

<sup>1</sup> **A comprehensive proteomic and  
2 bioinformatics analysis of human  
3 spinal cord injury plasma identifies  
4 proteins associated with the  
5 complement cascade as potential  
6 prognostic indicators of neurological  
7 outcome**

<sup>8</sup> **1 Abstract**

<sup>9</sup> Introduction

<sup>10</sup> Spinal Cord Injury (SCI) is a major cause of disability, with complications post-injury often leading  
<sup>11</sup> to life-long health issues with need of extensive treatment. Neurological outcome post-SCI can be  
<sup>12</sup> variable and difficult to predict, particularly in incomplete injured patients. The identification of  
<sup>13</sup> specific SCI biomarkers in blood, may be able to improve prognostics in the field. This study has  
<sup>14</sup> utilised proteomic and bioinformatics methodologies to investigate differentially expressed pro-  
<sup>15</sup> teins in plasma samples across human SCI cohorts with the aim of identifying prognostic biomark-  
<sup>16</sup> ers and biological pathway alterations that relate to neurological outcome.

<sup>17</sup> Methods and Materials

<sup>18</sup> Blood samples were taken, following informed consent, from ASIA impairment scale (AIS) grade C  
<sup>19</sup> "Improvers" (AIS grade improvement) and "Non-Improvers" (No AIS change), and AIS grade A and D  
<sup>20</sup> at <2 weeks ("Acute") and approx. 3 months ("Sub-acute") post-injury. The total protein concentra-  
<sup>21</sup> tion from each sample was extracted, with pooled samples being labelled and non-pooled samples  
<sup>22</sup> treated with ProteoMiner™ beads. Samples were then analysed using two 4-plex isobaric tag for  
<sup>23</sup> relative and absolute quantification (iTRAQ) analyses and a label-free experiment for comparison,  
<sup>24</sup> before quantifying with mass spectrometry. Proteomic datasets were analysed using **bioinfor-**  
<sup>25</sup> **matics...**

<sup>26</sup> Proteins of interest identified from this analysis were further validated by enzyme-linked im-  
<sup>27</sup> munosorbent assay (ELISA). OpenMS (version 2.6.0) was used to process the raw spectra data. R  
<sup>28</sup> (version 4.1.4) and in particular, the R packages MSstats (version 4.0.1), STRINGdb (version 2.4.2)  
<sup>29</sup> and pathview (version 1.32.0) were used for downstream analysis.

<sup>30</sup> Results

<sup>31</sup> The data demonstrated proteomic differences between the cohorts, with the results from the  
<sup>32</sup> iTRAQ approach supporting those of the label-free analysis. A total of 79 and 87 differentially  
<sup>33</sup> abundant proteins across AIS and longitudinal groups were identified from the iTRAQ and label-  
<sup>34</sup> free analyses, respectively. Alpha-2-macroglobulin (A2M), retinol binding protein 4 (RBP4), serum  
<sup>35</sup> amyloid A1 (SAA1), Peroxiredoxin 2, alipoprotein A1 (ApoA1) and several immunoglobulins were

36 identified as biologically relevant and differentially abundant, with potential as individual prognos-  
37 tic biomarkers of neurological outcome. Bioinformatics analyses revealed that the majority of dif-  
38 ferentially abundant proteins were components of the complement cascade and most interacted  
39 directly with the liver.

40 **Conclusions**

41 Many of the proteins of interest identified using proteomics were detected only in a single group  
42 and therefore have potential as a binary (present or absent) biomarkers. Additional investigations  
43 into the chronology of these proteins, and their levels in other tissues (cerebrospinal fluid in par-  
44 ticular) are needed to better understand the underlying pathophysiology, including any potentially  
45 modifiable targets. **The complement cascadde was confirmed using pathway analysis as...**

46 **2 Introduction**

47 Spinal cord injury (SCI) is the transient or permanent loss of normal spinal sensory, motor or au-  
48 tonomic function, and is a major cause of disability. Globally, SCI affects around 500,000 people  
49 each year and is most commonly the result of road traffic accidents or falls.(Crozier-Shaw, Den-  
50 ton, and Morris 2020) Patients typically require extensive medical, rehabilitative and social care at  
51 high financial cost to healthcare providers. The lifetime cost of care in the UK is estimated to be  
52 £1.12 million (mean value) per SCI, with the total cost of SCI in the UK to the NHS being £1.43 bil-  
53 lion in 2016.(McDaid et al. 2019) Individuals with SCI show markedly higher rates of mental illness  
54 relative to the general population.(Furlan, Gulasingam, and Craven 2017) Complications arising  
55 post-SCI can be long-lasting and often include pain, spasticity and cardiovascular disease, where  
56 the systemic inflammatory response that follows SCI can frequently result in organ complications,  
57 particularly in the liver and kidneys.(Gris, Hamilton, and Weaver 2008; X. Sun et al. 2016)

58 The recovery of neurological function post-SCI is highly variable, requiring any clinical trials to have  
59 an impractically large sample size to prove efficacy, hence the translation of novel efficacious ther-  
60 apies is challenging and expensive.(Spiess et al. 2009) Being able to more accurately predict patient  
61 outcomes would aid clinical decisions and facilitate future clinical trials. Therefore, novel biomark-  
62 ers that allow for stratification of injury severity and capacity for neurological recovery would be  
63 of high value to the field.

64 Biomarkers studies in SCI often investigate protein changes in cerebral spinal fluid (CSF) as the  
65 closer proximity of this medium is thought to be more reflective of the parenchymal injury.(Brian  
66 K. Kwon et al. 2019; Hulme et al. 2017) Whilst this makes CSF potentially more informative for  
67 elucidating the pathology of SCI, the repeated use of CSF for routine analysis presents challenges  
68 in clinical care due to the risk and expense associated with the invasiveness of the collection proce-  
69 dure. In contrast, systemic biomarkers measurable in the blood represent a source of information  
70 that can be accessed and interpreted both a lower cost and risk. Studies of traumatic brain injury  
71 have demonstrated that protein markers identified in CSF are also detectable in both plasma and  
72 serum.(Wang et al. 2018) More recently, circulating white blood cell populations have also been  
73 identified as potential SCI injury biomarkers, with a 2021 study showing that elevated levels of neu-  
74 trrophils were associated with no AIS grade conversion, while conversely an increase in lymphocytes  
75 during the first week post-SCI were associated with an AIS grade improvement.

76 A number of individual proteins have been shown to be altered in the bloods post-SCI, including  
77 multiple interleukins (IL), tumour necrosis factor alpha (TNF- $\alpha$ ) and C-reactive protein (CRP).(Segal  
78 et al. 1997; Hayes et al. 2002; Frost et al. 2005)

79 Further, changes in inflammatory marker levels detected in acute SCI patients were found to  
80 be mirrored in donor-matched blood and CSF, albeit at lower absolute concentrations systemi-

<sup>81</sup> cally.(Brian K. Kwon et al. 2010)

<sup>82</sup> Previously, we have shown that routinely collected blood measures associated with liver function  
<sup>83</sup> and inflammation added predictive value to AIS motor and sensor outcomes at discharge and 12-  
<sup>84</sup> months post-injury.(Bernardo Harrington et al. 2020; Brown et al. 2019) The current study uses  
<sup>85</sup> an unbiased shotgun proteomic approach to investigate differentially expressed proteins in SCI  
<sup>86</sup> patients, coupled with bioinformatics pathway and network analyses.

### <sup>87</sup> 3 Methods and Materials

**Table 1.** Patient demographics. ± denotes interquartile range

	n	Percent
<b>Polytrauma</b>		
Yes	16	41
No	23	59
<b>Gender</b>		
F	13	33
M	26	67
<b>Diabetes</b>		
Yes	7	18
No	32	82
<b>Neurological level</b>		
C	26	67
L	4	10
T	9	23
<b>AIS change</b>		
A	11	28
C	7	18
C->D	10	26
D	11	28
Age at injury (Median years±IQR)	53±26	-

#### <sup>88</sup> 3.1 Patients

<sup>89</sup> Blood samples were taken from SCI patients who had provided informed consent and in accordance  
<sup>90</sup> to ethical provided by the National Research Ethics Service [NRES] Committee North West  
<sup>91</sup> Liverpool East [11/NW/0876]. “Improvers” were defined as individuals who experienced an AIS  
<sup>92</sup> grade improvement from admission to a year post-injury, whereas “non-improvers” were defined  
<sup>93</sup> as patients who saw no change in AIS grade in the same period.

#### <sup>94</sup> 3.2 Plasma collection and storage

<sup>95</sup> Plasma samples were collected within 2 weeks of injury (acute) and at approximately 3 months  
<sup>96</sup> post-injury (subacute). Upon collection in EDTA (ethylenediaminetetraacetic acid) coated tubes  
<sup>97</sup> samples were centrifuged at 600g for 15 minutes, to pellet erythrocytes and the resultant plasma  
<sup>98</sup> fraction was aspirated and divided into aliquots for long-term storage in -80°C briefly and liquid  
<sup>99</sup> nitrogen in the longer term.

100 **3.3 Sample preparation and analysis using iTRAQ proteomics**

101 Thawed plasma samples ( $2\mu\text{l}$ ) each were diluted with distilled water ( $98\mu\text{l}$ ). Total protein was  
102 quantified using a Pierce™ 660nm Protein Assay (Thermo Fisher Scientific, Hemel Hempstead,  
103 UK)(Stoscheck 1987).

104 A total of  $100\text{mg}$  of plasma protein was taken from each sample and pooled equally to form a  
105 patient test group. For example, the AIS C improver group was pooled from 10 separate patient  
106 samples,  $10\text{mg}$  of protein per patient.

107 The pooled plasma samples were precipitated by incubation of the sample in six times the volume  
108 of chilled acetone for 1 hour at  $-20^\circ\text{C}$ . The samples were then centrifuged at 6,000G for 10 minutes  
109 at  $4^\circ\text{C}$ , and re-suspended in  $200\mu\text{l}$  of triethylammonium bicarbonate buffer. Sequencing Grade  
110 Modified Trypsin ( $10\mu\text{g}$ - $85\mu\text{g}$  of protein; Promega, Madison, WI, USA) was then added to the sam-  
111 ples for overnight digestion at  $37^\circ\text{C}$ . Peptides underwent reduction and alkylation (according to  
112 the manufacturer's instructions; Applied Biosystems, Bleiswijk, The Netherlands). Tryptic digests  
113 were labelled with iTRAQ tags (again according to the manufacturer's instructions for the iTRAQ  
114 kit), before being pooled into test groups and dried in a vacuum centrifuge. Two individual iTRAQ  
115 experiments were set up, the first to assess acute and sub-acute improvers or non-improvers and  
116 the second to assess acute improvers and non-improvers to AIS grade A and D patients. The follow-  
117 ing tags were used for each group of patient samples 114 tag - acute improvers, 115 tag - sub-acute  
118 improvers, 116 tag - acute non-improvers and 117 tag - sub-acute non-improvers for run 1 and 114  
119 tag - acute improvers, 115 tag - acute non-improvers, 116 tag - AIS grade A and 117 tag - AIS grade  
120 D for run 2.

121 **3.3.0.1 iTraq mass spectrometry analysis** The samples were analysed at the BSRC St. An-  
122 drews University Mass Spectrometry and Proteomics Facility using methods previously described.

123 A total of 12 SCX fractions were analysed by nano-electrospray ionisation-liquid chromatogra-  
124 phy/tandem mass spectrometry (LC-MS/MS) using a TripleTOF 5600 tandem mass spectrometer  
125 (AB Sciex, Framingham, MA, USA) as described previously.(Fuller et al. 2015)

126 **SECTION TO BE REWRITTEN**

127 Each fraction ( $10\mu\text{l}$ ) was then analysed by nanoflow LC-ESI-MSMS, as described previously.

128 Parent (MS) ions were accepted with a mass tolerance of 50 mDa and MSMS was conducted with  
129 a rolling collision energy (CE) inclusive of preset iTRAQ CE adjustments. Analyzed parent ions were  
130 then excluded from analysis for 13 s after 3 occurrences.

131 **3.3.1 Sample preparation and analysis using label-free proteomics**

132 No sample pooling was used, and so each of the 73 samples were maintained separately through-  
133 out protein equalisation, mass spectrometry, and label-free quantification steps. Thus, protein  
134 abundance was quantified for each sample, whereupon mean protein abundance across experi-  
135 mental groups was calculated to assess protein changes.

136 To reduce the dynamic range of proteins, ProteoMiner™ beads (BioRad, Hemel Hempstead, UK)  
137 were used.(Boschetti and Righetti 2008) Total protein was quantitated with a Pierce™ 660nm Pro-  
138 tein Assay (Thermo Fisher Scientific, Hemel Hempstead, UK), whereupon 5 mg of total protein was  
139 applied to ProteoMiner™ beads, and processed as described previously.(Stoscheck 1987)

140 **3.3.1.1 Label free mass spectrometry analysis** Tryptic peptides were subjected to LC-MC/MC  
141 via a 2-h gradient on a NanoAcuity™ ultraperformance LC (Waters, Manchester, UK) connected

142 to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Fisher Scientific Hemel Hempstead, UK)  
143 as described **previously**.

144 **REWRITE IN BRIEF**

145 The Q-Exactive was operated in a data dependent positive electrospray ionisation mode, automati-  
146 cally switching between full scan MS and MS/MS acquisition. Survey full scan MS spectra (*m/z*  
147 300–2000) were acquired in the Orbitrap with 70,000 resolution (*m/z* 200) following accumulation  
148 of ions to  $1 \times 10^6$  target value based on the predictive automatic gain control values from the previ-  
149 ous full scan. Dynamic exclusion was set to 20s, the 10 most intense multiply charged ions ( $z \geq 2$ )  
150 were sequentially isolated and fragmented in the octopole collision cell by higher energy colli-  
151 sional dissociation (HCD), with a fixed injection time of 100ms and 35,000 resolution. The follow-  
152 ing mass spectrometric conditions were used: spray voltage, 1.9kV, no sheath or axillary gas flow;  
153 normalised HCD collision energy 30%; heated capillary temperature, 250°C. MS/MS ion selection  
154 threshold was set to  $1 \times 10^4$  count and 2Da isolation width was set.

155 **3.3.2 iTraq OpenMS analysis**

156 TripleTOF 5600 tandem mass spectrometer output files produced in the ABSciex proprietary .wiff  
157 file format were converted to an open file format, .mzML for analysis with OpenMS (version 2.6.0).  
158 The docker image of ProteoWizard version 3.0.20287 was used for conversion, and peak picking  
159 was applied on conversion (Chambers et al. 2012). OpenMS version 2.6.0 was used for further anal-  
160 ysis.(Röst et al. 2016) Unless otherwise stated, default arguments were used. The 12 fraction files  
161 were merged and sorted by retention time. A decoy database was generated with DecoyDatabase  
162 and the -enzyme flag set to Trypsin, the human reference proteome was taken from Uniprot (Pro-  
163 teome ID: UP000005640, downloaded: 2020-10-01), as was the .fasta for porcine trypsin (Entry:  
164 P00761, downloaded: 2020-10-01).(The UniProt Consortium 2021)

165 The MSFQPlusAdapter was used to run the search. For the -fixed\_modifications "Methylthio (C)"  
166 and "iTRAQ4plex (N-term)" were passed due to the alkylating agent used in sample preparation  
167 and to account for the N-terminus modifications made by iTRAQ tags. "Oxidation (M)" was passed  
168 to -variable\_modifications to reflect the likely occurrence of methionine oxidation. To reflect the  
169 instrument the following flags were also set: -precursor\_mass\_tolerance 20 -enzyme Trypsin/P  
170 -protocol iTRAQ -instrument high\_res.

171 To annotate the search results PeptideIndexer and PSMFeatureExtractor were used. For peptide  
172 level score estimation and filtering PercolatorAdapter was used with the following arguments:  
173 -score\_type q-value -enzyme trypsinp. IDFFilter was used to filter to a peptide score of 0.05  
174 with -score:pep 0.05

175 IsobaricAnalyzer with -type itraq4plex was used with the merged .mzML files to assign protein-  
176 peptide identifications to features or consensus features with IDMapper. The files for each run  
177 output by IDMapper were then merged with FileMerger. Bayesian score estimation and protein  
178 inference was performed with Epifany and the following flags: -greedy\_group\_resolution  
179 remove\_proteins\_wo\_evidence -algorithm:keep\_best\_PSM\_only false Decoys were removed  
180 and 0.05 FDR filtering was done via IDFFilter with -score:protgroup 0.05 -remove\_decoys.  
181 Finally, IDConflictResolver was used to resolve ambiguous annotations of features with peptide  
182 identifications, before quantification with ProteinQuantifier.

183 **3.3.3 Label free OpenMS analysis**

184 For quantification, the raw spectra files were analysed via OpenMS (version 2.6.0) command line  
185 tools, with the workflow from the prior section (3.3.2) adapted to suit a label-free analysis. The  
186 files were first converted from the proprietary .Raw format to the open .mzML standard with the

187 FileConverter tool via the open-source ThermoRawFileParser.(Röst et al. 2016; Hulstaert et al.  
188 2020) Unless otherwise stated, default arguments were used throughout.

189 The decoy database generated in the prior section (iTRAQ OpenMS analysis) was also re-used. The  
190 CometAdapter was used to run the search.(Eng, Jahan, and Hoopmann 2013) Fixed modifications  
191 were set to "Carbamidomethyl (C)" and "Oxidation (M)" was set as a variable modification. To reflect  
192 the instrument the following flags were also set: -precursor\_mass\_tolerance 20 -isotope\_error  
193 0/1.

194 To annotate the identified peptides with proteins the PeptideIndexer tool was used. PeptideIndexer  
195 and PSMFeatureExtractor were used for annotation. For peptide level score estimation and fil-  
196 tering PercolatorAdapter was used with the following flags: -score\_type q-value -enzyme  
197 trypsin. IDFFilter was used to filter to a peptide score of 0.01 with -score:pep 0.01 followed  
198 by IDScoreSwitcher with the following flags: -new\_score "MS:1001493" -new\_score\_orientation  
199 lower\_better -new\_score\_type "pep" -old\_score "q-value". The ProteomicsLFQ was used for  
200 subsequent processing with the flags: -proteinFDR 0.05 -targeted\_only true. The -out\_msstats  
201 flag was also used to produce quantitative data for downstream statistical analysis with the R  
202 package MSstats.(Choi et al. 2014)

### 203 3.3.4 Network and pathway analysis

204 Protein interation networks were created using the Bioconductor package STRINGdb which pro-  
205 vides an R interface to STRING version 11.(Szklarczyk et al. 2019) Instantiation of the STRINGdb  
206 reference class was done with species and score\_threshold set to 9606, for *Homo sapiens*, and  
207 400 respectively. Clustering of networks with STRINGdb used the "fastgreedy" algorithm from the  
208 iGraph package.

209 The Bioconductor package ReactomePA, which employs the open-source, open access, manually  
210 curated and peer-reviewed pathway database Reactome was used for network analysis.(G. Yu and  
211 He 2016; Jassal et al. 2020)

### 212 3.3.5 Enzyme-linked immunosorbent assays

213 Four proteins identified by the iTRAQ analysis were measured by enzyme-linked immunoab-  
214 sorbent assay (ELISA) from non-pooled samples to validate the iTRAQ findings.

215 These proteins were alpha-2-macroglobulin (A2M), retinol binding protein 4 (RBP4), serum amy-  
216 loid A1 (SAA1) and apolipoprotein A1 (ApoA1). They were selected for their biological relevance  
217 and differential abundance between AIS C improvers and non-improvers, implying potential as  
218 biomarkers of neurological outcome prediction. A2M, RBP4 and SAA1 were assessed using a hu-  
219 man DuoSet® ELISAs (R&D Systems, Abingdon, UK). ApoA1 was assessed using a human Quan-  
220 tikine® ELISA (R&D Systems, Abingdon, UK). Samples were diluted 1:600,000 for A2M and RBP4,  
221 1:100 for SAA1 and 1:20,000 for ApoA1 in the respective assay kit diluent. Samples that were above  
222 the assay detection limit were rerun at 1:300 and 1:40,000 for SAA1 and ApoA1 respectively. All  
223 ELISAs were carried out according to the manufacturer's protocol. Protein concentrations were  
224 normalised to the sample dilution factor. Statistical analysis was performed using the statistical  
225 programming language R version 4.1.3 (2022-03-10). Pairwise t tests with bonferroni adjusted P-  
226 values with the R rstatix package were used to assess differential abundance.

227 **4 Results**

228 **4.1 Results**

229 Plasma from American Spinal Injury Association (ASIA) grade C SCI patients (total n=17) contrasting  
230 those who experienced an AISA grade conversion (n=10), and those who did not (n=7) collected  
231 within 2 weeks, and at approximately 3 months post-injury (Improvers n=9 vs Non-improvers n=6).  
232 Relative protein abundance in AIS grade A (n=10) and grade D (n=11) patients was also examined.

233 In the interest of brevity, only the plots of acute and subacute AIS C improvers VS non-improvers  
234 are included here, please see the supplemental data for the other comparisons (section 5.3.2).

