

1 INVESTIGATION OF THE BLOOD PROTEOME IN RESPONSE TO SPINAL CORD INJURY IN RODENT
2 MODELS

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14 **KEYWORDS:** Spinal cord injury; SCI; iTRAQ proteomics; complete transection SCI;
15 contusion SCI

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32 **ABSTRACT**

33 **STUDY DESIGN:** Explanatory and Mechanistic Study

34 **OBJECTIVES:** A better understanding of the 'whole-body' response following spinal cord injury (SCI)
35 is needed to guide future research aimed at developing novel therapeutic interventions and identifying
36 prognostic indicators for SCI. This study aimed to characterise the blood proteome following
37 contusion or complete SCI compared to a sham injury in rat models.

38 **SETTING:** United Kingdom

39 **METHODS:** Pooled blood samples from one and seven days after a contusion (serum; n=5) or from
40 14 days and 112 days post-complete transection SCI (plasma; n=8) and their sham-injured
41 counterparts were subjected to independent iTRAQ nanoflow liquid chromatography tandem mass-
42 spectrometry proteomic analyses. Pathway analyses of the proteins that were differentially abundant
43 between SCI and their matched sham injured counterparts were completed to indicate biological
44 pathways that may be changed in response to SCI.

45 **RESULTS:** Eleven and 42 proteins were differentially abundant ($\geq \pm 2.0FC$; $p \leq 0.05$) between the
46 contusion SCI and sham injured animals at 24h and seven days post-injury, respectively. Seven and
47 12 proteins were differentially abundant between complete and sham injured rats at 14 and 112 days
48 post-injury, respectively. Acute-phase response signalling and Liver X Receptor/Retinoic X Receptor
49 activation were identified as differentially regulated pathways in both models of SCI.

50 **CONCLUSIONS**

51 We have utilised longitudinal preclinical SCI models to provide an insight into the blood proteome
52 changes that result following SCI and to highlight a number of biological pathways of interest for
53 future studies.

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58 INTRODUCTION

59 Many clinical and scientific attempts have been made to better diagnose and to develop new
60 treatments to improve the prognosis of individuals following a spinal cord injury (SCI). There is
61 growing evidence that measures of some haematological indices post-injury relate with longer term
62 (12 month) clinical outcome measures in human SCI patients [1,2]. These studies indicate that over-
63 time the 'whole-body', multi-organ response to SCI contributes to long-term clinical outcome and that
64 proteins within the blood can be used to assess this phenomenon [1,2]. Therefore a better
65 understanding of the longitudinal protein changes within the blood following a SCI is needed to
66 develop novel interventions [3].

67 The majority of studies that have aimed to understand protein changes that occur following a SCI,
68 have focused upon changes within the SC tissue itself [3]. We have previously reviewed the few
69 studies that have assessed the protein response to SCI in either the blood or cerebrospinal fluid
70 (CSF) of SCI animal models or humans with a SCI [3]. It is particularly desirable to identify markers
71 that can be measured systemically as blood samples can be collected immediately after injury with
72 fewer associated risks compared to CSF samples. Having a better understanding of the proteome
73 response to SCI in blood, will aid in the development of novel interventions for SCI, as well as to
74 identify candidate biomarkers that may have the potential to better determine SCI severity or predict
75 clinical outcome of human with a SCI [4].

76 Several well-characterised pre-clinical SCI models exist which aim to represent the different common
77 human SCI situations, contusive (blunt force) or penetrative (stab) injuries. In animals contusion
78 injuries are commonly modelled using specialised devices that deliver an impact of calibrated force to
79 the surgically exposed spinal cord [5,6]. Penetrative injury, in humans more commonly leads to
80 complete SCI, in which a variable cross section of the spinal cord is transected [7]. Surgical exposure
81 and complete incision across all SC tracts is commonly performed in these animal models using
82 either microscissors or a scalpel blade under visual control [8]. Rodent models are invaluable in
83 studying the underlying mechanisms of SCI, as there is evidence that they undergo similar biological
84 processes to humans following injury [9] and allow for comparison to sham injured animals (via
85 surgical exposure of, but no injury to, the SC) whereas such comparator control groups are difficult to
86 identify in the human situation.

