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# Prospective clinical biomarkers of caspase-mediated apoptosis associated with neuronal and neurovascular damage following stroke and other severe brain injuries: Implications for chronic neurodegeneration

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## Abstract:

Acute brain injuries, including ischemic and hemorrhagic stroke, as well as traumatic brain injury (TBI), are major worldwide health concerns with very limited options for effective diagnosis and treatment. Stroke and TBI pose an increased risk for the development of chronic neurodegenerative diseases, notably chronic traumatic encephalopathy, Alzheimer's disease, and Parkinson's disease. The existence of premorbid neurodegenerative diseases can exacerbate the severity and prognosis of acute brain injuries. Apoptosis involving caspase-3 is one of the most common mechanisms involved in the etiopathology of both acute and chronic neurological and neurodegenerative diseases, suggesting a relationship between these disorders. Over the past two decades, several clinical biomarkers of apoptosis have been identified in cerebrospinal fluid and peripheral blood following ischemic stroke, intracerebral and subarachnoid hemorrhage, and TBI. These biomarkers include selected caspases, notably caspase-3 and its specific cleavage products such as caspase-cleaved cytokeratin-18, caspase-cleaved tau, and a caspase-specific 120 kDa  $\alpha$ II-spectrin breakdown product. The levels of these biomarkers might be a valuable tool for the identification of pathological pathways such as apoptosis and inflammation involved in injury progression, assessment of injury severity, and prediction of clinical outcomes. This review focuses on clinical studies involving biomarkers of caspase-3-mediated pathways, following stroke and TBI. The review further examines their prospective diagnostic utility, as well as clinical utility for improved personalized treatment of stroke and TBI patients and the development of prophylactic treatment chronic neurodegenerative disease.

## Keywords:

Caspase-3, caspase-cleaved cytokeratin-18, caspase-cleaved tau, stroke, traumatic brain injury,  $\alpha$ II-spectrin breakdown products

## Introduction

Acute brain injuries such as stroke and traumatic brain injury (TBI) are a global health problem with very limited treatment options. An estimated 15 million people sustain stroke and 10 million people sustain TBI annually worldwide.<sup>[1-7]</sup> In the United

States alone, approximately 1.5 million people are affected by TBI and over 700,000 people are affected by stroke annually, with an estimated mortality and long-term disability rate of over 50,000 and 90,000 persons per year from TBI.<sup>[8-14]</sup> An estimated 130,000 Americans die from stroke annually.<sup>[2-4]</sup>

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Stroke is classified into two main types: ischemic stroke and hemorrhagic stroke, including intracerebral hemorrhage and subarachnoid hemorrhage (SAH).<sup>[15-17]</sup> Although ischemic stroke accounts for over 85% of all stroke cases, hemorrhagic stroke also imposes a comparable health burden.<sup>[18,19]</sup> TBI and stroke share several common features such as cerebral ischemia, excitotoxicity, and neuroinflammation, suggesting the presence of similar molecular mechanisms in the etiopathology of these disorders. For example, a postmortem study in TBI patients suggested that intracerebral hemorrhage is a common feature of severe TBI and secondary cerebral ischemia is a major factor associated with the most severely impaired outcomes after TBI.<sup>[13]</sup> Moreover, TBI and stroke are major risk factors in the development of chronic neurodegenerative disorders and diseases such as posttraumatic and poststroke epilepsies,<sup>[20]</sup> chronic traumatic encephalopathy (CTE),<sup>[21]</sup> as well as Alzheimer's and Parkinson's diseases (AD and PD, respectively).<sup>[22-25]</sup> Recent prospective clinical studies have shown that cerebral ischemia is a frequent comorbid condition of AD with the presence of cerebrovascular pathology in up to 84% of AD patients<sup>[26-28]</sup> and stroke survivors also have elevated incidence of AD.<sup>[29-32]</sup> Furthermore, there is evidence that indicates stroke and AD exacerbate the severity and prognosis of each other.<sup>[30,33,34]</sup>

Stroke, TBI, and other neurodegenerative diseases are characterized by the presence of delayed, progressive neuronal apoptosis involving caspase activation, suggesting a possible link between pathological molecular mechanisms and prospective targets for treatment. Early detection of specific apoptotic pathways using relevant biomarkers present in the cerebrospinal fluid (CSF) or peripheral blood would provide valuable information about progression of the neurodegenerative processes following brain injuries and suggest possible treatment interventions. The main focus of this review is to provide the current information on clinical studies of biomarkers involved in caspase-3-mediated apoptosis following TBI and stroke by focusing on the association of these biomarkers with clinical outcomes. The possible cellular and molecular mechanisms associated with these biomarkers and their clinical implications are also discussed.

## Pathways Involved in Cell Death Following Brain Injuries and Neurodegenerative Diseases: Role of Apoptosis

### Role of apoptosis in the central nervous system

Neuronal cell death is involved in the etiopathology of many brain injuries, neurological disorders, and neurodegenerative disease including stroke, TBI, AD, and PD.<sup>[35]</sup> Necrosis and apoptosis are two major mechanisms of cell death in the central

nervous system (CNS) with distinct physiological and pathophysiological features, biochemical pathways, and histological descriptions.<sup>[36-38]</sup> Necrosis generally occurs in direct response to a pathological stimulus, such as excitotoxicity generated during acute brain injuries or chronic neurological disorders and diseases by activation of a calpain-mediated cell death pathway.<sup>[37]</sup> In contrast, apoptosis is involved in both physiological and pathophysiological processes and can result in selective cell death in response to a specific death stimulus. During apoptosis, cell death is caused by a tightly regulated biochemical cascade involving activation of caspases.<sup>[39,40]</sup>

Although both calpain- and caspase-mediated cell death mechanisms often coexist, in neurological disorders such as cerebral ischemia and brain trauma, necrosis and apoptosis are differentially involved in the etiopathology of these disorders and characterized by different spatiotemporal representations.<sup>[36]</sup> Following acute brain injuries such as cerebral ischemia and brain trauma, necrosis plays a major role in the cell death within injured areas (e.g., infarct core and contusion zone, respectively), resulting in the formation of primarily irreversible brain lesions, in contrast to apoptosis which can extend delayed cell death into potentially treatable perilesional areas, often referred as penumbra. Thus, taking into account the brain's very limited capacity for neurogenesis and regeneration,<sup>[38,40-44]</sup> apoptotic cell death pathways may represent potential targets for therapeutic treatment of brain injuries and stroke.<sup>[45]</sup> In the CNS, necrosis primarily occurs in neurons whereas apoptosis is present in both in neuronal and nonneuronal cells. Understanding of the detailed molecular mechanisms and recognition of spatiotemporal profiles of apoptotic pathways in different acute brain injuries and neurodegenerative diseases are an important step for target-based development of novel therapeutic strategies for acute injury as well as neurological disorders.

Under physiological conditions, apoptosis plays an important role in maintaining the integrity and functionality of the CNS and peripheral nervous system during development, as well as neuro- and synapto-genesis and synaptic function and plasticity, by inducing a cell death sequence, or apoptotic cascade, in selected old or damaged cells while leaving surrounding cells intact.<sup>[46-50]</sup> Neuronal apoptosis in the embryonic brain is a highly, genetically regulated process which plays a significant role for normal CNS development and function.<sup>[50-53]</sup> However, abnormal apoptosis resulting in excessive neuronal and glial cell death and disrupted synaptic function plays an important role in the progression of brain injury and neurodegenerative diseases.<sup>[39]</sup>

## Apoptotic pathways and caspase activation

Genetic and molecular determinants of apoptotic cell death pathways are well understood. Seminal studies performed in the nematode *Caenorhabditis elegans* identified four major genes controlling programmed cell death (*ced-3*, *ced-4*, *egl-1*, and *ced-9*). The orchestrated expression of these genes and their involvement in the cell death process laid a foundation for our current understanding of apoptosis in more complex organisms.<sup>[39,40,54-58]</sup> In mammals and other vertebrates, cellular apoptosis is regulated by expression of caspase proteases which are related to the *ced-3* gene of *C. elegans*. Fourteen vertebrate caspases have been described to date, eleven of which are expressed in humans.<sup>[59]</sup> Activation of caspases plays a major role in apoptotic events in acute and chronic neurological disorders, such as stroke, TBI, and other neurodegenerative diseases.<sup>[39,60-62]</sup>

