomarkers will be developed (Figure 3). Ideal biomarkers will provide information on the pathobiology of TBI and facilitate better stratification of patients by their severity of injury,

better monitoring of the progression of secondary damage, response to treatment/intervention, and prediction of outcome. Although the initial characterization of biomarkers will be mainly based on spinal fluid analysis (Figure 3), methods for measurement of such biomarkers in blood (plasma or serum) will be developed. The development of accessible and reliable biomarkers is likely to change the way clinical studies of head injury are conducted, resulting in more mechanism driven, optimally timed therapies.

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### BIBLIOGRAPHY

1. *Consensus conference. Rehabilitation of persons with traumatic brain injury. NIH Consensus Development Panel on Rehabilitation of Persons With Traumatic Brain Injury. Jama, 1999. 282(10): p. 974-83.*
2. Alexander, M.P., *Mild traumatic brain injury: pathophysiology, natural history, and clinical management. Neurology, 1995. 45(7): p. 1253-60.*
3. Anderson RE, H.L., Nilsson O, Dijlai-Merzoug R, Settergen G., *High serum S100B levels for trauma patients without head injuries. Neurosurgery, 2001. 49(5): p. 1272-3.*
4. Bartus, R.T., P.J. Elliott, N.J. Hayward, R.L. Dean, S. Harbeson, J.A. Straub, Z. Li, and J.C. Powers, *Calpain as a novel target for treating acute neurodegenerative disorders. Neurol Res, 1995. 17(4): p. 249-58.*
5. Bartus, R.T., N.J. Hayward, P.J. Elliott, S.D. Sawyer, K.L. Baker, R.L. Dean, A. Akiyama, J.A. Straub, S.L. Harbeson, Z. Li, and et al., *Calpain inhibitor AK295 protects*

- neurons from focal brain ischemia. Effects of postocclusion intra-arterial administration.* PG - 2265-70. Stroke, 1994. **25**(11).
6. Beer, R., G. Franz, A. Srinivasan, R.L. Hayes, B.R. Pike, J.K. Newcomb, X. Zhao, E. Schmutzhard, W. Poewe, and A. Kampfl, *Temporal profile and cell subtype distribution of activated caspase-3 following experimental traumatic brain injury.* J Neurochem, 2000. **75**(3): p. 1264-73.
  7. Behrens, M.M., J.L. Martinez, C. Moratilla, and J. Renart, *Apoptosis induced by protein kinase C inhibition in a neuroblastoma cell line.* Cell Growth Differ, 1995. **6**(11): p. 1375-80.
  8. Bellamy, R., *The causes of death in conventional land warfares: implications for combat casualty research.* Military Medicine, 1984. **149**: p. 55-62.
  9. Bernath, E.e.a., *Spontaneous Cytoskeletal Protein Proteolysis in Aged Wistar Rat Brains.* In Preparation.
  10. Bonfoco, E., D. Krainc, M. Ankarcrona, P. Nicotera, and S.A. Lipton, *Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7162-6.
  11. Buki, A., D.O. Okonkwo, K.K. Wang, and J.T. Povlishock, *Cytochrome c release and caspase activation in traumatic axonal injury.* J Neurosci, 2000. **20**(8): p. 2825-34.
  12. CDC, *Fact Book.* 2000.
  13. Chang, L.K., G.V. Putcha, M. Deshmukh, and E.M. Johnson, *Mitochondrial involvement in the point of no return in neuronal apoptosis.* Biochimie, 2002. **84**(2-3): p. 223-31.
  14. Choi, D.W., *Ischemia-induced neuronal apoptosis.* Curr Opin Neurobiol, 1996. **6**(5): p. 667-72.

15. Choi SC, B.R., *Design and statistical issues in multicenter trials of severe head injury*. Neurological Research, 2001. **Mar-Apr**(23(2-3)): p. 190-2.
16. Clark, R.S., P.M. Kochanek, P.D. Adelson, M.J. Bell, J.A. Carcillo, M. Chen, S.R. Wisniewski, K. Janesko, M.J. Whalen, and S.H. Graham, *Increases in bcl-2 protein in cerebrospinal fluid and evidence for programmed cell death in infants and children after severe traumatic brain injury*. J Pediatr, 2000. **137**(2): p. 197-204.
17. Clark, R.S., P.M. Kochanek, M. Chen, S.C. Watkins, D.W. Marion, J. Chen, R.L. Hamilton, J.E. Loeffert, and S.H. Graham, *Increases in Bcl-2 and cleavage of caspase-1 and caspase-3 in human brain after head injury*. Faseb J, 1999. **13**(8): p. 813-21.
18. Clark, R.S., P.M. Kochanek, S.C. Watkins, M. Chen, C.E. Dixon, N.A. Seidberg, J. Melick, J.E. Loeffert, P.D. Nathaniel, K.L. Jin, and S.H. Graham, *Caspase-3 mediated neuronal death after traumatic brain injury in rats*. J Neurochem, 2000. **74**(2): p. 740-53.
19. Cohen, G.M., *Caspases: the executioners of apoptosis*. Biochem J, 1997. **326 ( Pt 1)**: p. 1-16.
20. Colicos, M.A. and P.K. Dash, *Apoptotic morphology of dentate gyrus granule cells following experimental cortical impact injury in rats: possible role in spatial memory deficits*. Brain Res, 1996. **739**(1-2): p. 120-31.
21. Conti, A.C., R. Raghupathi, J.Q. Trojanowski, and T.K. McIntosh, *Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period*. J Neurosci, 1998. **18**(15): p. 5663-72.
22. Cooper, E., *Neuron-specific enolase*. Int J Biol Markers, 1994(4): p. 205-210.
23. Copin, J.C., Y. Li, L. Reola, and P.H. Chan, *Trolox and 6,7-dinitroquinoxaline-2,3-dione prevent necrosis but not apoptosis in cultured neurons subjected to oxygen deprivation*. Brain Res, 1998. **784**(1-2): p. 25-36.