87 Proteomics is a powerful analytical tool that can be used to profile large numbers of proteins in an
88 untargeted manner. We have assessed the blood proteome of rat models of both a contusion and a
89 complete transection SCI to determine whether we can detect differences between SC injured and
90 sham injured rodents.. Consideration of the blood proteome in such pre-clinical injury models could
91 provide a more in-depth understanding of the biochemical response to SCI compared to sham injury
92 and how these responses change over time in each independent model.

93 **METHODS**

94 **Animals used in the study**

95 All experimental procedures were approved by the Ethical Review Panel of the University of Glasgow
96 and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and adhering to our
97 recommended good practice for SCI rodent models [10]. Blood samples were collected surplus to the
98 study outcomes and were used in this study to maximise the output from animal experiments in the
99 spirit of the 3R's of animal use (replacement, refinement and reduction) [11]. Blood samples were
100 obtained at 1 day (n=5) or 7 days (n=5) after a contusion injury performed at the C6 level [12] or at 14
101 days (n=8) or 112 days (n=8) after a complete spinal cord transection performed at the T9 level [13].
102 Control blood samples were obtained from animals that underwent sham surgery performed at the
103 same spinal level. Further details of the animal experiments are included in the supplementary
104 material (appendix 1).

105 **Isobaric Tag for Relative and Absolute Quantitation proteomics**

106 Two independent proteomic experiments were performed; one for the contusion SCI (serum
107 samples) and the other for the complete SCI (plasma samples) models. Briefly, samples were pooled
108 from the different injury and timepoint groups and prepared and analysed using isobaric tagging for
109 relative and absolute quantitation (iTRAQ) proteomics, as described previously [14] and detailed in
110 the supplementary material (appendix 1). Proteins which were differentially abundant between the
111 SCI and matched sham counterparts were selected where $p < 0.05$ and the fold change was $\geq \pm 2.0$ FC.

112 **Pathway analysis of proteomic datasets**

113 Proteins were analysed using the pathway enrichment and topological analysis tools in Ingenuity
114 (Qiagen, US) [15] to identify and visualise the canonical pathways which are differentially effected
115 between SCI and sham injured rats at each of the timepoints of the study and in the different SCI
116 models. Functional annotations that were assigned a p-value > 0.05 , as assessed using a Fisher's
117 exact test were removed from the list.

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RESULTS

Differential Abundance of Proteins in the Contusion versus Sham Injured Animals

All the proteins identified in the proteomic analysis of serum from contusion SCI or sham counterparts are included in supplementary proteomic table 1. Eleven proteins demonstrated ≥ 2.0 fold differential abundance in the serum of contusion SC injured compared to sham injured rats at one day post-injury (Table 1). At seven days post-injury 42 proteins were differentially abundant between contusion and sham injured rodents (Table 1). Three of these proteins demonstrated common changes in abundance at both timepoints; Fibrinogen alpha chain isoform 1 and haemoglobin subunit alpha-1/2 had increased and C9 Protein had decreased abundance across time i.e. at one day and seven days post-injury (Table 1). Supplementary proteomic tables 2 and 3 detail all the proteins identified with ≥ 2 peptides and any ratio between SCI and sham counterparts at 24 hours and seven days post-injury, respectively.

Differential Abundance of Proteins in the Complete versus Sham Injured Animals

Supplementary proteomic table 5 details all the identified proteins following iTRAQ proteomic analysis of plasma from the complete SCI and sham counterpart animals. In serum collected at 14 days post injury, a comparison of complete SC injured compared to sham injured rats identified seven proteins that were differentially abundant ($\pm 2.0FC$), three of which increased in abundance after SCI (Table 2). Twelve serum proteins demonstrated a differential abundance at 112 days after a SCI compared to sham injury, with two proteins showing increased abundance and 10 showing decreased abundance (Table 2). Supplementary proteomic tables 6 and 7 detail all the proteins identified with ≥ 2 peptides and any ratio between SCI and sham counterparts at 14 days and 112 days post-injury, respectively.

Biological Functions associated with the Protein Changes Identified

In an aim to better interpret what the large numbers of identified protein changes related to in terms of biological function, pathway analysis was performed. Biological pathways which had significant numbers of differentially abundant proteins connected with them (determined by Fisher's exact test) could be identified.

Several functional pathways were associated with the protein changes identified in the plasma of rats with a contusion SCI compared to sham injured rats in the acute (1 day) phase of injury (Figure 1).