235 **4.1.1 Comparing OpenMS and ProteinPilot**

236 The AIS A group had 56 and 26 more abundant and 9 and 6 less abundant proteins respectively.  
237 Acutely, AIS C improvers relative to AIS A and D had 21 and 53 more abundant and 46 and 12 less  
238 abundant for OpenMS, whereas ProteinPilot had 5 and 19 more abundant proteins, and 18 and 6  
239 less abundant.

240 **4.1.2 iTRAQ analyses**

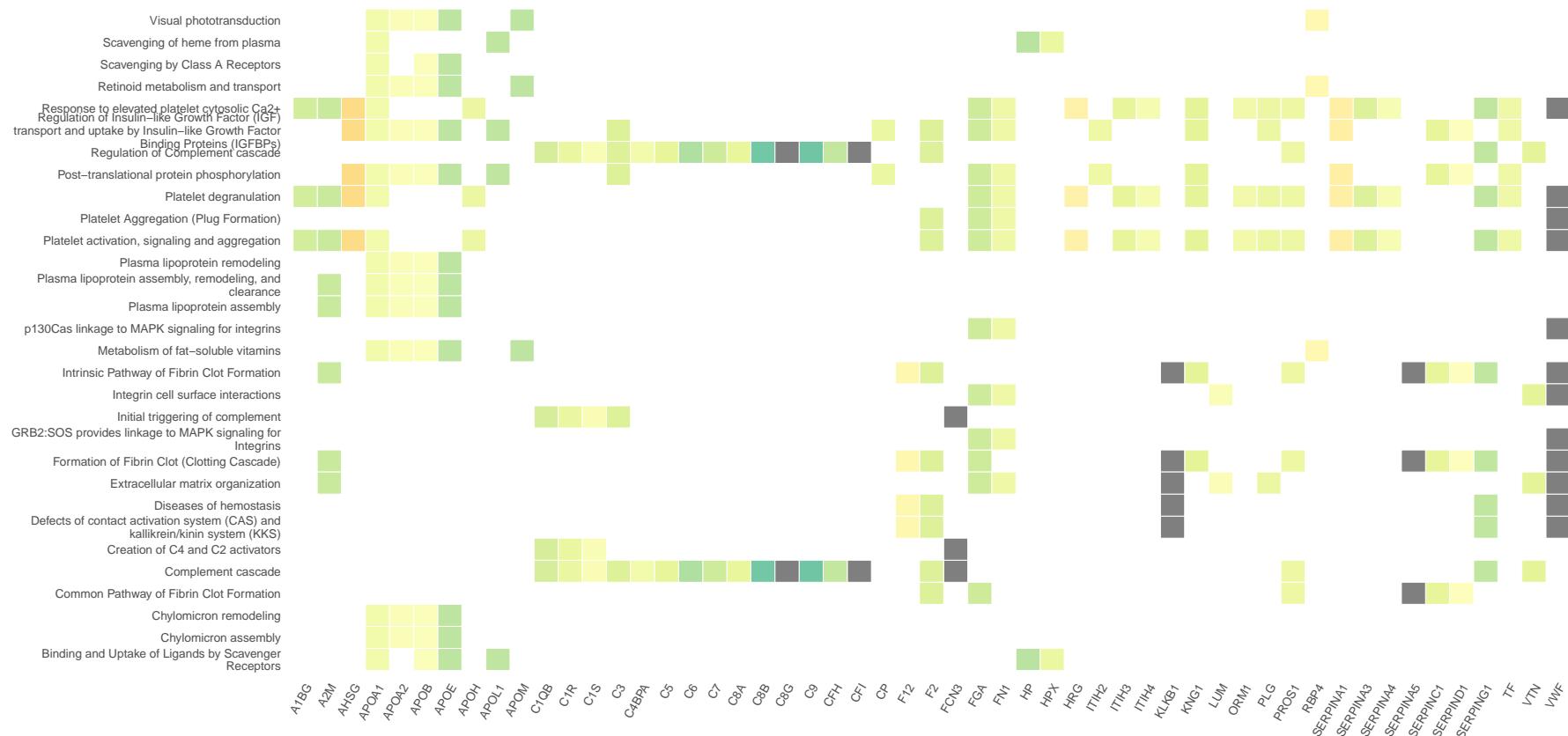
241 **4.1.3 Differential protein abundances**

242 AIS C improvers had 18 more abundant proteins and 49 less abundant proteins at the acute phase  
243 relative to non-improvers. Similarly, at the subacute phase, AIS C improvers had 34 more abun-  
244 dant proteins and 34 less abundant proteins relative to non-improvers. The AIS A group had 56  
245 more abundant and 9 less abundant proteins respectively relative to non-improvers. Acutely, AIS  
246 C improvers relative to AIS A and D had 21 and 53 more abundant and 46 and 12 less abundant  
247 proteins. Please see the appendix for a full list of protein changes.

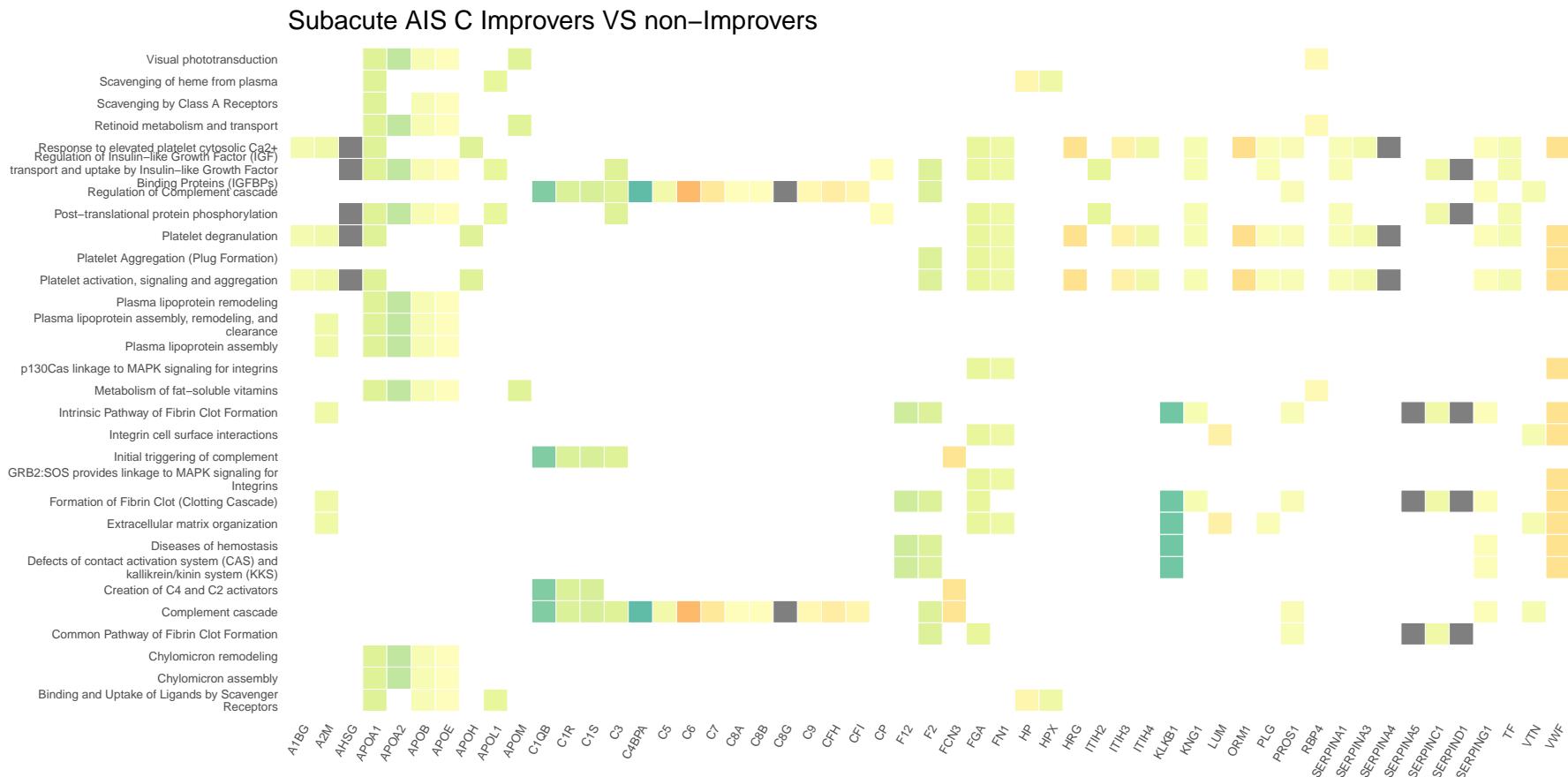
248 **4.1.4 Heatmaps**

249 The majority of the pathways associated with the proteins identified by these iTRAQ experiments  
250 are related to the complement cascade and platelet activity (Figure 1, 2, S1, S2, S3, S4, S5, S6, S7, S8).  
251 There are also several pathways implicated in metabolic processes, particularly with apolipopro-  
252 teins and retinoids.

### Acute AIS C Improvers VS non-Improvers



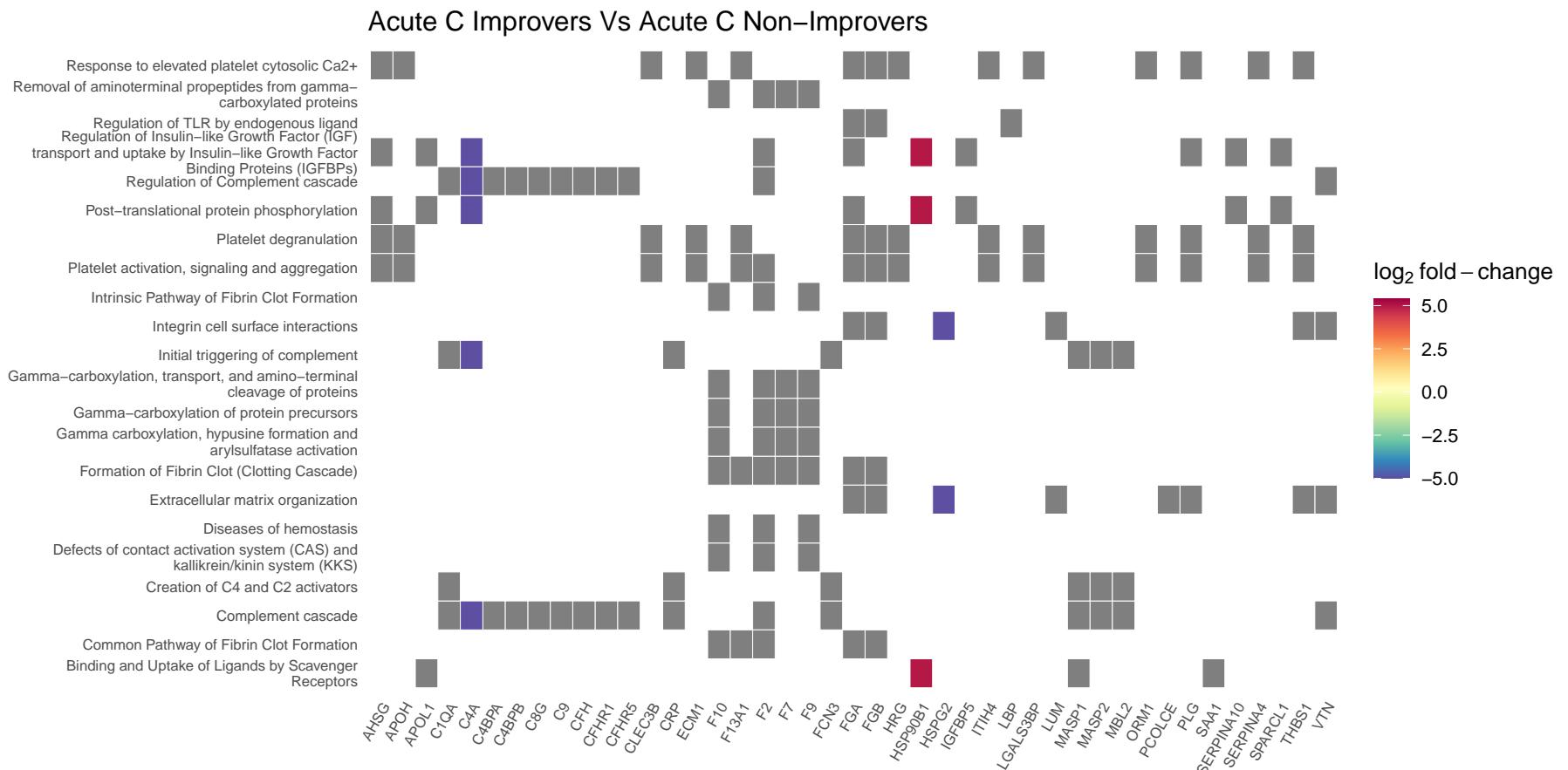
**Figure 1.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.



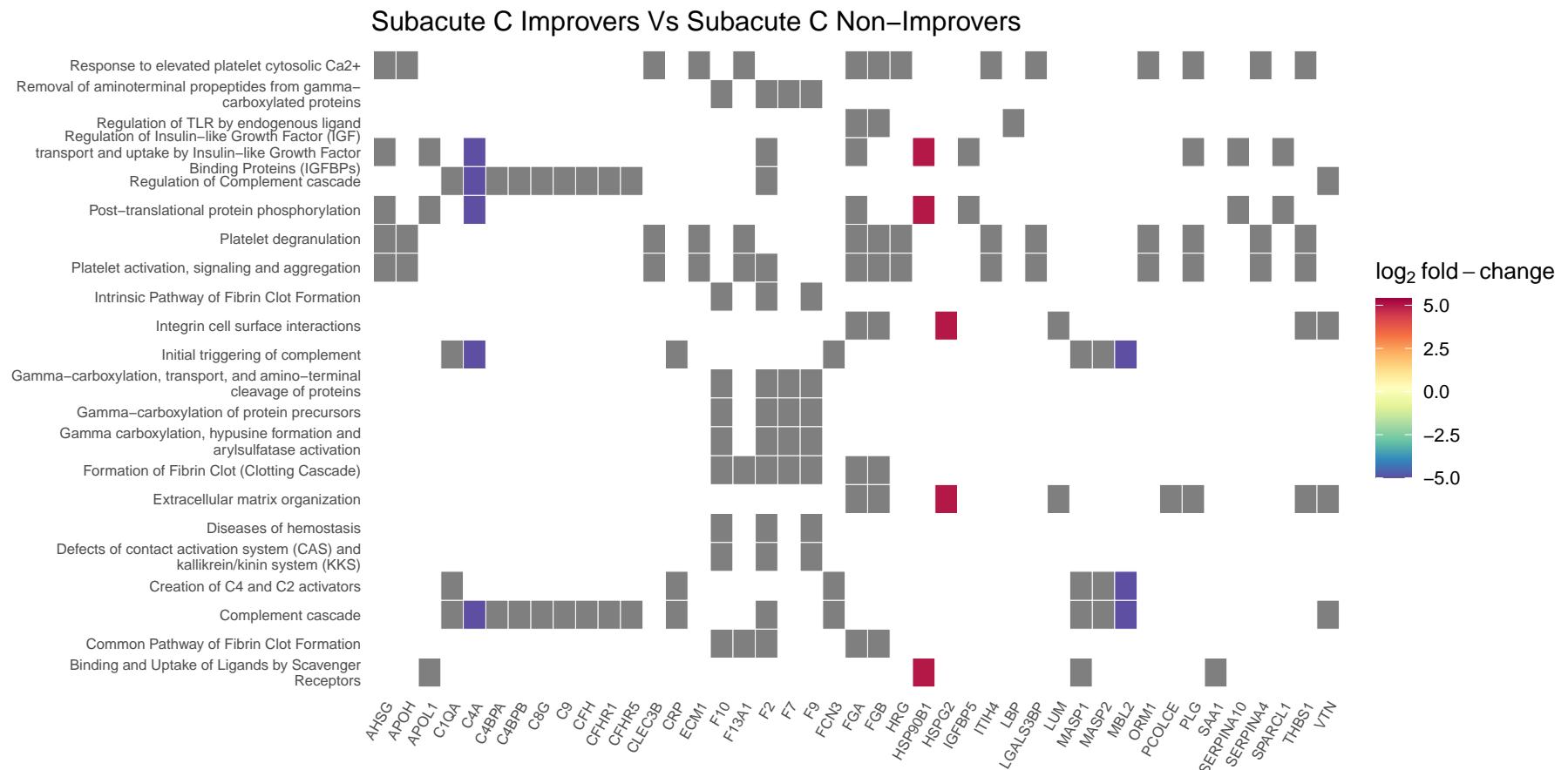
**Figure 2.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

<sup>253</sup> Similarly to the iTRAQ data, many of the Reactome pathways are associated with the complement cascade and platelets activation (Figures 3, 4, S9, S10, S11, S12, S13, S14, S15).

<sup>254</sup> Please see appendix section 5.6 for additional plots.



**Figure 3.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins not present in the comparison.

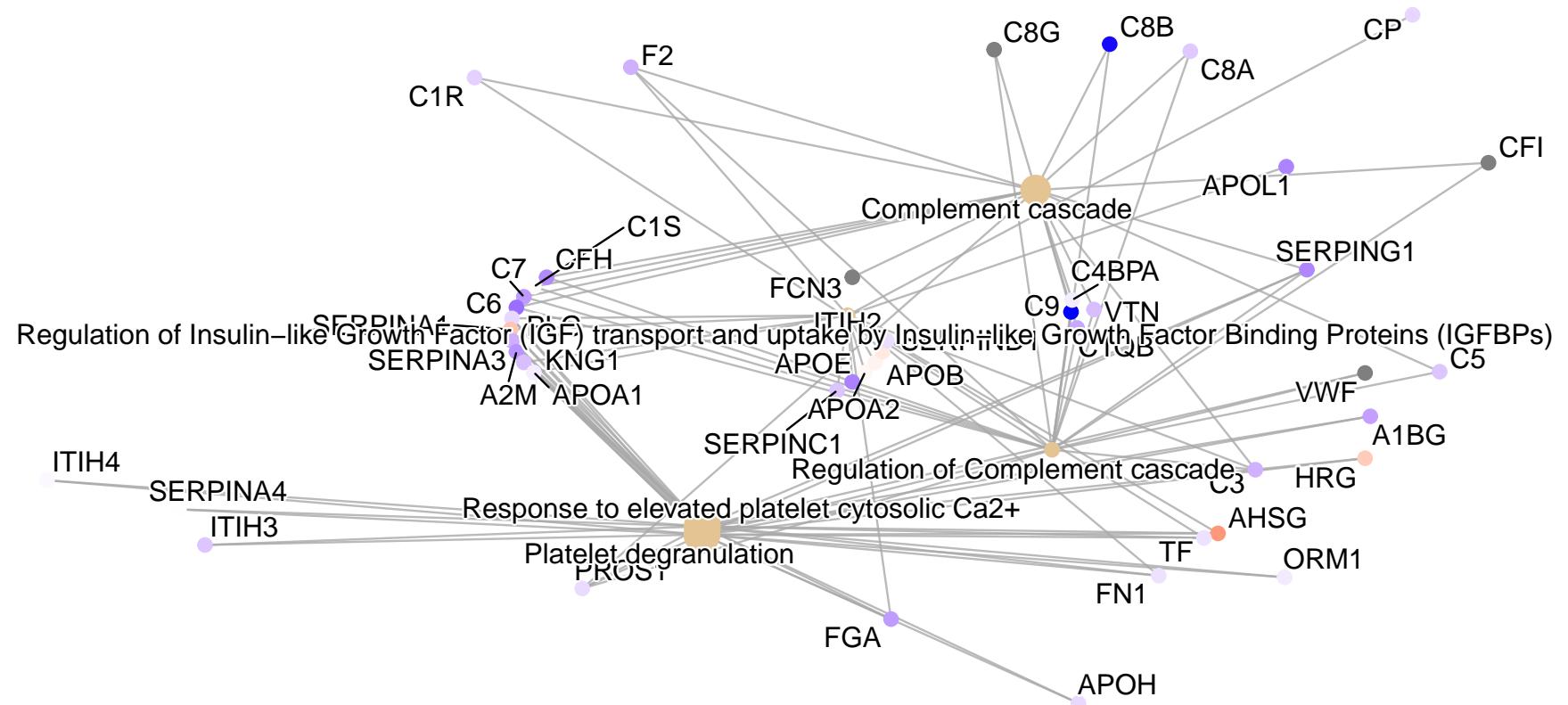


**Figure 4.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins not present in the comparison.

256   **4.1.5 Network analysis of Differentially Abundant Proteins between AIS C improvers and**  
257   **non-improvers**

