



# Protein Degradome of Spinal Cord Injury: Biomarkers and Potential Therapeutic Targets

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## Abstract

Degradomics is a proteomics sub-discipline whose goal is to identify and characterize protease-substrate repertoires. With the aim of deciphering and characterizing key signature breakdown products, degradomics emerged to define encryptic biomarker neoproteins specific to certain disease processes. Remarkable improvements in structural and analytical experimental methodologies as evident in research investigating cellular behavior in neuroscience and cancer have allowed the identification of specific degradomes, increasing our knowledge about proteases and their regulators and substrates along with their implications in health and disease. A physiologic balance between protein synthesis and degradation is sought with the activation of proteolytic enzymes such as calpains, caspases, cathepsins, and matrix metalloproteinases. Proteolysis is essential for development, growth, and regeneration; however, inappropriate and uncontrolled activation of the proteolytic system renders the diseased tissue susceptible to further neurotoxic processes. In this article, we aim to review the protease-substrate repertoires as well as emerging therapeutic interventions in spinal cord injury at the degradomic level. Several protease substrates and their breakdown products, essential for the neuronal structural integrity and functional capacity, have been characterized in neurotrauma including cytoskeletal proteins, neuronal extracellular matrix glycoproteins, cell junction proteins, and ion channels. Therefore, targeting exaggerated protease activity provides a potentially effective therapeutic approach in the management of protease-mediated neurotoxicity in reducing the extent of damage secondary to spinal cord injury.

**Keywords** Spinal cord injury · Degradomics · Protease · Breakdown · Biomarkers

## Introduction

Degradomics involves the study of proteases, which are enzymes responsible for the hydrolysis of peptide bonds, and breakdown products (BDPs) generated from the substrates they target [1]. While degradomic profiling may be done at the nucleic acid level using gene microarrays and RNA sequencing, protein-level degradomics using technologies in mass spectrometry is more representative of the true expression levels of proteins, their substrates, and the generated BDPs [2]. With unprecedented sensitivity and specificity, BDPs serve as encryptic neoproteins that could be further utilized in diagnosis, assessment, and therapeutic management as well as treatment and follow-up in several states of disease [3]. For this reason, several techniques [4] have emerged to decipher the degradomic profiles of increasing epidemics including neurotrauma, where uncontrolled protease activation contributes to neurotoxicity [5–7].

Spinal cord injury (SCI) is a devastating trauma to the spinal cord that often leads to an individual's partial or complete loss of

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motor and sensory capabilities with ensuing loss of independence and reduced life expectancy [8–10]. SCIs commonly occur in young individuals and constitute a burden on the injured and the society concurrently [11, 12]. Clinical decision-making is challenging especially in elderly patients with other comorbidities [13]. Similar to the relationship between brain trauma and depression, SCI patients are also susceptible to develop post-traumatic psychiatric consequences [14]. Primary SCI corresponds to the initial direct trauma to the spinal cord yielding shear forces on axons and ultimately structural irreversible defects that vary depending on severity [15]. Secondary injury represents the myriad of activated cellular and molecular processes that have been shown to escalate the initial injury and hinder recovery [16]. These secondary processes include alterations in neurovascular homeostasis [16], damage to the integrity of the blood-CNS barrier [17], formation of autoantibodies [18] and ion channelopathies [19], and production of free radicals [20], cytotoxic neurotransmitters, and proteolytic enzymes [21]. In the context of these microscopic events and the extent of gross structural defects, SCI treatment strategies are established to minimize or eliminate secondary injury [22–25]. Retrograde neuronal death and difficulty regenerating axons contribute largely to the severe neurological consequences of SCI and serve as the platform on which secondary injury and chronic demyelination and nerve fibrosis occur [26, 27]. The pathophysiological events and neurochemical mechanisms that mediate the pathogenesis of spinal cord trauma are complex. Identification and characterization of BDPs, generated by numerous proteases may advance our understanding of these mechanisms and can be used to guide drug development and clinical decision-making (Fig. 1).

In this article, we aim to review the degradomic profile of SCI and identify therapies targeting proteins involved in the proteolytic cascades. We will describe putative substrates of different proteases that show prominent breakdown signature markers involved in trauma to the spinal cord. These include cytoskeletal proteins, components of the neuronal extracellular matrix (ECM), and cellular junction proteins, as well as ion channels, whose proteolysis is mediated by several enzymes such as caspases, calpains, cathepsins, and matrix metalloproteinases (MMPs).

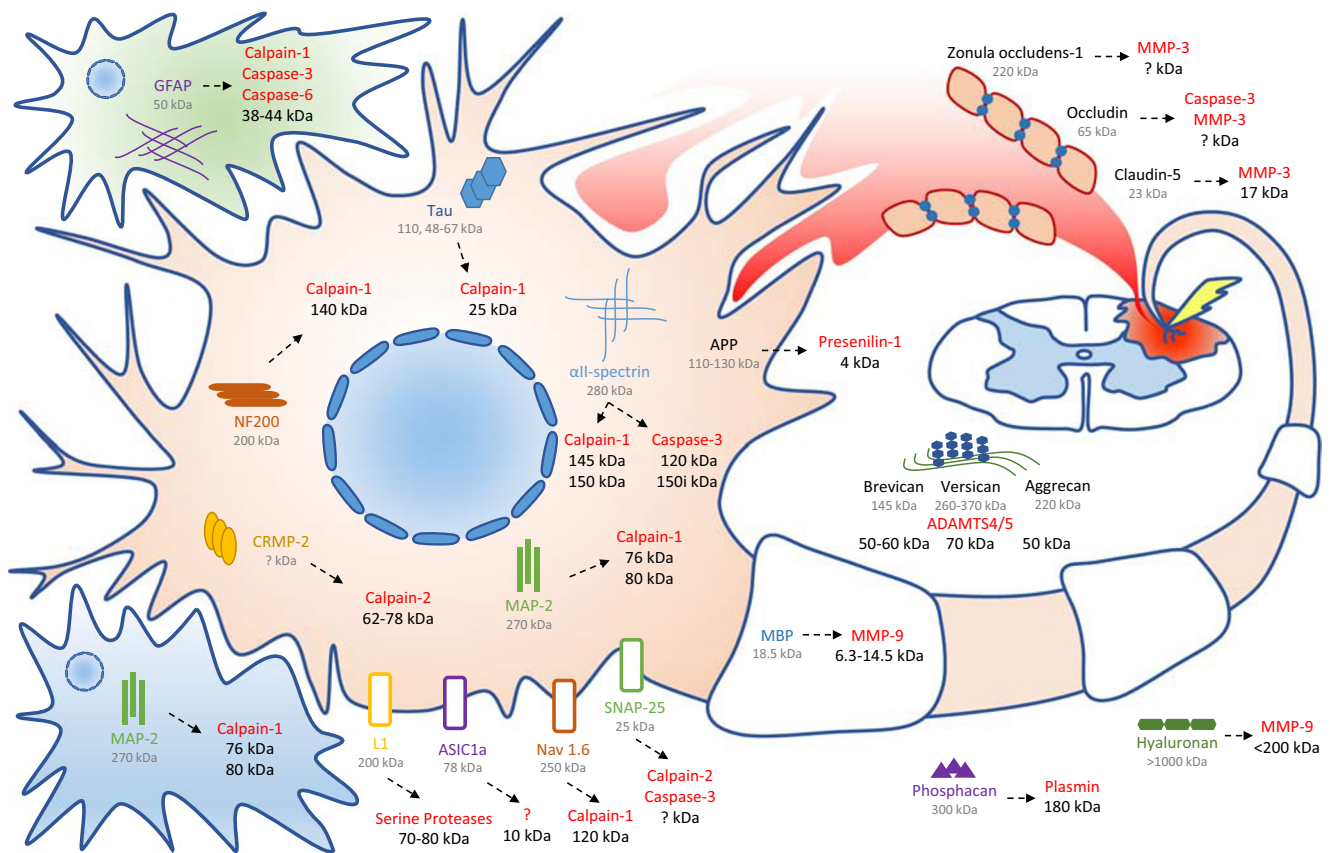
## Protease-Substrate Repertoires

Substrates subject to proteolysis have been extensively characterized in SCI. The roles of calpains, highly conserved nonlysosomal cysteine proteases, and caspases, cysteine-aspartic proteases, in the spinal cord became evident when Gill et al. studied acute atrophy of the diaphragm following hemisection of the cervical spinal cord in rats [28]. While type I and type IIa diaphragmatic muscle fibers had a reduced cross-sectional area (CSA) at day 1 post-SCI but not at day

7, type IIb muscle fibers had a reduction in CSA at both time points. This myoplasticity was found to be directly related to an increase in the activity of the proteolytic enzymes, caspases, and calpains. Caspase-3 was significantly elevated at day 1 and calpain-3 was elevated at day 7 post-SCI. This activation of caspases and calpains has been directly linked to the activity of circulating cytokines which activate proteolytic enzymes [29, 30]. Inhibition of calpain was not attempted, but as it was later uncovered, inhibition of proteases demonstrated a potential therapeutic approach towards minimizing the extent of injury [31].