Apoptotic cell death consists of a stereotypic biochemical pathway involving activation of different signal molecules and caspase proteases.<sup>[39]</sup> Caspase activation involves several cleavage steps and processing from procaspases or caspase precursors, comprising p10 and p20 subunits, to activate caspase heterotetramers consisting of two p10 and two p20 subunits derived from these procaspases. Functionally and structurally, caspases are categorized into upstream initiator caspases and downstream effector caspases. All initiator caspases have long N-terminal activation prodomains, and all effector caspases have short N-terminal activation prodomains. Further, initiator caspases are structurally subcategorized into two groups based on the presence of specific a long N-terminal procaspase activation domain, which also determine the caspase's specificity for signal molecules required for its activation including caspase-recruiting domain (caspases 1, 2, 4, 5, 9, 11, 12, and 13) or death-effector domain (caspases 8 and 10).<sup>[63-65]</sup> Activation of upstream initiator caspases precedes and is required for activation of downstream effector caspases.<sup>[63,64]</sup> Although many of these caspases are implicated in neurological disorders, they are differentially involved in cellular responses to the CNS injury and the etiopathology of neurodegenerative diseases, including both apoptotic and apoptosis-independent inflammatory pathways. The initiator caspases 2, 8, 9, and 10 and effector caspases 3, 6, and 7 are involved in apoptosis, whereas caspases 1, 4, 5, 11, 12, and 13 and caspase-14 are involved in cytokine activation and maturation, respectively.<sup>[64]</sup>

Apoptotic cell death includes three major stages: initiation, effector, and degradation phases.<sup>[39]</sup> Apoptotic processes might be initiated involving both caspase-dependent and caspase-independent pathways. The caspase-dependent pathways can be activated by

specific independent or converging intrinsic and extrinsic cell signaling mechanisms and involve specific caspases. The terminal phase of apoptotic cell death is initiated by cleavage of specific enzyme proteins by activated effector caspases such as poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase catalytic subunit (DNA-PKCS) resulting in the DNA degradation and fragmentation of cell nuclei.<sup>[66-69]</sup> In addition, effector caspase proteases, notably caspase-3, are involved in the cleavage of specific cytoskeletal proteins (discussed in detail later in this review) that also play critical roles in cell death in neurological and neurodegenerative disorders. On the other hand, caspase-1 activation leads to induction of nonapoptotic pathways resulting in the induction of inflammation and programmed cell death known as pyroptosis associated with the release of inflammatory cytokines. Simplified pathways involved in aforementioned mechanisms of cell death are presented in Figure 1.

## Intrinsic mechanisms of classic apoptosis

Apoptosis through intrinsic mechanisms is associated with the activation of highly regulated multi-step mitochondria-dependent pathways resulting in activation of executor caspases, leading to cleavage of several cellular proteins promoting cell death. The initiation phase of apoptosis is induced by several stressors such as oxidative stress, genetic mutations, deficits of growth factors, and excitotoxicity, which activate an intracellular biochemical cascade involving one or more different joints and/or mutually independent mechanisms including increases in the cytosolic levels of free calcium ions ( $\text{Ca}^{2+}$ ) and reactive oxygen species, upregulation of prostate apoptosis response-4 protein (Par-4), and translocation of proapoptotic proteins from B-cell lymphoma-2 (Bcl-2) family, such as Bcl-2-associated X-protein (Bax) and Bcl-2-associated death promoter (Bad) from the cytoplasm to the mitochondrial membrane, leading to initiation of the apoptotic cascade in mitochondria.<sup>[70,71]</sup> The effector phase of apoptosis involves increases in the mitochondrial levels of  $\text{Ca}^{2+}$  and reactive oxygen radicals, the formation of permeability transition pores in the mitochondrial membrane, which allows transportation of cytochrome c from the mitochondria into the cytosol where it forms a complex with apoptotic protease-activating factor 1 (Apaf-1) involving in the activation of an initiator caspase (i.e., caspase-9) leading to processing and activation of executor caspases (e.g., caspases 3 and 7) that initiate the degradation phase of apoptosis. The current published data indicate that apoptotic cell death following brain injuries and neurodegeneration is primarily associated with activation of caspase-3 although there are limited data for the involvement of caspase-7. Activation of the executor caspases involves multiple steps including cytochrome C-induced formation of an Apaf-1-caspase-9 apoptosome complex,<sup>[72]</sup> subsequent cleavage, and



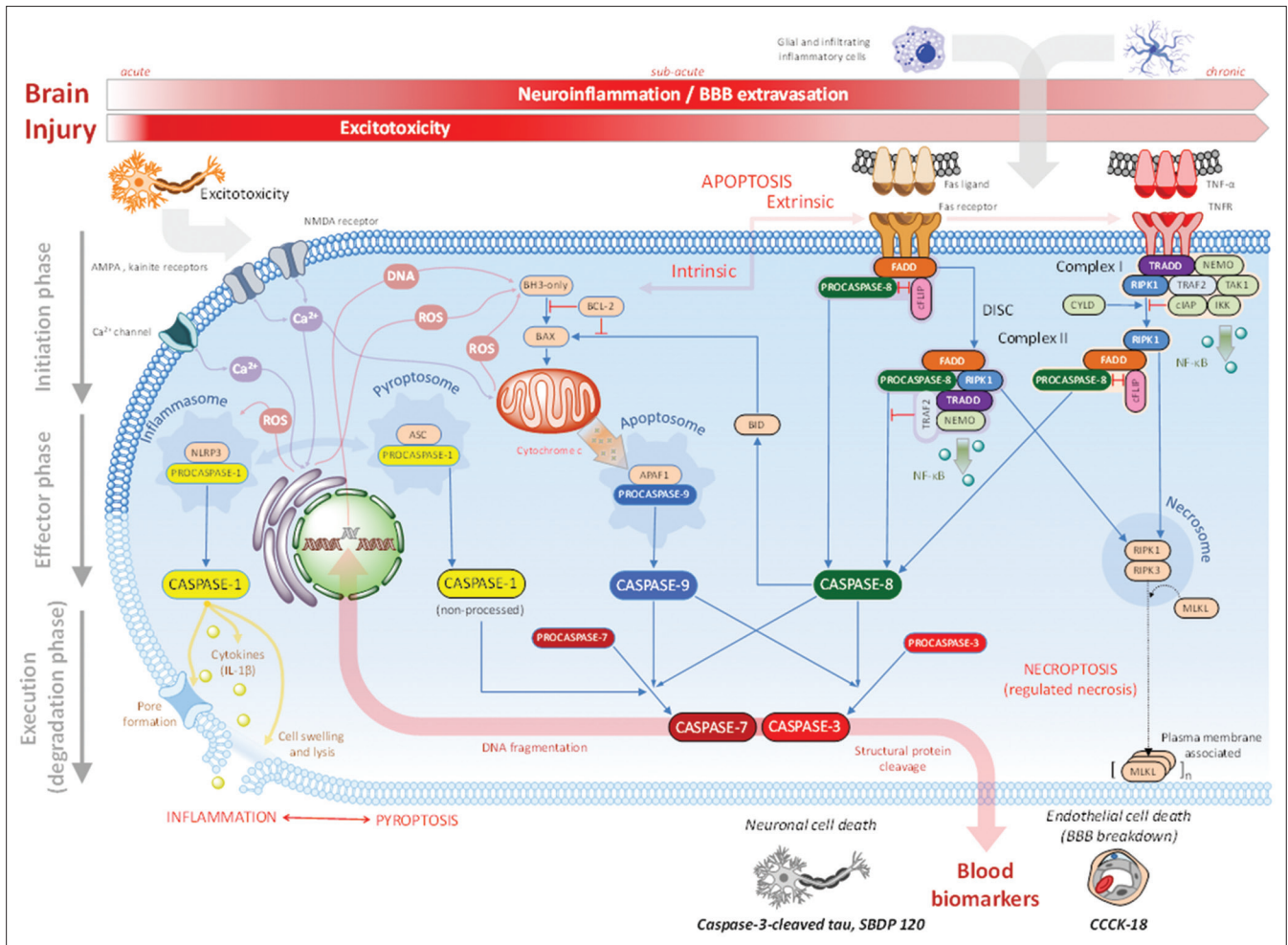


Figure 1: Cell death pathways following brain injury

processing of procaspase-3 and/or procaspase-7 resulting in activation of caspase-3 and/or caspase-7, effector caspase family members [Figure 1]. The execution caspases are involved in the cleavage of certain cellular proteins that result in the characteristic alteration of the plasma membrane structure and increased membrane permeability, and fragmentation of nuclear chromatin, all characteristics of apoptotic cell death.