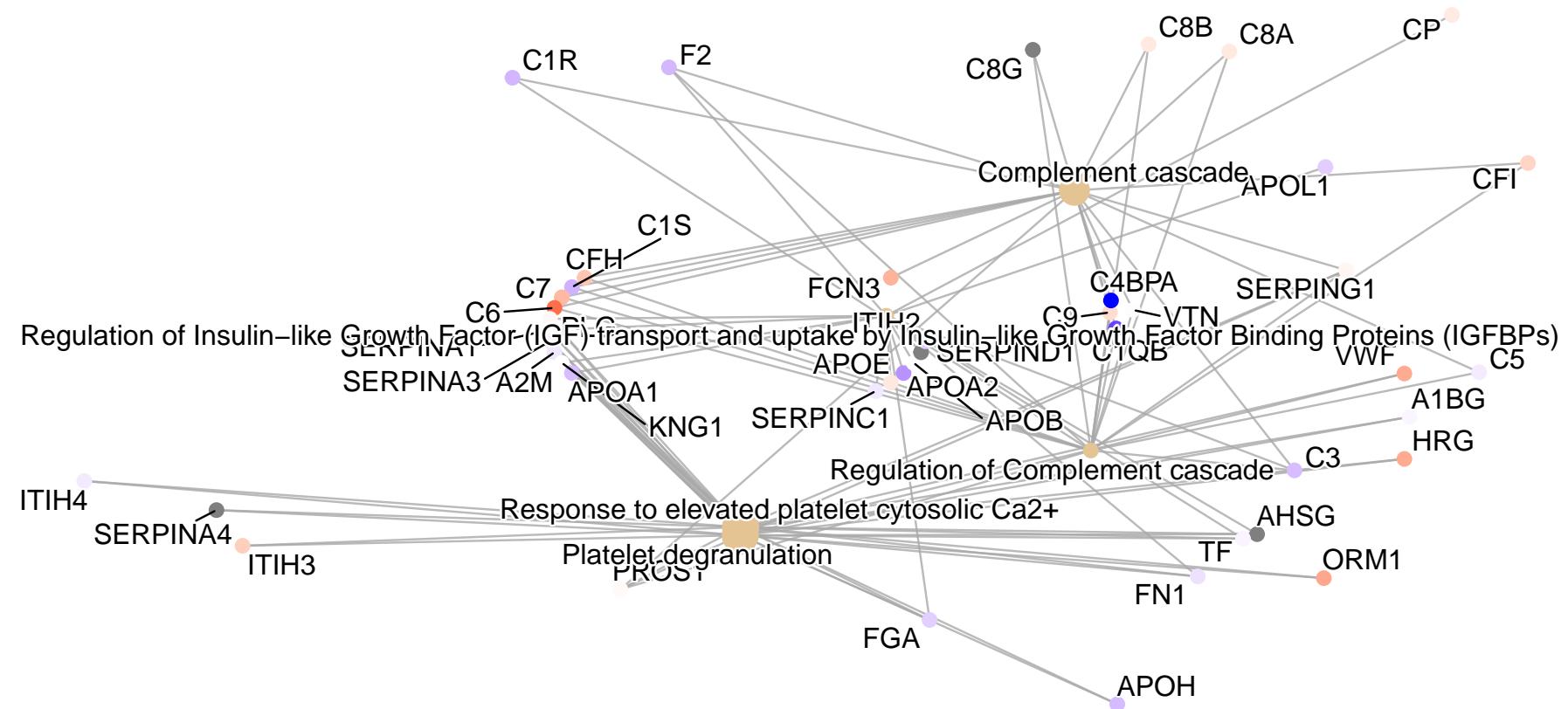
258   Similar to the heatmaps, network plots highlighted that the majority of proteins changes were  
259   associated with the complement cascade and pathways linked to platelet activity (Figure 5, 6, S16,  
260   S17, S18, S19, S20, S21, S22, S23). Several proteins were also associated with the regulation of  
261   insulin-like growth factor.

Acute AIS C Improvers VS non-Improvers



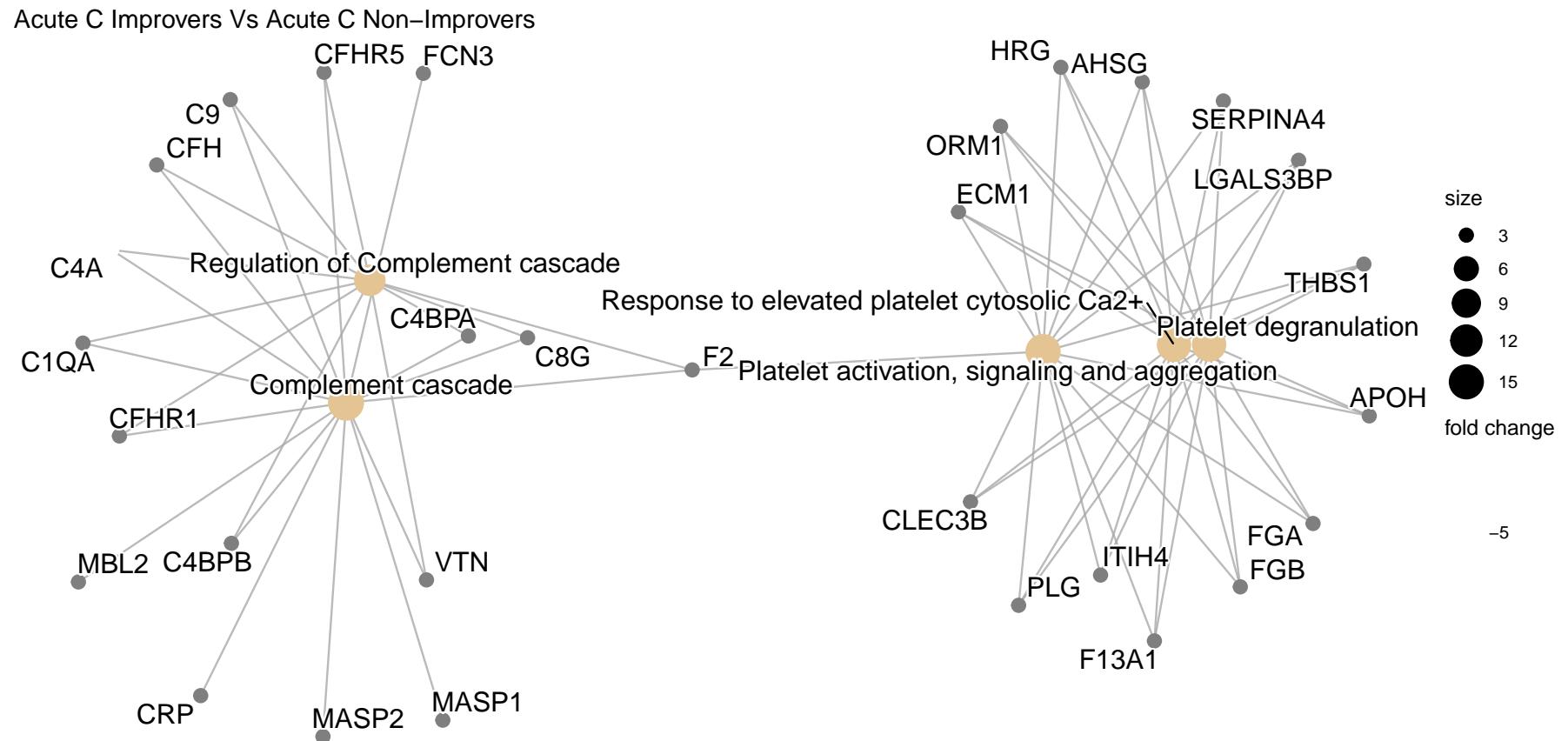
**Figure 5.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute AIS C Improvers VS non-Improvers



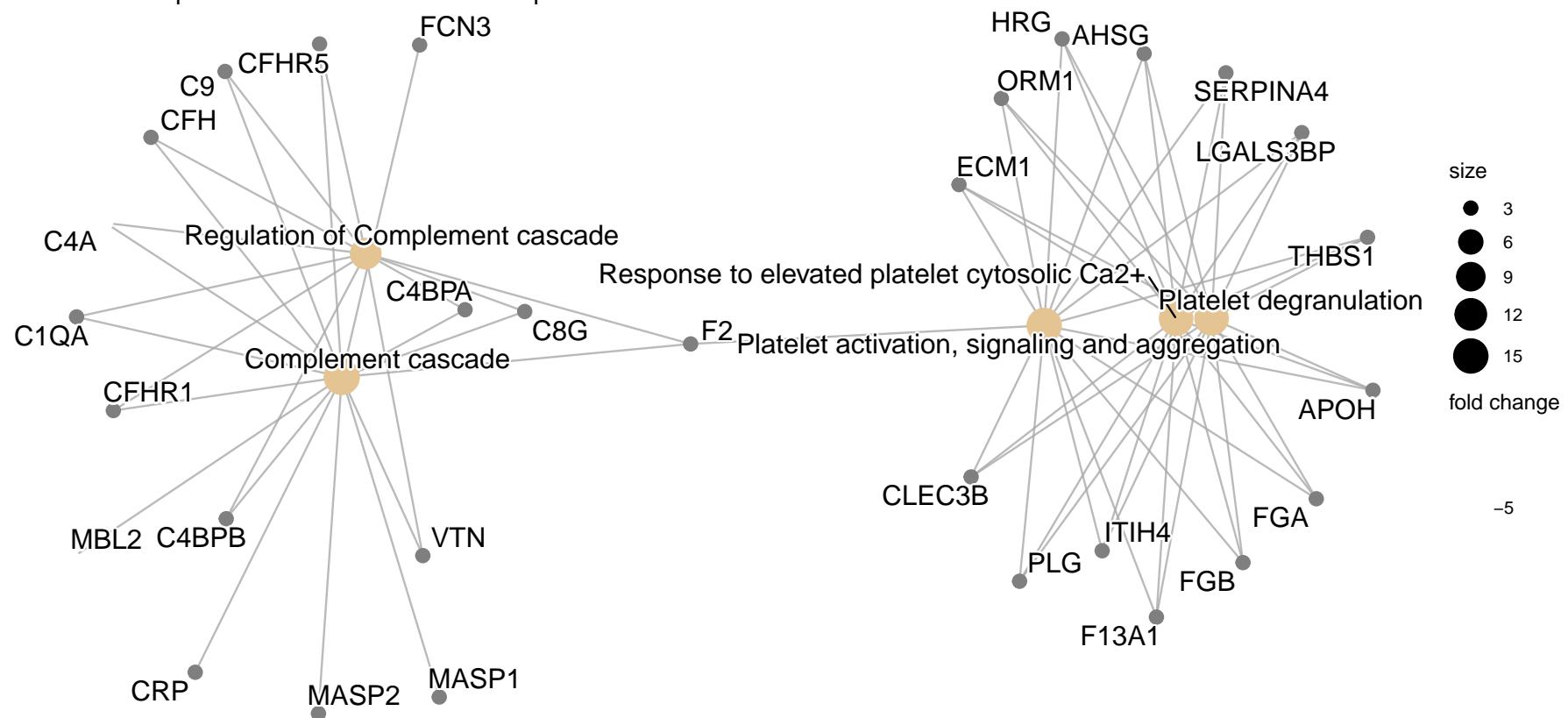
**Figure 6.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

- <sup>262</sup> Similarly to the heatmaps and the iTRAQ data, network plots derived using the label-free data  
<sup>263</sup> highlight the majority of differential proteins are associated with the complement cascade and  
<sup>264</sup> pathways linked to platelets (Figures 7, 8, S24, S25, S26, S27, S28, S29, S30).
- <sup>265</sup> Please see appendix section 5.7 for additional plots.



**Figure 7.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

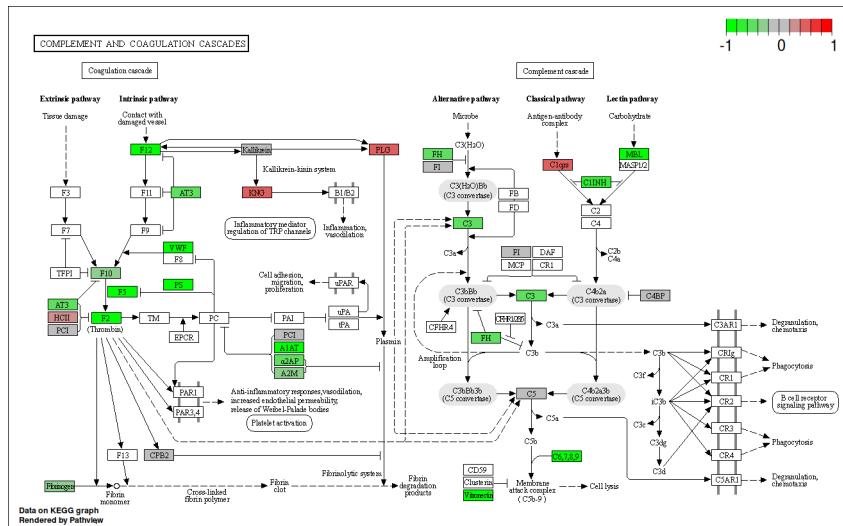
Subacute C Improvers Vs Subacute C Non-Improvers



**Figure 8.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

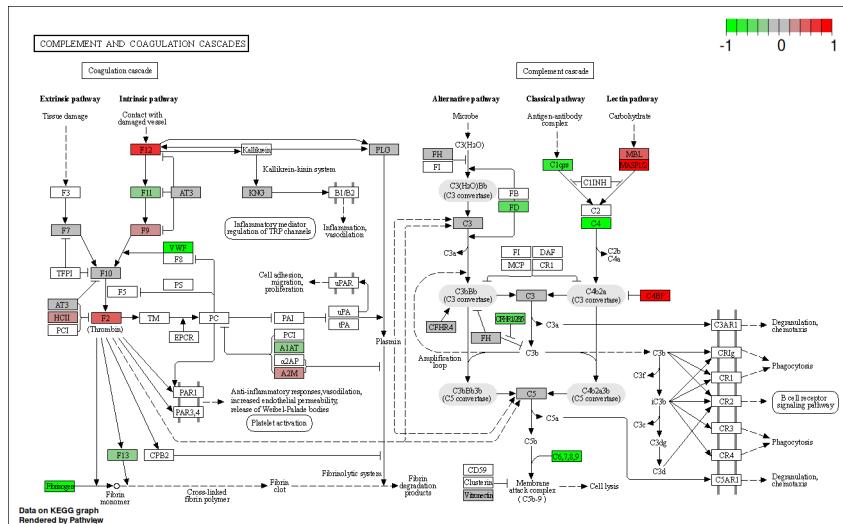
## 4.1.6 Pathway analysis of Differentially Abundant Proteins between AIS C improvers and non-improvers

Pathway analysis via the pathview R package returned the complement and coagulation cascade to be the sole significant KEGG pathway to derive from the OpenMS analysed data. The majority of the proteins present in this pathway were less abundant in the 2-week post-injury plasma of AIS C patients who experienced an AIS grade conversion and those who did not (Figure 9).



**Figure 9.** KEGG complement cascade pathway annotated with log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Similarly to the iTRAQ pathway analysis, the label free data analysed via the pathview R package returned the complement and coagulation cascade to be the sole significant KEGG pathway derived from the OpenMS analysed data. The majority of the proteins present in this pathway were less abundant 2-weeks post-injury in the plasma of AIS C patients who experienced an AIS grade conversion than those who did not (Figure 10).

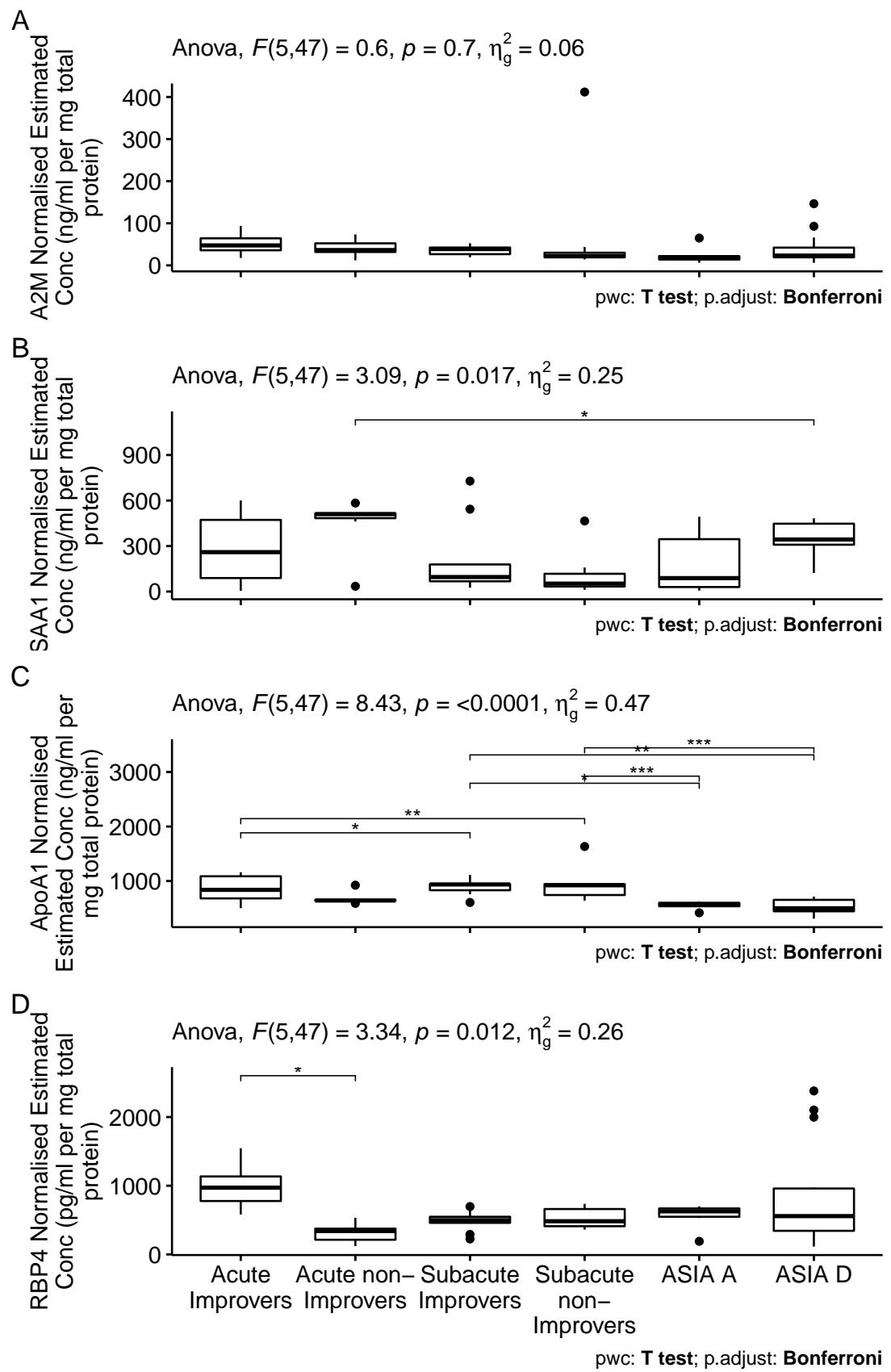


**Figure 10.** KEGG complement cascade pathway annotated with log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

### **4.1.7 Validation of Proteomic Data using ELISA**

278 No statistically significant difference between groups for A2M abundance in plasma via DuoSet®  
279 ELISAs, though there were outliers in the AIS A and D groups, and particularly in the AIS C patients  
280 at 3-months who did not experience an AIS grade conversion (Figure 11).

281 A significant difference was found between AIS C non-improvers at 2-weeks and AIS D for SAA1,  
282 with outliers in AIS C non-improvers at 2-weeks, and both AIS C improvers and non-improvers at  
283 3-months post-injury (Figure 11). For ApoA1 plasma abundance estimated via Quantikine® ELISAs,  
284 statistically significant differences were found between AIS C improvers at 2-weeks and both AIS C  
285 improvers and non-improvers at 3-months, AIS C 3-month improvers and AIS A and D, and AIS C  
286 3-month non-improvers and AIS A and D (Figure 11). A statistically significant difference was also  
287 found between AIS C improvers and non-improvers at 2-weeks post-injury for RBP4 (Figure 11).



**Figure 11.** Normalised estimated concentration of  $\alpha$ -2-macroglobulin (A), serum amyloid A1 (B), apolipoprotein A1 (C) and retinol binding protein 4 (D). Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled iTRAQ samples was assayed and pairwise t-tests with bonferroni adjusted P-values were performed to assess differential abundance.

288    **4.1.8 STRINGdb plots**

289    Network interaction plots generated from the OpenMS processed data via STRINGdb revealed that  
290    all test groups contained similar proteins, albeit with different abundances, with no distinct group-  
291    specific networks observed (Figures S31, S32, S33, S34, S35, S36, S37, S38 and S39).

292    Network interaction plots generated of the significant proteins via STRINGdb revealed that all groups  
293    contained similarly smaller networks, with many proteins with no known interactions in the STRING  
294    database (Figures S40, S41, S42, S43, S44, S45, S46, S47, S48).

295    **4.1.9 Volcano plots**

296    The mean number of down-regulated and up-regulated significant proteins in each group is 10.6,  
297    and 6.8. Between AIS C improvers and non-improvers, 8 and 4 proteins were up- and down-  
298    regulated acutely, whereas 6 and 6 were up- and down-regulated subacutely (Figures S49 and  
299    S50). Longitudinally, AIS C acute improvers had 10 up-regulated and 7 down-regulated proteins  
300    relative to subacute improvers, while for non-improvers 6 and 12 were up- and down-regulated  
301    respectively (Figures S51 and S52).

302    **4.1.10 Comparing iTRAQ and label-free proteins**

303    A total of 87 and 79 unique proteins were identified across the label-free and iTRAQ experiments  
304    respectively, with a modest overlap of 26 proteins found using both techniques.