Previously, Veeravalli et al. summarized the collection of proteases identified post-SCI [21]. Besides the previously discussed cysteine proteases, the roles of aspartyl proteases and serine proteases were also described. Aspartyl proteases involved in SCI include cathepsin-B [32] and cathepsin-D [33, 34]. Not long time ago, the first description on the involvement of cathepsin-B in SCI by Ellis et al. demonstrated a significant increase of the 37-kDa cathepsin-B proenzyme protein at 48, 72, and 168 h and its active form at 72 and 168 h post-injury in contusion SCI in rats [35]. In parallel, the role of cathepsin-D first described by Hashimoto et al. using microarray analysis where the 31-kDa active cathepsin-D was mainly detected in microglia at 3 and 7 days [36] suggesting its role in phagocytosis and lysosomal activation during neurotrauma [34]. The endoplasmic reticulum (ER) which is protected by autophagy [37] is stressed in SCI where cathepsin-D is acutely reduced while ER stress-associated caspase-12 and caspase-3 are co-expressed facilitating stress-induced apoptosis [38]. Consistently, ER protein 29 (ERp29) that gradually recovered at 28 days after SCI downregulated caspase-3 expression likely via the Erk-1/PI3K pathway [39], whereas adenosine downregulated caspase-12 [40].

MMPs are also implicated in SCI. MMPs are proteases involved in numerous physiological processes (e.g., embryogenesis, wound healing, neuronal growth, plasticity) and whose expression is dependent on growth factors and cytokines [41]. Dysregulation of MMPs results in detrimental outcomes in disease, as remarkably studied in cancer and cardiovascular homeostasis [42]. Using post-mortem samples of control and traumatized human spinal cords, an increase in the level of MMP-1 early on following SCI, and an early and brief rise of MMP-2 at the lesion core in macrophages were detected [43]. This rise in MMP-1 was also similarly described in animal studies [44]. For MMP-2, it was noted to rise at 7 days post-SCI in a mouse model of SCI by Goussev et al. [45] and 5 days post-SCI in a rat model by de Castro et al. [46] and serves to control the extent of glial scarring [47–49]. To study the role of MMP-9 in limiting functional recovery following SCI, Noble et al. studied the activity of MMP-9 on MMP-null mice versus wild-type mice with SCI created by a T8 weight-drop contusive injury [50].



**Fig. 1** Cytoskeletal, cell junction, extracellular matrix, ion channels and other membrane proteins cleaved by several proteases that are implicated in the pathogenesis of SCI. Beige-colored cell represents a neuron, dark

beige-colored cell represents endothelial cells with damaged blood-spinal cord barrier, green-colored cell represents an astrocyte, and blue-colored cell represents microglia

Decreased neutrophil infiltration, blood-spinal cord barrier (BSCB) stability, and better locomotor activity in MMP-null mice were observed [50]. For MMP-12, spinal cord compression in MMP-12-null mice showed improved motor recovery compared with their wild-type counterparts, and motor strength was higher at 14, 21, and 28 days post-SCI [51]. A delayed expression of MMP-9 and MMP-12 was noted as long as 24 days after SCI, and a limited presence of tissue inhibitors of metalloproteinases (TIMPs) throughout as well [43]. TIMPs are endogenous inhibitors that influence MMP role in the degradation of collagen and other ECM components [52]. At the therapeutic level, intrathecal injection of a selective MMP-2/MMP-9 inhibitor, SB-3CT, reduced dye extravasation and apoptotic cell death [53]. Hence, these studies bring to light the variety of MMPs involved in neuronal remodeling as well as the similarity of SCI mechanisms in both animal and human specimens with temporal alterations of the MMP protease-substrate repertoire.

The expression of glutamine synthetase (GS), whose role in SCI has been studied by Zou et al. as a promoter of proteolysis involved in reactive transformation and glial scar formation, is a hallmark of reactive astrocytosis [54]. GS is highly expressed in astrocytes and usually plays an important role in neuroprotection by converting the cytotoxic compounds

glutamate and ammonia into glutamine [55, 56]. GS was found to be downregulated acutely post-SCI while upregulated at 7 days [54, 57]. Using a T9 weight-drop SCI model, an increase in astrocyte migration into the lesion site in GS knockdown mice and an increase in membrane type 1-MMP (MT1-MMP) activation as well as inhibited expression of integrin  $\beta$ 1 were noted [54]. MT1-MMP-mediated degradation of GS post-SCI leads to increased cell migration and enhanced glial scar formation, as well as increased susceptibility of neurons to neurotoxicity via glutamate and ammonia [54]. GS was found elevated in microglia/macrophages at day 7 post-injury likely to compensate for inhibited astrocytic function [58]. This suggests that MMPs and subsequent Schwann cell functions are regulated by astrocytic GS whose downregulation can ameliorate secondary demyelination and inflammatory damage in the acute phase of SCI thereby representing a promising therapeutic approach for SCI [58]. Serine proteases include tissue-type plasminogen activator (tPA) and urokinase PA (uPA), which reduce the activation of macrophages and microglia and thus improve functional recovery [59]. Neuroserpin (tPA inhibitor), neuropsin (kallikrein-like serine protease) knockout, and liver X receptor agonists were found to attenuate I $\kappa$ B- $\alpha$  degradation, demyelination, and oligodendrocyte death [60–62].

The protease-substrate repertoire is also determined by the activity of the ubiquitin-proteasome system (UPS). This system represents many factors including ubiquitin ligases, ubiquitin hydrolases, ubiquitin, and ubiquitin-like molecules besides the proteasome itself [63]. Ubiquitination tags proteins and modulates several aspects of cellular functions and is activated in SCI [64]. Phosphorylation of ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) and conjugated ubiquitin are significantly increased acutely after injury, attributed in part to 26S proteasome-dependent degradation [65]. Noteworthy, the transformative effects of UCH-L1 lie in its ability to promote neurogenesis and regulate the differentiation of neuronal progenitor cells [66]. Ubiquitin-specific protease 4 (USP4) was downregulated in a T9 contusion injury SCI rats promoting microglial activation and subsequent neuronal inflammation [67]. Both of Src-associated in mitosis (Sam68), an RNA-binding protein, and sentrin-specific protease 3 (SENP3), a ubiquitin-like protease, were co-localized with caspase-3 post-SCI mediating proteolysis and decreasing cyclin D1 protein, the outcomes that were reversed using Sam68 siRNA [68, 69]. Treating SCI rats with hydroxysafflor yellow A (HSYA), possessed potent neuroprotective effects as evident by improved motor function besides reduced release of key mediators of inflammation such as TNF- $\alpha$ , IL-6, iNOS, COX-2, and NF- $\kappa$ B along with attenuation of caspase-3-associated oxidative and mitochondrial stress [70]. An unusual protease, a 50-kDa asparaginyl endopeptidase, proved essential in neuronal recovery after spinal cord injury in zebrafish due to its involvement in cell migration, proliferation, and ECM turnover [71]. Recently, MRI scans and pressure-volume catheterizations revealed contractile dysfunction, decreased cardiomyocyte size, and increased expression of angiotensin II receptors 5 and 12 weeks post-SCI attributed to the activation of the UPS [72, 73]. A handful number of preclinical trials on the use of drugs targeting the UPS such as phosphodiesterase-4 inhibitors and dibutyryl-cAMP yielded favorable findings [74].

In parallel, extensive transcriptomic and proteomic bioinformatics analysis in animal models of SCI revealed overlooked and potentially therapeutic targets which included cathepsins A, H, Z, gamma-secretase, and proteasome protease PSMB10 [75]. Amyloid precursor protein (APP) is a membrane protein involved in synaptogenesis and iron transport and infamous for its involvement in the formation of amyloid plaques by presenilin 1 (PS1), the catalytic subunit of gamma-secretase, in Alzheimer's disease [76]. In a T8-level cord hemisection, PS1 immunopositive cells and the expression level of full-length APP (95–130 kDa) as well as amyloid- $\beta$  remarkably elevated on day 1 after injury, whereas the mature APP (110–130 kDa) increased relatively more than the immature counterpart [77]. Thus, it might be beneficial to develop gamma-secretase inhibitors besides inhibitors of cathepsins and PSMB10. In fact, treatment with carfilzomib,

proteasome inhibitor which interacts with PSMB10, exerts a neuroprotective effect after thoracic transection SCI in rats [78]. While very few protease inhibitors (such as nafamostat mesilate) have been tested, none of the anti-cathepsin drugs have been attempted in preclinical or clinical studies of SCI. Treatment with recombinant secretory leukocyte protease inhibitor was found to improve locomotor activity and reduce secondary injury [79]. Indeed, nafamostat mesilate, which is used clinically in the treatment of pancreatitis, was shown to substantially increase in spared tissue and to reduce cytokine levels and improve functional recovery in a contusion SCI rat model [80]. Interestingly, nafamostat mesilate and mesencephalic astrocyte-derived neurotrophic factor (MANF) reduced caspase-3-mediated apoptosis [81]. Caspase-3 drives cells towards programmed cell death by activating the DNA fragmentation factor that stimulates endonucleases to cleave nuclear DNA [82]. Mass spectrometry-based applications unveiled proteomic and phosphoproteomic differential protein expression following SCI that included heat-shock proteins, glycolytic enzymes, antioxidants, and proteins involved in cytoskeletal arrangements, cell signaling, and DNA damage as well as protein degradation [64]. In an attempt to identify therapeutic targets, Alawieh et al. employed network analysis of the SCI interactome revealing that neuronal growth factor (NGF), caspase-3, and H-Ras are the most central proteins involved in the pathogenesis of SCI [83]. Having identified numerous proteins and BDPs [84] through big data [85], it is therefore imperative to investigate the effect of other protease inhibitors and cathepsin blockers on the molecular and functional profiles post-SCI. The nature and regulation of caspases, calpains [86], MMPs [87], cathepsins [88], tPA [89], and the UPS [90] have been previously discussed elsewhere.