24. Cunningham, R.T., M. Watt, J. Winder, S. McKinstry, J.T. Lawson, C.F. Johnston, S.A. Hawkins, and K.D. Buchanan, *Serum neurone-specific enolase as an indicator of stroke volume*. Eur J Clin Invest, 1996. **26**(4): p. 298-303.
25. Cunningham, R.T., I.S. Young, J. Winder, M.J. O'Kane, S. McKinstry, C.F. Johnston, O.M. Dolan, S.A. Hawkins, and K.D. Buchanan, *Serum neurone specific enolase (NSE) levels as an indicator of neuronal damage in patients with cerebral infarction*. Eur J Clin Invest, 1991. **21**(5): p. 497-500.
26. Dauberschmidt, e.a., *Changes of neuron-specific enolase concentration in plasma after cardiac arrest and resuscitation*. Mol Chem Neuropathol, 1991. **14**: p. 237-245.
27. Dirnagl U, C.I., and Moskowitz MA, *Pathology of ischaemic stroke: an integrated view*. TINS, 1999. **22**(9): p. 391-397.
28. Donato, R., *Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type*. Biochim Biophys Acta., 1999. **1450**: p. 191-231.
29. Eberle, e.a., *Rapid prearrest cooling is associated with elevated S100 levels immediately after deep hypothermic circulatory arrest in adults*. Anesth Analg, 1997. **84**(4S(SCA12)).
30. Egon M.R. Doppenberg, S.C.C., and Ross Bullock, *Clinical trials in TBI, what can we learn from previous studies*. Annals of the New York Academy of Sciences, 1997. **825**: p. 305-322.
31. Eldadah, B.A., A.G. Yakovlev, and A.I. Faden, *The role of CED-3-related cysteine proteases in apoptosis of cerebellar granule cells*. PG - 6105-13. J Neurosci, 1997. **17**(16).
32. Ellis, E.F., J.S. McKinney, K.A. Willoughby, S. Liang, and J.T. Povlishock, *A new model for rapid stretch-induced injury of cells in culture: characterization of the model using astrocytes*. J Neurotrauma, 1995. **12**(3): p. 325-39.

33. Fischer, I., G. Romano-Clarke, and F. Grynspan, *Calpain-mediated proteolysis of microtubule associated proteins MAP1B and MAP2 in developing brain*. *Neurochem Res*, 1991. **16**(8): p. 891-8.
34. Flint, J.P., BR; Hayes,RL ; Moffett, J.R. Dave, JR; X.-C.M. Lu, and F.C. and Tortella., *Accumulation of calpain and caspase-3 cleaved alpha-II spectrin breakdown products in CSF after middle cerebral artery occulsion in rats*. *J. Neurotrauma*. (in press). 2002 (In Press).
35. Fraser, A. and G. Evan, *A license to kill*. *Cell*, 1996. **85**(6): p. 781-4.
36. Friederer BM, Z.I., Goodman SR, *Brain spectrin (240/235) and brain spectrin (240/235E): two distinct spectrin subtypes with different locations within mammalian neural cells*. *J Cell Biol*, 1986. **Jun**(102(6)): p. 2088-97.
37. Goodman SR, Z.W., Clark MB, Zagon IS, Barker JE, and Bloom ML, *Brain spectrin: of mice and men*. *Brain Res Bull*, 1995. **36**: p. 593-606.
38. Gottron, F.J., H.S. Ying, and D.W. Choi, *Caspase inhibition selectively reduces the apoptotic component of oxygen-glucose deprivation-induced cortical neuronal cell death*. *Mol Cell Neurosci*, 1997. **9**(3): p. 159-69.
39. Gwag, B.J., D. Lobner, J.Y. Koh, M.B. Wie, and D.W. Choi, *Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro*. *Neuroscience*, 1995. **68**(3): p. 615-9.
40. Haimoto, H., S. Hosoda, and K. Kato, *Differential distribution of immunoreactive S100-alpha and S100-beta proteins in normal nonnervous human tissues*. *Lab Invest*, 1987. **57**(5): p. 489-98.
41. Hill, M.D., G. Jackowski, N. Bayer, M. Lawrence, and R. Jaeschke, *Biochemical markers in acute ischemic stroke*. *Cmaj*, 2000. **162**(8): p. 1139-40.