305    **5 Discussion**

306    This is the first study, to our knowledge, to comprehensively investigate the plasma proteome in  
307    SCI patients whose AIS scores either improved or did not improve post injury and also to compare  
308    these to AIS grade A and D patients. We have used two proteomic techniques allowing us to profile  
309    both high and low abundance proteins, in order to identify proteins which may have potential to  
310    predict neurological improvement within the acute setting. Moreover, this data can better inform  
311    us of the biology underlying neurological improvement or stability in a cohort of patients being  
312    conservatively managed post SCI.

313    This study has highlighted a number of proteins that may be able to discriminate in, the acute  
314    phase following injury, between AIS grade C patients who either improve or do not improve by  
315    an AIS grade following SCI. The most promising of these is Retinol Binding Protein 4 (RBP4) which  
316    was demonstrated to be increased in non-improvers compared to improvers in the acute phase.  
317    Further this change could be confirmed using ELISA, which may provide a more clinically useful  
318    means of assessing this protein on a wide scale.

319    RBP4 is synthesised in the liver and binds retinol that is released following vitamin A deficiency.(P.  
320    A. Peterson 1971) Once delivered to target cells, retinol can either be converted to retinaldehyde,  
321    which is required for functional vision, or oxidised to retinoic acid, which is a ligand for nuclear  
322    receptors, thus regulating gene expression.(Lane and Bailey 2005; J. E. Balmer and Blomhoff 2002)  
323    The role of retinoid signalling in spinal cord and motor neuron differentiation, including develop-  
324    ment of regions of the spinal cord has been outlined, and implies a possible involvement in main-  
325    taining motor neuron integrity.(Colbert et al. 1995; Sockanathan and Jessell 1998) The mRNA of a  
326    rodent homologue of RBP was found to be up-regulated at 24 hours post-SCI and may promote  
327    cell proliferation and regeneration by increasing retinoid metabolism.(Song et al. 2001; Hurst et  
328    al. 1999)

329    Another study of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease, comparing

330 gene expression between post-mortem spinal cord samples of ALS and controls also observed  
331 up-regulation of RBP1 in ALS spinal cord.(Malaspina, Kaushik, and Belleroche 2001) Furthermore,  
332 a transgenic mouse study reported retinoid signalling may contribute to the retained plasticity  
333 and regenerative potential of the mature spinal cord.(Haskell et al. 2002) The results found here  
334 support these findings for AIS C improvers relative to non-improvers as improver had increased  
335 levels of RBP4. Whether this is due to increased expression or due to higher vitamin A intake is  
336 unclear from this data, though at 3-months post-injury this is still the case even though patients  
337 diets could be more similar throughout hospital admission.

338 Alongside RBP4, a number of other protein abundance differences across the different biological  
339 comparisons were identified in proteins associated with liver function. Our previous work investi-  
340 gating the potential of routinely measured haematological analytes for predicting neurological  
341 outcome in SCI patients also highlighted several proteins that were linked with liver function; thus  
342 providing further support to this theory.(Brown et al. 2019; Bernardo Harrington et al. 2020) The  
343 pathway analysis specifically indicated that the acute phase response (APR) is implicated.

344 The APR is the body's first response to injury or infections, including SCI. This systemic response  
345 is largely coordinated by factors released from the liver, but the APRs effects extend to multiple  
346 peripheral organs including the kidneys, lungs and spleen.(Bao et al. 2012; S. J. Campbell, Zahid, et  
347 al. 2008; Fleming et al. 2012; Gris, Hamilton, and Weaver 2008) This hepatic response is typically  
348 transient and quickly fades, but prolonged liver inflammation and pathology has been observed in  
349 rodent SCI models.(Goodus et al. 2018; Sauerbeck et al. 2014) Basic liver functions are chronically  
350 impaired by SCI, including metabolising carbohydrates, fats and proteins, storage of minerals vi-  
351 tamins and glycogen and filtering blood from the digestive tract.(García-López et al. 2007; DeLeve  
352 2007; Farkas and Gater 2018; Chow et al. 2012; Sauerbeck et al. 2014) The acute (1-7 days) liver  
353 response to SCI is well documented; the inflammatory cytokines including TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and  
354 IL-6, released at the injury site, reach the liver through the bloodstream.(Fleming et al. 2012; Hundt  
355 et al. 2011) This provokes the liver to enter the APR and produce acute phase proteins thus stimu-  
356 lating a greater immune response.(Anthony and Couch 2014; Fleming et al. 2012) The hepatocytes  
357 that make up the majority of the liver biomass, express receptors that bind the aforementioned  
358 inflammatory cytokines; similarly the hepatic macrophage Kupffer cells also bind these cytokines,  
359 complement proteins and lipopolysaccharide (LPS) and swiftly remove microorganisms, endotox-  
360 ins and other debris from the blood.(C.-Y. Yang et al. 2013; Szalai et al. 2000; Crispe 2016; S. J.  
361 Campbell et al. 2005)

362 Furthermore, it has been suggested that liver inflammation and Kupffer cells activity promote re-  
363 cruitment of leukocytes to the injury site in brain or spinal trauma, potentially enhancing CNS in-  
364 jury.(Anthony and Couch 2014; S. J. Campbell et al. 2005) For example, a rodent study demon-  
365 strated depletion of Kupffer cells prior to injury resulted in few neutrophils infiltrating the injury  
366 site.(S. J. Campbell, Zahid, et al. 2008; S. J. Campbell, Anthony, et al. 2008)

367 • MH NOTE: PRX-2 was one of the most promising proteins from the label-free data. We didn't  
368 do any elisas for it, but I think we can argue that the label-free is more statistically robust due  
369 to the biological and technical replicates. We could argue that it may not have turned up in  
370 the iTRAQ as we didn't use the proteominer beads too I suppose

371 Another protein this data highlights is Peroxiredoxin 2 (PRX-2), which was detected acutely in AIS  
372 C improvers and AIS D patients, and subacutely in AIS A and AIS D. Peroxiredoxins are a large  
373 and highly conserved family of enzymes that reduce peroxides. PRX-2 is highly abundant in RBCs  
374 and intracellularly serves as an important anti-oxidant role in various cell types, including neu-  
375 rons.(Low, Hampton, and Winterbourn 2008) By contrast, extracellular PRX-2 has been suggested  
376 to act as an inflammatory DAMP, leading microglia and macrophages to release a plethora of pro-  
377 inflammatory factors.(Salzano et al. 2014; Garcia-Bonilla and Iadecola 2012; Shichita et al. 2012)

378 An *in vitro* primary neurons and microglia co-culture study reported PRX-2 activating microglia via  
379 TLR-4, potentially leading to neuronal apoptosis.(Y. Lu et al. 2018) A mouse study found over-  
380 expression of PRX-2 attenuated oxidative stress and neuronal apoptosis following subarachnoid  
381 haemorrhage.(Y. Lu et al. 2019) Over-expression of PRX-2 is speculated to protect again ischaemic  
382 neuronal injury by modulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase  
383 (ASK) 1 signalling complex.(Gan et al. 2012) Several molecular chaperones can interact with ASK1,  
384 including thioredoxin and TNF receptor-associated factor 6.(Matsuzawa et al. 2005) The disso-  
385 ciation of the thioredoxin-ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free  
386 radicals, whereupon its antioxidant activity is reduced. This inactivation can be reversed by the  
387 thioredoxin-thioredoxin reductase system, whereby oxidised PRX-2 can regain its activity by re-  
388 ducing thioredoxin, leading to the dissociation of the thioredoxin-ASK1 complex.(Rhee and Woo  
389 2011) Additionally, oxidised PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding  
390 domain on ASK1.(S. Y. Kim, Kim, and Lee 2008)

391 The presence of PRX-2 in acute AIS C improvers and absence in acute C non-improvers suggests the  
392 protein could indicate a more protective action against oxidative stress, and implies the protein has  
393 potential value as a biomarker of functional outcomes. Similarly, PRX-2 may be acting as a healthy  
394 response to trauma-induced oxidative stress in both acute AIS D, although the persistence to the  
395 subacute time-point is less clear. Likewise, the presence of PRX-2 in AIS A subacutely, but not  
396 acutely is more perplexing. It should be noted that as plasma was used and cells were lysed, there  
397 is no distinction between intracellular and extracellular PRX-2 in this data. Perhaps in the more  
398 severe AIS A injury, secondary injuries, including oxidative stress, are greater and so persist to the  
399 subacute time-point. The acute absence may be a result of an overwhelmed physiology unable to  
400 respond or prioritise managing oxidative stress.

401 Pathway analysis from both the iTRAQ and label-free experiments identified the complement and  
402 coagulation cascades as a significant pathway. More broadly, the trend in this data is for proteins  
403 in the complement pathway is lower abundance, or inhibitory proteins such as C4BP to be more  
404 abundant, in the acute improvers. C3 for instance, cleavage of which is vital for complement acti-  
405 vation, was less abundant in acute AIS C improvers relative to non-improvers. This is in line with  
406 a genetic C3 knockout study in mice which reported better neurological scores 2 days post-injury,  
407 reduced residual consolidated neurological deficit at 21 days and display minor change inreduced  
408 gliosis (20% decrease at 1h timepoint) but a three-to-fourfold decrease in neutrophil infiltration,  
409 resulting in enhanced regeneration of axons.(Qiao et al. 2006) Another study using a similar C3  
410 knockout model reported improved neurological scores at acute and long-term time points.(Guo  
411 et al. 2010)

412 This result implies the complement cascade is a particularly important component of a differential  
413 response to injury which ultimately leads to greater functional recovery. Given the complexity  
414 of the complement cascade and the limited time points in this study, further work is needed to  
415 elucidate which facets of the cascade are outcome modifying, and at which stages post-injury.  
416 The small number of statistically significant proteins speaks to the variability of human plasma  
417 samples, and is likely exacerbated by the inconstant timing of sample collection relative to injury.  
418 Post-hoc power analysis of the data reveals that to identify a 2.5 fold change with an FDR of 0.5 and  
419 a power of 0.9, 14 biological replicates would be needed, in contrast to the 7-11 replicates used  
420 across groups here.

421 Thus, a repeat of this experiment with a larger sample size will likely reveal many more proteins of  
422 potential interest. Furthermore, a metabolomic analysis with a similar sample size would greatly  
423 compliment this work, particularly with regards to investigating further links to the liver. Addi-  
424 tional investigation of the key proteins, RPB4 and PRX-2 in particular, but also the complement  
425 cascade more broadly would also be valuable. Quantitation of these proteins both at more acute  
426 and chronic time points would be of greatest immediate interest.

- 427 • MH note: Haven't added anything about comparing vendor proprietary software and  
428 openMS. I go back on forth thinking that could easily be a paper onto itself. Maybe just add  
429 a line or two mentioning OpenMS was chosen as it performed similarly to the proprietary  
430 stuff and allows for more openness and reproducibility  
431 • small point, but would it be worth adding subsection to the discussion as I did in the thesis?  
432 I think it makes it a more pleasant read personally

433 **5.1 THE FOLLOW IS COPIED FROM THESIS**

434 **5.2 iTRAQ discussion**

435 This work builds on the previous chapters (??) modelling of routine bloods by analysing the plasma  
436 proteome of SCI patients grouped by injury severity and improver status. In addition to continuing  
437 the pursuit of novel biomarkers of SCI, the link between the liver and neurological recovery hinted  
438 at in the aforementioned chapter is examined here.

439 **5.2.1 ProteinPilot and OpenMS**

440 Mass spectrometry is a major technique used in several fields, including metabolomics, lipidomics,  
441 interactomics and proteomics, each of which demands a variety of differing approaches to data  
442 acquisition and analysis. Multiple separation methods (liquid chromatography, gas chromatog-  
443 raphy), fragmentation methods (electron-capture dissociation, electron-transfer dissociation,  
444 collision-induced dissociation, etc.) and acquisition strategies (targeted, data-dependent and  
445 data-independent) are used in any combination. With quantification there are different label-  
446 free, isotopic or isobaric labelling approaches to employ. Finally the data analysis may require  
447 a database search, as in proteomics and metabolomics, spectral library search or a targeted  
448 analysis, depending on the experiment. This complexity necessitates a multi-interdependent-step  
449 workflow tailored to the given experiment.

450 The manufacturers of mass spectrometers often offer software tailored to their instruments which  
451 is often used in the literature. However, the source code for these software suits is not pub-  
452 licly available, and indeed manufacturers often boast of their particular inscrutable proprietary  
453 algorithms, often related to peak picking. This combination of complexity and opacity in analy-  
454 sis methodology can make it extremely difficult to reproducible results from other labs, or even  
455 analysis from one's own lab. ("Devil in the Details" 2011)

456 To address this issue many open-source (meaning the source code is publicly available) software  
457 packages which may perform one or several steps of a complex analysis workflow have been devel-  
458 oped. This issue here is that incorporating multiple software packages together can be both time-  
459 consuming and error-prone, and require significant maintenance and documentation to maintain  
460 reproducibility.

461 The OpenMS project aims to address these challenges by providing a flexible software environ-  
462 ment, with both pre-assembled workflows that aim to provide best-practices, and allow for more  
463 granular control with both command line and Python scripting interfaces. OpenMS is also inte-  
464 grated with graphical workflow systems such as KNIME and Galaxy, increasing the accessibility of  
465 the platform. (Berthold et al. 2009; Goecks et al. 2010)

466 Here we used both the vendor provided proprietary ProteinPilot and OpenMS to analyse two 4-  
467 plex iTRAQ experiments. We observe that both approaches produce similar results, with a similar  
468 number of total proteins identified, a large degree of overlap in the specific proteins identified,  
469 and similar fold changes (Figures ?? and ??). As the results are similar we choose to focus on the  
470 OpenMS results due to aforementioned superior reproducibility.

471    **5.2.2 Proteins identified**

472    A total of 79 proteins were identified across both runs for OpenMS, many of which are related in  
473    function. (Figure ??). Here we explore the potential these proteins have as biomarkers of SCI.

474    **5.2.2.1 Alpha-2-macroglobulin** A2M is an inhibitor of an unusually diverse array of proteinases  
475    by a unique 'trapping' mechanism. The protein achieves this with a peptide stretch, called the  
476    "bait region", which contains specific cleavage sites for different proteinases. When a proteinase  
477    cleaves the bait region, a conformational change is induced whereby A2M traps the proteinase.  
478    The entrapped enzyme retains active against low molecular weight substrates, whereas activity  
479    against high molecular weight substrates is greatly reduced. Following cleavage in the bait region, a  
480    thioester bond is hydrolysed and mediates the covalent binding of the protein to the proteinase.(P.  
481    K. Hall et al. 1981; Sottrup-Jensen et al. 1984) A2M is unique in its ability to inhibit virtually any  
482    protease regardless of its specificity, origin or catalytic mechanism.(Khan 2004; Lin et al. 2012)

483    Alpha macroglobulins are an integral part of innate immunity and thus are evolutionarily con-  
484    served.(**buresova\_iram2-macroglobulin\_2009?**) Alpha macroglobulins have significant primary  
485    sequence homology with complement components C3, C4 and C5. The A2M-proteinase complex  
486    is cleared from circulation primarily by receptors on hepatocytes.(Bond, Cianciolo, and Pizzo 2007;  
487    Travis and Salvesen 1983) The mammalian receptor for proteinase-reacted A2M is a low-density  
488    lipoprotein receptor related protein.(Fujiyoshi et al. 2011; Larios and Marzolo 2012; Wyatt and  
489    Wilson 2013)

490    A2Ms definitive function is the delivery of proteinase to an endocytotic proteinase clearance path-  
491    way. A2Ms trap the proteinases released by granulocytes and other cells during inflammation and  
492    also regulate the extracellular proteolytic activity resulting from clotting and fibrinolysis. A2M can  
493    also help protect against pathogens as it can trap proteinases from non-human origins as well.  
494    A2M can be recognised and phagocytosed by macrophages and hepatocytes, and it has been pro-  
495    posed to aid in the clearance of defensins and other peptide mediators in inflamed tissues, thus  
496    contributing to the regulation and containment of inflammation.(Rehman, Ahsan, and Khan 2013)

497    Myelin basic protein is released into the circulation following traumatic injury and A2M has been  
498    seen to be the only major myelin basic protein-binding protein in human plasma, suggesting A2M  
499    protects the immunogenic protein from degradation by proteases and help in its clearance from  
500    circulation.(Gunnarsson and Jensen 1998) A study looking at male infertility after SCI with pro-  
501    teomics found A2M to be elevated approximately 3-fold in the sperm plasma of SCI patients relative  
502    to normal controls.(Silva et al. 2016)

503    We observe A2M to be less abundant in AIS C improvers, within 2-weeks post injury and at 3-  
504    months, albeit to a lesser extent (Tables S1 and S2). Similarly, A2M was more abundant in AIS As  
505    relative to all groups, and whilst A2M was less abundant in AIS C improvers at 2-weeks compared  
506    to AIS Ds, AIS C non-improvers had more A2M than AIS Ds. (Table S1). With less A2M there would  
507    be more protease activity in these individuals, which may aid in the clearance of damaged tissue,  
508    and in particular may lessen the development of an astroglial scar, thus aiding repair. However,  
509    glial scarring is not entirely negative, the primary benefit it offers is minimising the extent of sec-  
510    ondary damage to neighbouring areas by functioning as a barrier around the injury site. Animal  
511    studies have demonstrated that prevention of astroglial scar formation following CNS injury leads  
512    to greater lesion size and poorer function outcomes.(Anderson et al. 2016; Wilhelmsson et al.  
513    2006) Interestingly, a rat study using quantitative liquid chromatography-mass spectrometry with  
514    CSF, found A2M to be more abundant in moderately injured animals compared to more severe  
515    injuries.(Lubieniecka et al. 2011)

516 **5.2.2.2 Apolipoproteins** We found ApoA1, ApoA2, ApoH, ApoL1 and ApoM to be less abundant  
517 in AIS improvers at both time points, whereas ApoA4 was more abundant at both time points (Ta-  
518 bles S1 and S2). ApoA1 is the main protein component of high-density lipoproteins (HDL). Plasma  
519 HDL include two main apolipoproteins, these being ApoA1 and ApoA2 (~70% and ~20% of total  
520 HDL protein content respectively), but some HDL particles can also contain small amounts of other  
521 apolipoproteins, including ApoA4, ApoA5, ApoC, ApoD, ApoE, ApoJ and ApoL. The primary function  
522 of HDL in plasma is the transport of cholesterol, which can have dietary origins, but also be pro-  
523 duced endogenously in the liver.

524 **5.2.2.2.1 HDL Activity** HDLs have serve a wide range of functions, including contributing to  
525 anti-inflammatory activity. They can limit chemokine secretion from multiple cells types including  
526 endothelial cells and monocytes.(Cockerill Gillian W. et al. 1995; Vorst et al. 2013; Bursill Christina  
527 A. et al. 2010) Rats injected with ApoA1 showed significant reduction in expression of CCR2 and  
528 CX<sub>3</sub>CR1, the receptors for chemokines of the same name, which play a role in leukocyte migration.  
529 (Bursill Christina A. et al. 2010)

530 HDL is also associated with protection from oxidative damage, also inhibiting the potentially  
531 atherogenic oxidised LDL formation.(Anatol, Sandrine, and John 2003) The exact mechanisms of  
532 these antioxidant effect is still actively researched, the enzyme paraoxonase-1, which is present on  
533 HDL particles are likely important.(Mackness, Durrington, and Mackness 2004) Apolipoproteins,  
534 including ApoA4 and ApoAE also have antioxidant properties, for example phospholipid hydroper-  
535 oxidase can be reduced by methionine residues of ApoA1, forming redox-inactive phospholipid  
536 hydroxides.(Christison, Rye, and Stocker 1995; Zerrad-Saadi Amal et al. 2009)

537 HDLs can also suppress proliferation of haematopoietic stem cells, thus reducing leucocytosis and  
538 moncytosis.(Yvan-Charvet et al. 2010) Furthermore, HDLs are implicated in the transport of mi-  
539 croRNAs, though the mechanisms of loading the microRNAs and their biological significance is still  
540 under study.(Vickers et al. 2011)

541 ApoE was less abundant in AIS C improvers within 2-weeks and more abundant at 3-months, and  
542 more abundant in more severe injury, such as AIS A relative to D or C and in AIS C relative to D  
543 (Table S1). ApoE is primarily produced by hepatocytes in the liver, but second-most in the brain,  
544 synthesised in and secreted by astrocytes, and has been found to an important determinant in  
545 response to types of CNS injuries in both animal and human studies.(Teasdale et al. 1997; Poirier  
546 1994) A key function of ApoE is as a ligand for the LDL receptor family of proteins, which mediate  
547 trafficking of cholesterol to neurons, which is vital for axonal growth, and for synapse formation  
548 and remodelling.(Xu, Finkelstein, and Adlard 2014) Additionally, ApoE is implicated in the clearance  
549 of neuronal apoptotic bodies.(Elliott et al. 2007) In humans there are three variants/alleles of ApoE:  
550 ApoE2, ApoE3 and ApoE4, which have a frequency of 8.4%, 77.9% and 13.7% globally.(C.-C. Liu et  
551 al. 2013) The variant proteins differ by one or two amino acids and have been found to result in  
552 substantial physiological alterations.(Mahley and Rall 2000; Jha et al. 2008) The presence of the  
553 ApoE4 variant has been linked to worse outcomes in SCI and TBI.(Jha et al. 2008; C. Sun et al. 2011;  
554 Smith et al. 2006; Friedman et al. 1999) More specifically, the SCI study reported significantly lower  
555 change in the median AIS motor score compared the individuals without the ApoE4 allele during  
556 rehabilitation.(Jha et al. 2008)

557 Prior *in vivo* rodent studies have demonstrated up-regulation of ApoE following SCI and TBI, though  
558 ApoE is not observed in neurons of rodents under normal neuropathology, and they only posses  
559 a single ApoE allele.(Iwata et al. 2005; Seitz et al. 2003; Mahley, Weisgraber, and Huang 2006) A  
560 separate rodent study reported ApoE levels decreased for the first 3 days post-injury, and then in-  
561 creased peak expression at 7 days post-injury, a similar pattern to our results.(X. Yang et al. 2018)  
562 Furthermore, mouse studies have demonstrated replacement of ApoE in neurons with human

563 ApoE4 have impaired neurite outgrowth compared to replacement with ApoE2 or ApoE3, suggesting  
564 ApoE4 interferes with neuroplasticity.(Seitz et al. 2003; White et al. 2001) The underlying mech-  
565 anism/s by which ApoE and its alleles effect neuroplasticity is not currently known, but proposals  
566 have been made. One possibility is reduced lipid transport from astrocytes to neurons, poten-  
567 tially impeding the membrane generation required to support axon growth or dendrite sprouting.  
568 ApoE has anti-oxidant properties, so others have suggested impaired anti-oxidant activity may con-  
569 tribute. ApoE4 has been found to be both secreted less than ApoE2 or ApoE3, and to have inferior  
570 anti-oxidant abilities, lending some credence to this idea.(Mishra and Brinton 2018; Miyata and  
571 Smith 1996) Knowing this, whilst ApoE may make for a useful biomarker for SCI, it will be impor-  
572 tant that particular variants of ApoE a given patient has could be just as important, if not more so,  
573 than simple abundance.