## The SCI Degradome

The SCI degradome is the repertoire of proteases that cells and tissues coordinately regulate in order to modulate their local environment. The study of the proteolytic and degradomic activity related to SCI is hence important, as it provides insight both into the processes that occur post-SCI, and may help to identify promising targets for therapy to reduce neurological damage and induce regeneration. The degradomic profile of SCI is summarized in Table 1 and potential protein targets involved in the degradomics of SCI are presented in Table 2 and discussed below.

## Cytoskeletal Proteins

Calpains trigger sequences of neurochemical events leading to glutamate neurotoxicity including degradation of cytoskeletal

**Table 1** The degradomic profile of SCI

Precursor	Weight (kDa)	Location	Role	Proteolytic enzyme	Proteolytic product	SCI model	Animal	Ref.
APP	110–130	Cell membrane	Synapse formation and iron transport	Presenilin-1 of $\gamma$ -Secretase	4-kDa amyloid- $\beta$	T8 hemisection	Rats	[77]
$\alpha$ II-spectrin	280	Cytoplasm	Cytoskeletal protein, involved in maintaining structural integrity, development and stabilization of axons, and maintenance of polarization	Calpain-1	150-kDa SBDP	High-pressure air blasts to lower thoracic spine	Mice	[25, 91]
NF200	200	Cytoplasm	Intermediate filaments, stabilize neuronal structure and maintains axonal caliber	Calpain-1	150-kDa SBDP	T9–10 contusion injury	Rats	[38, 92]
Other neurofilaments: NF-L, NF-M, NF210, NF155, NF70	70 155 210	Cytoplasm		Caspase-3	150i-kDa SBDP 120-kDa SBDP			
MAP2	270	Cytoplasm		Calpain-1 Cathepsin D	140 kDa ?	T6–8 contusion injury Ischemia-reperfusion injury	Rats Rabbits	[93] [94, 95]
Tau	110 and 48–67 isoforms	Nucleus and cytoplasm	Cytoskeletal protein, involved in maintaining structural integrity A MAP cytoskeletal protein, involved in maintaining structural integrity, development and stabilization of axons, and maintenance of polarization	Calpain-1	76 kDa 80 kDa	T10 contusion injury	Rats	[91]
CRMP-2	?	Cytoplasm	Modulates microtubule growth	Calpain-1	25 kDa	10 g weight drop at T10	Rats	[96]
GFAP	50	Cytoplasm	Intermediate filament III protein. Role in cytoskeletal structure of glial cells, maintenance of their mechanical strength and support to neighboring neurons	Calpain-2 Caspase-3 Caspase-6	62 kDa 70 kDa 75 kDa 78 kDa 38–44 kDa	Cervical spinal cord transection	embryonic chick	[97]
SNAP-25	25	Presynaptic intracellular membrane	SNARE protein complex, involved in vesicular trafficking	Calpain-2 and caspase-3	?	T11 contusion injury	Rats	[99]
Hyaluronan	>1000	ECM	ECM, framework for the structure of the CNS	MMP-9	Sulfated proteoglycan <200 kDa Tenascin fragments <200 kDa	–	Mice	[100]
Aggrecan	220	ECM	Mediates chondrocyte-chondrocyte	ADAMTS4/5	50 kDa		Mice	[101, 102]

**Table 1** (continued)

Precursor	Weight (kDa)	Location	Role	Proteolytic enzyme	Proteolytic product	SCI model	Animal	Ref.
Versican	260–370	ECM	and chondrocyte-matrix interactions Mediates cell adhesion, migration, and proliferation ECM		70 kDa	Contusion injury at middle thoracic level		
Phosphacan	Brevican 50–60 kDa 300	145 ECM	ECM Chondroitin sulfate proteoglycan extracellular variant of a receptor-type protein tyrosine phosphatase	Plasmin	180 kDa	C1–2 transection	Rats	[103, 104]
Neural cell adhesion molecule L1	200	Cell membrane	Induces neuronal migration and survival, axon outgrowth and fasciculation, and myelination	Serine proteases (trypsin, plasmin, PC5a)	70 kDa 80 kDa	T7–9 electromagnetic compression injury	Mice	[105]
Zonula occludens-1	220	Cell membrane	Tight junction proteins, seal the paracellular pathway	MMP-3	?	T9–10 contusion injury	Mice	[106]
Occludin	65	Cell membrane		MMP-3	?			
Claudin-5	23	Cell membrane		MMP-3	17 kDa			
ASIC1a	78	Cell membrane	Sense reduced levels of extracellular pH	?	10 kDa	T9–11 clamp injury	Rats	[107]
Nav1.6	250	Cell membrane	Voltage-gated channel, initiate and propagate the action potential	Calpain-1	120 kDa	T9 transection	Rats	[108]
MBP	18.5	Myelin membranes	Myelination of nervous tissue	MMP-9	6.3 kDa, 7.0 kDa, 7.3 kDa, 8.3 kDa, 10.2 kDa, 14.5 kDa	Contusion injury	Rats	[109]

**Table 2** Therapeutic interventions in SCI at the degradomic level and the observed outcomes

Therapeutic intervention	Target	Outcome	SCI model	Animal	Ref.
Adenosine	⊕ GRP-78 ⊖ Caspase-12	⊖ Apoptotic cell death	Cross clamping of aorta inferior to renal artery	Rats	[40]
SB-3CT	⊖ MMP-2 ⊖ MMP-9	⊖ Apoptotic cell death ⊖ BSCB leak	T13 clip for 5 s	SOD1 transgenic rats	[53]
Neuroserpin	⊖ Serine proteases	⊕ LC3-II ⊕ p62 ⊖ Beclin-1 ⊖ Autophagosomes ⊕ Functional recovery ⊖ Demyelination ⊖ Oligodendrocyte death ⊖ Axonal degeneration ⊖ iNOS (130 kDa) ⊖ NF-κB (65 kDa) ⊖ IκB-α degradation ⊕ Functional recovery ⊖ Caspase-3 ⊖ Cyclin D1 ⊖ Apoptosis ⊖ Astrocytosis ⊖ Caspase-3 ⊖ SOD	T10 clamp for 1 min	Rats	[60]
Neuropsin knockout	–	⊖ Liver X receptors α (LXRα) and β (LXRβ)	L1 forceps compression injury	Mice	[61]
T0901317	⊕ Liver X receptors α (LXRα) and β (LXRβ)	⊖ NF-κB (65 kDa) ⊖ IκB-α degradation ⊕ Functional recovery ⊖ Caspase-3 ⊖ Cyclin D1 ⊖ Apoptosis ⊖ Astrocytosis ⊖ Caspase-3 ⊖ SOD	T5–8 clips for 1 min	Mice	[62]
Sam68 siRNA	⊖ Sam68	⊕ Functional recovery ⊖ Cyclin D1 ⊖ Apoptosis ⊖ Astrocytosis ⊖ Caspase-3 ⊖ SOD	10 g weight drop at T9 from 5 cm height	Rats	[68]
Hydroxysafflor yellow A (HSYA)	?	⊖ Apoptosis ⊖ NF-κB, inflammation and inflammatory cytokines ⊖ Oxidative stress ⊖ Tissue injury and edema ⊕ Recovery ⊖ H <sub>2</sub> O <sub>2</sub> -induced caspase-3/8 ⊖ H <sub>2</sub> O <sub>2</sub> -induced apoptosis ⊕ Akt/mTOR/p70S6K pathway ⊖ Nestin, NeuN, NSE, NF200 ⊖ Astroglisis ⊖ Locomotion ⊕ NF 160 and 200 kDa ⊕ Locomotor activity ⊕ Urinary function ⊕ Dendritic staining of the 270-kDa MAP2 ⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T9–10 clip for 1 min	Rats	[70]
Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP)	⊖ Reactive oxygen species	⊖ H <sub>2</sub> O <sub>2</sub> -induced caspase-3/8 ⊖ H <sub>2</sub> O <sub>2</sub> -induced apoptosis ⊕ Akt/mTOR/p70S6K pathway ⊖ Nestin, NeuN, NSE, NF200 ⊖ Astroglisis ⊖ Locomotion ⊕ NF 160 and 200 kDa ⊕ Locomotor activity ⊕ Urinary function ⊕ Dendritic staining of the 270-kDa MAP2 ⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T13 H2O2 injection	Rats	[110]
ATP	⊕ P2 nucleotide receptors	⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	50 g weight drop at T8–T10 from 12.5 cm height	Rats	[111]
Gonadotropin-releasing hormone	⊕ GnRH receptor	⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T10 balloon inflation for 5 mins	Ovariectomized rats	[112]
Riluzole	⊖ Glutamate release	⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T10 impact injury	Rats	[91]
PAR1 antagonists or PAR1 knockout	⊖ PAR1	⊖ Serine proteases (kallikerin 6) ⊕ Myelination ⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T11–T12 injection	Mice	[113]
Lipopeptide inhibitors or PAR1/2 knockout	⊖ PAR1/2	⊖ Serine proteases (kallikerin 6) ⊖ ERK1/2 pathway	T9 clip for 1 min	Mice	[114]