42. Horn, M., F. Seger, and W. Schlote, *Neuron-specific enolase in gerbil brain and serum after transient cerebral ischemia*. Stroke, 1995. **26**(2): p. 290-6; discussion 296-7.
43. Ingebrigtsen T, R.B., *Biochemical Serum Markers of TBI*. The Journal of Trauma Injury, Infection, and Critical Care, 2002. **52**: p. 798-808.
44. Ingebrigtsen T, R.B., Trumpy JH., *Management of minor head injury: the value of early CT and serum protein S-100 measurements*. Journal of Clinical Neuroscience, 1997. **4**: p. 29-33.
45. Irazuzta, J.E., G. de Courten-Myers, F.P. Zemlan, M.Y. Bekkedal, and J. Rossi, 3rd, *Serum cleaved Tau protein and neurobehavioral battery of tests as markers of brain injury in experimental bacterial meningitis*. Brain Res, 2001. **913**(1): p. 95-105.
46. Jacobs A, P.E., Ingels M, Put T, Bossuyt A, *One-year follow-up of technetium-99m-HMPAO SPECT in mild head injury*. J Nucl Med, 1996. **37**: p. 1605-1609.
47. Jenkins, L.W., K. Moszynski, B.G. Lyeth, W. Lewelt, D.S. DeWitt, A. Allen, C.E. Dixon, J.T. Povlishock, T.J. Majewski, G.L. Clifton, and et al., *Increased vulnerability of the mildly traumatized rat brain to cerebral ischemia: the use of controlled secondary ischemia as a research tool to identify common or different mechanisms contributing to mechanical and ischemic brain injury*. Brain Res, 1989. **477**(1-2): p. 211-24.
48. Johnson, E.M., Jr., L.J. Greenlund, P.T. Akins, and C.Y. Hsu, *Neuronal apoptosis: current understanding of molecular mechanisms and potential role in ischemic brain injury*. J Neurotrauma, 1995. **12**(5): p. 843-52.
49. Johnsson, P., *Markers of cerebral ischemia after cardiac surgery*. J Cardiothorac Vasc Anesth, 1996. **10**(1): p. 120-6.
50. Jonas, S., V. Aiyagari, D. Vieira, and M. Figueroa, *The failure of neuronal protective agents versus the success of thrombolysis in the treatment of ischemic stroke. The predictive value of animal models*. Ann N Y Acad Sci, 2001. **939**: p. 257-67.

51. Jonsson H, J.P., Hoglund P, Alling C, Blomquist S., *The elimination of S-100b and renal function after cardiac surgery.* J Cardiothorac Vasc Anesth, 2000. **14**: p. 698-701.
52. Jordan, J., M.F. Galindo, and R.J. Miller, *Role of calpain- and interleukin-1 beta converting enzyme-like proteases in the beta-amyloid-induced death of rat hippocampal neurons in culture.* J Neurochem, 1997. **68**(4): p. 1612-21.
53. Kaku, Y., Y. Yonekawa, T. Tsukahara, N. Ogata, T. Kimura, and T. Taniguchi, *Alterations of a 200 kDa neurofilament in the rat hippocampus after forebrain ischemia.* J Cereb Blood Flow Metab, 1993. **13**(3): p. 402-8.
54. Kampfl, A., R. Posmantur, R. Nixon, F. Grynspan, X. Zhao, S.J. Liu, J.K. Newcomb, G.L. Clifton, and R.L. Hayes, *mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury.* J Neurochem, 1996. **67**(4): p. 1575-83.
55. Kampfl, A., R.M. Posmantur, X. Zhao, E. Schmutzhard, G.L. Clifton, and R.L. Hayes, *Mechanisms of calpain proteolysis following traumatic brain injury: implications for pathology and therapy: implications for pathology and therapy: a review and update.* J Neurotrauma, 1997. **14**(3): p. 121-34.
56. Kampfl, A., J.S. Whitson, X. Zhao, R. Posmantur, G.L. Clifton, and R.L. Hayes, *Calpain inhibitors reduce depolarization induced loss of tau protein in primary septo-hippocampal cultures.* Neurosci Lett, 1995. **194**(3): p. 149-52.
57. Kampfl, A., X. Zhao, J.S. Whitson, R. Posmantur, C.E. Dixon, K. Yang, G.L. Clifton, and R.L. Hayes, *Calpain inhibitors protect against depolarization-induced neurofilament protein loss of septo-hippocampal neurons in culture.* Eur J Neurosci, 1996. **8**(2): p. 344-52.
58. Kant R, S.-S.L., Isaac G, Duffy J., *Tc-HMPAO SPECT in persistent post-concussion syndrome after mild head injury: comparison with MRI/CT.* Brain Injury, 1997(Feb; **11**(2):): p. 115-24.