574 **5.2.2.3 Serum Amyloid A1** SAA1 was less abundant in AIS C improvers at 2-weeks relative to  
575 non-improvers, but more abundance in plasma at 3-months (Table S1. SAA1 was also more abun-  
576 dant in AIS A relative to less severe injuries, and in AIS Cs relative to Ds (Table S1. SAA1 is a major  
577 acute-phase protein mainly produced in the liver by hepatocytes in response to infection, tissue  
578 injury and malignancy.(L. Sun and Ye 2016) SAA1 is a precursor of amyloid A (AA), the aberrant  
579 deposition of which leads to inflammatory amyloidosis.(Tape et al. 1988) There are 5 known SAA1  
580 variants, though currently, no indication of substantial functional differences have been identi-  
581 fied.(J. Lu et al. 2014) However, some alleles have been linked to disease, including increased amy-  
582 loidogenesis and tumour suppression.[van der Hilst et al. (2008); lung\_saa1\_2015]

583 During the APR, plasma levels of SAA increase up to 1000-fold, and so serves as a well-established  
584 clinical biomarker for inflammatory disorders.(Gabay and Kushner 1999) SAA isoforms produced  
585 by hepatocytes during an APR are swiftly released into the blood where they associate with HDL,  
586 displacing ApoA1 and becoming an apolipoprotein of HDL.(Banka et al. 1995; Benditt and Erik-  
587 sen 1977) Reverse cholesterol transport, whereby cholesterol in non-hepatic tissues is transported  
588 back to the liver, is conducted via plasma components such as HDL, ABCA1 and ABCG1. ApoA1 acts  
589 as an acceptor for cholesterol in this process, and studies have found that SAA in lipid-free form  
590 can similarly function as a cholesterol acceptor for ABCA1. Whilst SAA is thought to be an important  
591 facet of lipid metabolism, its role is likely complex as mice knockout studies which eliminate SAA1  
592 and SAA1 have shown little effect on cholesterol transport, HDL levels and ApoA1 clearance.(de  
593 Beer et al. 2010, 2011) These studies indicate that the *in vivo* functions of SAA related to lipid  
594 metabolism are more complex than prior *in vitro* studies implied.

595 SAA1 can both induce anti-inflammatory interleukin 10 (IL-10)-secreting neutrophils, but also pro-  
596 motes the interaction of invariant natural killer T cells with those neutrophils, which limits their  
597 suppressive activity by diminishing the production of IL-10 and enhancing the production of IL-12,  
598 indicating that SAA1 can have both pro- and anti-inflammatory effects.(Santo et al. 2010) There has  
599 however been conflicting results reported of SAA's cytokine induction abilities, and some studies  
600 have suggested that recombinant human SAA1 provided by some vendors may have additional  
601 cytokine-inducing activity due to the altered amino acid sequence.(M.-H. Kim et al. 2013)

602 Macrophages are a major source of SAA in inflammatory tissues, and elevated SAA production has  
603 been observed in rheumatoid arthritis, Crohn's disease, Type 2 diabetes and atherosclerosis.(Marzi  
604 et al. 2013; Dong et al. 2011; Vallon et al. 2001; C, F, and B 1997; Meek, Urieli-Shoval, and Benditt  
605 1994) SAA binding to HDL was reported to increase affinity for macrophages whilst decreasing  
606 affinity for hepatocytes.(R. Kisilevsky and Subrahmanyam 1992) This change is thought to favour  
607 the removal of cholesterol from site of inflammation.(R. Kisilevsky 1991) SAA inhibits the binding of  
608 the scavenger receptor SR-BI and cholesterol efflux is enhanced in a SR-BI-dependent manner.(Cai  
609 et al. 2005; van der Westhuyzen et al. 2005) It has been suggested that the SR-BI-mediated re-  
610 uptake of cholesterol underpins the role of SAA in cholesterol recycling during tissue repair, where

611 a great deal of cholesterol is required.(Robert Kisilevsky and Manley 2012)

612 In blood circulation SAA1 may also function as a immune opsonin for increased neutrophil up-  
613 take of Gram-negative bacteria.(Shah, Hari-Dass, and Raynes 2006) Both human and mouse SAA  
614 proteins have been found to bind retinol with nanomolar affinity that limits bacterial burden in  
615 tissues after acute infection.(Derebe et al. 2014) Retinol is important to the body's response to mi-  
616 crobial infection, so SAA may also have a role in limiting bacterial burden, particularly in the liver,  
617 spleen and intestine. The aforementioned study demonstrated that mice lacking in both SAA1 and  
618 SAA2 have a higher bacterial burden in the liver and spleen following infection.(Derebe et al. 2014)  
619 All 3 SAA isoforms are found in intestinal epithelium, which is exposed to the gut microbiome, in  
620 mice. The anti-bacterial properties of SAA isoforms may therefore explain the role of SAA as an  
621 acute-phase protein that protects the host in tissues and organs exposed to bacteria.

622 **5.2.2.4 Retinol-binding protein 4 (RBP4)** In plasma within 2-weeks post-injury, RBP4 was less  
623 abundant in AIS C improvers relative to AIS D and A, and more abundant in AIS C non-improvers  
624 again, relative to AIS D and A (Table S1. Similarly, AIS A plasma had more RBP4 compared to AIS  
625 D, and AIS C improvers were also more abundant in RBP4 compared to non-improvers at both  
626 2-weeks and 3-months post-injury (Table S1).

627 Vitamin A is a collective term for a group of fat-soluble compounds with a range of essential bio-  
628 logical activities including aspects of growth, vision and metabolism.(Blomhoff and Blomhoff 2006)  
629 Following dietary absorption, vitamin A is ferried from the intestine, with chylomicrons as retinyl  
630 esters, to tissues for immediate use or the liver for storage in hepatic stellate cells. A subsequent  
631 dietary deficiency of vitamin A will result in these liver stores being mobilised by hydrolysing the  
632 retinyl esters to release retinol. The retinol is then bound by RBP4, which is also mainly synthesised  
633 in the liver, and secreted into circulation from hepatocytes, whereupon it is bound by an additional  
634 transport protein, transthyretin.(P. A. Peterson 1971) The membrane plasma protein STRA6 facil-  
635 itates retinol transport from RBPs across the cell membrane.(Berry et al. 2012) Once delivered  
636 to target cells, retinol can either be converted to retinaldehyde, which is required for functional  
637 vision, or oxidised to retinoic acid, which is a ligand for nuclear receptors, thus regulating gene  
638 expression.(Lane and Bailey 2005; J. E. Balmer and Blomhoff 2002)

639 RBPs are localised in the ventral region, associated with motor neurons, in the mammalian de-  
640 veloping neural tube.(Pierani et al. 1999; Maden, Ong, and Chytil 1990) The role of retinoid sig-  
641 nalling in spinal cord and motor neuron differentiation, including development of regions of the  
642 spinal cord has been outlined, and implies a possible involvement in maintaining motor neuron  
643 integrity.(Colbert et al. 1995; Sockanathan and Jessell 1998)

644 The mRNA of a rodent homologue of RBP, named cytosolic retinol binding protein, was found to  
645 be up-regulated at 24 hours post-SCI and may promote cell proliferation and regeneration by in-  
646 creasing retinoid metabolism.(Song et al. 2001; Hurst et al. 1999) Another study of amyotrophic  
647 lateral sclerosis (ALS), a neurodegenerative disease, comparing gene expression between post-  
648 mortem spinal cord samples of ALS and controls also observed up-regulation of RBP1 in ALS spinal  
649 cord.(Malaspina, Kaushik, and Belleroche 2001) Furthermore, a transgenic mouse study reported  
650 retinoid signalling may contribute to the retained plasticity and regenerative potential of the ma-  
651 ture spinal cord.(Haskell et al. 2002)

652 The results found here support these findings for AIS C improvers relative to non-improvers as  
653 improver had increased levels of RBP4. Whether this is due to increased expression or due to  
654 higher vitamin A intake is unclear from this data, though at 3-months post-injury this is still the  
655 case even though patients diets could be more similar throughout hospital admission.

656    **5.2.3 Metabolism and SCI**

657    **5.2.3.1 Acute phase response** The bodies first response to injury or infections, including SCI,  
658    is often referred to as the “acute phase response” (APR), which is non-specific, innate reaction  
659    that precedes more specific and situational immune reactions.(Gordon and Koj 1985; Gruys et  
660    al. 2005) This systemic response is largely coordinated by factors released from the liver, but the  
661    APRs effects extend to multiple peripheral organs including the kidneys, lungs and spleen.(Bao et  
662    al. 2012; S. J. Campbell, Zahid, et al. 2008; Fleming et al. 2012; Gris, Hamilton, and Weaver 2008)  
663    This hepatic response is typically transient and quickly fades, but prolonged liver inflammation and  
664    pathology has been observed in rodent SCI models.(Goodus et al. 2018; Sauerbeck et al. 2014)

665    Basic liver functions are chronically impaired by SCI, including metabolising carbohydrates, fats  
666    and proteins, storage of minerals vitamins and glycogen and filtering blood from the digestive  
667    tract.(García-López et al. 2007; DeLeve 2007; Farkas and Gater 2018; Chow et al. 2012; Sauerbeck  
668    et al. 2014) This is likely related to the elevated incidence of metabolic disease in the SCI cohort,  
669    including insulin resistance, impaired glucose tolerance and cardiovascular disease.(Bauman and  
670    Spungen 2001; Maruyama et al. 2008; Lee et al. 2004; J. Myers, Lee, and Kiratli 2007) Long-term  
671    survival is noticeably lower relative to the general population and, whilst mortality in the first 2  
672    year following SCI has decreased in recent decades, long-term survival has not.(Strauss et al. 2006;  
673    Shavelle et al. 2015) More recently, a longitudinal study found SCI patients had a significantly higher  
674    incidence of acute pancreatitis relative to a matched healthy cohort.(Ho, Yeh, and Pan 2021)

675    The acute (1-7 days) liver response to SCI is well documented; the inflammatory cytokines in-  
676    cluding TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-6, released at the injury site, reach the liver through the blood-  
677    stream.(Fleming et al. 2012; Hundt et al. 2011) This provokes the liver to enter the APR and pro-  
678    duce acute phase proteins (APPs) thus stimulating a greater immune response.(Anthony and Couch  
679    2014; Fleming et al. 2012) The hepatocytes that make up the majority of the liver biomass, express  
680    receptors that bind the aforementioned inflammatory cytokines; similarly the hepatic macrophage  
681    Kupffer cells also bind these cytokines, complement proteins and lipopolysaccharide (LPS) and  
682    swiftly remove microorganisms, endotoxins and other debris from the blood.(C.-Y. Yang et al. 2013;  
683    Szalai et al. 2000; Crispe 2016; S. J. Campbell et al. 2005) Hepatic stellate cells act as sensors of  
684    tissue integrity by exposure to signals of oxidative stress, danger/pathogen associated molecu-  
685    lar patterns (DAMPs/PAMPs), chemokines/cytokines and factors secreted from neighbour hepatic  
686    cells, and can stimulate innate immunity by releasing cytokines and as antigen presenting cells  
687    during the APR. (Weiskirchen and Tacke 2014; Fujita and Narumiya 2016)

688    SCI studies in rodent and canine models have found the APPs serum amyloid (SA) A, SAP, CRP, fib-  
689    rinogen, haptoglobin and a1-antichymotrypsin are elevated 4-24 hours post-injury in blood.(Pepys  
690    and Baltz 1983; Gabay and Kushner 1999; J. C. E. Hall et al. 2012; Steel and Whitehead 1994) In ro-  
691    dents, hepatic CD68 mRNA is observed to be elevated within 24 hours post-SCI and CD68+ Kupffer  
692    cell numbers increase during the first 7 days post-SCI.(Sauerbeck et al. 2014)

693    Furthermore, it has been suggested that liver inflammation and Kupffer cells activity promote re-  
694    cruitment of leukocytes to the injury site in brain or spinal trauma, potentially enhancing CNS in-  
695    jury.(Anthony and Couch 2014; S. J. Campbell et al. 2005) For example, a rodent study demon-  
696    strated depletion of Kupffer cells prior to injury resulted in few neutrophils infiltrating the injury  
697    site.(S. J. Campbell, Zahid, et al. 2008; S. J. Campbell, Anthony, et al. 2008)

698    **5.2.4 Microbiome & SCI**

699    Circulating factors from the injury site are not the only potential driver of hepatic inflammation.  
700    Within 24 hours post-SCI in rodents tight junctions between epithelial cells become more perme-  
701    able, thus allowing gut bacteria and the endotoxins they can produce to enter the bloodstream.(J.

702 Liu et al. 2004) This will reach the liver through the portal vein where Kupffer cells function as a  
703 “first line of defence”.(Jenne and Kubes 2013; M. L. Balmer et al. 2014) It has been proposed that  
704 elevated LPS+ endotoxins caused by the post-SCI “leaky gut” causes acute liver inflammation by  
705 overloading hepatic filtrations capacity, allowing microbes to bypass the liver and elicit systemic  
706 inflammation.(J. Liu et al. 2004; O’Connor et al. 2018) The binding of LPS to Kupffer cells results  
707 in the production of a range of growth factors, including TNF- $\alpha$ , multiple interleukins and reactive  
708 oxygen species (ROS), stimulating bone-marrow-derived monocytes and neutrophils to infiltrate  
709 the liver.(S. A. Myers et al. 2019; Milosevic et al. 2019; Kazankov et al. 2019) A rodent study found  
710 transcription factors for tight junctions down-regulated following SCI, and that application of pro-  
711 biotics improved neurological outcomes.(Kigerl, Mostacada, and Popovich 2018; Kigerl et al. 2016)  
712 Human studies of the microbiome post-SCI have also demonstrated dysbiosis, both chronically  
713 and more acutely post-injury.(Zhang et al. 2018; Gungor et al. 2016; Bazzocchi et al. 2021)

#### 714 5.2.5 Drivers of liver steatosis

715 Steatosis, the abnormal retention of lipids within cells or organs, most commonly associated with  
716 the liver, has been observed to increase in rodents during the first week post-injury.(Sauerbeck  
717 et al. 2014) The liver takes up circulating fatty acids, and when levels exceed the oxidative and  
718 secretory limits of the liver, hepatocytes store the excess as triglycerides.(Diraison and Beylot 1998)  
719 Adipose tissue lipolysis during elevated sympathetic activity leading to spikes in circulating fatty  
720 acids has been reported in human subjects following SCI.(Karlsson 1999)

721 *De novo* lipogenesis occurring within the liver can also drive hepatic steatosis.(Lavoie and Gau-  
722 thier 2006) Ceramides are lipid signalling molecules and regulators of apoptosis and inflamma-  
723 tion; they can contribute to insulin resistance, oxidative stress and inflammation-induce liver adi-  
724 posity through sustained Toll-like-receptor(TRL)-4 activation.(Schilling et al. 2013; Bhargava and  
725 Lee 2012; Pagadala et al. 2012) If released into the circulatory system, ceramides can cause CNS  
726 toxicity, including oxidative damage and changes to the aggregation of proteins associated with  
727 diseases such as Parkinson’s, Huntington’s and Alzheimer’s.(Pagadala et al. 2012; Vidaurre et al.  
728 2014; Czubowicz et al. 2019) Mature and precursors of hepatic ceramides and enzymes which  
729 contribute to ceramide synthesis are elevated by 1 day post-injury.(Sauerbeck et al. 2014) Endo-  
730 toxins can also stimulate the synthesis of ceramides and so the aforementioned “leaky gut” may  
731 also contribute to this elevation.(Chang et al. 2011) Ceramide synthesis and lipogenesis genes are  
732 also stimulated by TNF- $\alpha$ , which, as touched on in the general introduction (??), has been found  
733 to be elevated post-SCI, and associated with differential neurological recovery.(Davies, Hayes, and  
734 Dekaban 2007; Hasturk et al. 2009; Biglari et al. 2015; Sauerbeck et al. 2014; Bikman 2012)

#### 735 5.2.6 Chronic liver inflammation in SCI

736 The hepatic APR and associated inflammation that typically follows bodily trauma, subsequently  
737 rapidly subsides, whereas post-SCI this hepatic inflammation persists chronically. This chronic  
738 phase may be due in part to long-term changes in intestinal permeability via fewer tight junc-  
739 tions in intestinal epithelial cells, resulting in gut dysbiosis.(Milosevic et al. 2019; O’Connor et al.  
740 2018; Kigerl, Mostacada, and Popovich 2018; Kigerl et al. 2016) Bacterial translocation and gut  
741 dysbiosis can be the result of non-mechanical intestinal obstruction, impaired intestinal motility  
742 and systemic immune suppression, all of which are potential complications of SCI.(Balzan et al.  
743 2007) Specifically, butyrate-producing bacteria have been found to be reduced in SCI relative to a  
744 healthy cohort.(Gungor et al. 2016) Butyrate is known to modulate epithelial differentiation and  
745 cell growth, and suppress macrophages, including CNS inflammation, thus the reduction in bu-  
746 tyrate from bacteria may contribute to recovery post-SCI, though links to the liver specifically have  
747 not yet been studied.(H. J. Kim et al. 2007; Arpaia et al. 2013; Park et al. 2005; P. S. Chen et al. 2007)

748 LPS is another potential modulator of post-SCI chronic liver physiology. Kupffer cells, hepatic en-  
749 dothelial cells and hepatocytes all participate in the clearance of LPS via CD14- and TLR4-dependent  
750 mechanisms.(Mimura et al. 1995; van Oosten et al. 2001; Vodovotz et al. 2001) LPS induced the  
751 release of factors such as TNF- $\alpha$

### 752 **5.2.7 Longitudinal metabolic health**

753 Prior work has found at least 25% of acute SCI patients to be obese, which is well known to induce  
754 low-level systemic inflammation, and that this cohort has significantly worse outcomes compared  
755 to non-obese SCI patients (Stenson et al. 2011). Alcohol abuse has also been associated with  
756 poorer SCI neurological outcomes (Elliot et al. 2002). Furthermore, advancing age is associated with  
757 increased liver inflammation and the SCI population has followed the general populations ageing  
758 trend (Bertolotti et al. 2014; Y. Chen, He, and DeVivo 2016). Taken together, it is not unreasonable  
759 to assume that a large number of SCI patients may have pre-existing liver inflammation at injury.  
760 This may be an important differentiator that contributes to the degree of neurological recovery  
761 a given patient may experience. Future experiments investigating neurological outcomes of SCI  
762 may benefit from establishing parameters of metabolic health, including the composition of the  
763 microbiome, as close to injury as possible, and potentially monitoring changes in these parameters  
764 longitudinally.