**Table 2** (continued)

Therapeutic intervention	Target	Outcome	SCI model	Animal	Ref.
Brain-derived neurotrophic factor (BDNF) or PAR2 knockout	⊖ TrkB receptor signaling through cAMP, ERK1/2 pathway	⊖ Thrombin-mediated cerebellar toxicity ⊖ Glutamate neurotoxicity ⊖ PAR2-mediated downregulation of proteolipid protein (PLP)	L2–3 clip	Mice	[115]
Pifithrin- $\mu$ (PFT- $\mu$ )	⊖ p53	⊖ Accelerated myelination ⊖ Demyelination and oligodendrocyte apoptosis	L1 compression injury	Rats	[116]
CR2-Crry or C3 knockout	⊖ Sites of C3 deposition	⊖ Caspase-3/12, E2F1, cytochrome C ⊖ Caspase-mediated necrosis ⊖ Demyelination	T12.5 g weight drop injury	Mice	[117]
CX3CR1 knockout	–	⊖ Neutrophil infiltration ⊖ IL-6, IL-1 $\beta$ release ⊖ Ly6C <sup>lo</sup> /iNOS <sup>+</sup> macrophages characterized by high proteolytic activity degrading injured and intact tissues	T9–10 contusion injury (0.5 mm over 30 ms)	Mice	[118]
tPA	⊖ ADAMTS-4	⊖ CSPG degradation ⊖ Axonal sprouting	T10–11 balloon inflation for 30 s	Rats	[119]
tPA or tPA + ChABC	⊖ Plasminogen (tPA) ⊖ CSPG deglycosylation (ChABC)	⊖ Locomotion ⊖ Neurite regrowth ⊖ Sensory functions	T8–10 impact injury	Mice	[120]
T1 knockout	–	⊖ Motor function ⊖ Heparan sulfate degradation	T10 impact injury	Mice	[121]
RPTP $\sigma$	⊖ Cathepsin-B	⊖ Axonal growth	Contusion injury	Rats	[122]
RhoAi	⊖ RhoA	⊖ Axonal growth ⊖ Axonal regeneration	T7–11 balloon inflation injury	Rats	[123]
Simvastatin	⊖ HMG-CoA reductase, mTOR pathway	⊖ Synaptogenesis ⊖ Locomotion ⊖ Autophagy ⊖ BDNF and GDNF	10 g weight drop at T9–10 from 25 mm height	Rats	[124]
EGF and bFGF	⊖ Neural progenitor cells	⊖ Caspase-3 ⊖ Apoptosis ⊖ Loss of Nissl bodies ⊖ Neuronal sparing ⊖ Motoneuron synapses ⊖ Corticospinal tract nerve fibers	T8–9 balloon inflation for 5 mins	Rats	[125]
PTP $\sigma$ peptide inhibitor	⊖ PTP $\sigma$	⊖ Angiogenesis ⊖ Serotonergic innervation ⊖ Locomotion	T8 impact injury	Rats	[126]
Cerebrolysin	⊖ Growth factor receptors	⊖ Urinary function ⊖ BSCB breakdown and cord edema ⊖ Leakage of plasma proteins ⊖ Number of injured neurons	T10–11 longitudinal incision	Rats	[127]
Duloxetine, phenelzine, sulfate and tacrine	⊖ Neural cell adhesion molecule L1	⊖ 90-, 70-, and 32-kDa L1 fragments		Mice	[128]

**Table 2** (continued)

Therapeutic intervention	Target	Outcome	SCI model	Animal	Ref.
MMP3 siRNA or N-isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid	⊖ MMP3	⊕ Motor recovery ⊕ Remyelination ⊕ Neuronal survival ⊖ Astroglia ⊖ BSCB permeability ⊕ Tight junctions (occluding, zonula occludens-1) ⊕ Functional recovery	T7–9 compression injury	Mice	[106]
DI-3-n-butylphthalide (NBP)	⊕ HIF-1 $\alpha$ , VEGF expression	⊖ Degradation of adherens ( $\beta$ -catenin, p120-catenin) and tight junction (claudin-5, occludin) proteins ⊖ Caspase-12 (38 kDa, active form) ⊖ Endoplasmic reticulum stress ⊖ EIF 2 $\alpha$ /p-EIF 2 $\alpha$ , ATF-4/6, CHOP, PDI, GRP78, XBP-1 ⊕ Locomotion ⊖ Caspase-3 ⊖ NF- $\kappa$ B ⊕ Bcl-2 ⊕ Autophagy ⊖ Apoptotic cell death ⊖ Inflammation ⊕ Autophagy ⊕ Functional recovery ⊖ MMP-9 ⊖ TNF- $\alpha$ ⊖ ICAM-1 ⊖ Neutrophil infiltration ⊖ Tight junction degradation ⊖ BSCB leak ⊕ Functional recovery ⊕ Cathepsin D ⊕ Lysosomal-associated membrane protein 1 (LAMP1) Restoration of autophagic flux ⊕ Lysosomal biogenesis ⊖ Neuronal apoptosis ⊕ Functional recovery	T9 clip for 2 mins	Rats	[129]
Metformin	⊕ 5' AMP-activated protein kinase (AMPK), mTOR/p70S6K pathway	⊕ Locomotion ⊖ Caspase-3 ⊖ NF- $\kappa$ B ⊕ Bcl-2 ⊕ Autophagy ⊖ Apoptotic cell death ⊖ Inflammation ⊕ Autophagy ⊕ Functional recovery ⊖ MMP-9 ⊖ TNF- $\alpha$ ⊖ ICAM-1 ⊖ Neutrophil infiltration ⊖ Tight junction degradation ⊖ BSCB leak ⊕ Functional recovery ⊕ Cathepsin D ⊕ Lysosomal-associated membrane protein 1 (LAMP1)	T9 contusion injury	Rats	[130]
Netrin-1 (both studies same things)	⊕ Netrin receptor, AMPK/mTOR pathway	⊕ Functional recovery ⊕ Cathepsin D ⊕ Lysosomal-associated membrane protein 1 (LAMP1) Restoration of autophagic flux ⊕ Lysosomal biogenesis ⊖ Neuronal apoptosis ⊕ Functional recovery	T9 clip for 1 min	Rats	[131]
Erythropoietin	⊕ AMPK/mTOR pathway	⊕ Loss of motor neurons ⊕ Functional recovery ⊖ BSCB breakdown: degradation of AJ and TJ ⊕ Locomotion ⊖ BSCB breakdown ⊖ Histological injury of neurons	10 g weight drop at T9 from 2.5 cm height	Rats	[132], [133]
EGF	⊕ Rac1, PI3K/Akt pathway	⊕ Loss of motor neurons ⊕ Functional recovery ⊖ BSCB breakdown: degradation of AJ and TJ ⊕ Locomotion ⊖ BSCB breakdown ⊖ Histological injury of neurons	T7 to T10 clamp for 1 min	Rats	[134]
TiO2-nanowired growth hormone (NW/GH)	⊕ IGF-1	⊕ Loss of motor neurons ⊕ Functional recovery ⊖ BSCB breakdown: degradation of AJ and TJ ⊕ Locomotion ⊖ BSCB breakdown ⊖ Histological injury of neurons	T9 contusion injury	Rats	[135]
			T10–11 incisional injury	Rats	[136]

**Table 2** (continued)

Therapeutic intervention	Target	Outcome	SCI model	Animal	Ref.
N-benzyl-N-ethyl-2-(7,8-oxo-2-phenyl-9H-purin-9-yl)acetamide (ZBD-2)	⊕ Translocator protein 18, P13K/Akt pathway	⊖ Bax/Bcl-2 ratio ⊖ Locomotion ⊖ Tissue injury ⊖ preserved white matter ⊖ Oxidative stress ⊖ MMP-9 ⊖ SUR1 and TrpM4 ⊖ Zona occludens-1 and occluding degradation ⊖ BSCB leak and hemorrhage ⊖ Inflammation	T7–9 clip for 20 s	Mice	[137]
17β-estradiol (E2)	⊕ Estrogen receptor	⊖ SUR1 and TrpM4 ⊖ Zona occludens-1 and occluding degradation ⊖ BSCB leak and hemorrhage ⊖ Inflammation	T9–10 contusion injury	Rats	[138]
miR-7-1	⊖ L-type Ca <sup>2+</sup> channel protein alpha 1C (Cpα1C)	⊕ Neuroprotective functions of estrogen receptor agonists ⊖ Apoptosis (Bax) ⊖ MMP-9 ⊖ Tight junction degradation ⊖ SUR1 and TrpM4 ⊖ BSCB leak and hemorrhage ⊖ Inflammation ⊖ Apoptosis	Calcium ionophore insult	VSC4.1 motoneurons	[139]
Mithramycin A (MA)	⊖ Specificity protein 1 (Sp1)	⊖ Apoptosis (Bax) ⊖ MMP-9 ⊖ Tight junction degradation ⊖ SUR1 and TrpM4 ⊖ BSCB leak and hemorrhage ⊖ Inflammation ⊖ Apoptosis ⊖ Functional recovery	T9 impact injury	Rats	[140]
Anti-Ly6G or clodronate-liposomes	⊖ Neutrophils (anti-Ly6G) ⊖ Monocytes (clodronate-liposomes)	⊖ Oxidative stress and lipid peroxidation ⊖ Functional recovery ⊖ MMP-9	2 g weight drop at T9 from 5 cm height	Mice	[141]
fluoxetine	⊖ Serotonin reuptake	⊖ Oxidative stress and lipid peroxidation ⊖ Functional recovery ⊖ MMP-2/9/12 ⊖ Grox, MIP1α and 1β (chemokines) ⊖ BSCB breakdown ⊖ Extracellular matrix degradation ⊖ Inflammation ⊖ Apoptosis ⊖ Functional recovery	T9 impact injury	Mice	[142]
Fluoxetine and vitamin C	⊖ Serotonin reuptake (fluoxetine) ⊖ Oxidants (vitamin C)	⊖ Functional recovery ⊖ MMP-9 ⊖ Superoxides ⊖ BSCB leak ⊖ Extracellular matrix degradation ⊖ Inflammation ⊖ Apoptosis ⊖ Locomotion	T9 impact injury	Rats	[143]
Ghrelin	⊕ Growth hormone secretagogue receptor 1a (GHS-R1a)	⊖ MMP-9 ⊖ SUR1 and TrpM4 ⊖ BSCB breakdown ⊖ Inflammation	T9 impact injury	Rats	[144]
BQ123 or BQ788	⊖ Endothelin receptor A (BQ123) ⊖ Endothelin receptor B (BQ788)	⊖ Inflammation ⊖ Hemeoxygenase-1 ⊖ MMP-9 ⊖ Inflammation	2 g weight drop at T9 from 5 cm height	Mice	[145]