**Extrinsic mechanisms of classic apoptosis and the convergence of extrinsic and intrinsic pathways**

Certain caspases (e.g. caspases 8 and 10) leading to activation of executor caspase-3 or caspase-7 might be involved in the apoptotic pathways induced by activation of surface death receptors, the processes primarily independent of mitochondrial signaling (i.e., cytochrome C-mediated Apaf-1 activation).<sup>[73]</sup> Currently known death receptor pathways that have been linked with subsequent cell death as a consequence of neurovascular disorders, including stroke, TBI, and neurodegenerative diseases, are largely a result of the family of cytokine receptors, known as tumor necrosis factor receptors (TNFRs) which associate with extracellular signaling ligands

such as cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Fas. Subsequently, specific adaptor proteins including tumor necrosis factor receptor type 1-associated death domain (TRADD) and Fas-associated protein with death domain (FADD) can form death-initiating signaling complexes (DISCs) inside the cytoplasm that can initiate a subsequent cascade of caspase activation [Figure 1].

Expression of Fas and/or Fas ligand has been reported in stroke<sup>[74,75]</sup> and severe TBI patients<sup>[76-78]</sup> and documented in preclinical models of brain ischemia<sup>[79-81]</sup> and TBI.<sup>[82-85]</sup> Upregulation of TNF- $\alpha$  has been reported in clinical stroke studies including ischemic<sup>[86-88]</sup> and animal TBI models.<sup>[83-85,89,90]</sup> TNF- $\alpha$  might contribute to neuronal injury as well as exert protective effects.<sup>[91]</sup> Clinical data also suggest that, following brain injuries, extrinsic pathways are more delayed as compared to intrinsic pathways. For example, a significant association between upregulation of caspase3 and soluble Fas levels has been reported on day 5 after TBI.<sup>[77]</sup>

In addition, activation of TNFR1 and further processing of formation of receptor-interacting protein kinase

1 (RIPK1) and the RIPK3 complex, along with the recruitment of mixed lineage kinase domain-like, lead to activation of another highly regulated and genetically controlled necrotic cell death mechanisms often referred to as necroptosis (to distinguish with nonregulated necrosis).<sup>[92]</sup>

Extrinsic apoptotic mechanisms are also associated with the release of nuclear factor  $\kappa$ -light-chain-enhancer of activated B-cells (NF- $\kappa$ B), a transcription factor that regulates the expression of a wide array of immune response genes which could trigger differential pathways including upregulation mitogen-activated protein kinases<sup>[93]</sup> and subsequent activation of pro-inflammatory cytokines.<sup>[91,94]</sup> Time-dependent NF- $\kappa$ B upregulation has been reported in TBI patients<sup>[95,96]</sup> and animal TBI models.<sup>[89,90,97-100]</sup> In TBI, NF- $\kappa$ B plays a key role in astrocytic swelling and edema formation that might further worsen brain injury.<sup>[99,100]</sup>

However, there are differences between the levels of caspase-8 produced following activation of death receptor and DISC formation between cell types, so-called cell Type I and cell Type II, involving different apoptotic mechanisms.<sup>[101,102]</sup> The major pathway of death receptor activation in the cell Type I amount involves caspase-8 production sufficient for direct cleavage of procaspase and activation of executor caspases 3 and 7, whereas in the cell Type II, the smaller amount of DISCs and active caspase-8 production triggers activation of intrinsic mitochondria-dependent pathway of executor caspase activation<sup>[101,102]</sup> through cleavage of Bid, a Bcl-2-intracting protein involved in activation of apoptotic signaling in mitochondria and cytochrome c release.<sup>[103,104]</sup> Bid cleavage associated with activation of both caspases 8 and 9 has been reported in preclinical TBI model, suggesting the involvement of convergent intrinsic and extrinsic mechanisms in brain injury progression.<sup>[105]</sup>

### Role of Caspase-mediated Apoptosis in Neurodegenerative Disorders: Possible Link between Acute Brain Injury and Chronic Neurodegeneration

Recent clinical and experimental data indicate that TBI and other acute brain injuries share many common features with neurodegenerative disorders, including chronic inflammatory and neurovascular pathologies and apoptosis.<sup>[39,106-108]</sup> The molecular and cellular mechanisms triggering the development of these pathologies and their progression following brain injuries are poorly understood; caspase-mediated apoptotic pathways associated with irregular accumulation of different tau are considered one possible factor linking development of neurodegenerative disease following acute brain injury.

Upregulation of several caspases has been demonstrated in human AD brain and genetic animal AD models including caspases 1, 3, 6, 7, 8 and 9.<sup>[109-113]</sup> Activation of several caspases has been reported in transgenic animal AD models.<sup>[114,115]</sup> Activated caspase-3 has long been implicated in AD pathophysiology, and its expression in different cell types including neurons, astrocytes, and blood vessels exhibited a high degree of colocalization with neurofibrillary tangles and senile plaques.<sup>[116]</sup> Human studies have reported increased caspases 3 and 9 immunoreactivity in AD brain tissue as compared to controls<sup>[112,116]</sup> though another study failed to find statistically significant differences in caspases 3 and 9 levels between AD and controls.<sup>[111]</sup> Postmortem studies suggest that, in AD, a principle caspase pathway contributing to neuronal loss resulting from caspase-8-mediated induction of caspase-3 and/or caspase-7.<sup>[111,117]</sup> Further, data indicate that increased caspase-3 activity and neuronal apoptosis appear in perivascular regions, an observation suggestive of involvement of caspase-3 in cerebrovascular injury.<sup>[118]</sup>

Clinical and experimental data indicate that caspase-3-mediated apoptosis plays a key role in cleavage of tau, an initial process underlying formation of fibrillary tangles and amyloid plaques that are commonly observed in AD and other neurodegenerative disorders<sup>[106,112,119-122]</sup> and two the most recognized hallmarks of neurodegeneration.<sup>[123,124]</sup> Numerous publications have suggested that caspase-3-cleaved tau found in neurofibrillary tangles might be one of the earliest biomarkers of AD.<sup>[106,120,121,125]</sup> Recent studies have also demonstrated increased levels of caspase-3 cleaved tau in brain extracts of patients with CTE<sup>[126]</sup> and in serum of both AD and TBI patients.<sup>[127,128]</sup> In addition, caspase-3 activity is associated with proteolytic degradation of cytoskeletal proteins, such as  $\alpha$ II-spectrin, further contributing to neuronal pathology in human TBI and animals models,<sup>[129,130]</sup> and preclinical evidence suggests that this pathology might be exacerbated by the presence of existing neurodegenerative disease.<sup>[131]</sup>

### Caspase Activation and Apoptosis in Traumatic Brain Injury and Stroke

Caspase-mediated apoptotic cell death has long been demonstrated in animal models of brain injuries including stroke and TBI.<sup>[132,133]</sup> Experimental and clinical studies have also indicated that following cerebral ischemia and TBI, neural cellular death involves both caspase-mediated apoptotic and calpain-mediated necrotic cell death mechanisms.<sup>[132,134-138]</sup> Further, numerous clinical and preclinical studies have shown that caspases are involved in the pathophysiology of many neurological disorders through complex apoptotic and inflammatory pathways.

The seminal study performed by Friedlander *et al.* first demonstrated involvement of caspase-1 in an animal model of ischemic stroke.<sup>[139]</sup> Caspase-1 is an upstream initiator caspase and plays an important role in inflammatory responses, neuronal apoptosis, and neurodegeneration following brain injuries.<sup>[137,139-142]</sup> Further preclinical and clinical studies have shown activation of different caspases following cerebral ischemia (caspases 1, 3, 8, 9, and 11)<sup>[143-145]</sup> and TBI (caspases 1, 3, 6, 7, 8, 9 and 12).<sup>[146-153]</sup> Caspase-dependent pathways have also been revealed in acute subdural hematoma.<sup>[154]</sup>

Although there are similarities in caspase involvement in acute brain injuries and neurodegenerative diseases, mechanisms activation of certain caspases might differ and represent specific pathways characteristic of these conditions. For example, a preclinical rodent study suggests that caspase-2 is involved neuronal degeneration in a mouse AD model<sup>[115]</sup> but not involved in ischemic brain damage following experimental stroke.<sup>[145]</sup>

The activation of different caspases in these studies suggests involvement of both intrinsic and extrinsic apoptotic mechanisms as well as caspase-1-mediated inflammation and pyroptotic cell death in both TBI and stroke although these mechanisms might be involved differently depending on postinjury time point and injury phenotype.