59. Kaya, S.S., A. Mahmood, Y. Li, E. Yavuz, M. Goksel, and M. Chopp, *Apoptosis and expression of p53 response proteins and cyclin D1 after cortical impact in rat brain*. Brain Res, 1999. **818**(1): p. 23-33.
60. Kesler, S.R., H.F. Adams, and E.D. Bigler, *SPECT, MR and quantitative MR imaging: correlates with neuropsychological and psychological outcome in traumatic brain injury*. Brain Inj, 2000. **14**(10): p. 851-7.
61. Kochanek, P.M., R.S. Clark, R.A. Ruppel, and C.E. Dixon, *Cerebral resuscitation after traumatic brain injury and cardiopulmonary arrest in infants and children in the new millennium*. Pediatr Clin North Am, 2001. **48**(3): p. 661-81.
62. Lammie, G.A., I.R. Piper, D. Thomson, and F. Brannan, *Neuropathologic characterization of a rodent model of closed head injury--addition of clinically relevant secondary insults does not significantly potentiate brain damage*. J Neurotrauma, 1999. **16**(7): p. 603-15.
63. Laskowitz, e.a., *Serum Markers of Cerebral Ischemia*. Journal of Stroke and Cerebrovascular Diseases, 1998. **7**(4 (July-August)): p. 234-241.
64. Levi, L., J.N. Guilburd, A. Lemberger, J.F. Soustiel, and M. Feinsod, *Diffuse axonal injury: analysis of 100 patients with radiological signs*. Neurosurgery, 1990. **27**(3): p. 429-32.
65. Leviton, A. and O. Dammann, *Brain damage markers in children. Neurobiological and clinical aspects*. Acta Paediatr, 2002. **91**(1): p. 9-13.
66. Lyeth, B.G., L.W. Jenkins, R.J. Hamm, C.E. Dixon, L.L. Phillips, G.L. Clifton, H.F. Young, and R.L. Hayes, *Prolonged memory impairment in the absence of hippocampal cell death following traumatic brain injury in the rat*. Brain Res, 1990. **526**(2): p. 249-58.
67. Martens, P., *Serum neuron-specific enolase as a prognostic marker for irreversible brain damage in comatose cardiac arrest survivors*. Acad Emerg Med, 1996. **3**: p. 126-131.

68. Martens, P., A. Raabe, and P. Johnsson, *Serum S-100 and neuron-specific enolase for prediction of regaining consciousness after global cerebral ischemia*. Stroke, 1998. **29**(11): p. 2363-6.
69. Martin, S.J., G.A. O'Brien, W.K. Nishioka, A.J. McGahon, A. Mahboubi, T.C. Saido, and D.R. Green, *Proteolysis of fodrin (non-erythroid spectrin) during apoptosis*. J Biol Chem, 1995. **270**(12): p. 6425-8.
70. Missler, U., M. Wiesmann, C. Friedrich, and M. Kaps, *S-100 protein and neuron-specific enolase concentrations in blood as indicators of infarction volume and prognosis in acute ischemic stroke*. Stroke, 1997. **28**(10): p. 1956-60.
71. Missler U, W.M., Wittmann G, Magerkurth O, Hagenstrom H., *Measurement of glial fibrillary acidic protein in human blood: analytical method and preliminary clinical results*. Clin Chem, 1999. **45**: p. 138-141.
72. Mitchener, A., D.J. Wyper, J. Patterson, D.M. Hadley, J.T. Wilson, L.C. Scott, M. Jones, and G.M. Teasdale, *SPECT, CT, and MRI in head injury: acute abnormalities followed up at six months*. J Neurol Neurosurg Psychiatry, 1997. **62**(6): p. 633-6.
73. Miura, M., H. Zhu, R. Rotello, E.A. Hartweg, and J. Yuan, *Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3*. Cell, 1993. **75**(4): p. 653-60.
74. Moore, B., *A soluble protein characteristic of the nervous system*. Biochem Biophys Res Commun, 1965. **19**: p. 739-744.
75. Morioka, M., K. Fukunaga, S. Yasugawa, S. Nagahiro, Y. Ushio, and E. Miyamoto, *Regional and temporal alterations in Ca<sup>2+</sup>/calmodulin-dependent protein kinase II and calcineurin in the hippocampus of rat brain after transient forebrain ischemia*. J Neurochem, 1992. **58**(5): p. 1798-809.

76. Nagy, Z., L. Simon, and Z. Bori, [*Regulatory mechanisms in focal cerebral ischemia. New possibilities in neuroprotective therapy*]. *Ideggyogy Sz*, 2002. **55**(3-4): p. 73-85.
77. Narayan, R.K., M.E. Michel, B. Ansell, A. Baethmann, A. Biegon, M.B. Bracken, M.R. Bullock, S.C. Choi, G.L. Clifton, C.F. Contant, W.M. Coplin, W.D. Dietrich, J. Ghajar, S.M. Grady, R.G. Grossman, E.D. Hall, W. Heetderks, D.A. Hovda, J. Jallo, R.L. Katz, N. Knoller, P.M. Kochanek, A.I. Maas, J. Majde, D.W. Marion, A. Marmarou, L.F. Marshall, T.K. McIntosh, E. Miller, N. Mohberg, J.P. Muizelaar, L.H. Pitts, P. Quinn, G. Riesenfeld, C.S. Robertson, K.I. Strauss, G. Teasdale, N. Temkin, R. Tuma, C. Wade, M.D. Walker, M. Weinrich, J. Whyte, J. Wilberger, A.B. Young, and L. Yurkewicz, *Clinical trials in head injury*. *J Neurotrauma*, 2002. **19**(5): p. 503-57.
78. Nath, R., K.J. Raser, K. McGinnis, R. Nadimpalli, D. Stafford, and K.K. Wang, *Effects of ICE-like protease and calpain inhibitors on neuronal apoptosis*. *Neuroreport*, 1996. **8**(1): p. 249-55.
79. Nath, R., K.J. Raser, D. Stafford, I. Hajimohammadreza, A. Posner, H. Allen, R.V. Talanian, P. Yuen, R.B. Gilbertsen, and K.K. Wang, *Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis*. *Biochem J*, 1996. **319** ( Pt 3): p. 683-90.
80. Neumar, R.W., F.H. Meng, A.M. Mills, Y.A. Xu, C. Zhang, F.A. Welsh, and R. Siman, *Calpain activity in the rat brain after transient forebrain ischemia*. *Exp Neurol*, 2001. **170**(1): p. 27-35.
81. Newcomb, J.K., A. Kampfl, R.M. Posmantur, X. Zhao, B.R. Pike, S.J. Liu, G.L. Clifton, and R.L. Hayes, *Immunohistochemical study of calpain-mediated breakdown products to alpha-spectrin following controlled cortical impact injury in the rat*. *J Neurotrauma*, 1997. **14**(6): p. 369-83.