**Table 2** (continued)

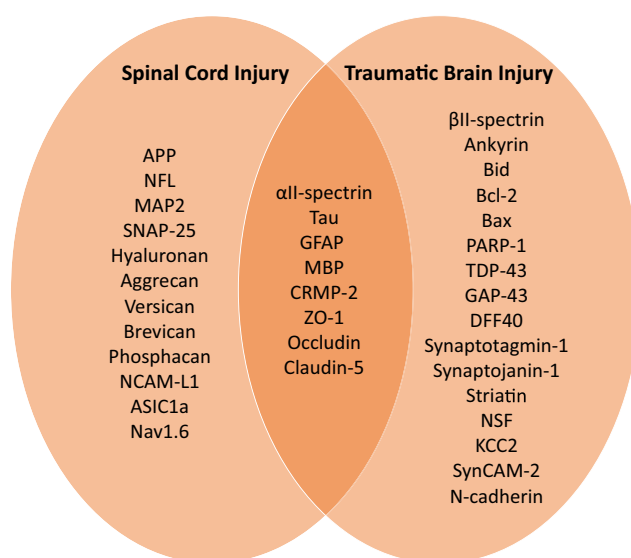
Therapeutic intervention	Target	Outcome	SCI model	Animal	Ref.
MMP-8i	⊖ MMP-8	⊖ Oxidative stress ⊖ Locomotion ⊖ TNF- $\alpha$ ⊖ IL-6 ⊖ iNOS ⊖ Degradation of occluding and zonula occludens-1 ⊖ BSCB breakdown ⊖ Inflammation ⊖ Caspase-3 ⊖ Neuronal apoptosis ⊖ Bladder bleeding ⊖ Hindlimb motor function ⊖ Calpain-mediated spectrin breakdown ⊖ Structural damage ⊖ Functional recovery ⊖ Calpain-mediated apoptosis ⊖ Edema ⊖ Functional recovery ⊖ Locomotion ⊖ Tissue loss ⊖ Ischemia ⊖ Spasticity ⊖ Neuropathic pain	T10 impact injury	Rats	[146]
Omega-conotoxin (MVIIC)	⊖ N (Ca <sub>v</sub> 2.1) and P/Q (Ca <sub>v</sub> 2.2) voltage-dependent calcium channels (VDCC) ⊖ Na <sup>+</sup> -Ca <sup>2+</sup> exchanger		T12 extradural compression	Rats	[147]
Bepridil or KB-R7943	⊖ Calpain		Compression injury	Rats	[148]
AK 295	⊖ Calpain		T8–10 weight drop injury	Rats	[149]
PcTx1	⊖ ASIC1a		T10 impact injury	Rats	[107]
MDL28170 Riluzole	⊖ Calpain (MDL28170) ⊖ Persistent sodium current (Riluzole) ⊖ Potassium-chloride cotransporter (KCC2)		T9 complete transection	Rats	[108]
Prochlorperazine	⊖ Potassium-chloride cotransporter (KCC2)		T8 transection	Rats	[150]
4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide	⊖ 5-HT <sub>2A</sub> receptors		T8 left hemisection	Rats	[151]
Bumetanide	⊖ Cl <sup>-</sup> regulatory protein Na <sup>+</sup> -K <sup>+</sup> -Cl <sup>-</sup> 1 (NKCC1)		10 g weight drop at T9 from 12.5 mm height	Rats	[152]
Tamoxifen	⊖ Estrogen receptor modulator	⊖ Motor recovery ⊖ Oligodendrocyte death and demyelination ⊖ Cavitation (by upregulation of AQP4) ⊖ Locomotion ⊖ White matter tissue sparing ⊖ Neuronal survival ⊖ Olig-2 ⊖ Reactive gliosis	T10 impact injury	Rats	[153,

154]

⊖ Denotes downregulation of the target or decrease in the observed outcome. ⊕ Denotes upregulation of the target or increase in the observed outcome

proteins while caspases are mainly implicated in the induction of apoptosis in neurons and glial cells [21]. Del Mar et al. examined how axons can be damaged by acute traumatic events, and took advantage of the extensive white matter of the spinal cord [25]. Antibodies to  $\alpha$ II-spectrin breakdown product (SBDP150) revealed calpain-mediated degradation of  $\alpha$ II-spectrin (280 kDa) into a 150-kDa fragment following injury afflicted via high-pressure air blast injury to the lower thoracic spine in mice. Immunolabeling SBDP150 at 3 and 6 h post-SCI revealed an acute significant increase in SBDP150 following blast injury [25, 91]. Xiong et al. detected 145-kDa and 150-kDa SBDPs generated from the activity of calpain-1 in addition to a caspase-3-generated 150i-kDa SBDP in rats subjected to contusion spinal cord injury at the T-10 level [155]. Similarly, Liu et al. detected SBDP145/150 at day 1 after a T8 weight-drop SCI in rats [38]. In addition to SBDP150/145, SBDP120 was recently detected in traumatized spinal cord tissue but only until 7 days post-injury [92]. Another key cytoskeletal component is ankyrin-G whose breakdown products have been described in brain trauma but not in SCI yet [156]. Interestingly, the same  $\alpha$ II-spectrin fragments emerged in traumatic brain injury as previously summarized by our lab (Fig. 2) [5]. The activation of calpain is related to an accumulation of peroxynitrite (PN) following SCI [157]. PN is formed by nitric oxide synthase-generated nitric oxide radical and superoxide radical, which seems to play a significant role in post-traumatic oxidative damage [158]. H<sub>2</sub>O<sub>2</sub>-induced apoptosis activating caspases was reduced with the catalytic antioxidant Mn 4-benzoic acid porphyrin [110]. An increased activity of calpain-1 peaking at 2 h post-injury was observed along with its proteolytic protein BDPs and loss of dephosphorylated neurofilament (NF200), a 200,000 molecular weight protein important for stabilizing

and maintaining neuronal structure, generating 140,000 fragments [93, 159]. NF-M and NF-L are altered by oxidative processes and subsequent cathepsin-D activation in SCI, which were significantly upregulated and downregulated, respectively, after 24 h [94, 95]. Therapeutically, exogenous ATP administration augmented the Akt/mTOR/p70S6K signaling pathway increasing the expression of nestin, neuronal nuclei (NeuN), neuron-specific enolase (NSE), and NF200 [111]. Administration of gonadotropin-releasing hormone restored locomotor activity and 160- and 200-kDa neurofilaments [112]. Riluzole, a glutamate release inhibitor, proved to be a potential therapeutic agent after a significant decrease in the gradual loss of neuronal/microglial staining of the 270-kDa microtubule-associated protein 2 (MAP2) following riluzole treatment in an impactor drop T10 spinal cord injury in rats during which activation of calpain-1 at 1 h was evident by the autolysis of its 80-kDa form to 76 kDa [91]. Calpain-1 also degraded 110- and 48–67-kDa tau isoforms, MAP proteins, into a 25-kDa BDP [96, 160, 161], whereas BDPs ranging from 22 to 48 kDa were reported in brain injury [5]. Caspase-3 also cleaves tau (not shown in Fig. 1) in animal models of aging and Alzheimer's disease but there is little evidence of cleaved tau in SCI models so far. Collapsin-response-mediator protein-2 (CRMP-2), known to modulate microtubule growth, was found in multiple forms including 62, 70, 75, and 78 kDa following SCI [97] compared with only a calpain-2-mediated 55-kDa fragment as well as an MMP-9 25-kDa BDP in brain trauma [5]. As for glial fibrillary acidic protein (GFAP), an intermediate filament present in astrocytes and Schwann cells, Yokobori et al. studied its role and reported a calpain-1-mediated cleavage of GFAP of molecular weight 50 kDa into 38–44-kDa fragments following SCI [98]. Similar GFAP BDP protein weights have been reported in both spinal cord and brain injuries [5]. GFAP elevations is, in part, mediated via protease activated receptor 2 (PAR2) [162], whereas oligodendrocyte activity is dependent on kallikrein 6, a serine protease, and activation of PAR1 [113] and PAR2 [114]. Although the kallikrein-kinin is less studied in SCI, it has been subject to preclinical studies in other neurological diseases [163]. Inhibition of PAR1 or PAR2 using lipopeptide inhibitors reduced ERK1/2 signaling and subsequent neurotoxicity [114]. PAR2 knockout mice exhibited a pro-myelinating phenotype translated as enhancements in myelin production after SCI, as evident with the higher levels of both proteolipid protein (PLP) and myelin basic protein (MBP), cAMP, ERK1/2 along with increased oligodendrocyte number [115]. PAR2 acts as a suppressor of myelin production and may be a useful target for therapies aimed at myelin regeneration after neurotrauma. Consistently, Ma et al. reported increased expression levels of p53, E2F1 (a transcription factor), caspase-3, caspase-12, and cytochrome C followed by myelin swelling and breakdown in compression SCI in rats, all of which were reversed