### 765 **5.2.8 Validation of results**

766 The ELISAs used to validate the proteomic data often did not demonstrate significant differences  
767 between the groups (Figures ??, ??, ?? and ??). This may be in part to the individual variability of  
768 the samples. However, the trends of the data do largely reflect those found in the iTRAQ data, sug-  
769 gesting that with greater statistical power there may be a more robust validation. Furthermore, the  
770 ApoA1 ELISAs resulted in the most significant differences, and was the only Quantikine® kit used  
771 (Figure ??). As the Quantikine® kits are highly optimised, including for use with plasma, whereas  
772 the DuoSet®s, which were used for the other proteins, are not. Future studies should therefore  
773 consider either simply using Quantikine® kits, or ensure good optimisation of the DuoSet® kits  
774 in advance. These results are also corroborated by a recent label-free proteomic SCI study, using  
775 a rodent model, which reported similar proteins associated with complement cascade, including  
776 A2M and C3.(Yao et al. 2021)

### 777 **5.2.9 Conclusion**

778 This work shows that proteins associated with the complement cascade, and apolipoproteins in  
779 particular, have potential as prognostic biomarkers for SCI. For some of these biomarkers, ApoE  
780 in particular, it may not be pure abundance, but also the particular allele of the patient that may  
781 provide valuable insight. However, the relatively small number of proteins identified here is a lim-  
782 itation, likely due to highly abundant proteins impacting the dynamic range of the samples. The  
783 pooling of samples also obscures individual variability in protein abundance. Subsequent pro-  
784 teomics experiments using label-free techniques, and depletion of highly abundant proteins may  
785 allow for more in-depth pathway analysis. These results, in concert with the prior chapters find-  
786 ings (??), provide further evidence of a link between metabolic function and functional neurological  
787 recovery post-SCI. Further work is needed elucidate the precise biochemistry at play, and perhaps  
788 more importantly, whether modulation of these pathways has the potential to improve outcomes.  
789 Experiments that closely monitor the liver, modify diet and analyse metabolites, particularly longi-  
790 tudinally post-injury, would all give further insight into this relationship.

791 **5.3 thesis label-free discussion**

792 As outlined previously (5.2.9), two key limitations of the iTRAQ experiments were the pooling of  
793 samples, which prevents statistically robust group-wise comparisons, and the high dynamic range  
794 of protein abundances in plasma potentially obscuring less abundant proteins. This work seeks  
795 to address these factors by a combination of Proteominer™ beads to shrink the dynamic range of  
796 protein abundances, and by not pooling samples.

797 **5.3.1 Proteins identified**

798 A total of 87 proteins were identified, many of which were only detected in one group. Proteins  
799 only present in limited groups could be highly suited for use as biomarkers as binary indicators are  
800 much simpler to test for, and suggest more dramatic biological differences. Here we explore the  
801 potential these proteins have a biomarkers of SCI.

802 **5.3.1.0.1 Peroxiredoxins** Peroxiredoxins are a large and highly conserved family of enzymes  
803 that reduce peroxides. Peroxiredoxin 2 (PRX-2) is highly abundant in RBCs and intracellularly serves  
804 as an important anti-oxidant role in various cell types, including neurons.(Low, Hampton, and  
805 Winterbourn 2008) By contrast, extracellular PRX-2 has been suggested to act as an inflamma-  
806 tory DAMP, leading microglia and macrophages to release a plethora of pro-inflammatory fac-  
807 tors.(Salzano et al. 2014; Garcia-Bonilla and Iadecola 2012; Shichita et al. 2012) An *in vitro* primary  
808 neurons and microglia co-culture study reported PRX-2 activating microglia via TLR-4, potentially  
809 leading to neuronal apoptosis.(Y. Lu et al. 2018) A mouse study found over-expression of PRX-2 at-  
810 tenuated oxidative stress and neuronal apoptosis following subarachnoid haemorrhage.(Y. Lu et al.  
811 2019) Over-expression of PRX-2 is speculated to protect again ischaemic neuronal injury by mod-  
812 ulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase (ASK) 1 signalling com-  
813 plex.(Gan et al. 2012) Several molecular chaperones can interact with ASK1, including thioredoxin  
814 and TNF receptor-associated factor 6.(Matsuzawa et al. 2005) The dissociation of the thioredoxin-  
815 ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free radicals, whereupon its an-  
816 tioxidant activity is reduced. This inactivation can be reversed by the thioredoxin-thioredoxin  
817 reductase system, whereby oxidised PRX-2 can regain its activity by reducing thioredoxin, leading  
818 to the dissociation of the thioredoxin-ASK1 complex.(Rhee and Woo 2011) Additionally, oxidised  
819 PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding domain on ASK1.(S. Y. Kim,  
820 Kim, and Lee 2008)

821 PRX-2 was found to be present in AIS C improvers and AIS D patients acutely, and in AIS A and  
822 D patients subacutely. The differences in abundance between these groups was not statistically  
823 significant, though acute AIS D had less PRX-2 relative to subacute AIS D ( $\log_2$  fold change -1.9) and  
824 subacute AIS A also had less abundant PRX-2 relative to subacute AIS D ( $\log_2$  fold change -1.7). The  
825 presence of PRX-2 in acute AIS C improvers and absence in acute C non-improvers suggests the  
826 protein could indicate a more protective action against oxidative stress, and implies the protein  
827 has potential value as a biomarker of functional outcomes. Similarly, PRX-2 may be acting as a  
828 healthy response to trauma-induced oxidative stress in both acute AIS D, although the persistence  
829 to the subacute time-point is less clear. Likewise, the presence of PRX-2 in AIS A subacutely, but not  
830 acutely is more perplexing. It should be noted that as plasma was used and cells lysed, so there  
831 is no distinguishing between intracellular and extracellular PRX-2. Perhaps in the more severe AIS  
832 A injury, secondary injuries, including oxidative stress, are greater and so persist to the subacute  
833 time-point. The acute absence may be a result of an overwhelmed physiology unable to respond  
834 or prioritise managing oxidative stress.

835 **5.3.1.1 Neuroinflammation post-SCI** The neuro-inflammatory response begins immediately  
836 post-trauma, and involves a complex series of events that can persist well into the chronic phase.

837 The sudden emergence of necrotic cell debris and associated DAMPs lead surviving CNS-resident  
838 cells to produce cytokines, complement factors and ROS. Within minutes CNS cells at the lesion site  
839 have been found to secrete several pro-inflammatory mediators, including TNF- $\alpha$  and interleukins,  
840 in both rodent models and human patients with SCI.(Pineau and Lacroix 2006; Chandrasekar et al.  
841 2017; Dalgard et al. 2012; Bastien et al. 2015) The resulting inflammatory response occurs in  
842 parallel to the mechanical destruction of the blood-spinal cord barrier, and the development of  
843 tissue oedema and ischaemia combine to propagate damage to parts of the cord spared by the  
844 initial trauma.(Maikos and Shreiber 2007; Ahuja et al. 2017)

845 The microglial population at the lesion site have been observed to be significantly depleted  
846 immediately post-injury, due to death via both the apoptosis and mechanical injury in a rodent  
847 model.(Bellver-Landete et al. 2019) Surviving microglia change in shape and migration patterns,  
848 and begin to produce ROS, oxidative metabolites and pro-inflammatory cytokines.(Pineau and  
849 Lacroix 2006; Bastien and Lacroix 2014) These cells can associate with damaged axons rapidly  
850 post-injury, but are thought to not actively phagocytose these cells until approximately 4 days  
851 post-trauma.(Bellver-Landete et al. 2019; Pineau and Lacroix 2006; Greenhalgh and David 2014)

852 The following hours and days post-injury are characterised by a substantive complement sys-  
853 tem activation and sequential leukocyte migration from the periphery into the injured neural  
854 parenchyma.(Brennan et al. 2015; S. L. Peterson and Anderson 2014; Qiao et al. 2006) Curiously,  
855 though the breakdown of the BSCB would presumably allow unrestricted access of circulating  
856 leukocytes into the injured cord segment, recruitment of these cells remains a highly controlled  
857 process.(Beck et al. 2010; Brennan et al. 2019) A mouse study reported lymphocytes, which  
858 account for approximately 80% of circulating leukocytes, only enter the cord in substantial  
859 numbers at least several weeks to months post-injury.(Beck et al. 2010) Early infiltrate is instead  
860 largely comprised of myeloid cells, predominantly neutrophils, which are a minority of circulating  
861 cells but are the swiftest peripheral responders to SCI, with studies detecting them at the lesion  
862 site within 4 hours of injury.(Wright et al. 2010) Neutrophil numbers have been reported to peak  
863 at 1 day post-trauma, but also to remain at the site for a minimum of 42 days post-injury.(Okada  
864 2016; Kigerl, McGaughy, and Popovich 2006)

865 This neutrophil recruitment is often viewed as principally detrimental to recovery following SCI, but  
866 also wound healing more generally. A recent study found circulating neutrophil numbers in ad-  
867 mission bloods from human SCI patients were negatively correlated with patient outcomes at dis-  
868 charge.(Brennan et al. 2019) The same study utilising a contusive SCI mouse model, showed the ex-  
869 tent of neutrophil presence at the lesion site inversely correlated with neurological outcomes, and  
870 depletion of said cells with an antibody against Ly6G improver recovery of motor function.(Brennan  
871 et al. 2019) However, other studies have suggested neutrophil activity which potentially benefits  
872 SCI recovery. A transgenic mouse contusion model study showed over-expression of secretory  
873 leukocyte protease inhibitor, which can arise from neutrophils and activated macrophages, im-  
874 proved locomotive functional outcomes, and reduced markers of secondary injury.(Ghasemlou  
875 et al. 2010) Another study, using a peripheral nerve injury mouse model, reported neutrophil  
876 infiltration and associated cytokine/chemokine production was vital for clearance of myelin de-  
877 bris.(Lindborg, Mack, and Zigmond 2017) Additionally, another study using a mouse contusion  
878 model found increased lesion sizes and impaired neurological outcomes following neutrophil de-  
879 pleition, though the Gr-1 antibody used also depletes inflammatory monocytes, muddying the pic-  
880 ture somewhat.(Stirling et al. 2009) Regardless, it is clear that the complexity of the role neutrophils  
881 play in the SCI response extends beyond any simple binary beneficial/harmful distinction.

882 Moving forward in the SCI pathology, newly proliferated and recruited microglia begin ac-  
883 tively phagocytosing necrotic cell debris, and begin accumulating around the lesion epicen-  
884 tre.(Greenhalgh and David 2014; Bellver-Landete et al. 2019; Pineau and Lacroix 2006) The  
885 presence of microglia appears to be vital, particularly during the first week post-SCI, as depletion

886 via the colony stimulating factor-1 inhibitor PLX5622 has been linked to substantially worsened  
887 functional outcomes.(Bellver-Landete et al. 2019; Brennan et al. 2018) Relatedly, another  
888 mouse SCI model study found early enhancement of microglial activation can reduce secondary  
889 pathology.(Stirling et al. 2014)

890 Circulating inflammatory monocytes are also recruited during the first days post-trauma. Adoptive  
891 transfer experiments have shown recruitment to pick up at approximately 3 days post-injury,  
892 and peak at 7 days.(Blomster et al. 2013) Whilst monocyte turnover at the lesion appears to be  
893 high, infiltrating monocyte-derived macrophages remain at the site of weeks to months post-  
894 trauma.(Blomster et al. 2013; Shechter et al. 2009) Interestingly, the timing of monocyte recruit-  
895 ment appears to be delayed relative to non-neurological tissue injury. For instance, monocytes  
896 are reported to be rapidly recruited to the heart following a myocardial infarction, as early as 1 day  
897 post-injury, and their numbers return to baseline by roughly 16 days post-injury.(Nahrendorf et al.  
898 2007)

899 Owing to the diversity of monocyte subsets and macrophage phenotypes, a complete un-  
900 derstanding of their role with respect to SCI pathology is still lacking, and requires under-  
901 active research.(David and Kroner 2011) Some polarisation states associated with recruited  
902 macrophages are thought to be implicated in propagating secondary injury via fibrotic scar  
903 formation and demyelination of axons.(Kigerl et al. 2009; Popovich et al. 1999; Zhu et al. 2015)  
904 Similarly, several studies have reported a reduction in infiltration of monocytes/macrophages  
905 is associated with better SCI outcomes.(Kigerl et al. 2009; Zhu et al. 2015; Horn et al. 2008)  
906 Conversely, others have found depletion o circulating monocytes/macrophages significantly  
907 increased lesion size and results in worse function outcome, with restoration of blood monocyte  
908 numbers attenuating this phenotype.(Shechter et al. 2009) More recent *in vitro* studies suggested  
909 blood-derived macrophages can suppress microglial phagocytosis without reducing microglial  
910 proliferation and extension of processes.(Greenhalgh and David 2014; Greenhalgh et al. 2018)  
911 This literature represents and ongoing controversy over the role of monocytes/macrophages in  
912 relation to recovery post-SCI. Importantly, many of these studies are based on somewhat crude  
913 depletion of cell types, with little discrimination paid toward any potential subpopulations and/or  
914 cell polarisation status. Given the shear complexity of the pathology at play, more nuanced  
915 approaches will likely be needed in future studies to paint a more complete picture.

916 B cell recruitment is yet wave of immune cell infiltration, thought to occur several days post-injury.  
917 These cells can form follicle-like structures in combination with T cells, microglia and macrophages  
918 from roughly 28 days post-trauma, and remain present and the lesion well into the chronic phase  
919 of SCI.(Ankeny, Guan, and Popovich 2009) Whilst the extent of B cell presence has been reported  
920 to vary between animals, they have been correlated with self-reactive antibodies that recognise  
921 epitopes within protein homogenates of the spinal cord.(G. Sun et al. 2017) Adoptive transfer  
922 experiments in a mouse model isolated antibodies from SCI mice, and found injected them into  
923 the neural parenchyma of naïve animals induced significant damage, whereas mice lacking B cells  
924 have improved recovery post-SCI.(Ankeny, Guan, and Popovich 2009)

925 Move evidence is needed to establish whether these self-reactive antibodies precede an autoim-  
926 mune event, or signify a autoimmune disease. Alternatively, they may serve as a mechanism for  
927 opsonisation and debris clearance from the lesion site.(Nagele et al. 2013) Naturally occurring  
928 autoantibodies with well-established role in tissue regeneration and repair have been found to  
929 be elevated following SCI.(Palmers et al. 2016; Arevalo-Martin et al. 2018) Much like the afore-  
930 mentioned monocyte/macrophage controversy, it should be pointed out that any positive effects  
931 of these autoantibodies does not preclude any simultaneous negative impacts which could be  
932 modulated. For instance, another study reported naturally occurring IgM antibodies contribute to  
933 secondary injury during the more acute phase post-SCI.(Narang et al. 2017)

934 Neuro-inflammation is less understood at the chronic phase of SCI, as most studies focus on the  
935 first hours and days post-injury. By this stage, the glial scar has established a well-defined border  
936 between the lesion core and the healthy tissue flanking it.(Sofroniew and Vinters 2010) Infiltrating  
937 immune cells are largely restricted to within the lesion itself, as opposed to the surrounding spared  
938 tissue. B and T cells, macrophages and neutrophils have all been detected here many months post-  
939 trauma.(Beck et al. 2010; Ankeny, Guan, and Popovich 2009; Prüss et al. 2011) The chronic phase is  
940 also marked by substantial metabolic dysfunction, characterised by reduced lipid metabolites and  
941 increased oxidative stress, in addition to elevated pro-inflammatory mediators.(Dulin et al. 2013)  
  
942 There are fewer studies that attempt to elucidate the underlying mechanisms driving this non-  
943 resolving inflammatory response in the chronic phase of SCI. One study suggested communication  
944 with infiltrating monocytes suppresses chronic microglial activation and inflammation after  
945 SCI.(Greenhalgh et al. 2018) Interruption of this communication was linked to worsened function  
946 outcomes, implying the initial microglial response to trauma may be beneficial, their pro-  
947 tracted activation can eventually become detrimental.(Bellver-Landete et al. 2019; Greenhalgh et  
948 al. 2018) Furthermore, a rodent model study of chronic SCI, found use of the anti-inflammatory  
949 drug licoferone, applied daily for 1 month at 8 months post-injury, observed some improvement  
950 to metabolic functions, but no benefit to locomotor function.(Dulin et al. 2013) To summarise, un-  
951 derstanding of persistent inflammation during the chronic phase of SCI is lacking, and particularly  
952 complicated by the plateaus in locomotive recovery that typically occurs well before the chronic  
953 SCI phase is reached. Thus, there is a need for further studies to uncover the role of the various  
954 immune cell populations with respect to ongoing neurological dysfunction and pathology during  
955 the chronic phase of SCI.

956 **5.3.1.1.1 Intravenous immunoglobulin** Intravenous immunoglobulin (IVIG) is increasingly  
957 used as an immunomodulatory strategy for managing acute neurological conditions, including  
958 neurotrauma. Originally developed as an antibody replacement therapy for immunodeficiency  
959 disorders, IVIG is a product comprised primarily of immunoglobulin G (IgG) taken from the blood  
960 plasma of healthy donors.(Bayry, Negi, and Kaveri 2011; Schwab and Nimmerjahn 2013) IVIG  
961 therapy was found to increase platelet number in idiopathic thrombocytopenic purpura (ITP)  
962 patients, which lead to an interest in using it as an immunomodulatory therapy.(Imbach et al.  
963 1981) Its potent effects and limited side effects have lead high-dose IVIG therapy to be commonly  
964 used in a plethora of inflammatory and autoimmune disorders, including ITP, arthritis, Kawasaki's  
965 syndrome and Guillain-Barré syndrome.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et  
966 al. 1998)

967 Some recent research using a contusive SCI mouse model has reported promising results of high-  
968 dose IVIG as a therapeutic for SCI.(Brennan et al. 2016) The study found that a clinical dose of  
969 IVIG (0.5-2g/kg body weight) lead to a 30-40% reduction in lesion size, and reductions in demyeli-  
970 nation, central canal dilation, and axonal degeneration, though doses below 0.5g/kg were ineffec-  
971 tive.(Brennan et al. 2016) The same study also found albumin treatment did not produce the same  
972 effects as IVIG, suggesting simple protein loading is not the causative mechanism. Likewise, rodent  
973 studies utilising purified human IgG in a high-level (C7-T1) clip aneurysm model, and another lower-  
974 level (T9) contusion SCI study, reported similar improvements.(Nguyen et al. 2012; Chio et al. 2019;  
975 Gok et al. 2009) Additionally, a Phase I/IIa clinical trial aiming to explore the safety and efficacy of  
976 IVIG therapy in human SCI patients is approved and underway (ACTRN12616001385437). How-  
977 ever, whilst there are several pre-clinical studies reporting IVIG treatment can benefit outcomes in  
978 CNS injury from a range of neurological conditions, the exact mechanism/s behind any potential  
979 neuroprotective effects of IVIG for SCI are currently unclear.(Tzekou and Fehlings 2014)

980 In TBI mouse models, animals treated with IVIG were shown to have improved neurobehavioural  
981 outcomes, and a reduction in neuronal degeneration both acutely and chronically, relative to

982 vehicle-treated controls in rotarod and Morris water maze experiments.(Jeong et al. 2014) Further  
983 mouse studies using cerebral artery occlusion, a model of stroke, reported high-dose IVIG signif-  
984 icantly reduced infarct volumes, neurological impairment and mortality rates.(Arumugam et al.  
985 2007; Widiapradja et al. 2012) Under condition of BBB/BSCB compromise, IVIG has been found to  
986 enter the neural parenchyma within hours of injury.(Brennan et al. 2016; Arumugam et al. 2007)  
987 SCI studies have found IVIG to localise to oligodendrocytes, astrocytes, neurons, macrophages,  
988 microglia, pericytes and blood vessels.(Brennan et al. 2016; Chio et al. 2019) Additionally, reduc-  
989 tions in immune cells, as indicated by F4/80<sup>+</sup> microglia/macrophages and polymorphonuclear  
990 cells in brain and spinal injury models respectively, have also been reported.(Jeong et al. 2014;  
991 Nguyen et al. 2012; Chio et al. 2019) Relatedly, the aforementioned SCI IVIG mouse study found  
992 reduced CD68<sup>+</sup> macrophages at and surrounding the lesion 35 days post-injury.(Brennan et al.  
993 2016) Importantly, these studies do not differentiate between resident microglial and infiltrating  
994 monocytes/macrophages. Thus, further research is needed to understand the influence of IVIG  
995 on both recruitment and activation states of these cell subsets.