82. Newcomb, J.K., X. Zhao, B.R. Pike, and R.L. Hayes, *Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat.* Exp Neurol, 1999. **158**(1): p. 76-88.
83. NIH, *Rehabilitation of persons with traumatic brain injury.* NIH Consens Statement, 1998. **16**(1): p. 1-41.
84. Nishida, A., K. Emoto, M. Shimizu, T. Uozumi, and S. Yamawaki, *Brain ischemia decreases phosphatidylcholine-phospholipase D but not phosphatidylinositol-phospholipase C in rats.* PG - 1247-51. Stroke, 1994. **25**(6).
85. Nitatori, T., N. Sato, S. Waguri, Y. Karasawa, H. Araki, K. Shibana, E. Kominami, and Y. Uchiyama, *Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis.* J Neurosci, 1995. **15**(2): p. 1001-11.
86. Nygaard O. Langbakk B, R.B., *Neuro-specific enolase concentrations in serum and cerebrospinal fluid in patients with no previous history of neurological disorder.* Scand J Clin Lab Invest., 1998. **58**: p. 183-186.
87. Ogata, N., Y. Yonekawa, W. Taki, R. Kannagi, T. Murachi, T. Hamakubo, and H. Kikuchi, *Degradation of neurofilament protein in cerebral ischemia.* J Neurosurg, 1989. **70**(1): p. 103-7.
88. Paterakis, K., A.H. Karantanas, A. Komnos, and Z. Volikas, *Outcome of patients with diffuse axonal injury: the significance and prognostic value of MRI in the acute phase.* J Trauma, 2000. **49**(6): p. 1071-5.
89. Perini, F., M. Morra, M. Alecci, E. Galloni, M. Marchi, and V. Toso, *Temporal profile of serum anti-inflammatory and pro-inflammatory interleukins in acute ischemic stroke patients.* Neurol Sci, 2001. **22**(4): p. 289-96.

90. Pike, B.R., J. Flint, S. Dutta, E. Johnson, K.K. Wang, and R.L. Hayes, *Accumulation of non-erythroid alpha II-spectrin and calpain-cleaved alpha II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats*. J Neurochem, 2001. **78**(6): p. 1297-306.
91. Pike, B.R., X. Zhao, J.K. Newcomb, C.C. Glenn, D.K. Anderson, and R.L. Hayes, *Stretch injury causes calpain and caspase-3 activation and necrotic and apoptotic cell death in septo-hippocampal cell cultures*. J Neurotrauma, 2000. **17**(4): p. 283-98.
92. Pike, B.R., X. Zhao, J.K. Newcomb, R.M. Posmantur, K.K. Wang, and R.L. Hayes, *Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury*. Neuroreport, 1998. **9**(11): p. 2437-42.
93. Pike, B.R., X. Zhao, J.K. Newcomb, K.K. Wang, R.M. Posmantur, and R.L. Hayes, *Temporal relationships between de novo protein synthesis, calpain and caspase 3-like protease activation, and DNA fragmentation during apoptosis in septo-hippocampal cultures*. PG - 505-20. J Neurosci Res, 1998. **52**(5).
94. Posmantur, R., R.L. Hayes, C.E. Dixon, and W.C. Taft, *Neurofilament 68 and neurofilament 200 protein levels decrease after traumatic brain injury*. J Neurotrauma, 1994. **11**(5): p. 533-45.
95. Posmantur, R., A. Kampfl, R. Siman, J. Liu, X. Zhao, G.L. Clifton, and R.L. Hayes, *A calpain inhibitor attenuates cortical cytoskeletal protein loss after experimental traumatic brain injury in the rat*. Neuroscience, 1997. **77**(3): p. 875-88.
96. Posmantur, R.M., J.K. Newcomb, A. Kampfl, and R.L. Hayes, *Light and confocal microscopic studies of evolutionary changes in neurofilament proteins following cortical impact injury in the rat*. Exp Neurol, 2000. **161**(1): p. 15-26.
97. Posmantur, R.M., X. Zhao, A. Kampfl, G.L. Clifton, and R.L. Hayes, *Immunoblot analyses of the relative contributions of cysteine and aspartic proteases to neurofilament*