The most significant functional pathways were: acute phase response signalling ($p = 1.1 \times 10^{-13}$;

148 predicted inhibition); liver X receptor/ retinoic X receptor (LXR/RXR) Activation ($p = 1.8 \times 10^{-10}$);
149 coagulation system ($p = 9.6 \times 10^{-9}$) and intrinsic prothrombin activation pathway ($p = 1.1 \times 10^{-6}$). Several
150 of these pathways were still effected at a more sub-acute phase of injury (7-days post-injury); namely,
151 LXR/RXR activation ($p = 6.0 \times 10^{-17}$), acute phase response signalling ($p = 1.6 \times 10^{-13}$) and the
152 coagulation system ($p = 7.2 \times 10^{-15}$). Other pathways, however, were indicated as altered only at seven
153 days post-injury, such as the extrinsic prothrombin activation pathway ($p = 5.9 \times 10^{-12}$) (Figure 1).

154 The same analyses were performed based on the protein changes detected within the serum of
155 rodents with a complete SCI. Pathways that were associated with protein changes seen at two weeks
156 post-injury included LXR/RXR activation ($p = 3.5 \times 10^{-12}$) and acute phase-response signalling ($p = 3.0$
157 $\times 10^{-9}$), the complement system ($p = 4.1 \times 10^{-4}$) and Clatherin-mediated endocytosis signalling ($p = 4.27$
158 $\times 10^{-4}$) (Figure 1). Clatherin mediated endocytosis was also altered based upon protein changes seen
159 at 112 days following a complete injury ($p = 1.1 \times 10^{-4}$), along with acute phase response signalling (p
160 $= 1.5 \times 10^{-8}$), the extrinsic prothrombin activation pathway ($p = 3.0 \times 10^{-5}$) and the coagulation system (p
161 $= 1.5 \times 10^{-4}$) (Figure 1).

162 These biological pathways may provide targets for future work aimed at targeting and/or developing
163 therapies for SCI specific responses. Furthermore, these analyses help to convey whether specific
164 biological functions are altered over time.

DISCUSSION

We have presented one of the first studies to assess how the blood proteome changes after SCI in both contusive and complete transection SCI models. This investigation has presented a unique opportunity to determine the blood protein response to SCI in order to greater understand the mechanisms underlying SCI and repair.

One of the limitations of this study is that blood samples were collected from animals at different timepoints following SCI and different blood fractions were collected (plasma and serum). Therefore, this study does not aim to directly compare between the two different models but rather to demonstrate that following SCI there are protein changes within the blood, which can be detected in either blood fraction: serum or plasma. The range of timepoints in these pre-clinical models has allowed for the blood proteome to be assessed longitudinally, from the acute phase (1 day post-contusion SCI) to the chronic phase (112 day post-complete SCI) of injury. These proteomic analyses highlight that there is a differential response to SCI compared to sham injury , which can be detected in the blood in these rodent models over-time.

Pathway analyses were used to provide a better understanding of what the proteome changes might relate to in terms of biological response to injury. These analyses allow for the assessment of biological functional changes which present a more global response within the blood, and therefore commonality in functional pathway changes across time, irrespective of the blood fraction and injury severity (contusion or complete) indicates that these functional pathways are likely a specific response to SCI and not sham injury and can be taken forward for further study with much greater confidence. Moreover when the results from the two rodent models are assessed independently, these novel proteomic datasets present scientists with an understanding of the systemic response to two widely used SCI models.

In the contusion model, only 11 proteins showed differential abundance, based on our selection criteria, between the injured and the sham injured animals at 24 hours post-injury. This limited number of alterations based on our fold change and p-value cut-offs, along with the large number of common proteins identified in both the injured and sham sera, indicates that many of the protein changes that do occur as an immediate response to injury could be common to both SCI and sham injuries. Of the proteins that were most highly differentially abundant in SCI compared with sham injury at this acute

194 timepoint, several were associated with the haematological system and haemostasis e.g. fibrinogen
195 alpha chain isoform 1; haemoglobin subunit alpha 1/2; an observation that is strengthened by our
196 pathway analysis, which also associated protein changes in this timeframe with the coagulation
197 system and the intrinsic prothrombin activation pathway. These findings indicate that there is a
198 response to SCI which can be detected within the blood immediately after injury and highlights the
199 potential of assessing proteins which are not associated with the SCI itself, e.g. neuronal or glial
200 related proteins, but rather that there may be a 'whole-body', multi-organ response that is different in
201 response to SCI compared with sham injury. Interestingly, by seven days post-injury high numbers of
202 differentially abundant proteins were seen, many of which displayed large FC differences in
203 abundance, thus indicating that there may be value in measuring blood proteins through the acute
204 phase of injury.