**Fig. 2** Comparative degradomics of spinal cord injury and traumatic brain injury

upon treatment with pifithrin- $\mu$ , a p53 inhibitor [116]. Interestingly, the level of GFAP BDPs was severity-dependent in spinal cord tissue where severe SCI yielded higher levels of 4-h, 24-h, and 7-day GFAP BDPs [92]. GFAP was also subject to degradation by caspase-3 but to a lesser extent compared with calpain. Clinically, mass spectrometry proteomic analysis of plasma samples from SCI patients also showed autoantibodies against GFAP indicating BSCB breakdown [164]. Identified GFAP BDPs included novel 36- and 42-kDa fragments detected at 2–4 weeks but not < 48 h. Synaptosomal-associated protein 25 kDa (SNAP-25), a membrane protein essential for neurotransmitter release at synaptic junctions, is degraded in SCI where reduced levels were associated with sensory and locomotor functions and provides a potential target gene for neural regeneration [99, 165]. SNAP-25 is susceptible to degradation by calpain-2 and caspase-3 [166]. Further mass spectrometry proteomic analysis revealed dysregulation of other cytoskeletal proteins including dynein light chain 1 (increase), tubulin beta-5 chain (increase), and F-actin capping protein subunit beta (decrease) 8 h post-injury [167], whereas  $\alpha$ -tubulin (increase), dynamin 1 (decrease), and the novel fascin (decrease) all of which remained stable until 12 h when they started to change expression peaking at 24 h before returning to baseline at 48 h [94].

## Extracellular Matrix

Our current understanding on how neuroinflammation affects the synthesis and deposition of ECM continues to evolve in CNS injury. SCI causes an inflammatory response that breaks down the BSCB precipitating leukocytic infiltration and edema of the spinal cord [100]. The ECM plays a crucial role in regulating cellular infiltration and the inflammatory response by releasing alarmins, ECM proteins triggering and amplifying the inflammatory reaction, and other peptides to activate the immune system by sequestering or presenting pro-inflammatory molecules to the damaged tissue [168]. Inhibition of complement receptor 2 (CR2) using CR2-Crry resulted in remarkable decrease in leukocytosis, demyelination, and necrosis after a weight-drop SCI in mice [117], whereas deficient chemokine CX3CR1 signaling stimulated recovery [118]. Following SCI, certain components of the ECM are degraded, while others are significantly upregulated. In a study designed to investigate the regulation of the ECM post-SCI, Gaudet and Popovich found that MMPs activity is increased following SCI in mice, causing remarkable degradation of hyaluronan (HA) (> 1000 kDa) and release of sulfated proteoglycans (PG) and tenascin fragments [100]. This is thought to reduce the potential of post-SCI repair whereby uncontrolled activation of MMP-9 [169] and MMP-12 [51] have been linked to limited recovery. MMP cleavage of HA results in fragmentation of high-molecular-weight HA into

low-molecular-weight HA with fragments of < 200 kDa. Noteworthy, levels of HA remain stable due to turnover, i.e., simultaneous production and degradation, up until day 5 after which HA release takes over resulting in gliosis [170]. Utilizing these findings in therapy, studies have shown that the application of HA improves recovery following SCI in rat models by reducing macrophage density, and gliosis as well as deposition of proteoglycans [100, 171]. On the other hand, among the biomarkers detected in CSF from SCI rats are inter-alpha-trypsin inhibitors (Itih1, Itih3, and Itih4), serine protease inhibitors whose role is to stabilize ECM and regulate inflammation by binding HA [172]. Free HA thus accumulates modulating myelination and regeneration during CNS repair [170, 173].

ECM and its glycosylation patterns have been heavily studied in brain cancers [174] and have extended to include research in traumatic injuries and secondary neuroinflammation [175]. Axonal growth of the mature CNS is inhibited mostly by molecules known as chondroitin sulfate proteoglycans (CSPGs), which include aggrecan (220 kDa), versican (260–370 kDa), and brevican (145 kDa) [176], and others such as NG2, neurocan, and phosphacan [177]. Microscopically, neuron axonal tips fail to regenerate as they become embedded with an inhibitory extracellular matrix, mainly with the increased perineuronal scarring by CSPGs [178, 179] and expression of angiopoietins during endothelial barrier breakdown [180]. While acute immunolabeling studies following SCI showed increased expression of neurocan, brevican, and versican, expression of phosphacan was decreased but later recovered and peaked at 2 months [177]. The decrease in phosphacan levels may be attributed to the activity of proteolytic enzymes that degrade the 300-kDa phosphacan such as plasmin, which also targets neurocan [103, 104]. Nevertheless, both increases and reductions of phosphacan levels have been reported likely due to the dynamic state of the traumatized matrix. Demircan et al. investigated the acute proteolysis of CSPGs in *Adams4*<sup>-/-</sup> and *Adams5*<sup>-/-</sup> knockout after SCI in mice. ADAMTS proteoglycanases, which include ADAMTS4 and ADAMTS5, degrade CSPGs and represent a potential target to reduce the inhibition of axonal growth post-SCI induced by a weight-drop technique. ADAMTS-derived 50–60-kDa aggrecan and 50-kDa brevican fragments were observed in *Adams4*<sup>-/-</sup> and *Adams5*<sup>-/-</sup> mice, but they seemed resilient to proteolysis of versican [101, 102]. This indicates that proteolysis of certain CSPGs, aggrecan and brevican, is compensated in *Adams4*<sup>-/-</sup> or *Adams5*<sup>-/-</sup> mice, whereas versican is susceptible to degradation by ADAMTS proteoglycanase family members during SCI [102]. Neutralizing CSPGs using tPA demonstrated neuroplasticity following SCI via activation of ADAMTS-4 [119, 120]. Conversely, antagonizing metalloprotease 17 (ADAM17) impaired EGFR signaling, microglia recovery and functional outcomes following SCI [181]. A study by

Shields et al. attempted to study the chronic effect of chondroitinase ABC (ChABC), an enzyme that degrades CSPGs, on promoting axonal regeneration following a C3 dorsal hemisection SCI [176]. Five ChABC injections administered intrathecally showed a decrease in expression of CSPGs, as well as a 2–3-fold increase in axonal growth in rats treated with ChABC compared with wild-type rats [176, 182]. Knockout of N-acetylgalactosaminyltransferase-1 involved in chondroitin sulfate biosynthesis resulted in relatively more recovery along with an intrinsic induction of axon regeneration in SCI mice as compared with treatment with ChABC [121]. Computational carbohydrate microarray modeling methodologies have allowed the identification of glycosaminoglycan-protein interactions and potential structural target sites [183]. Indeed, epitopes on chondroitin sulfate interact with growth factors and neurotrophins to guide the development and maintenance of the vertebrate nervous system [183]. Recently, Tran et al. developed protein tyrosine phosphatase sigma (RPTPsigma), an intracellular peptide modulator of the cognate receptor of CSPGs that was shown to significantly improve coordination and sensorimotor function in SCI rats [122]. Although the exact mechanism is speculative, RPTPsigma enhanced cathepsin-B activity digesting CSPGs. Employing antisense morpholino oligonucleotides to knockdown chondroitin-4-sulfotransferase-1 resulted in accelerated regeneration and improved locomotion [184]. RhoA GTPase inhibitors were found to induce neurite outgrowth and synaptogenesis in a rat SCI model likely mediated via the transmembrane proteoglycan N2 [123]. Likewise, simvastatin [124], epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) [125] reduced CSPG and caspase-3 levels in SCI rat models [185] with elevation of heat-shock protein (Hsp) 70 and Hsp25 [186]. Consistently, Petrovic et al. recently demonstrated neuroprotection and decreased cell death biomarker apoptosis-inducing factor (AIF) upon treatment with celastrol that also induced Hsp70 in a rat model of SCI [187]. Hsp70 plays an anti-apoptotic role by its ability to inhibit both Apaf-1-mediated caspase-dependent and AIF-mediated caspase-independent apoptosis [188, 189]. Activation of the protein tyrosine phosphatase  $\sigma$ , receptor for the inhibitory CSPG, restored serotonergic innervation and functional capacity in an impact injury SCI rat model [126, 190]. Cerebrolysin, a mixture of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF) attenuated edema and BSCB permeability. [127, 171, 191] Taken together, these observations support a potential therapeutic benefit of CSPG inhibitors following SCI.