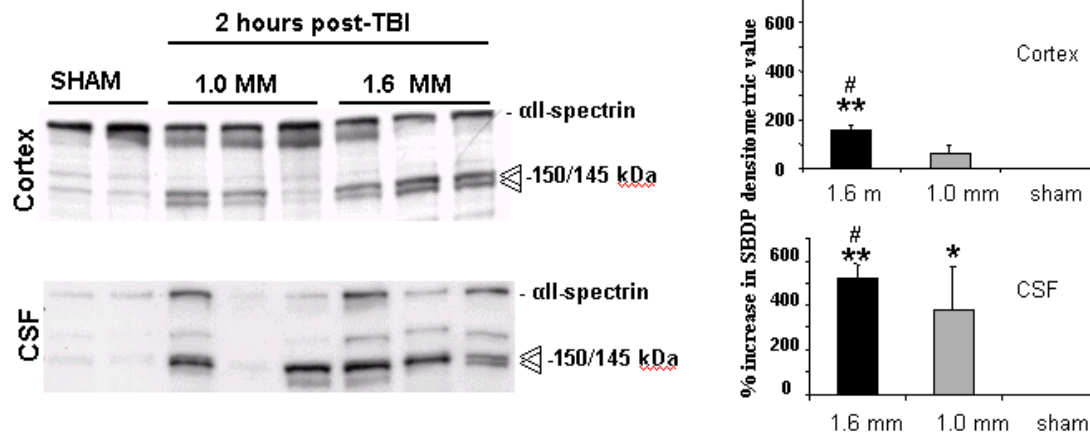
- breakdown products following experimental brain injury in rats.* Neurochem Res, 1998. **23**(10): p. 1265-76.
98. Raabe A, G.C., Keller M, Dohnert J, Sorge O, Seifert V., *Correlation of computed tomography findings and serum brain damage markers following severe head injury.* Acta Neurochir (Wein), 1998. **140**: p. 787-792.
99. Raabe A, G.C., Seifert V., *Serum markers of brain damage and outcome prediction in patients after severe head injury.* BR J Neurosurg, 1999. **13**: p. 56-59.
100. Raabe, A., D.K. Menon, S. Gupta, M. Czosnyka, and J.D. Pickard, *Jugular venous and arterial concentrations of serum S-100B protein in patients with severe head injury: a pilot study.* J Neurol Neurosurg Psychiatry, 1998. **65**(6): p. 930-2.
101. Raghupathi, R., A.C. Conti, D.I. Graham, S. Krajewski, J.C. Reed, M.S. Grady, J.Q. Trojanowski, and T.K. McIntosh, *Mild traumatic brain injury induces apoptotic cell death in the cortex that is preceded by decreases in cellular Bcl-2 immunoreactivity.* Neuroscience, 2002. **110**(4): p. 605-16.
102. Raghupathi, R. and S.S. Margulies, *Traumatic axonal injury after closed head injury in the neonatal pig.* J Neurotrauma, 2002. **19**(7): p. 843-53.
103. Ringger NC, e.a., *CSF Calpain-induced spectrin breakdown products predict injury magnitude and chronic lesion sized in a rat model of TBI.* In Preparation.
104. Ringleb, P.A., P.D. Schellinger, C. Schranz, and W. Hacke, *Thrombolytic therapy within 3 to 6 hours after onset of ischemic stroke: useful or harmful?* Stroke, 2002. **33**(5): p. 1437-41.
105. Rink, A., K.M. Fung, J.Q. Trojanowski, V.M. Lee, E. Neugebauer, and T.K. McIntosh, *Evidence of apoptotic cell death after experimental traumatic brain injury in the rat.* Am J Pathol, 1995. **147**(6): p. 1575-83.

106. Roine, e.a., *Neurological outcome after out-of-hospital cardiac arrest. Prediction by cerebrospinal fluid enzyme analysis*. Arch Neurol, 1989. **46**: p. 753-756.
107. Romner B, I.T., Kongstad P, Borgesen SE, *Traumatic brain injury: serum S-100 measurements related to neuroradiological findings*. J Neurotrauma, 2000. **17**: p. 641-647.
108. Ross SA, C.R., Johnston CF, Rowlands BJ, *Neuron-specific enolase as an aid to outcome prediction in head injury*. BR J Neurosurg, 1996. **10**: p. 471-476.
109. Saatman, K.E., D. Bozyczko-Coyne, V. Marcy, R. Siman, and T.K. McIntosh, *Prolonged calpain-mediated spectrin breakdown occurs regionally following experimental brain injury in the rat*. J Neuropathol Exp Neurol, 1996. **55**(7): p. 850-60.
110. Saido, T.C., H. Sorimachi, and K. Suzuki, *Calpain: new perspectives in molecular diversity and physiological-pathological involvement*. Faseb J, 1994. **8**(11): p. 814-22.
111. Scheidler, M.G., B.L. Shultz, L. Schall, and H.R. Ford, *Risk factors and predictors of mortality in children after ejection from motor vehicle crashes*. J Trauma, 2000. **49**(5): p. 864-8.
112. Schwartz, S.M., J.Y. Duffy, J.M. Pearl, and D.P. Nelson, *Cellular and molecular aspects of myocardial dysfunction*. Crit Care Med, 2001. **29**(10 Suppl): p. S214-9.
113. Sellman, M., T. Ivert, G. Ronquist, K. Caesarini, L. Persson, and B.K. Semb, *Central nervous system damage during cardiac surgery assessed by 3 different biochemical markers in cerebrospinal fluid*. Scand J Thorac Cardiovasc Surg, 1992. **26**(1): p. 39-45.
114. Shaw GJ, J.E., Zemlan FP, *Serum cleaved tau protein levels and clinical outcome in adult patients with closed head injury*. Ann Emerg Med, 2002. **Mar**(39): p. 254-7.
115. Singhal, A., A.J. Baker, G.M. Hare, F.X. Reinders, L.C. Schlichter, and R.J. Moulton, *Association between Cerebrospinal Fluid Interleukin-6 Concentrations and Outcome after Severe Human Traumatic Brain Injury*. J Neurotrauma, 2002. **19**(8): p. 929-37.