The pathways involved in caspase activation following cerebral ischemia include translocation of Bcl-2 family proteins<sup>[155-157]</sup> and cytochrome c release,<sup>[143-145]</sup> suggesting involvement of intrinsic apoptotic mechanisms. Intrinsic cytochrome c and Bcl-2 mediated apoptotic pathways involving activation of caspases 9 and 3 have been also reported in adult<sup>[152,158,159]</sup> and pediatric<sup>[159-161]</sup> clinical TBI studies.

Activation of caspase-3 through caspase-8-mediated pathways in TBI<sup>[78,151]</sup> and stroke<sup>[86,88,162]</sup> pathology suggests involvement of extrinsic apoptotic mechanisms in these disorders. Notably, similar pathways are involved in neurodegenerative disease.<sup>[111,117]</sup> Involvement of extrinsic apoptotic mechanisms is also suggested from clinical studies showing correlations of caspase-3 and/or caspase-8 activation with upregulation of death receptors (e.g., Fas) and death receptors ligands (e.g., TNF- $\alpha$ ) in stroke<sup>[74,86-88]</sup> and TBI<sup>[77,151]</sup> patients. Similar findings were reported in preclinical models of stroke<sup>[143,163-165]</sup> and TBI.<sup>[82,147,151,166]</sup>

Acute neuronal apoptosis after TBI was observed primarily within the injury site, whereas delayed neuronal apoptosis lasting days and weeks after TBI occurred mainly in remote, indirectly impacted regions.<sup>[167-169]</sup>

## Involvement of Different Cellular Types and Mechanisms in Caspase-mediated Apoptosis in Stroke and Traumatic Brain Injury

Although neuronal cell death plays a major role in brain dysfunction following brain injuries and neurodegenerative diseases, the current data indicate that apoptosis in nonneuronal cell types plays an important role in the progression of these disorders. In addition, activation of caspases following brain injuries can exacerbate inflammation and affect glial function by induction of apoptosis and activation of microglia. Both neuronal and glial apoptosis have been reported in several experimental and clinical studies including acute CNS injuries such as stroke,<sup>[170]</sup> TBI, and spinal cord injuries,<sup>[136]</sup> as well as chronic neurodegenerative diseases. Activated caspase-3 upregulation at acute time points after experimental TBI was observed primarily in neurons and to a lesser extent, in astrocytes and oligodendrocytes.<sup>[146,147]</sup>

Expression of cleaved caspases 8 and 3 have been reported in Iba1-positive microglia in animal models of cerebral ischemia and in CD68-positive microglia/macrophages in ischemic human stroke.<sup>[162,171,172]</sup> Other published data suggest that TBI-induced white matter degeneration and myelin loss in corpus callosum may result from oligodendrocyte apoptosis.<sup>[173-175]</sup> An increased number of activated caspase-3-immunopositive oligodendrocytes in the corpus callosum was observed starting at 48 h after injury and remained elevated for up to 3 weeks following fluid percussion injury in rats. This activated caspase-3 upregulation was associated with decreased numbers of healthy oligodendrocytes, suggesting that apoptosis had occurred.<sup>[173-175]</sup>

## Caspase Inhibition as a Novel Neuroprotective Strategy for Stroke and Traumatic Brain Injury

Preclinical evidence indicates that delayed activation of caspase-mediated apoptosis following stroke, TBI, intracerebral hemorrhage, and SAH primarily occurs in potentially treatable penumbral and perilesional areas providing potential therapeutic opportunities for targeting apoptotic pathways to limit the expansion of brain lesions.<sup>[45,176]</sup>

Preclinical studies using pharmacological inhibition with pan- and selective caspase inhibitors, and using genetically modified caspase-deficient (e.g., caspases 1 and 11) animals, have reported that decreasing activity of selected caspases improves neurological deficits and provides neuroprotection from cerebral ischemia primarily in penumbral regions,<sup>[139,143,145,155,177-180]</sup> following acute subdural hematomas.<sup>[154]</sup>



## Apoptotic Biomarkers and Their Association with Brain Injury Outcomes

Over the past two decades, several clinical studies have examined biomarkers of apoptosis following acute brain injuries. These biomarkers include the caspase proteases, notably caspase-3, and their specific cleavage products such as caspase-cleaved cytokeratin-18 (CCK-18), caspase-cleaved tau, and caspase-specific  $\alpha$ II-spectrin breakdown products (SBDPs). Clinical studies focusing on the detection of apoptotic biomarkers in brain tissue and biofluids are summarized in Table 1.

Increases in the levels of certain caspases following brain injuries are suggestive of involvement of pathological pathways such as apoptosis and inflammation, as well as injury severity. Similarly, levels of caspase-specific cleavage products such as CCK-18, caspase-cleaved tau, and caspase-specific SBDP120 are both indicative of cellular involvement of specific cell types and also provide information on injury mechanisms. In addition, use of a panel comprising cells-specific glial and neuronal biomarkers of brain injuries would provide additional valuable information on the injury mechanisms in the involvement of different cell populations making it possible for evidence-based diagnostics and personalized treatment of stroke and TBI patients.<sup>[203,204]</sup>

### Caspase-3 as a Major Biomarker of Brain Apoptosis following Stroke and Traumatic Brain Injury

Experimental and clinical studies have provided evidence that activated caspase-3 is a key player in cellular death following acute brain injuries and might be involved in the progression of chronic neurodegenerative processes. In preclinical TBI models, the upregulation of activated caspase-3 in the ipsilateral cortex was observed from 6 to 72 h with maximal increase at 48 h after controlled cortical impact, whereas no evidence of caspase-3 activation was observed in the ipsilateral hippocampus and contralateral cortex and hippocampus up to 14 days after injury.<sup>[146,147]</sup> Similarly, acute increase of cleaved-caspase-3 in neurons has been observed in the injured cortex after fluid-percussion injury.<sup>[205]</sup> In a model of surgical brain injury, the upregulation of caspase-3 was observed mainly in neurons within the injured cortex, and this upregulation was transient, peaking at 5 days and then gradually decreased within the next 3 weeks.<sup>[206]</sup>

Numerous clinical studies have demonstrated upregulation of caspase-3 following ischemic and hemorrhagic strokes and TBI in postmortem and surgically removed brain tissues,<sup>[74,77,95,157-159,162,171,172,180,195]</sup> CSF,<sup>[77,197]</sup> and blood plasma.<sup>[86-88,181-183]</sup> Other major

caspases that are upregulated following activation after injuries include caspase-1,<sup>[158,191]</sup> caspase-7,<sup>[86,148]</sup> and caspase-8.<sup>[75,78,88]</sup> Immunohistochemical studies revealed that caspase-1 upregulation was observed in the brain tissue, blood vessels, T-lymphocytes, and CD68positive macrophages; these caspase-1 increases were associated with Bcl-2, interleukin 1  $\beta$ , and NLRP3 levels.<sup>[158,191]</sup> Caspase-3 upregulation was observed in neurons<sup>[157,180]</sup> and in CD68-positive cells, including infiltrating macrophages and microglia.<sup>[162,171,172]</sup> Caspase8 was primarily expressed in neurons<sup>[78]</sup> and caspase-7 was expressed in astrocytes, neurons, and other glial and infiltrated inflammatory cells.<sup>[148]</sup>

Caspase3 upregulation was associated with other apoptotic and cellular injury markers including caspase-3 substrates DNA-PKCS and PARP, end-product of PARP activity poly (ADP-ribose), phosphorylated c-Jun N-terminal kinases 1 and 2, and terminal transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL), an indicator of DNA fragmentation.<sup>[74,157,172]</sup>

Increases in caspases 3 and 8 expression and activity in both TBI and stroke samples have been reported in association with changes in TNF- $\alpha$ , NF- $\kappa$ B, and Fas levels,<sup>[74,75,77,78,95]</sup> suggesting the involvement of caspase-8 and death receptors in activation of caspase-3 following injury.<sup>[73]</sup>