205 Studying the plasma proteome of rats following a complete injury demonstrated that up to 112 days
206 after injury protein differences can be observed between SCI and sham injured animals. These
207 proteins can provide an insight into the potential reparative and/or degenerative processes that
208 continue longitudinally post-SCI. As with the contusion model, our pathway analyses have highlighted
209 that changes within the blood occur in response to SCI, such as in the extrinsic prothrombin activation
210 pathway and the coagulation system, and this model highlights that these may go on being implicated
211 for a long period of time following injury.. We have previously demonstrated that red blood cell (RBC)
212 measures (RBCs, haematocrit and mean cell haemoglobin) in the blood of humans with SCI at 7±4
213 days post- SCI correlate with initial AIS motor and sensory scores [1], confirming that systemic
214 release of red blood cell measures may be indicative of injury severity.

215 Changes in acute phase response signalling were identified at both the sub-acute (14 day) and
216 chronic (112 day) timepoints following complete injury and the acute (1 day) and sub-acute (7 day)
217 timepoints following contusion injury, highlighting that changes in the acute phase response continue
218 longitudinally post-SCI. Acute phase response signalling is the first systemic response to trauma [16],
219 therefore it is unsurprising that in the case of SCI, this signalling pathway is activated. This signalling
220 cascade is triggered by inflammatory cytokines which leads to drastically changed protein synthesis
221 by the liver and resultant release of the acute phase proteins [16]. Therefore this signalling pathway
222 provides an attractive target for novel therapies, potentially by targeting upstream inflammatory

cytokines using biologic therapies [17]. Changes in this pathway, along with identified changes in LXR/RXR activation, add strength to our previous findings [1] also highlighting the importance of considering the effect of SCI on other organ systems aside from the nervous system, such as the liver, in contributing to clinical outcome following SCI. In future studies, it would be interesting to assess whether differences in acute-phase response signalling in the different phases of injury exist between individuals with varying injury severities and/or who either do or do not show long-term neurological improvements.

This study presents the first unbiased assessment of the blood proteome in rodent models of SCI. A large number of proteins have been identified which, with further study, may have the potential to inform on the type and severity of injury from the acute through to the chronic phase of SCI. Moreover, we have identified biological mechanisms that are associated with these protein changes, thus providing a better understanding of the blood proteome changes that occur in response to SCI.

STATEMENT OF ETHICS

We certify that all applicable Institutional and governmental regulations concerning the ethical use of human volunteers/animals were followed during the course of this research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CHH, HRF, KTW & JR conceptualised and designed the study. CHH, SLS, CHB & JR collected data which was then analysed and interpreted by CHH, HRF, KTW, SLS, CHB & JR. CHH, HRF, JR, AO, SLS, CHB & KTW drafted the manuscript, critically revised and approved the final article. Funding for the study was obtained by KTW, HRF & AO. All authors read and approved the final manuscript.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [18] partner repository with the dataset identified PXD021137.

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323 **FIGURES**

324 Figure 1: Heat map demonstrating canonical pathways that were most significantly enriched in spinal
325 cord injured compared to sham injured rats. The significance of the association of a given
326 canonical pathway and the differentially abundant proteins was measured using Fisher's exact
327 test. The heat map demonstrates the strength of the $-\log P$ value related to the Fisher's exact
328 test for each pathway in each biological comparator group.

329 **TABLES**

330 Table 1: Protein changes in rat blood following contusion spinal cord injury compared to sham injury
331 at either 1 day or 7 days following injury

332 Table 2: Protein changes in rat blood following complete spinal cord injury compared to sham injury at
333 either 14 days or 112 days following injury.