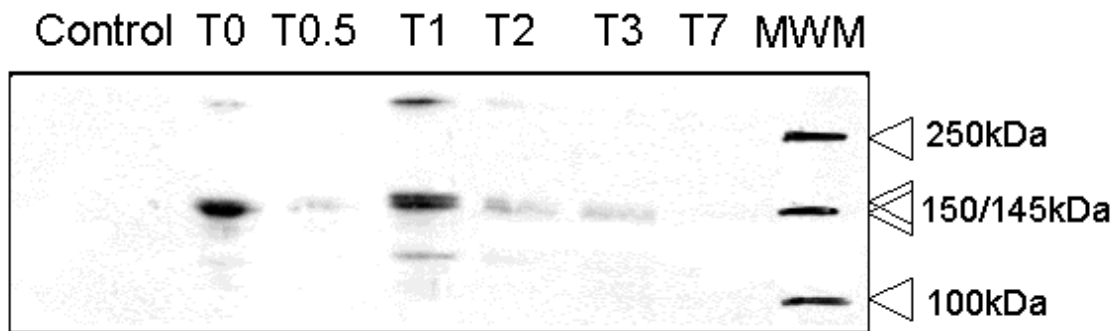
116. Sloviter, R.S., *Hippocampal pathology and pathophysiology in temporal lobe epilepsy*. Neurologia, 1996. **11 Suppl 4**: p. 29-32.
117. Squier, M.K., A.C. Miller, A.M. Malkinson, and J.J. Cohen, *Calpain activation in apoptosis*. J Cell Physiol, 1994. **159**(2): p. 229-37.
118. Suzuki, K., H. Sorimachi, T. Yoshizawa, K. Kinbara, and S. Ishiura, *Calpain: novel family members, activation, and physiologic function*. Biol Chem Hoppe Seyler, 1995. **376**(9): p. 523-9.
119. Thomaidou, D., M.C. Mione, J.F. Cavanagh, and J.G. Parnavelas, *Apoptosis and its relation to the cell cycle in the developing cerebral cortex*. J Neurosci, 1997. **17**(3): p. 1075-85.
120. Usui A, K.K., Abe T, Murase M, Tanaka M, Takeuchi E, *S-100 $\alpha$  protein in blood and urine during open-heart surgery*. Clin Chem, 1989. **35**: p. 1942-1944.
121. van Engelen BG, L.K., Gabreels FJM, Wevers RA, van Geel WJA, Borm GF, *Age-related changes of neuron-specific enolase, S-100 protein, and myelin basic protein concentrations in cerebrospinal fluid*. Clin Chem, 1992. **38**: p. 813-816.
122. Vanags, D.M., M.I. Porn-Ares, S. Coppola, D.H. Burgess, and S. Orrenius, *Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis*. J Biol Chem, 1996. **271**(49): p. 31075-85.
123. Villa, P.G., W.J. Henzel, M. Sensenbrenner, C.E. Henderson, and B. Pettmann, *Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis*. J Cell Sci, 1998. **111 ( Pt 6)**: p. 713-22.
124. Wang, K.K., R. Posmantur, R. Nath, K. McGinnis, M. Whitton, R.V. Talanian, S.B. Glantz, and J.S. Morrow, *Simultaneous degradation of alphaII- and betaII-spectrin by caspase 3 (CPP32) in apoptotic cells*. J Biol Chem, 1998. **273**(35): p. 22490-7.

125. Wang, K.K., A. Villalobo, and B.D. Roufogalis, *Calmodulin-binding proteins as calpain substrates*. Biochem J, 1989. **262**(3): p. 693-706.
- 125a. Wang, K.K.W. (2000) Calpain and Caspase: Can You Tell the Difference. Trends Neurosci. 23, 20-26.
126. Wang, K.K. and P.W. Yuen, *Calpain inhibition: an overview of its therapeutic potential*. Trends Pharmacol Sci, 1994. **15**(11): p. 412-9.
127. Westaby, S., P. Johnsson, A.J. Parry, S. Blomqvist, J.O. Solem, C. Alling, R. Pillai, D.P. Taggart, C. Grebenik, and E. Stahl, *Serum S100 protein: a potential marker for cerebral events during cardiopulmonary bypass*. Ann Thorac Surg, 1996. **61**(1): p. 88-92.
128. Woertgen C, R.R., Holzschuh M, Metz C, Brawanski A, *Comparison of clinical, radiologic, and serum marker as prognostic factors after severe head injury*. J Trauma, 1999. **47**: p. 1126-1130.
129. Woertgen C, R.R., Holzschuh M, Metz C, Brawanski A, *Comparison of serial S-100 and NSSE serum measurement after severe head injury*. Acta Neurochir (Wein). **71**: p. 1161-1165.
130. Yakovlev, A.G., S.M. Knoblach, L. Fan, G.B. Fox, R. Goodnight, and A.I. Faden, *Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury*. J Neurosci, 1997. **17**(19): p. 7415-24.
131. Yamazaki Y, Y.K., Morii S, Kitahara T, Ohwada T., *Diagnostic significance of serum neuron-specific enolase and myelin basic protein assay in patients with acute head injury*. Surg Neurol, 1997. **43**: p. 267-271.
132. Yang, K., W.C. Taft, C.E. Dixon, R.K. Yu, and R.L. Hayes, *Endogenous phosphorylation of a 61,000 dalton hippocampal protein increases following traumatic brain injury*. PG - 523-32. J Neurotrauma, 1994. **11**(5).