996 **5.3.1.1.2 Speculative mechanisms of action for IVIG in SCI** As IVIG is made from pooled anti-  
997 bodies taken from thousands of donors, it includes a vast repertoire of antibodies specific against  
998 millions of unique antigens, allowing for a diverse variety of effects in differing disease contexts.  
999 Whilst there is extensive research of IVIG and autoimmune disorders, such as Guillain-Barré syn-  
1000 drome, the immune pathology found in the acute phase of CNS injury is not typically considered  
1001 to be driven by autoimmune processes.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et  
1002 al. 1998) There may be some overlap in therapeutic mechanism, but it seems more likely any  
1003 benefits are conferred through modulation of the innate rather than adaptive immune responses.  
1004 The potential mechanisms of IVIG can be split between those mediated via the IgG constant (Fc)  
1005 fragment, which binds the Fc receptors, and the F(ab')<sub>2</sub> fragment, which governs antigen recogni-  
1006 tion.(Schwab and Nimmerjahn 2013) In the context of neurological diseases, mechanisms related  
1007 to F(ab')<sub>2</sub> are thought to potentially bind and therefore neutralise cell surface receptors, comple-  
1008 ment, cytokines and autoantibodies. By contrast, Fc-dependent mechanisms are speculated to in-  
1009 clude regulation of Fc receptor expression, saturation of the neonatal Fc receptor, block activation  
1010 of Fc receptors, and modulate T cells.(Schwab and Nimmerjahn 2013; Lünemann, Nimmerjahn,  
1011 and Dalakas 2015; Dalakas 2014) Furthermore, models of neurological injury suggest both F(ab')<sub>2</sub>  
1012 and Fc-dependent signalling cascades could be involved in the modulation of several chemokines  
1013 and cytokines.(Dalakas 2014)

1014 Modulation via the variable F(ab')<sub>2</sub> region

1015 Self-reactive antibodies have been found circulating in both chronic rodent SCI models and hu-  
1016 man patients 1 year post-injury.(Ankeny, Guan, and Popovich 2009; Hayes et al. 2002) Whilst some  
1017 studies have suggested potential relevance of naturally occurring autoantibodies (germline en-  
1018 coded and produced by B1 cells) in acute SCI, it remains unclear whether IVIG treatment may have  
1019 any impact on them.(Palmers et al. 2016; Narang et al. 2017) The impact or lack thereof of IVIG on  
1020 chronic phase SCI autoimmunity also remains to be seen.

1021 A separate potential F(ab')<sub>2</sub>-dependent mechanism involves the neutralisation of the cell death  
1022 mediator Fas (AKA CD95). Studies of Lyell's syndrome, a disorder whereby active Fas ligand binds  
1023 Fas present on keratinocytes, inducing apoptosis, reported IVIG therapy completely inhibited Fas  
1024 ligand-induced cell death both *in vitro* and in human patients.(Viard et al. 1998; Altnauer et al.  
1025 2003) Importantly, IVIG blocked Fas, as opposed to Fas ligand, in these studies, as this result was  
1026 only observed with cells pre-treated with IVIG. Incubation of IVIG with soluble Fas ligand did not  
1027 attenuate cell death, implying IVIG contains antibodies specific to Fas.(Viard et al. 1998; Altnauer  
1028 et al. 2003) This modulatory effect of the Fas-Fas ligand pathway may have relevance in SCI, as a  
1029 study using knock-out mice lacking Fas showed a reduction in both apoptosis at the lesion site and

1030 glial scarring, and improved motor function post-SCI.(Sobrido-Cameán and Barreiro-Iglesias 2018;  
1031 W. R. Yu and Fehlings 2011) Neurons and glial cells from post-mortem human patients were found  
1032 to be more Fas- and Fas ligand-positive, but this was limited to the acute phase of SCI, and not  
1033 observed chronically, suggesting this pathway is more significant immediately post-injury.(W. R. Yu  
1034 and Fehlings 2011) Therefore, acute IVIG treatment could act by attenuating secondary cell death  
1035 by blocking Fas, thus disrupting this pathway.

1036 Conversely, agonistic anti-Fas antibodies have also been reported within IVIG prepara-  
1037 tions.(Altnauer et al. 2003) Whilst it remains unknown how these agents may act in SCI,  
1038 one could postulate a benefit if they induce apoptosis in circulating leukocytes, which could  
1039 otherwise do harm.(Schneider et al. 2017) Supporting this, papers have found reductions in poly-  
1040 morphonuclear cell populations within the lesion at 1 day post-injury in rodent models.(Nguyen  
1041 et al. 2012; Chio et al. 2019; Gok et al. 2009) However, IVIG-induced apoptosis has only been  
1042 observed in human leukocytes, not in rodents, casting doubt on this idea.(Altnauer et al. 2003;  
1043 Schneider et al. 2017) Alternatively, the reduced recruitment could be a result of IVIG regulating  
1044 the expression of adhesion molecules or molecules involved in leukocyte trafficking. A feline  
1045 ischaemia-reperfusion injury model study found IVIG to down-regulate expression of integrins  
1046 on leukocyte cell surfaces, inhibiting adhesion and subsequent extravasation of the cells into the  
1047 damaged site.(Gill et al. 2005) Again however, these findings are contradicted by an experimental  
1048 stroke study where IVIG was found to increase leukocyte and platelet trafficking to the injury,  
1049 leading to formation of aggregates within cerebral vasculature.(Lapointe et al. 2004)

1050 Finally,  $F(ab')_2$  may act by complement scavenging. Both *in vitro* and *in vivo* studies have found the  
1051 non-antigen-binding regions of  $F(ab')_2$  can bind and neutralise the complement activation prod-  
1052 ucts C3a and C5a, thus preventing complement-mediated tissue damage.(Milan Basta et al. 2003;  
1053 M. Basta et al. 1989) Multiple studies utilising various models of CNS injury have reported IVIG  
1054 attenuating complement.(Brennan et al. 2016; Arumugam et al. 2007) Specifically in SCI, IVIG was  
1055 found to reduce levels of the complement activation products C3b and C5a within the damaged  
1056 cord.(Brennan et al. 2016) Similarly, an experimental stroke study reported IVIG reducing C3b lev-  
1057 els in the infarct area.(Arumugam et al. 2007) Interestingly, whilst this study found IgG able to  
1058 bind mouse C3b, supporting the hypothetical neutralisation of complement activation products,  
1059 they also found IVIG able to attenuate oxygen deprivation-induced production of C3 itself in pri-  
1060 mary neuron cultures. This seems to suggest IVIG is able to scavenge both secreted complemen-  
1061 t activation products, and their local production.(Arumugam et al. 2007)

#### 1062 Modulation via the constant Fc region

1063 With respect to the Fc region, this portion normally binds to  $Fc\gamma$  receptors ( $Fc\gamma$ Rs), which are  
1064 present on most leukocytes and resident CNS cells. Many  $Fc\gamma$ Rs act as activating receptors, such as  
1065 inducing phagocytosis in response to opsonised targets, or as an inhibitory receptor that dampens  
1066 effector cell responses.(Schwab and Nimmerjahn 2013) A given cell's response to an immunoglobu-  
1067 lin isotype is determined by the combination of which  $Fc\gamma$ Rs are expressed by said cell. Myeloid cell  
1068 all express some combination of these activating  $Fc\gamma$ Rs, as do some innate lymphoid cells which  
1069 do not express more classical antigen receptors, such as natural killer cells, whereas T and B cells  
1070 do not.(Perussia et al. 1989) The inhibitory  $Fc\gamma$ RIIb receptor is also expressed on myeloid cells, in  
1071 addition to B cells, but not natural killer cells or resting T cells.(Bruhns and Jönsson 2015) Whilst  
1072 there is debate over the expression and function of  $Fc\gamma$ Rs in neurons, *in vitro* work with neuronal  
1073 cultures has detected mRNA for all  $Fc\gamma$ Rs.(Thom et al. 2017) Astrocytes, microglia and oligoden-  
1074 drocyte precursors have also been found to express  $Fc\gamma$ R, and up-regulate them under some disease  
1075 states.(Thom et al. 2017)

1076 Studies utilising just the Fc fragment have been found to be equally effective as normal IVIG in  
1077 several non-neurological autoimmune diseases, including nephrotoxic nephritis, ITP and K/BxN

1078 arthritis models, suggesting Fc $\gamma$ Rs play a key role in the mechanism of IVIG.(Samuelsson, Towers,  
1079 and Ravetch 2001; I. K. Campbell et al. 2014; Kaneko et al. 2006) With respect to CNS injury, some  
1080 evidence suggesting a role of Fc $\gamma$ Rs comes from a mouse study with animals lacking the common  
1081  $\gamma$ -chain, and thus no functional Fc $\gamma$ Rs, which were found to be protected from experimental stroke  
1082 and SCI.(Ankeny, Guan, and Popovich 2009; Komine-Kobayashi et al. 2004)

1083 Within the context of antibody-mediated autoimmune disorders, high-does IVIG may saturate Fc  
1084 receptor and reduce the half-life of pathogenic endogenous IgG.(Schwab and Nimmerjahn 2013)

1085 **5.3.1.1.3 Immunoglobulins** Several immunoglobulin components were identified here, includ-  
1086 ing 3  $\lambda$  variable precursors (3-19, 3-10 and 2-18), 3 heavy variable precursors (3-15, 1-69 and 1-24)  
1087 and 2 heavy constant gamma regions (2 and 4). For the  $\lambda$  variable precursors, acute AIS C improvers  
1088 the precursors 3-19 and 3-10 were detected, whereas 3-10 and 2-18 were detected in acute C non-  
1089 improvers. That acute C non-improvers expressed the 2-18 precursor whilst the improvers did  
1090 not, suggests potential as a biomarker of poorer functional outcomes. It is difficult to comment  
1091 on the biological mechanisms that may be at play here from this data, but one could infer that  
1092 it is indicative of either a more robust, or a more maladaptive, immune response to the trauma.  
1093 Given that the injuries are of the same severity by AIS grade, the latter seems more likely, though  
1094 again, further research is needed to highlight the precise nature of this difference. Interestingly,  
1095 whilst the acute C improvers do not express precursor 2-18, both the subacute C improvers and  
1096 non-improvers, and subacute As do, whereas acute or subacute Ds do not, seemingly implying this  
1097 precursor is also indicative of more severe injury in the latter phases of SCI.

1098 In addition of acute C improvers, subacute As and acute Ds also express the 3-19 precursor, with  
1099 subacute As possessing the greatest abundance. Again, this would seem to suggest this marker  
1100 is indicative of positive outcomes or less severe injury in the acute phase, but may be more detri-  
1101 mental in the latter phases. The final  $\lambda$  precursor, 3-10, is present in acute As, subacute As and  
1102 both subacute C groups as well as the aforementioned acute C improvers. The curious absence  
1103 of 3-10 in both AIS D groups and C non-improvers groups suggests the marker is implicated in a  
1104 more beneficial response, but perhaps this is limited to more severe injuries.

1105 With respect to the immunoglobulin heavy variable precursors, 3-15 was present in all groups  
1106 except acute As and acute C non-improvers, though there was insufficient power to confidently  
1107 compare the fold change of groups expressing 3-15. Another heavy variable precursor, 1-69, was  
1108 expressed in subacute As, both acute and subacute C improvers, and both acute and subacute  
1109 Ds. The final heavy variable precursor, 1-24, was found in all groups except acute C improvers and  
1110 non-improvers.

1111 For the two immunoglobulin heavy constant  $\gamma$ s, 4 was significant in acute C improvers and non-  
1112 improvers, relative to subacute As, whereas  $\gamma$  2 was only significant in acute C improvers relative to  
1113 subacute Ds. Both acute C improvers and non-improvers had a lower abundance of  $\gamma$  4 relative to  
1114 subacute As (-2.2 and -2.7 respectively), whilst  $\gamma$  2 had a -1.8 fold change between acute C improvers  
1115 and subacute Ds.

### 1116 **5.3.2 Conclusion**

1117 Much like the iTRAQ experiments (5.2.9), the majority of proteins identified are functionally asso-  
1118 ciated with the complement cascade. Unlike the iTRAQ however, many of the proteins where only  
1119 detected in one group of the pairwise comparisons, suggesting greater suitability as biomarkers.  
1120 PRX-2, a protein associated with oxidative stress, is of particular interest, both as a biomarker for  
1121 improvement in acute AIS C patients, but also mechanistically in relation to functional recovery.  
1122 Furthermore, several immunoglobulins were identified as differentially abundant, though further  
1123 *in vitro/vivo* work is needed to elucidate the pathophysiological relevance of each precursor. The

<sup>1124</sup> λ 2-18 and 3-10 precursors are of particular relevance to acute and subacute AIS C improvement  
<sup>1125</sup> respectively, and both are of interest longitudinally in AIS As, with 2-18 potentially being linked to  
<sup>1126</sup> severity of injury.

<sup>1127</sup> The small number of statistically significant proteins speaks to the variability of human samples,  
<sup>1128</sup> and is likely exacerbated by the inconstant timing of sample collection relative to injury. Post-hoc  
<sup>1129</sup> power analysis of the data reveals that to identify a 2.5 fold change with an FDR of 0.5 and a power  
<sup>1130</sup> of 0.9, 14 biological replicates would be needed, in contrast to the 7-11 replicates used across  
<sup>1131</sup> groups here. Thus, a repeat of this experiment with a larger sample size will likely reveal many  
<sup>1132</sup> more proteins of potential interest. Furthermore, a metabolomic analysis with a similar sample  
<sup>1133</sup> size would greatly compliment this work, particularly with regards to investigating further links to  
<sup>1134</sup> the liver.