Elevated plasma caspase-3 levels and caspase-3/7 activity in stroke patients have been reported in both acute<sup>[86,181]</sup> and late phases of stroke for up to 6 months after cerebral injury.<sup>[88]</sup> A study by Montaner *et al.* has shown that a combination of caspase-3 and d-dimer might be a promising biochemical strategy for rapid diagnosis of stroke.<sup>[183]</sup> Acute increases in caspase-3 levels were associated with infarct growth and short- and long-term neurological outcomes.<sup>[181]</sup> Significant increases in acute caspase-3/7 activity in blood were observed only in patients with gray matter lesions, suggesting that apoptosis occurs primarily in neurons.<sup>[86]</sup> However, spatiotemporal analysis of cleaved caspase-8 and-3 expression in postmortem brain tissue of stroke patients suggests that changes in their activities following cerebral ischemia can also occur in microglia/macrophages.<sup>[162]</sup> In addition, acute caspase-3/7 activation in blood plasma and blood levels of caspase-3 and 8 in late phase of stroke were significantly correlated with TNF- $\alpha$  levels in blood plasma<sup>[86,88]</sup> and platelets.<sup>[87]</sup>

A recent study by Wang *et al.* revealed that caspase-3 activation at admission and at day 3 in aneurysmal SAH patients was increased in those patients who had an unfavorable outcome or died. These levels were highly associated with the severity of injury and prognosis

**Table 1: Expression of apoptotic biomarkers following acute brain injuries**

Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
Ischemic stroke and ischemia-reperfusion injury <sup>[172]</sup>	Caspase-3 (activated)	12 h to 9 days	11 patients with atherothrombotic brain infarct 11 patients with cardiac arrest resuscitation (ischemia-reperfusion injury) (46-95 years)	Brain tissue (postmortem) (IHC)	Caspase-3 upregulation in macrophages/microglia and limited upregulation in neurons Differential caspase-3 upregulation in transient ischemia-reperfusion injury and permanent cerebral ischemia (atherothrombotic brain infarcts) Association of caspase-3 upregulation with caspase-3-cleaved PARP (89 kDa)
Ischemic stroke <sup>[180]</sup>	Caspase-3	4, 8, 16, 24, 48, 72, 130, 192 h	48 patients (40-70 years, average age 60.8 years)	Brain tissue (postmortem) ( <i>in situ</i> hybridization and IHC)	Time-dependent caspase3 upregulation in hippocampus (CA1) Association of caspase-3 upregulation with TUNEL and loss of MAP-2
Ischemic stroke <sup>[157]</sup>	Caspase-3 (activated)	2-37 days	18 patients (51-86 years)	Brain tissue (postmortem and ipsi- and contra-lateral) (Western blot and IHC)	Activated caspase-3 upregulation in neurons in the infarct area Association of activated caspase-3 upregulation with TUNEL and p-JNK The presence of Bcl-2 in penumbra of gray matter significantly correlated with shorter survival time
Ischemic stroke <sup>[74]</sup>	Caspase-3 (activated)	15 h to 18 days	13 patients 2 controls (41-89 years)	Brain tissue (postmortem, peri-infarct area) (IHC)	Cytoplasmic activated caspase-3 immunopositivity correlated with death receptor Fas Nuclear and cytoplasmic PARP-1 differentially correlated with increasing neuronal necrosis in the peri-infarct area
Ischemic stroke <sup>[181]</sup>	Caspase-3	12 and 24 h from stroke onset and 2 h after tPA	116 tPA-treated stroke patients (mean age 71.8±11.3 years) 40 healthy control subjects (mean age 60.5±9.4 years)	Blood plasma	Plasma caspase-3 levels were higher in stroke patients versus the control group throughout the acute phase of stroke Plasma caspase-3 level at 24 h was associated with poorer short- and long-term neurological outcomes and positively correlated with infarct volume
Ischemic stroke <sup>[86]</sup>	Caspase-3/7 activity	Acute	7 patients 4 controls (39-82 years)	Blood plasma	Relative caspase-3/7 activity in plasma was significantly correlated with the soluble TNF- $\alpha$ levels but not with the membrane-associated TNF- $\alpha$ levels or density of plasma DNA
Ischemic Stroke <sup>[87]</sup>	Caspase-3 expression	Acute	60 patients (55-70 years, mean age 57.9±10.2 years) 45 controls (mean age 51.05±9.07 years)	Blood plasma Platelets (Western blot and qRT-PCR)	Stroke patients had significantly increased in plasma TNF- $\alpha$ and platelet levels of annexin-V, CD62p, cytochrome-c, and caspase3 gene expression as compared to the controls
Stroke <sup>[171]</sup>	Caspase-3	18 h-several month	35 patients (46-95 years)	Brain tissue (postmortem) (IHC)	Caspase-3 upregulation in neurons during the first 2 days after stroke Caspase-3 upregulation in infiltrating macrophages during between 3 days and 3 weeks after stroke. Association of caspase-3 upregulation with other apoptotic markers, PAR, PARP, DNA-PKCS, and TUNEL

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**Table 1: Contd...**

Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
Stroke and stroke mimics <sup>[182,183]</sup>	Caspase-3	24 h from symptom onset	915 stroke (mean age 72.63±12.46 years) 90 stroke mimics patients (mean age 69.57±17.13 years)	Blood plasma >1.96 ng/mL	The best combination of biomarkers in the model predictive probability of stroke was caspase-3 and d-dimer as compared to combinations of RAGE, MMP-9, S100B, brain natriuretic peptide, neurotrophin-3, and chimeric secretagogen
Stroke <sup>[88]</sup>	Caspase-3 Caspase-8	>6 months	40 patients (average age 63±2.1 years) 40 controls (average age 56±1.6 years)	Blood plasma Caspase-3 Patients: 142.9±16.6 pg/mL Controls: 28.9±0.87 pg/mL Caspase-8 Patients: 64.6±16.9 pg/mL Controls: 50.1±1.3 pg/mL	Caspase-3 and caspase-8 levels were significantly higher in stroke patients as compared to controls Stroke patients with dyslipidemia had significantly higher caspase-3 and caspase-8 levels than stroke patients without dyslipidemia and control groups. Caspase-3 and caspase-8 were significantly correlated with TNF- $\alpha$
Stroke <sup>[162]</sup>	Caspase-3 Caspase-8	Days after stroke	9 subjects with two stroke events (average age 75±9 years) 5 controls (average age 75±11 years)	Brain tissue (postmortem)	Expression of cleaved caspase-8 and caspase-3 in CD68-positive cells could only be found in the area of second stroke Cleaved caspase-8 and caspase-3 expressions correlated with the time of stroke onset
Stroke <sup>[184]</sup>	Caspase - cleaved tau	Admission, 1 and 7 days	19 patients (average age 61±13 years)	Serum 104.39±24.95 ng/mL (7 days, NIHSS $\geq$ 6) 42.86±6.53 ng/mL (7 days, NIHSS $\leq$ 5)	Significant difference in caspase-cleaved tau levels between patients with a good outcome (NIHSS $\leq$ 5) and patients with a poor outcome (NIHSS $\geq$ 6) Significant elevation caspase-cleaved tau level at day 7 for patients with a poor outcome as compared to patients with a good outcome No significant difference in caspase-cleaved tau levels at admission between the patients with good and poor outcomes
Multiple cerebral infarcts and AD <sup>[75]</sup>	Caspase-8	Postmortem	3 stroke patients 6 AD patients 7 age-matched controls (72-96 years)	Brain tissue (postmortem) (IHC)	Activated caspase-8 protein and Fas-positive neurons were found only in the AD brains and not in the other groups
Transient cerebral ischemia <sup>[185]</sup>	Caspase-3	Cardiac arrest or severe hypotension	23 patients with a history of transient hypoxic attacks 11 control (cases with similar systemic diseases) (38-91 years, mean age 70.6±12.7 years)	Brain tissue (postmortem)	Upregulation of caspase-3 and redistribution cytochrome c in a region-specific manner with marked activation in the selectively vulnerable hippocampal areas Increases in TUNEL-positive cells predominantly during the first 3 days after ischemia in the regions of greatest susceptibility to hypoxic injury
Perinatal hypoxic-ischemic brain injury <sup>[186]</sup>	Caspase-3	Postmortem	6 patients	Brain tissue (postmortem) (IHC)	Caspase-3 activation and apoptosis in pontosubicular neuron necrosis