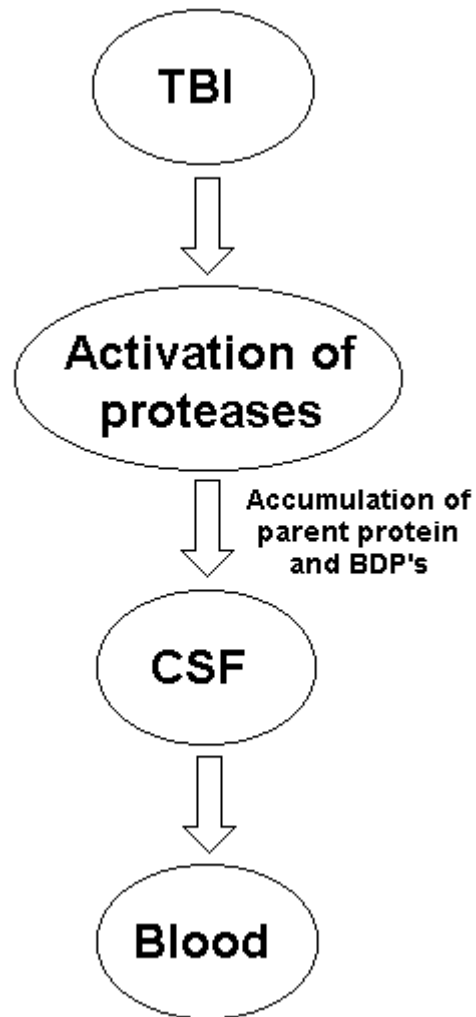
133. Ytrebo LM, N.G., Korvald C, et al, *Renal elimination of protein S-100beta in piggs with acute encephalopathy*. Scand J Clin Lab Invest., 2001. **61**: p. 217-225.
134. Zauner, A. and R. Bullock, *The role of excitatory amino acids in severe brain trauma: opportunities for therapy: a review*. J Neurotrauma, 1995. **12**(4): p. 547-54.
135. Zemlan, F.P., E.C. Jauch, J.J. Mulchahey, S.P. Gabbita, W.S. Rosenberg, S.G. Speciale, and M. Zuccarello, *C-tau biomarker of neuronal damage in severe brain injured patients: association with elevated intracranial pressure and clinical outcome*. Brain Res, 2002. **947**(1): p. 131-9.



**Figure 1. Accumulation of calpain-specific SBDPs is sensitive to injury magnitude after controlled cortical impact.** Animals were injured using a model of controlled cortical impact at two magnitudes of injury. Accumulation of brain cortex and spinal fluid levels of  $\alpha$ II-SBDP were measured by densitometric analysis at 2 hours post-injury. The 1.6 mm injury magnitude produced the highest levels of  $\alpha$ II-SBDP accumulation in cortex and CSF compared to 1.0 mm injury and to sham-injured controls. However, statistical significance ( $p < 0.05$ ) between 1.6 mm and 1.0 mm injuries was only achieved at 2 hours but not at 6 or 24 hours post-injury (data not shown). This finding could indicate a critical window for discriminating injury magnitude using  $\alpha$ II-SBDP as a biomarker. CSF levels of  $\alpha$ II-SBDP were greater in CSF than in cortex, particularly for the 1.6 mm injury. This most likely reflects a greater ratio of SBDP to total protein in CSF relative to cortex and indicates that low abundance proteins may be more easily detected in CSF after brain injury. ( $n = 6$  per group).



**Figure 2.** Western blot analysis of cerebrospinal fluid (CSF) samples from a 9 year old patient diagnosed with severe head injury.  $\alpha$ II-spectrin breakdown products reflecting caspase-3 (150kDa and 120kDa bands) and calpain (150kDa and 145kDa bands) activity were identified as early as 12 hours after injury and persisted for at least 3 days after injury. Increased protein expression 1 day after injury may reflect clinical evidence of increased brain swelling and intracranial hypertension. CSF from a child with chronic hydrocephalus and no acute brain injury was used as control. T0= admission to the intensive care unit; T0.5= 12 hours after injury; T1= 24 hours after injury; T2: 2 days after injury; T3: 3 days after injury; T7= 7 days after injury; MWM= molecular weight marker.



**Figure 3.** Development of biomarkers of protease activity. Brain injury leads to activation of proteases after traumatic brain injury. Proteolytic processing of cytoskeletal proteins (i.e.  $\alpha$ -II-spectrin) leads to accumulation of both the parent protein as well as signature break down products (BDP's). Initial characterization will most likely require analysis of cerebrospinal fluid (CSF). Antibodies will then be developed so that an easily accessible biomarker of protease activity is available for sampling from peripheral blood.