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Key:
-logP value

$\times 10^{20} - \times 10^{30}$

$\times 10^{10} - \times 10^{19}$

$\times 10^5 - \times 10^9$

$\times 10^{2.5} - \times 10^{4.9}$

$\times 10^1 - \times 10^{2.4}$

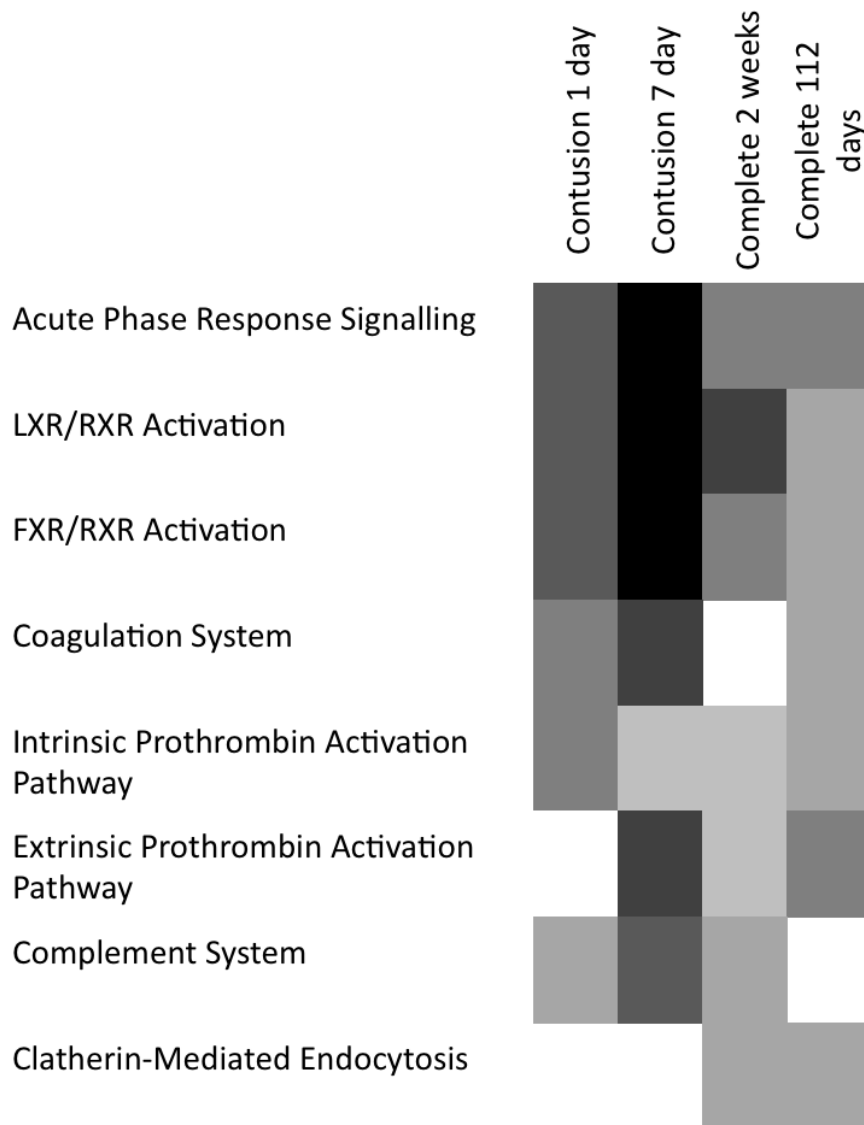


Table 1: Protein changes in rat blood following contusion spinal cord injury compared to sham injury at either 1 day or 7 days following injury

	Name	Protein ID	1 day SCI : 1 day sham	7 days SCI : 7 day sham	FDR (p-value) (a) 1 day SC: 1 day sham; (b) 7 day SC: 7 day sham
Up in SCI	Fibrinogen alpha chain isoform 1	FGA	8.3	10.5	(a) 0.0007; (b) 0.0005
	Hemoglobin subunit alpha-1/2	HBA-1	8.3	3.8	(a) 0.0002; (b) 0.001
	Beta-2-glycoprotein 1	APOH	3.5		0.03
	Angiotensinogen	AGT/SERPINA8	2.6		0.02
	Immunoglobulin kappa light chain variable region	IGKV		12.9	0.03
	Prealpha-2-macroglobulin	A2M		10.8	0.03
	Fibrinogen beta chain precursor	FGB		7.9	0.02
	Myosin-11	MYH-11		6.0	0.04
	Fibrinogen gamma chain	FGG		5.75	9.48x10 ⁻⁹
	Serine protease inhibitor A3M	Serpina3m		3.4	0.001
	Antithrombin-III	SERPINA1		2.6	0.0002
	LOC500183 protein	LOC500183		2.5	0.03
	Interleukin-1 receptor accessory protein isoform b	IL1RAPb		2.4	0.01
	Heparin cofactor 2	SERPIND1		2.4	0.01
	Gamma-2a immunoglobulin heavy chain	Igg-2a		2.4	0.002
	Attractin	ATRN		2.3	0.04
	Immunoglobulin gamma-2b, partial	Igh-1a		2.2	0.006
	Antithrombin-III	SERPINC1	-3.9		2.72E-05
	Complement component 4, gene 2	C4b	-3.5		0.04
	Gelsolin	GSN	-3.4		0.0002
	Complement C5	C5	-3.3		2.07E-08
	C9 protein	C9	-2.8	-8.5	(a) 0.0001; (b) 6.87E-05
	Serum amyloid P-component	APCS	-2.5		0.002
	Plasma kallikrein	KLKB1	-2.4		0.005
	Ab1-018	Ab1-018		-20.5	0.0003
	C-reactive protein	CRP		-16.1	0.005
	Alpha-1-acid glycoprotein	ORM1		-13.7	0.01
	Vitamin D-binding protein	Gc		-9.8	1.17E-09
	C4b-binding protein alpha chain	C4BP1		-9.6	1.99E-05
	Inter-alpha-inhibitor H4 heavy chain	ITI4		-9.4	3.88E-05
	Apolipoprotein E	APOE		-8.1	0.0002
	Alpha-1-antiproteinase	SERPINA1		-7.8	0.0004
	Complement factor H	CFH		-7.5	1.77E-11
	Complement component C6	C6		-6.3	6.26E-06