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Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
Perinatal hypoxic-ischemic brain injury <sup>[187]</sup>	Caspase-3	Postmortem	16 patients (0.5 h to 20 days)	Brain tissue (postmortem) (IHC, immunoblot)	Caspase-3 activation and upregulation of apoptotic markers (TUNEL, Bcl-2, Bcl-x) in pontosubicular neuron necrosis Number of apoptotic cells in premature brains was significantly more than in mature brains
Focal ischemia <sup>[188]</sup>	Caspase-3	<24, 24-72, >72 h	19 patients (1.5 week to 16 years) 5 controls (2 months to 13 years)	Brain tissue (postmortem)	Cell death (TUNEL) continues to occur for >3 days postischemic insult Cell death in the penumbra of subacute infarcts is partially caspase-3 independent and may be attributed to nitric oxide
Neurologic injury in infants with complex congenital heart disease <sup>[189]</sup>	SBDP120	N/A	14 patients (infants) with open- and closed-heart surgery	Serum	Peak SBDP120 and SBDP150 significantly increased following open heart surgery as compared to closed heart surgery with different temporal profiles
Intracerebral hemorrhage <sup>[190]</sup>	CCCK-18	5.3±2.1 h	102 patients 102 controls (average age 63.9±9.8 years)	Serum Patients: 245.8±108.3U/L Controls: 23.6±18.1U/L	NIHSS score and hematoma volume were independent predictors of high CCCK-18 levels CCCK-18 was identified as an independent predictor of 6-month mortality and unfavorable outcome
Cerebral aneurysm <sup>[191]</sup>	Caspase-1	Acute	36 patients (19 ruptured and 17 unruptured) 4 controls (18-65 years)	Aneurysmal domes (surgically removed) Middle cerebral artery (control, postmortem) (IHC)	Differential caspase-1 expression in ruptured and unruptured aneurysms Association of caspase-1 with IL-1 $\alpha$ , NLRP3, ASC in T-lymphocytes and CD68positive in macrophages.
SAH (aneurysmal) <sup>[192]</sup>	Caspase-3	Admission, 1, 2, 3, 5 and 7 days	118 patients 118 controls (>18 years)	Serum Detectable (>0.1 ng/mL) at days 0-5 in all patients Undetectable (<0.1 ng/mL) at day 7 in 20 patients and in the control group	Serum caspase-3 activity was transiently increased (peaked at day 3) after aneurysmal SAH and was substantially higher in patients than in controls Caspase-3 activity was higher in patients suffering from death or an unfavorable outcome (GOS, WFNS, modified Fisher scores, and possessed high areas under receiver operating characteristic curve) Caspase-3 activities at admission and at day 3 predicted poor outcome independently of age, WFNS scores and modified Fisher scores
SAH (aneurysmal) <sup>[193]</sup>	CCCK-18	Admission	128 patients 128 controls (23-70 years, average age 41.6±11.6 years)	Blood plasma Patients: 235.1±86.8 U/L Controls: 25.6±23.4 U/L	CCCK-18 levels were markedly higher in patients with an unfavorable outcome (WFNS and modified Fisher scores) and nonsurvivors CCCK-18 was identified as an independent prognostic predictor for mortality and unfavorable outcome and CCCK-18 had similar predictive values compared to WFNS and modified Fisher scores

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Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
SAH (aneurysmal) <sup>[194]</sup>	SBDP120	Every 6 h (up to 72 h)	20 patients 1 control (34-77 years)	CSF	SBDP120 and calpain-mediated SBDPs were significantly increased in patients suffering aneurysmal SAH The concentration of SBDPs was found to increase significantly over baseline level up to 12 h before the onset of cerebral arterial vasospasm
TBI (severe) <sup>[76]</sup>	Caspase-1	0-10 days	67 patients (0.1-16, median age 6 years) 19 controls (without trauma or meningitis) (0.1-12 years, median age 1.7 years)	CSF	Caspase-1 and Fas were increased in CSF after TBI Increased CSF cytochrome c was independently associated with inflicted TBI ( $P=0.0001$ ) and female gender ( $P=0.04$ ), but not age, GCS score, or survival
TBI <sup>[158]</sup>	Caspase-1 (cleaved) Caspase-3 (cleaved)	Acute phase of TBI	8 patients (21-57 years, average age 35.9±4.4 years) 6 controls (16-77 years, average age 52.2±9.0 years)	Brain tissue (TBI, surgically removed) Brain tissue (control, postmortem)	Cleavage of caspase-1, up-regulation and cleavage of caspase-3, evidence for DNA fragmentation with both apoptotic and necrotic morphologies and an increase in Bcl-2 but not Bcl-xL or Bax were found in tissue from TBI patients compared with controls
TBI <sup>[159]</sup>	Caspase-3	54±49 h 82±56 h 109±95 h	29 patients 3 controls (epilepsy) (12-72 years, mean age 28.3±15.3 years)	Brain tissue (TBI peri-ischemic zone, surgically removed)	Significant caspase-3 upregulation in patients who died (GOS score 1, $n=12$ ) as compared to patients experienced a good outcome (GOS score 4 or 5, $n=17$ patients) Bcl-2 (negative) and caspase-3 (positive) are independent predictors of poor outcome
TBI <sup>[95]</sup>	Caspase-3	0, 12, 24, 48, 72, 168, 264, and 480 h	39 patients (dead from TBI) 8 controls (dead from none TBI reasons) (average age 34.8 years)	Brain tissue (postmortem) (IHC)	Marked caspase-3 upregulation in every injured group compared to controls Time-depending upregulation of NF- $\kappa$ B in 168-480 h groups after TBI
TBI <sup>[195]</sup>	Caspase-3	Few minutes to 126 days	56 patients (14-86 years, average age 47.3 years)	Brain tissue (postmortem) (IHC and <i>in situ</i> labeling)	Caspase-3 upregulation in cortical neurons was detectable 80 min after TBI Caspase-3 upregulation in glial cells was detectable 80 min after TBI Cerebral apoptosis was significantly associated with TBI compared to control
TBI <sup>[196]</sup>	Caspase-3	0-96 h	12 patients (18-81 years, mean age 42 years) 2 controls (sudden death) (mean age 52 years)	Brain tissue (postmortem) (RT-PCR)	Significant increase in caspase-3 and TrkB in the cerebellum of patients with short survival times as compared to controls
TBI <sup>[197]</sup>	Caspase-3 (activated)	1-14 days	27 patients (average age 36.1±12.9 years) 7 controls (average age 41.6±9.8 years)	CSF	Caspase-3 activity was detected in 31 (27.4%) CSF samples with highest values (>5.5 $\mu$ M/min) at day 2-5 after TBI

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Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
TBI (severe) <sup>[77]</sup>	Caspase-3	1, 2, 3, 5, 7, and 10 days	14 patients (5-69 years, mean age 25.3±15.4 years)	CSF	Caspase-3 and Bcl-2 activities significantly increased in CSF of patients Significant correlation of caspase-3 associated with increased ICP and cerebral perfusion pressure Significant association between caspase-3 and soluble Fas on day 5 after TBI
TBI (severe) <sup>[198]</sup>	Caspase-3 (activated)	1 day	112 patients 81 survivors (median age 46 years, IQRs 27-60 years) 31 nonsurvivors (median age 63 years IQRs 53-75 years)	Serum	Multiple logistic regression analysis showed that serum caspase-3 levels >0.20 ng/mL were associated with mortality at 30 days in TBI patients controlling for Marshall CT classification, age and GCS (OR=7.99; 95% CI=2.116-36.744; P=0.001)
TBI <sup>[148]</sup>	Caspase-7	Acute	16 patients (mean age 40.4±10.1 years) 6 controls (mean age 52.2±21.3 years)	Brain tissue (postmortem) (Western blot and IHC)	Significant increases in pro-caspase-7 and 20 kD proteolytic fragment in TBI patients compared to controls Caspase-7 expression included astrocytes, neurons, and possibly other glial cell types and infiltrated inflammatory cells
TBI (severe) <sup>[78]</sup>	Caspase-8	Acute phase of TBI	17 patients (16-64 years, mean age 40±17 years) 6 controls (16-77 years, mean age 52±22 years)	Brain tissue (TBI, surgically removed) Brain tissue (control, postmortem)	Significant increases in caspase-8 mRNA and protein in TBI patients as compared to controls Caspase-8 protein was predominately expressed in neurons Proteolytic 20 kDa fragments of caspase-8 were detected only in TBI patients Association of caspase-8 with Fas in TBI patients
TBI (severe) <sup>[152]</sup>	Caspase-9	2-26 h (catheter placement) 24, 48 and 72 h (intervals)	9 patients (18-62 years; median age, 40 years) 5 controls (orthopedic)	CSF (ELISA)	Cytochrome c was detected in 18 (51.4%) samples (0.44±0.632, mean±SD) Activated caspase-9 was detected in 10 (28.6%) samples (0.28±0.39 ng/mL, mean±SD) Control CSF samples had no detectable levels of either marker Activated caspase-9 shows weak correlation with poor neurological outcome
TBI (severe) <sup>[199]</sup>	CCCK-18	Admission	100 patients 73 survivors (median age 66 years IQRs 45-76 years) 27 nonsurvivors (median age 47 years IQRs 32-67 years)	Serum >201 u/L	CCCK-18 levels are associated with 30-day mortality