ECM components fibronectin, laminin, and collagen IV, which are cleaved by mast cell protease 6, are upregulated in protease 6-deficient SCI mice [192] in parallel to increased TNF-alpha, IL-10, and IL-13 in protease 4-deficient mice

[193], and an overexpression of alarmins amplifying SCI secondary inflammation [194], expression of 75- and 100-kDa Dab-2 isoforms mediating cell-ECM pathways in glial cells [195], and elevation of proteolytic enzymes such as kallikreins, acrosin, myeloblastin, neutrophil elastase, and protease inhibitors such as alpha-2-macroglobulin (A2M) and pregnancy zone protein (PZP) [196]. TNF-alpha mediates activation of caspase-3 and caspase-8 post-SCI, initiating inflammatory and apoptotic pathways [197]. In terms of extravasation, the 200-kDa neural cell adhesion molecule L1 is cleaved by several enzymes generating a 70-kDa L1 fragment at 1 week post-injury [105] that induces L1-dependent neuro-proliferative, migration, survival, and myelination processes [198]. Kataria et al. identified small organic compounds as agonists of cell adhesion molecule L1 including duloxetine, phenelzine sulfate, and tacrine that serve as candidates for translational research in SCI [128]. Kallikrein 6 activation of PAR1 promoted vacuolating myelopathy and loss of MBP [113]. In a compression SCI mice model, adenovirus-mediated delivery of a proteolytically active MBP resulted in restoration of the neuro-regenerative changes as compared with proteolytically inactive MBP [199]. This highlights the innovative therapeutic platforms that may be utilized in the delivery of potential immuno-modulatory agents in an attempt to reduce tissue injury and promote a faster recovery [200].

## Cell Junction Proteins

Three MMPs, mainly MMP-2, MMP-9, and MMP-12, are proteases involved in turnover of cellular junction proteins. MMP-2 is implicated in impaired wound healing and CNS-blood vascular instability [201]. MMP-9 contributes to the disruption of the BSCB post-SCI and is responsible for the degradation of gelatin, collagen IV, V, XI, elastin, vitronectin, MBP, and other substrates [21, 50]. MMP-12, on the other hand, modulates migration of microglia into the traumatized site, leading to acute and chronic inflammation [21]. Moreover, the active 48-kDa MMP-3 emerged to play a significant role in BSCB breakdown following SCI with a peak expression at 1 day post-injury [106]. MMP-3 yielded higher levels of activated MMP-2 and MMP-9. In the moderate T9–T10 contusion SCI mice, alteration of cell junction protein levels was observed where zonula occludens-1 (220 kDa) decreased at 4–8 h, occludin (65 kDa) decreased beyond day 1, and claudin-5 (23 kDa) decreased slightly at 4–8 h with the emergence of a 17-kDa form beyond day 1 [106]. Knockout of MMP-3 or its inhibition using an MMP-3 siRNA resulted in higher levels of zonula occludens-1, occludin, and claudin-5 [106]. Cai et al. showed that the most significant period of tight junction protein loss is 6–12 h, whereas cytokines and chemokines increased in the hyper-acute period (3 h), in an oxygen-glucose deprivation/re-oxygenation model of SCI

[202]. Di-3-n-butylphthalide limited the expression of ER stress-associated proteins and degradation of adherens junction and tight junction proteins such as occludin, claudin-5,  $\beta$ -catenin, and p120-catenin [129]. Given the findings on the use of metformin in traumatic brain injury and cerebral ischemia, Zhang et al. recently showed that metformin protects the integrity of BSCB by preventing the proteolysis of tight junction proteins, occludin, claudin-5, and zonula occludens-1 along with reduced caspase-3 expression [130, 131, 203]. Metformin resulted in a downregulation of AMP-activated protein kinase (AMPK)-dependent MMP-9, ICAM-1, and neutrophil infiltration [131]. Netrin-1, a chemotropic factor with a neuroprotective role in brain ischemia, improved functional recovery after SCI by stimulating autophagy flux through inhibition of mTOR via activation of AMPK/mTOR signaling pathway, decreased caspase-3-positive neurons but increased cathepsin-D [132, 133]. Similar findings on the BSCB were reported upon treatment with erythropoietin, [134] epidermal growth factor, [135] TiO<sub>2</sub>-nanowired growth hormone [136], N-benzyl-N-ethyl-2-(7,8-oxo-2-phenyl-9H-purin-9-yl) acetamide [137], 17 $\beta$ -estradiol [138], and its agonist miR-7-1 [139], whereas NK1 receptor blockade proved to be ineffective in alleviating BSCB permeability [204]. Recently, intraperitoneal treatment with mithramycin A [140], an anti-cancer drug, and with anti-Ly6G that depletes neutrophils and monocytes attenuated BSCB breakdown by inhibiting MMP-9 expression known to degrade tight junction proteins [141]. Similarly, fluoxetine [142], vitamin C [143], and ghrelin [144] ameliorated the loss of occludin and zonula occludens-1 by inhibiting MMP activation. Since endothelin receptors guide leukocyte infiltration, blockade of these receptors reduced MMP-9 and stimulated hemoxygenase-1, a protective protease, following SCI in mice [145]. Likewise, MMP-8 inhibitor reduced the decrease in occludin and zonula occludens-1 protein expression in a moderately traumatized rat spinal cord [146] supporting the involvement of MMP-8 in inflammation and degradation of tight junctions [205]. As a result of the aforementioned, inhibition of MMPs proves to be a promising therapeutic intervention in SCI [49, 206–208].

## Ion Channels

As part of the secondary injury to the spinal cord, transient channelopathies precipitate alteration of cellular transmembrane potential and ion gradients. Indeed, cellular uptake of lucifer yellow showed that the severity of injury correlates with the degree of plasma membrane damage resulting in pericellular blebbing [209]. Calpains, cathepsins, caspases, and other cysteine proteases require intracellular calcium for activation [210]. Since calcium influx is involved in SCI neurotoxicity, the hypothesis of using calcium channel blockers as a neuroprotective intervention yielded favorable outcomes

[211]. Omega-conotoxin, a selective voltage-dependent calcium channel inhibitor, attenuated caspase-3 and subsequent neuronal cell death in a weight-compression SCI rat model [147]. Calcium-free perfusate abolished glutamate-mediated white matter damage [212]. The G protein-coupled P2Y<sub>2</sub> receptors found on astrocytes increase intracellular calcium and stimulate cell migration and proliferation [213]. In SCI, the 42-kDa P2Y<sub>2</sub> was upregulated, whereas the 62-kDa P2Y<sub>2</sub> was downregulated likely due to internalization rather than degradation [214, 215]. Inhibition of sodium-calcium exchanger using bepridil or KB-R7943 demonstrated improved functional recovery and decreased degradation of calpain-targeted spectrin proteins [148]. Inhibition of calpain using AK 295 improved neurologic function in SCI rats and limited calpain-dependent apoptosis [149]. mAcid-sensing ion channel 1a (ASIC1a), a proton-gated ion channel that mediates sodium and calcium influx, found on most neurons is activated in conditions of acute hypoxia in SCI inducing mitochondrial dysfunction and activation of apoptotic pathways [216]. Inhibition of ASIC1a using the spider-venom peptide PcTx1 improved functional outcomes in thoracic SCI rat model after which degadomic BDPs up to 10 kDa persisted till day 4 post-injury [107]. Inhibition of L-type calcium channel protein  $\alpha$ 1C using miR-7-1 triggered cell survival signaling and potentiated estrogen neuroprotection likely via downregulation of Bax and upregulation of Bcl-2 [139].

Targeting ion channels is instrumental not only to reduce SCI pathophysiology but also to control symptomology post-SCI. Persistent sodium current (I-NaP) underlies spasticity observed following SCI [108]. This was found to be related to calpain-mediated proteolysis of the 250-kDa voltage-gated sodium (Nav) 1.6 channels into a 120-kDa band in spinal cord transected rats leading to increased I-NaP [108]. This is in contrast to a downregulation of Nav 1.1 as soon as 3 h [165] and an upregulation of Nav 1.3 channels following a transient decrease at 48 h after impact [217, 218]. Treatment with riluzole or inhibition of calpain-1 and calpain-2 activity using MDL28170 reduced Nav channel cleavage and I-NaP and relieved spasticity. Thus, a tight relationship between the proteolysis of Nav 1.6 channels by calpain, the upregulation of I-NaP, and the observed spasticity following SCI, which demonstrates the potential of alleviating spasticity post-SCI through inhibition of calpain-mediated proteolysis of Nav 1.6 channels [219]. Likewise, activation of the potassium-chloride cotransporter (KCC2) using prochlorperazine alleviated SCI-induced spasticity [150, 220]. Activation of 5-HT<sub>2A</sub> receptors using (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide increased expression of KCC2 and reduced neuropathic pain [151]. Since MMPs destroy the myelin sheath, it was assumed that the resulting instability activates the nociceptive circuitry in A $\beta$ -fibers and results in pain from mild tactile stimulation [221].