	Fibronectin 1	FN1		-5.9	1.80E-07
	Major urinary protein	Mup		-5.8	0.002
	Complement C1S subcomponent	C1s		-5.8	0.0009
	Plasma protease C1 inhibitor	SERPING1		-5.5	0.0008
	Clusterin	Clu		-4.9	0.009
	Apolipoprotein C-I	APOC1		-4.5	0.02
	Apolipoprotein B-100	APOB		-4.1	6.44 x10 ⁻¹⁴ 6
	Fetub protein	FETUB		-3.5	0.001
	Apolipoprotein A-I preproprotein	Apoa1		-3.0	0.008
	Angiotensinogen	Agt		-2.7	0.027
	Afamin, isoform CRA_c	Afm		-2.6	9.02 x10 ⁻⁸
	Complement C4	C4		-2.3	0.004
	Lumican	LUM		-2.2	0.05
	Complement component C7	C7		-2.2	6.08 x10 ⁻⁵
	Pigment epithelium-derived factor	SERPINF1		-2.1	0.002
	Vitamin K-dependent protein S	PROS1		-2.0	0.002

Abbreviations: FDR, False discovery rate; SCI, spinal cord injury

Table 2: Protein changes in rat blood following complete spinal cord injury compared to sham injury at either 14 days or 112 days following injury.

	Protein	Protein ID	14 day SCI: 14 day Sham	112 day SCI: 112 day Sham	FDR (P-value)(a) 14 day SCI: 14 day sham; (b) 112 day SCI: 112 day sham
Up after SCI	14-3-3 protein zeta/delta	YWHAZ	14.5		0.03
	Ceruloplasmin	CP		2.0	0.001
	Serine protease inhibitor A3K	SERPINA3k	2.1		0.04
	Serine protease inhibitor A3L	SERPINA3L	2.1		0.008
	Serum amyloid P-component	APCS		1.6	0.04
Down after SCI	Actin, cytoplasmic 2	ACTG1		-3.2	0.0001
	Alpha-1-inhibitor 3	A1i3	-3	-4.6	(a) 0.0001; (b) 2.2x10 ⁻⁶
	Alpha-2-HS-glycoprotein	AHSG	-3.6		0.009
	Apolipoprotein A-IV	APOA4		-2.6	8.1x10 ⁻⁶
	Apolipoprotein B-100	APOB		-2.1	0.001
	Fibrinogen beta chain	FGB		-1.5	0.007
	Fibrinogen gamma chain	FGC		-1.6	0.006
	Fibronectin	FN1	-2.9	-4.1	(a) 1.3x10 ⁻⁷ ; (b) 6.2x10 ⁻¹¹
	Gelsolin	GSN		-2.3	0.04
	Murinoglobulin-1	Mug1		-3	4.5x10 ⁻⁵
	Murinoglobulin-2	Mug2		-1.7	0.0008
	Vitamin D-binding protein	GC	-4.7		7.9x10 ⁻⁵

Abbreviations: FDR, False discovery rate; SCI, spinal cord injury