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**Table 1: Contd...**

Clinical study	Biomarker	Time points	Subjects, group size, and age	Matrix/levels	Results
TBI (severe) <sup>[200]</sup>	SBDP120	Acute	12 TBI patients 9 control #1 (SAH, IVH, brain tumor) 5 control #2 (diagnostic lumbar puncture) (18-70 years)	CSF (ventricular)	Nonerythroid $\alpha$ -spectrin and SBDPs occurred more frequently and their level was significantly higher in the CSF of TBI patients than in other pathological conditions associated with raised ICP
TBI (severe) <sup>[201]</sup>	SBDP120	6, 12, 24, 48, 72, 96, and 120 h	41 patients (18-67 years, mean age 38 years)	CSF	Calpain and caspase-3 mediated SBDP levels in CSF were significantly increased in TBI patients at several time points after injury as compared to control subjects. The time course of calpain-mediated SBDP150 and SBDP145 differed from that of caspase-3 mediated SBDP120 during the postinjury period examined. Mean SBDP densitometry values measured early after injury correlated with severity of injury, CT findings, and 6-month outcome
TBI (severe) <sup>[202]</sup>	SBDP120	Admission and every 6 h up to 7 days	40 patients (18-82 years, mean age 41.5 $\pm$ 3.17 years) 24 controls (23-83 years, mean age 56.2 $\pm$ 4.42 years)	CSF >17.55 ng/mL	SBDP120 release was more accurate 24 h after injury. Within 24 h after injury, SBDP145 CSF concentrations significantly correlated with GCS scores, while SBDP120 levels correlated with age. SBDP levels were significantly higher in patients who died than in those who survived. SBDP145 levels (>6 ng/mL) and SBDP120 levels (>17.55 ng/mL) strongly predicted death
TBI (sport concussion) <sup>[127]</sup>	Caspase - cleaved tau	1, 12, 36 and 144 h	288 adult ice hockey players (35 concussion)	Serum	Serum levels of caspase - cleaved tau were significantly higher in postconcussion samples compared with preseason

IHC: Immunohistochemistry, PARP: Poly (ADP-ribose) polymerase, TUNEL: Transferase-mediated dUTP-digoxigenin nick end-labeling, p-JNK: Phosphorylated c-Jun N-terminal kinases, tPA: Tissue plasminogen activator, TNF- $\alpha$ : Tumor necrosis factor-alpha, qRT-PCR: Quantitative reverse transcription polymerase chain reaction, PKCS: Protein kinase catalytic subunit, MMP: Metalloproteinase, NIHSS: National Institute Health Stroke Scale, AD: Alzheimer's disease, SBDPs: Spectrin breakdown products, N/A: Not available, CCKK: Caspase-cleaved cytokeratin, IL-1 $\beta$ : Interleukin 1 beta, GOS: Glasgow Outcome Scale, WFNS: World Federation of Neurological Surgeon, CSF: Cerebrospinal fluid, SAH: Subarachnoid hemorrhage, TBI: Traumatic brain injury, GCS: Glasgow Coma Scale, NF- $\kappa$ B: Nuclear factor kappa B, OR: Odds ratio, CT: Computed tomography, SD: Standard deviation, IQRs: Interquartile ranges, ICP: Intracranial pressure, CI: Confidence interval, IVH: Intraventricular hemorrhage, RAGE: Receptor for advanced glycation end products, MAP-2: microtubule associated protein-2

after SAH independently of age.<sup>[192]</sup> Lorente *et al.* have recently reported that increased serum levels of caspase-3 are associated with increased mortality in patients with severe TBI.<sup>[199]</sup>

### Caspase-3-mediated Pathways in Developing Brain following Traumatic Brain Injury and Hypoxic-ischemic Brain Injuries

It is well recognized that neuronal apoptosis in the developing brain is a physiological process controlled within normal CNS function which involves activation of Bcl-2-mediated upregulation of caspases 9 and 3.<sup>[50,53]</sup> Thus, the pediatric patient population,

especially neonates, might be more vulnerable to apoptotic cell death than adults.<sup>[52]</sup> Clinical studies in pediatric stroke (e.g., perinatal hypoxic-ischemic brain injury),<sup>[186,187]</sup> neurological injury associated with congenital heart disease surgery,<sup>[189]</sup> and TBI<sup>[76,160]</sup> have demonstrated increases in the caspase-3 and other apoptotic markers (e.g., TUNEL, Bcl-2, Bcl-x, cytochrome c) including those associated with caspase-3 activity such as SBDP120 and fractin, a caspase-specific actin cleavage product, in brain injured patients compared to controls. These data suggest that normal apoptotic pathways involved in CNS development are disrupted following ischemic and traumatic brain injuries that may affect long-term neurological outcome.<sup>[50,51]</sup> Askalan *et al.* have also suggested that cell death in the penumbra

of subacute infarcts following focal brain ischemia in children is partially caspase-3 independent and may be attributed to nitric oxide.<sup>[188]</sup> Several preclinical studies, using multiple animal models of neonatal brain injury, have documented involvement of apoptosis in neural cell death and potentially protective effects of selective caspase-3 inhibition to reduce neonatal hypoxic-ischemic brain injury.<sup>[207-214]</sup> However, a study using genetically modified mice lacking caspase-3 gene (Casp3<sup>-</sup>mice) had shown worsened outcomes in these mice after neonatal hypoxic-ischemic brain injury compared to wild-type controls, suggesting a protective role of caspase-3 in the developing brain.<sup>[215]</sup> In contrast, improved outcomes following ischemia-reperfusion brain injury have been reported in adult Casp3<sup>-</sup>mice.<sup>[216]</sup> Taken together, the aforementioned preclinical studies in wild-type rodent species and caspase-3 knockout mice suggest complex and differential roles of caspase-3 in adult and developing brain.

### Caspase-cleaved Products as Biomarkers of Brain Apoptosis following Stroke and Traumatic Brain Injury

#### Caspase-cleaved cytokeratin-18

The protein cytokeratin-18, an intermediate filament cytoskeletal protein primarily expressed in epithelial cells, is a well-recognized caspase substrate that is cleaved during epithelial cell apoptosis resulting in the production and release into circulation of its major cleavage fragment CCK-18.<sup>[217]</sup> A pilot study by Lorente *et al.* has shown that serum levels of CCK-18 are associated with mortality in patients with severe TBI.<sup>[199]</sup> Two recent studies have also demonstrated that blood levels of CCK-18 were increased in patients with intracerebral hemorrhage<sup>[190]</sup> and aneurysmal SAH,<sup>[193]</sup> and the increased levels were associated with poor short- and long-term neurological outcomes and mortality. A study by Gu *et al.* has also shown that increased serum CCK-18 levels following intracerebral hemorrhage were associated with neurological deficits and hematoma volume.<sup>[190]</sup>

#### Calpain- and caspase-mediated spectrin breakdown products

$\alpha$ II-Spectrin is a major axonal cytoskeletal protein and a major substrate for both calpain and caspase-3 proteases following brain injuries. Degradation of  $\alpha$ II-spectrin is an important component of necrotic and apoptotic cell death, respectively.<sup>[218]</sup> Moreover,  $\alpha$ II-spectrin cleavage by caspase and caspase proteases produce signature cleavage products including SBDP120 and 145 kDa and 150 kDa  $\alpha$ II-SBDP (SBDP145 and SBDP150) resulted primarily from caspase-3-mediated (apoptosis) and calpain-mediated (necrosis) proteolysis, respectively.<sup>[219,220]</sup> However, there is evidence that SBDP150 might be

associated with activities of both calpain and caspase-3 proteases.<sup>[221]</sup> Moreover, a recent experimental study using *in vitro* primary rat cerebrocortical cell cultures under apoptotic, necrotic, and excitotoxic conditions together with an *in vivo* rat TBI model suggests that breakdown of  $\beta$ II-spectrin, another important neuronal cytoskeletal protein, by caspase-3 and calpain-mediated proteolysis, contributes to cell death following brain injuries and that protease-specific signature  $\beta$ II-SBDPs may serve as biomarkers indicative of neuronal cell death mechanism.<sup>[222]</sup>