Following peripheral nervous system damage, MMPs act on small unmyelinated C-nociceptive and thinly myelinated A $\delta$ -afferents, resulting in the loss of electrical stability and damaging the structural and functional integrities of the myelin sheath, the process that was hypothesized to cause pain [222]. Liu et al. studied the role of MMPs in initiating pain post-SCI in rats [109]. Using mass spectrometry, the degradation of MBP by MMP-9 generated MBP84–104 and MBP68–86 degradomic peptides elevated during the first week after impact, with a variety of fragments with 14.5-kDa, 10.2-kDa, 8.3-kDa, 7.3-kDa, 7.0-kDa, and 6.3-kDa masses [109]. Compared with brain injury, a narrower spectrum of MBP BDPs were found limited to only the 8- and 10-kDa fragments [5]. The injection of the degradomic peptides into a healthy spine caused mechanical allodynia similar to that caused by SCI. Thus, this study implicated MBP BDPs as propagators of pain following SCI under the direct influence of MMP-9, whose inhibition seems to have potential therapeutic benefit, and whose activity peaks 1 day post-SCI [45, 46, 48–50, 109, 182, 223, 224]. Treatment with bumetanide, a chloride regulatory protein sodium-potassium-chloride (NKCC1) inhibitor, in a contusion SCI rat model increases withdrawal latency time and proves to be a potential candidate for alleviating NKCC1-mediated spinal cord edema and neuropathic pain [152]. Consistent with the fact that estrogen is neuroprotective in SCI [139], tamoxifen, a selective estrogen receptor modulator that readily crosses the BBB, reduced the extent of demyelination, upregulated aquaporin 4 (AQP4) water channels implicated in edema formation [153] and improved locomotor recovery [154]. Interestingly, higher AQP4 levels were associated with better functional recovery after SCI [225] while AQP4 knockout impaired locomotor recovery [226]. Other ion channels known to be altered in SCI but whose BDPs remain to be uncovered include Kv1.1/2 (axonal conduction), Kv1.4 (oligodendrocyte proliferation), NCX (import calcium and downstream inflammatory cascades), NMDA (neuron death), AMPA (cytoskeletal degradation and subsequent myelin disruption), and GABA- $\alpha/\beta$  receptors (membrane hyperpolarization). Inhibition of these channels by 4-AP (potassium channels), lidocaine (voltage-gated sodium channels), pregabalin (voltage-gated calcium channel with  $\alpha 2\delta 1$  subunit), gabapentin (NMDA receptor), and baclofen (GABA- $\beta$  receptor) yielded favorable outcomes in SCI clinical trials [211, 227].

### Perspective: The Clinical Endpoint

The ultimate goal of biomarker discovery is accurate diagnosis and effective treatment. Over the past decades, a growing interest emerged in understanding the chain of events post-primary injury in terms of cell to cell communication and spread of the insult to the nearby healthy tissue. Generating

a bedside unique profile of biomarkers for every patient should enhance patient characterization (e.g., severity and pathophysiology of the secondary injury), enable targeted and personalized management and treatment plan, and optimize prognosis and outcome prediction. While biomarkers themselves are not directly involved in therapy, we, however, hope to reduce either the generation of these biomarkers by inhibiting the proteases by which they are generated or the effect of these neoproteins on downstream pro-inflammatory pathways. The translational potential of biomarker research requires multidisciplinary input, from molecular biologists and analytical chemists to biomedical engineers, healthcare personnel, and policy makers. In the developing world, the lack of sophisticated technology makes of a point-of-care biomarker assay a highly needed and an efficient method to aid bedside clinical decisions. Despite animal models of SCI have substantially increased our knowledge, further studies with valid and reproducible traumatic SCI models as well as reliable statistical plans are warranted to delineate the temporal and spatial alterations of SCI biomarkers and to support clinical translation. Other challenges include the lack of correlation between animal models of SCI and their clinical counterparts besides the altered bioavailability of pharmacological agents within the CNS milieu itself, researchers still believe that new associations will eventually be uncovered. As it stands, establishing guidelines for biomarkers in the care of patients with SCI is far-fetched.

Numerous therapies have been tested preclinically ranging from immunotherapies that target mTOR signaling to anti-neurotoxic therapies targeting the effect of excess glutamate such as NMDA-receptor antagonists. The increasing number of available therapies raises the question which is the most worthy to invest in. The consistent mild to moderate effect of existing therapies on functional outcomes make it challenging to decide which of the listed interventions are worthwhile to pursue. Potentially promising therapies are direct inhibitors of proteases such as AK 295 inhibiting calpain [53] and SB-3CT inhibiting MMP-2/MMP-9 [149]. However, since a surge of calcium influx is the main trigger of protease activation in the first place, therefore, calcium channels are promising upstream targets [148]. Additionally, some features such as dosage, treatment duration, and route of administration may favor a specific therapeutic intervention over another. For example, Li et al. administered neuroserpin intrathecally [60], whereas Zheng et al. injected EGF subcutaneously [135]. Altogether, further comparative clinical trials are needed to determine treatment superiority.

Technical nuances are challenging to overcome in proteomics since researchers tend to focus on proteins whose roles are already defined leading to overlooked potentially revolutionary biomarkers, such as microRNAs and bioactive lipids, which are kept unidentified. Specifically, for lipids, these were originally understood to act as metabolites in the

de novo biosynthesis of phospholipids; however, recent evidence suggests that lysophospholipids carry signaling properties or growth factor-like effects. Nowadays, quantitative high-resolution two-dimensional (2D-PAGE-isoelectric focusing and SDS-PAGE) protein separation technique followed by matrix-assisted laser desorption/ionization (MALDI)-TOF mass spectrometry  $\pm$  nano ESI-MS/MS is the most commonly used and have yielded the identification of novel biomarkers in SCI. Relying on complementary systems biology is helpful in translating the altered proteins into a predictable model with a representative global network. Taking it one step further from a 2-dimensional identification, a 3-dimensional MALDI imaging to assess molecular and cellular processes in both spatial and temporal dimensions resulted in the identification of key systems biology-based functional pathways. Technologies aiming at deciphering protein-protein interactions such as the two-hybrid screening assay, become instrumental in further validating protease-substrate repertoires. These techniques have been rarely used in the context of experimental SCI but have yielded chemo-dynamic insights into the interaction of caspases with their substrates at the basic level. This proteomic profiling often includes the high-throughput protein microarrays to elucidate biochemical activities with unprecedented scale which also allows simultaneous functional screening of high numbers of proteins. Other proteomic approaches include liquid-phase isoelectric focusing (IEF) as well as isobaric tag for relative and absolute quantitation (iTRAQ) and multidimensional protein identification technology (MudPIT) both of which can be combined with liquid chromatography to separate protein complexes and concentrate low-abundance proteins. The majority of the techniques used by the studies reviewed here are limited to western blotting proteome analysis highlighting the need to integrate sophisticated structural and biochemical methodologies in SCI degradomics. Additionally, Liu et al. have utilized a novel high-throughput immunoblotting (HTIB) approach which embeds  $\sim$ 1000 monoclonal antibodies (PowerBlot<sup>TM</sup>) screening for proteins at their native molecular weight and their potential breakdown constituting the degradome profile in the context of brain injury; however, this approach has not been assessed in SCI [166]. Integrated spatio-temporal big data leads to a better understanding of the injury biochemistry. Nevertheless, with the advanced technology used in biomarker characterization such as mass spectrometry and high-throughput proteomics including large-scale analyses in both bottom-up (peptide-level) and top-down (protein-level) proteomics, employing a more comprehensive approach is mandated to fully elucidate differential profiles of proteins involved in SCI. Big data stratification is therefore a highly regarded option that may be utilized for identification of diagnostic biomarkers as well as exploring potential therapeutic targets.

## Concluding Remarks

A large body of research has been dedicated to elucidate the degradomic natural history of SCI. Proteolysis is essential for numerous cellular functions including cell-cycle progression, cellular differentiation, tissue regeneration, apoptosis, and aging; however, a dysregulated proteolytic cascade results in detrimental pathophysiological conditions. Calpains, caspases, MMPs, cysteine proteases, aspartyl proteases, GS and serine proteases, have all been implicated SCI, with some being upregulated and others downregulated. There has been an increasing abundance of published biomarker profiles of SCI summarizing inflammatory cytokines [228] and neuroglial peptides [229] as well as microRNAs [230], yet this is the first to detail the degradomic profile of SCI, the pathogenesis of encryptic neoprotein generation while simultaneously highlighting interventions with promising acute and long-term outcomes. In this review, the protease-substrate repertoires and various pharmacological interventions have been reviewed in the scope of neurotrauma. Fragmentation of key proteins suggests an underlying mechanism of pathogenesis throughout the course of SCI. It is worth mentioning that the surveyed degradomic profile is not SCI-specific and more caution should be observed when assessing these degradomic profiles due to their overlap with other neurodegenerative disorders. In addition, we have previously reported biomarkers in other diseases of the CNS such as multiple sclerosis as well as traumatic brain injury that substantially overlap with SCI. Being a relatively new field, the translational and therapeutic applications of this knowledge obtained so far warrants further research. As a promising omics field, degradomics provides key signature BDPs generated from the fragmentation of bioactive molecules. Degradomics therefore presents a new path for the identification and characterization of encryptic biomarker neoproteins in addition to key targets for drug design and development.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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