Experimental data obtained in animal models demonstrated an increase in the levels of both caspase-3 and calpain-specific SBDPs in brain and CSF after experimental ischemia<sup>[223-225]</sup> and preclinical models of TBI.<sup>[130]</sup> Interestingly, increased levels of SBDP120 after experimental TBI were observed in the triple transgenic AD mice (3xTg-AD) compared to wild-type controls of the same background.<sup>[131]</sup> Clinical studies in severe TBI have confirmed utility of SBDP120 as a sensitive biomarker of caspase-3 activation exclusively associated with apoptotic cell death.<sup>[129]</sup> Other studies have confirmed the utility of SBDP145 and SBDP150 as highly useful biomarkers of calpain activation primarily associated with necrosis.<sup>[218]</sup> In addition, both caspase-3 and calpain-specific SBDPs (i.e., SBDP120, SBDP145, and SBDP150) are currently thought to be biomarkers associated with an increased intracranial pressure.<sup>[129,226]</sup>

In patients with TBI, increases in CSF concentrations of different SBDPs were correlated with a severe TBI diagnosis. The temporal profiles of SBDP145 and SBDP120 suggested that neuronal cell death within the first 72 h is mostly due to necrosis, whereas delayed cell death after 72 h after brain trauma is primarily due to apoptosis.<sup>[129,201]</sup> Differential temporal increases in the serum levels of SBDP120 and SBDP150 have been reported in infants with congenital heart disease following open heart surgery, suggesting that SBDPs could be developed as biomarkers for brain necrosis and apoptosis.<sup>[189]</sup> Significant increases in SBDP120 and SBDP150 levels were shown in the CSF of SAH patients, and these SBDPs, when used in a biomarker panel, were significantly correlated with brain infarction, cerebral vasospasm, and generally poor outcomes.<sup>[194,227]</sup>

#### Caspase-cleaved tau

Tau is a structural protein that belongs to the neuron-specific Type II microtubule-associated protein family and is predominantly expressed in neurons and to a lesser extent in astrocytes and oligodendrocytes. Pathological formation of insoluble tau aggregates is implicated in the etiopathology of a class of neurodegenerative diseases, including CTE, AD, and PD, referred to also as tauopathies.<sup>[228]</sup> The detailed molecular mechanisms of the formation of tau aggregates and their

roles in progression of neurodegenerative disorders are still not completely understood. Hypothetical mechanisms of tau aggregate formation include its abnormal modification by hyperphosphorylation<sup>[229,230]</sup> and caspase-3-mediated cleavage.<sup>[59,112]</sup>

CSF and serum levels of total tau and its hyperphosphorylated form have long been considered as promising biomarkers of brain disorders primarily associated with neurodegeneration characteristic of chronic neurodegenerative disease<sup>[231,232]</sup> and acute brain injuries such as ischemic and hemorrhagic strokes and TBI.<sup>[233-240]</sup>

In ischemic stroke patients, CSF and blood tau protein levels transiently increase after 24 h within the 1<sup>st</sup> week of symptom onset and returned to control levels after 3–5 months. The acute increases in tau concentrations in samples collected between 5 and 10 days after stroke onset were associated with clinical stroke severity, stroke outcomes, and prognosis.<sup>[233-235,241,242]</sup>

Clinical data obtained in hemorrhagic stroke and TBI patients showed that increases in CSF and serum tau levels are detectable in samples collected at admission,<sup>[237,240,243]</sup> suggesting that following intracerebral hemorrhage and TBI, the increases in tau concentration appeared at an earlier time point after injury than the increases in tau observed in the ischemic stroke where tau was undetectable in most samples at 24 h after symptom onset.<sup>[242]</sup> Acute serum and CSF tau protein concentrations in TBI patients are correlated with short- and long-term outcomes.<sup>[237,240]</sup> Similarly, serum concentrations of tau in samples collected from hemorrhagic stroke patients at admission were predictive of mortality and poor 3-month neurological outcomes.<sup>[243]</sup> Significant increases in the serum concentration of caspase-cleaved tau were observed in ischemic stroke patients with poor outcomes for patients at day 7 after stroke onset compared to the caspase-cleaved tau concentrations in these patients measured at admission and compared to the caspase-cleaved tau concentrations in patients with favorable outcomes and controls.<sup>[184]</sup> In addition, increased levels of caspase-3-cleaved tau is a candidate biomarker associated with increased intracranial pressure following TBI.<sup>[226]</sup> Significant increases in serum concentrations of caspase-cleaved tau were observed in athletes after concussion as compared to the caspase-cleaved tau concentrations in preseason samples.<sup>[127]</sup>

### Clinical Implication of Biomarkers Related to Caspase-3-mediated Pathways in Acute Brain Injuries and Chronic Degeneration

Apoptosis is a common feature of acute brain injuries and many neurological disorders and neurodegenerative

disorders associated with inflammatory and neurovascular pathologies. Apoptosis is involved in the irregular accumulation of different isoforms of tau, blood–brain barrier dysfunction, and abnormal angiogenesis.<sup>[39,106-108]</sup> Caspase-3 upregulation in neuronal, glial, and infiltrating inflammatory cells contributes to the overall pathology following stroke and TBI in humans. Caspase-3-mediated apoptosis plays a key role in cleavage of cytoskeletal proteins<sup>[59]</sup> that can further contribute to chronic axonal and microvascular damage.<sup>[153,244,245]</sup> Clinical and experimental data indicate that the increased levels of a specific caspase-3 proteolytic product, SBDP120, are associated axonal damage following TBI pathology in humans, and these processes are accelerated in AD-like animal models suggesting a possible link between mechanisms involved in chronic axonal damage in these disorders.<sup>[129,131]</sup>

Increased levels of several tau isoforms including hyperphosphorylated and caspase-3-cleaved tau are considered hallmarks of neurodegeneration.<sup>[123,124]</sup> The presence of caspase-3-cleaved in neurofibrillary tangles is one of the earliest events in the tangle pathology of AD, leading to formation of amyloid plaques.<sup>[112,121,122]</sup> Thus, tau isoforms are considered to be one of the earliest biomarkers of AD.<sup>[106,120,121,125]</sup> Increased levels of caspase-cleaved tau were observed in brain extracts of CTE patients<sup>[126]</sup> and in serum of both TBI, stroke, and patients with AD.<sup>[127,128,184]</sup> Thus, abnormal tau processing following stroke and TBI<sup>[127,184]</sup> might be an initial step triggering formation of fibrillary tangles and amyloid plaques that are commonly observed in AD and other neurodegenerative disorders.<sup>[106,119,120]</sup>

## Conclusion

The current data provide strong experimental and clinical evidence that activation of caspase-3 following acute brain injuries including ischemic and hemorrhagic stroke and TBI is involved in the etiopathology of these disorders by inducing neuronal and glial cell death and degradation of cytoskeletal proteins that might affect neuronal and microvascular function and further trigger pathological processes underlying the development of chronic neurodegenerative diseases. The levels of biomarkers associated with caspase-3 activity in CSF and peripheral blood, including the levels of caspase-3 and other selected caspases such as products of caspase-3-mediated cleavage of cell-specific epithelial (e.g., CCK-18) and neuronal (e.g., SBDP120, caspase-3-cleaved tau) proteins, might provide valuable information for assessment of injury severity and mechanism and predict clinical outcomes. In light of the critical role of cleaved caspase-3 in the accumulation of caspase-3-cleaved tau, an early marker of neurodegenerative processes, the caspase-3-mediated pathway may be a promising target

for development of novel therapeutic strategies for the treatment of stroke and TBI.

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## Conflicts of interest

Ronald L. Hayes owns stock, receives compensation from and is an executive officer of Banyan Biomarkers, Inc., and, as such, may benefit financially as a result of the outcomes of this research or work reported in this publication.

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