1135 **Supplementary material**

1136 **5.4 Session Information**

```
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1138 ## platform      aarch64-apple-darwin20
1139 ## arch         aarch64
1140 ## os           darwin20
1141 ## system        aarch64, darwin20
1142 ## status
1143 ## major         4
1144 ## minor         1.3
1145 ## year          2022
1146 ## month         03
1147 ## day           10
1148 ## svn rev       81868
1149 ## language       R
1150 ## version.string R version 4.1.3 (2022-03-10)
1151 ## nickname      One Push-Up

1152 Packages Used

1153 package

1154 version

1155 date

1156 base

1157 4.1.3

1158 2022-03-18

1159 MSstats

1160 4.2.0

1161 2021-05-31

1162 STRINGdb

1163 2.6.5

1164 2020-01-10

1165 ReactomePA

1166 1.38.0

1167 2021-10-26

1168 rlang

1169 1.0.2

1170 2022-03-04

1171 bookdown

1172 0.26
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1175 0.5.2
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1181 1.2.0
1182 2021-10-19
1183 DiagrammeR
1184 1.0.9
1185 2022-03-04
1186 lubridate
1187 1.8.0
1188 2021-10-03
1189 patchwork
1190 1.1.1
1191 2020-12-15
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1194 2020-12-15
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1205 2.2.5
1206 2022-05-01
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1221 2020-09-19
1222 captioner
1223 2.2.3
1224 2015-07-15
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1263  2022-04-26
1264 ggplot2
1265  3.3.6
1266  2022-04-27
1267 tidyverse
1268  1.3.1
1269  2021-04-15
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1270    **5.5 Fold changes**

**Table S1.** OpenMS log<sub>2</sub> fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively.

gene	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C improvers acute vs subacute	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS A vs D	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D
A1BG	-0.9031824	-0.1017534	-0.6087849	0.1926441	0.2252650	0.7937347	-0.3497633	0.4439714	-0.5750284	0.2187064
A2M	-1.0385788	-0.2464392	-0.6760613	0.1160783	-1.2300968	1.4247538	-1.6029796	-0.1782258	-0.3728828	1.0518710
AFM	-0.3788476	-1.2248641	0.4815192	-0.3644973	0.5517904	1.1923601	-1.2566085	-0.0642484	-1.8083989	-0.6160388
AHSG	1.1794532	NA	-0.5545288	NA	NA	NA	NA	NA	NA	NA
AMBP	0.6562004	-0.3433433	0.8606588	-0.1388849	-0.9023293	NA	1.2037841	NA	2.1061134	NA
APCS	0.1498290	0.2108936	-0.0114011	0.0496636	NA	0.3557242	NA	NA	-0.0494567	0.3062675
APOA1	-0.1816744	-0.6923621	-0.2337557	-0.7444434	-0.7677301	0.6941282	-1.3172834	-0.6231553	-0.5495533	0.1445749
APOA2	0.0900143	-1.1461360	-0.6667620	-1.9029124	NA	NA	NA	NA	NA	NA
APOA4	0.1295961	0.9636781	-1.2312803	-0.3971983	-1.3254088	0.7876011	-1.3346720	-0.5470709	-0.0092632	0.7783379
APOB	0.1379231	-0.0164100	-0.6332751	-0.7876082	-0.8570393	0.5260041	-1.2345864	-0.7085823	-0.3775471	0.1484570
APOE	-1.2133754	0.2930673	-0.6884490	0.8179937	-0.9078302	0.7746514	-1.5477490	-0.7730977	-0.6399188	0.1347326
APOH	-0.3600286	-0.7024687	-0.6444887	-0.9867188	-0.9996639	2.8143614	-1.0091799	1.8051815	-0.0095159	2.8048455
APOL1	-1.1790763	-0.5193515	-1.0440264	-0.3843015	-0.1152769	0.5652696	0.1299333	0.6952029	0.2452102	0.8104799
APOM	-1.2167971	-0.6819883	0.6934807	1.2282895	NA	0.6561807	NA	NA	0.6664954	1.3226762
ATRN	NA	NA	-1.0062957	NA	NA	NA	NA	NA	NA	NA
AZGP1	1.2191679	1.0251503	0.0811400	-0.1128776	-3.3889514	-3.6440501	0.3702887	-3.2737614	3.7592401	0.1151900
C1QB	-0.8410072	-2.0020393	0.7071113	-0.4539208	-1.9729191	1.3563310	-2.0066282	-0.6502972	-0.0337090	1.3226219
C1R	-0.4335115	-0.7632158	0.0366498	-0.2930545	-0.1467491	0.7976066	0.3564300	1.1540366	0.5031791	1.3007857
C1S	0.0295224	-0.8193739	0.1679558	-0.6809404	NA	NA	NA	NA	NA	NA
C2	NA	NA	NA	NA	-2.5581036	2.5640965	-2.5952702	-0.0311737	-0.0371665	2.5269300
C3	-0.7440620	-0.6968585	0.0652375	0.1124410	-1.0730763	1.2388421	-2.1616420	-0.9227999	-1.0885657	0.1502764
C4BPA	-0.1810388	-2.4454980	1.6627662	-0.6016930	-1.2378707	1.5489731	-1.8448914	-0.2959183	-0.6070207	0.9419523
C5	-0.5447843	-0.2031226	0.9230001	1.2646617	-0.7200022	1.2710496	-1.6768797	-0.4058301	-0.9568775	0.3141721
C6	-1.3936214	1.7817023	-1.3097108	1.8656129	-3.0451914	1.7642372	-3.2550019	-1.4907647	-0.2098105	1.5544267
C7	-0.9642124	0.8848082	-0.7827165	1.0663041	0.9970185	0.0708650	-1.1136320	-1.0427670	-2.1106505	-2.0397855
C8A	-0.51117891	0.2736564	-0.7630145	0.0224310	-2.8108340	0.1731241	-2.1285385	-1.9554144	0.6822955	0.8554196
C8B	-2.1950427	0.2789045	-1.5954883	0.8784589	-1.8943958	-0.4802611	-0.9597537	-1.4400148	0.9346421	0.4543810
C8G	NA	NA	-1.6304866	NA	NA	NA	NA	NA	NA	NA
C9	-2.2199059	0.4534093	-1.9249790	0.7483361	-0.7345863	0.6495872	-3.2424254	-2.5928382	-2.5078391	-1.8582519
CD5L	-0.9293248	-0.6204735	-0.7145571	-0.4057058	-2.4642871	0.4482534	-2.3260120	-1.8777586	0.1382751	0.5865285
CFH	-1.1239737	0.7406948	-1.6480885	0.2165801	-1.0358708	0.1380093	-1.3260484	-1.1880391	-0.2901776	-0.1521683
CFI	NA	0.5359696	NA	1.2578110	NA	NA	NA	NA	NA	NA
CLU	-1.1958984	-0.8681850	-0.1721921	0.1555214	-1.3664377	0.8251962	-2.1976184	-1.3724222	-0.8311807	-0.0059845
CP	-0.3892064	0.2565411	-0.4537277	0.1920199	-0.6657547	0.4235353	-0.2695812	0.1539541	0.3961736	0.8197089
F12	0.4852010	-0.9397905	0.6702925	-0.7546990	-0.8534307	0.5549559	-1.3145850	-0.7596291	-0.4611543	0.0938016
F2	-0.7493082	-0.7563593	0.0982877	0.0912367	-0.5408805	1.1677146	-1.5476188	-0.3799042	-1.0067383	0.1609763
FCN3	NA	0.9644778	NA	NA	NA	NA	NA	NA	NA	NA

**Table S1.** OpenMS log<sub>2</sub> fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively. (continued)

gene	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C improvers acute vs subacute	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS A vs D	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D
FGA	-0.9591400	-0.5109050	0.4841704	0.9324054	-1.0155684	1.0486717	-1.4707952	-0.4221236	-0.4552268	0.5934449
FGB	-0.8339088	-0.1253771	0.0684287	0.7769604	-0.8343143	1.0951087	-1.4646547	-0.3695460	-0.6303405	0.4647683
FGG	-1.1432907	-0.0247316	-0.2978078	0.8207513	-0.7191139	0.7606622	-1.0780014	-0.3173392	-0.3588876	0.4017746
FN1	-0.2795610	-0.3153249	0.2899102	0.2541463	-0.5777631	1.1462731	-1.2550759	-0.1088028	-0.6773129	0.4689602
GC	-0.5583474	0.4050629	-0.7950103	0.1684001	-1.8700166	-0.2961353	-1.2641016	-1.5602369	0.6059149	0.3097797
GSN	0.0704855	0.0479440	-0.6709561	-0.6934976	NA	NA	NA	NA	NA	NA
HABP2	NA	NA	NA	NA	-0.5367242	1.4445961	-0.7070902	0.7375059	-0.1703660	1.2742301
HP	-1.2468596	0.5276209	-0.3488061	1.4256744	-0.6393503	0.9683391	-1.2963281	-0.3279890	-0.6569779	0.3113613
HPX	-0.4104644	-0.2880781	-0.7114901	-0.5891038	-0.3597680	0.9360243	-1.1034368	-0.1674125	-0.7436687	0.1923556
HRG	0.5979026	1.0672891	0.0321566	0.5015431	-0.7300739	0.6893699	-0.8231701	-0.1338002	-0.0930962	0.5962737
IGHA1	1.7635882	1.3476620	0.3628909	-0.0530353	-2.0152404	0.4328016	-2.2081140	-1.7753124	-0.1928737	0.2399280
IGHD	NA	NA	NA	NA	-2.4499647	0.4182281	-3.4284738	-3.0102457	-0.9785091	-0.5602810
IGHG1	-0.0855309	0.9292134	-0.4962961	0.5184482	-0.0970233	-1.8091062	0.4814333	-1.3276728	0.5784566	-1.2306496
IGHG2	0.9720422	0.3501681	0.4607992	-0.1610748	-0.6249433	-1.5106734	0.2705475	-1.2401258	0.8954908	-0.6151826
IGHG3	-0.1941508	1.4323226	-0.9309878	0.6954857	-1.8543540	-0.3927284	-1.8870246	-2.2797530	-0.0326705	-0.4253990
IGHM	-0.6318126	-0.8967300	-0.4174693	-0.6823867	-1.1741740	1.7915993	-2.3508710	-0.5592717	-1.1766971	0.6149023
IGKC	-0.0697458	0.0420359	-0.1150304	-0.0032487	-1.1868447	-0.2875492	-1.1765257	-1.4640749	0.0103190	-0.2772302
IGKV3D- 20	NA	NA	NA	NA	-0.3699302	-0.0536821	0.2114801	0.1577980	0.5814103	0.5277282
ITIH1	-0.9766570	0.7057133	-0.5211753	1.1611951	-0.6149247	0.5495684	-0.5039432	0.0456252	0.1109815	0.6605499
ITIH2	-0.3142692	-0.5283214	-0.2363320	-0.4503842	-0.7431549	0.6757214	-1.2136587	-0.5379373	-0.4705037	0.2052177
ITIH3	-0.5456033	0.6138901	0.3512683	1.5107617	-2.0564371	1.2902341	-1.8743188	-0.5840847	0.1821183	1.4723525
ITIH4	-0.0669542	-0.2189363	0.3808668	0.2288847	-1.0843698	0.9773070	-1.8198452	-0.8425382	-0.7354753	0.2418317
KLKB1	NA	-2.2093082	NA	-0.2713600	NA	NA	NA	NA	NA	NA
KNG1	-0.6198162	-0.0025326	-0.0676278	0.5496558	-0.6644071	0.8052877	0.0312278	0.8365155	0.6956349	1.5009226
LRG1	-0.7988007	0.2565104	0.1402188	1.1955298	-0.9515964	1.7017682	-2.1951046	-0.4933364	-1.2435082	0.4582600
LUM	0.0832323	0.6580097	-1.2635566	-0.6887792	NA	NA	NA	NA	NA	NA
ORM1	-0.1974770	1.1178187	-0.2240143	1.0912814	-1.9126407	1.6761382	-1.3025982	0.3735400	0.6100425	2.2861806
PGLYRP2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PLG	-0.3680073	0.0880557	-0.8410370	-0.3849741	-1.0701631	2.7112467	-2.8493306	-0.1380838	-1.7791675	0.9320793
PROS1	-0.3300860	0.0623958	-0.7963440	-0.4038621	-0.5089636	1.5349629	-3.8745298	-2.3395668	-3.3655662	-1.8306032
RBP4	0.4505693	0.4185795	-0.0211740	-0.0531638	-4.0971240	1.4352287	-2.9877294	-1.5525007	1.1093946	2.5446233
SAA1	-2.7778116	2.3463574	-0.5151865	4.6089825	-1.3858800	2.4855048	-2.5593861	-0.0738814	-1.1735062	1.3119986
SERPINA1	0.6825593	0.0481996	1.7824248	1.1480651	-0.0999129	-0.1558972	-1.3635079	-1.5194051	-1.2635950	-1.4194922
SERPINA3	-0.7582369	-0.1617666	0.1836958	0.7801661	-0.7417534	2.2311097	-2.0353461	0.1957637	-1.2935927	0.9375171
SERPINA4	0.0099121	NA	-1.0180116	NA	-1.4473701	NA	-0.6571525	NA	0.7902176	NA
SERPINAS	NA	NA	NA	0.2757029	NA	NA	NA	NA	NA	NA

**Table S1.** OpenMS log<sub>2</sub> fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively. (continued)

gene	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C improvers acute vs subacute	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS A vs D	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D
SERPINC1	-0.5553486	-0.2339361	-0.5421237	-0.2207112	-0.7720265	1.1066666	-1.3464506	-0.2397839	-0.5744241	0.5322425
SERPIND1	0.2536120	NA	0.0459257	NA	0.3050057	2.3844297	-1.6468854	0.7375442	-1.9518911	0.4325386
SERPING1	-1.1614755	0.1191571	-1.3510892	-0.0704566	-0.9301893	1.0766804	-1.0904641	-0.0137837	-0.1602748	0.9164056
TF	-0.2823635	-0.1105094	-0.4843676	-0.3125135	-0.7681926	0.5875721	-0.9945649	-0.4069929	-0.2263723	0.3611997
VTN	-0.6186100	-0.0323770	-0.2690009	0.3172321	-1.7234623	1.4918535	-2.1517604	-0.6599069	-0.4282982	1.0635554
VWF	NA	1.0585752	NA	1.3917877	-2.5662912	0.5161630	-1.9774026	-1.4612396	0.5888885	1.1050516

**Table S2.** ProteinPilot fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively.

Protein	AIS C improvers acute vs subacute	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D	AIS C	AIS A vs D
A1BG	-1.644372	-1.472312	NA	NA	NA	NA	NA	NA	NA	NA	NA
A2M	-6.137620	-9.908319	NA	1.380384	-5.861382	-3.467369	NA	1.659587	5.861382	3.564511	
AFM	NA	2.511886	NA	-4.055085	NA	NA	NA	NA	NA	NA	-3.499452
AHSG	NA	NA	NA	-2.249055	NA	NA	NA	NA	NA	NA	NA
APCS	NA	1.870682	NA	NA	NA	4.207266	1.721869	NA	NA	NA	NA
APOA1	-11.803206	-3.698282	NA	-3.250873	-2.884031	-2.884031	-3.801894	NA	-1.406047	NA	
APOA2	-14.321879	NA	NA	-4.965923	NA	NA	NA	NA	NA	NA	NA
APOA4	-11.587774	-5.915616	NA	-2.108628	-2.964831	-1.555966	-2.488857	1.870682	NA	NA	-1.629296
APOB	-2.443430	3.019952	NA	-6.025596	3.732502	-1.282331	1.367729	-4.742420	-2.805434	1.721869	
APOC1	NA	NA	NA	-4.528976	NA	NA	NA	NA	NA	NA	NA
APOC4	NA	NA	NA	NA	NA	1.318257	NA	4.920395	NA	-4.528976	
APOE	NA	NA	-1.527566	-1.753880	NA	-1.836538	-3.019952	-1.803018	-3.019952	NA	
AZGP1	2.269865	2.630268	3.597493	NA	1.819701	4.446313	NA	NA	NA	NA	-4.130475
C1QB	NA	NA	NA	NA	NA	-1.513561	NA	NA	NA	NA	NA
C1R	NA	NA	NA	NA	NA	-4.446313	NA	NA	NA	NA	NA
C3	2.754229	-1.940886	NA	3.981072	-2.398833	-4.365158	1.614359	-1.976970	3.597493	6.546362	
C4B	2.269865	-2.147830	-1.940886	2.654606	NA	NA	NA	NA	NA	NA	NA
C4BPA	NA	-1.419058	NA	NA	NA	NA	1.659587	-2.013724	NA	3.250873	
C5	1.737801	NA	NA	2.228435	NA	-2.333458	NA	-1.770109	NA	2.167704	
C6	1.887991	NA	NA	NA	NA	-2.070141	-2.805434	NA	NA	NA	NA
C9	NA	-2.421029	NA	9.908319	NA	-4.055085	NA	-1.499685	7.177943	9.375620	
CD5L	NA	-2.831392	-3.280953	NA	-1.819701	-1.819701	NA	NA	NA	NA	NA
CFB	NA	-1.674943	2.535129	4.285485	NA	-2.128139	2.032357	-1.690441	2.511886	4.055085	
CFH	NA	NA	NA	2.558586	NA	NA	NA	NA	2.333458	1.803018	
CFI	NA	NA	NA	NA	NA	NA	NA	NA	NA	2.269865	
CLU	NA	NA	NA	NA	NA	NA	NA	-2.582260	NA	NA	
CP	NA	NA	2.582260	3.019952	NA	NA	2.187762	NA	2.779713	NA	
F2	NA	NA	NA	NA	NA	NA	1.674943	NA	NA	1.527566	
FGA	3.467369	-1.644372	NA	12.133888	-3.531832	-2.654606	NA	NA	5.199960	4.092606	
FGB	3.280953	NA	2.443431	9.204495	-2.187762	-1.330454	2.654606	NA	5.248075	3.133286	
FGG	2.032357	-1.958845	NA	9.638290	-2.312065	-1.644372	4.325138	NA	9.204495	6.367955	

**Table S2.** ProteinPilot fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively. (continued)

Protein	AIS C improvers acute vs subacute	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D	AIS C	AIS A vs D
FN1	2.582260	2.228435	NA	NA	1.940886	-2.466039	1.472312	-4.875285	NA	3.404082	
GC	NA	NA	NA	NA	NA	NA	1.541700	NA	2.606154	2.398833	
GSN	-2.312065	NA	NA	-4.055085	-3.019952	NA	-4.365158	NA	NA	NA	
HBA1	NA	3.133286	NA	-4.017908	NA	NA	NA	NA	-2.654606	-2.535129	
HBB	NA	10.000000	NA	-15.995580	5.058247	2.167704	NA	NA	-6.137620	-2.558586	
HP	3.499452	NA	2.511886	13.427649	NA	-2.964831	NA	NA	4.092606	4.786301	
HPX	NA	-2.147830	NA	NA	NA	NA	1.995262	NA	2.208005	NA	
HRG	NA	NA	NA	NA	NA	3.531832	NA	3.908409	NA	NA	
IGHM	NA	-5.152286	-3.664376	NA	-5.199960	-4.655861	NA	NA	3.221069	2.937650	
IGKC	NA	NA	NA	NA	NA	1.753880	5.649370	1.786488	5.807644	NA	
ITIH1	NA	NA	NA	NA	NA	NA	NA	-3.597493	NA	NA	
ITIH2	NA	NA	NA	-1.629296	NA	-2.089296	-2.208005	-2.070141	-2.208005	NA	
ITIH3	NA	-2.051162	NA	2.466039	NA	NA	NA	NA	2.108628	2.630268	
ITIH4	1.819701	-2.312065	NA	3.104560	-1.836538	-3.104560	NA	-1.737801	2.376840	4.092606	
JCHAIN	NA	NA	-4.130475	NA	-5.011872	NA	NA	NA	NA	NA	
KNG1	NA	NA	NA	NA	NA	NA	2.754229	NA	NA	NA	
LPA	NA	NA	10.764652	14.723126	NA	NA	NA	NA	NA	NA	
LRG1	NA	-2.167704	NA	3.047895	-6.367955	-9.727472	NA	-1.629296	NA	3.311311	
LUM	-4.405549	NA	NA	-3.250873	NA	NA	NA	NA	NA	NA	
ORM1	NA	NA	16.904409	NA	NA	NA	3.630781	NA	NA	2.992265	
PLG	1.555966	NA	NA	NA	2.312065	1.870682	2.937650	NA	NA	NA	
RBP4	NA	5.495408	NA	NA	NA	NA	NA	NA	NA	NA	
SAA1	NA	NA	28.054337	51.522865	NA	NA	NA	NA	NA	NA	
SAA4	NA	NA	NA	NA	NA	-2.805434	NA	NA	NA	1.905461	
SERPINA1	NA	-2.333458	NA	7.585776	-2.754229	-5.597576	NA	-2.187762	3.221069	7.112135	
SERPINA3	2.108628	-1.737801	3.837072	12.705741	-1.976970	-5.915616	NA	-3.250873	4.325138	12.246162	
SERPIN C1	NA	NA	NA	NA	NA	NA	NA	-2.070141	NA	NA	
SERPIN D1	1.770109	NA	NA	NA	2.032357	NA	NA	NA	NA	NA	
SERPIN F1	NA	NA	NA	NA	NA	-4.365158	-5.248075	NA	NA	NA	
SERPIN F2	NA	NA	NA	NA	NA	-4.207266	NA	-3.467369	NA	NA	
SERPIN G1	NA	-2.535129	NA	2.964831	-1.836538	-4.365158	NA	-2.488857	2.187762	5.248075	

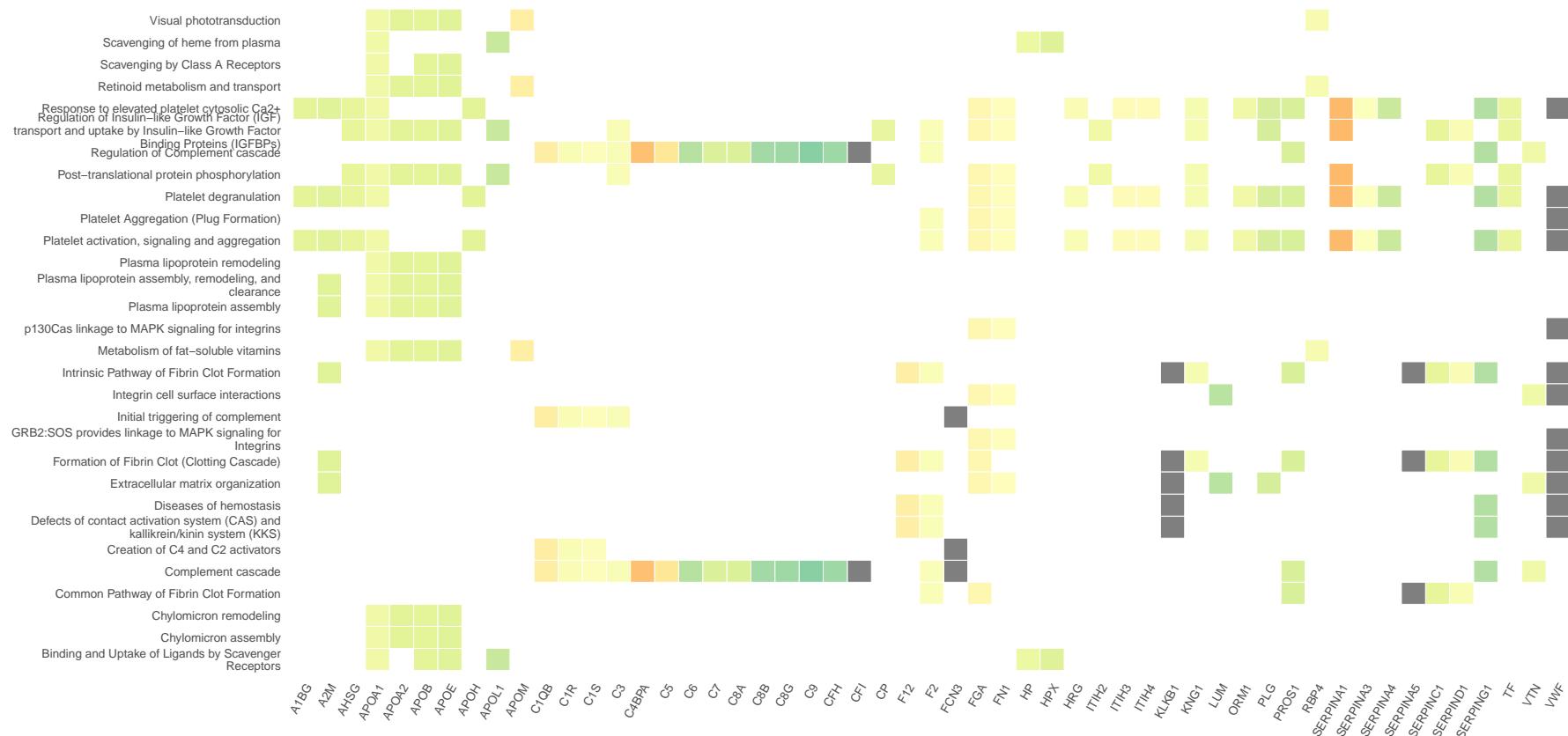
**Table S2.** ProteinPilot fold changes in the plasma proteome of SCI patients. 'Acute' and 'Subacute' samples collected within 2 week and approximately 3-months post-injury respectively. (continued)

Protein	AIS C improvers acute vs subacute	Acute AIS C improvers vs non-improvers	Subacute AIS C improvers vs non-improvers	AIS C non-improvers acute vs subacute	AIS C improvers vs non-improvers	AIS C improvers vs A	AIS C improvers vs D	AIS C non-improvers vs A	AIS C non-improvers vs D	AIS C	AIS A vs D
TF	-2.728978	NA	-1.527566	-5.445027	NA	NA	1.721869	NA	NA	NA	NA
TTN	NA	NA	NA	NA	NA	-1.706082	-2.208005	-1.770109	NA	NA	1.258925

1271 **5.6 Heatmaps**

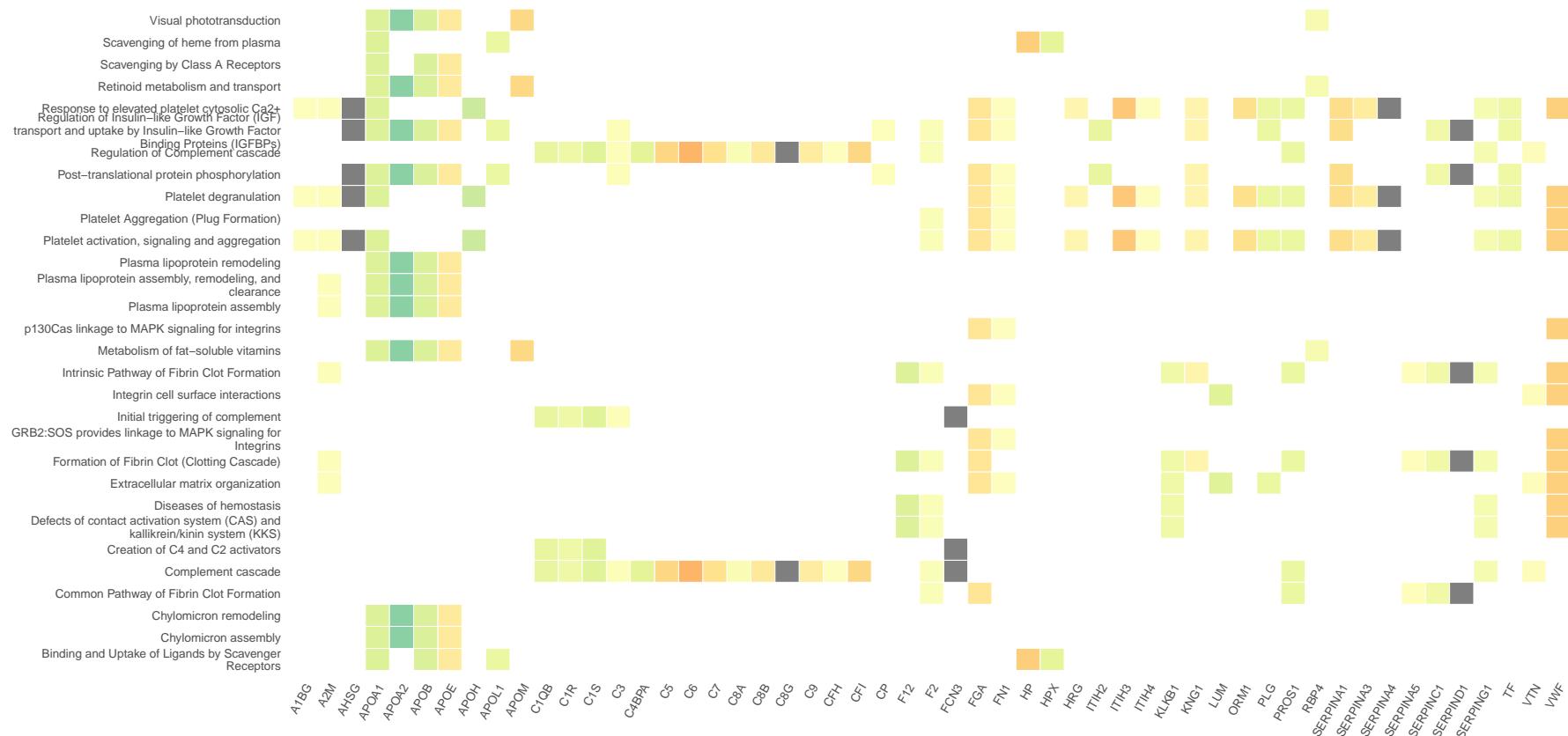
1272 **5.6.1 iTRAQ data**

### AIS C Improvers acute vs subacute



**Figure S1.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

### AIS C non-Improvers acute vs subacute

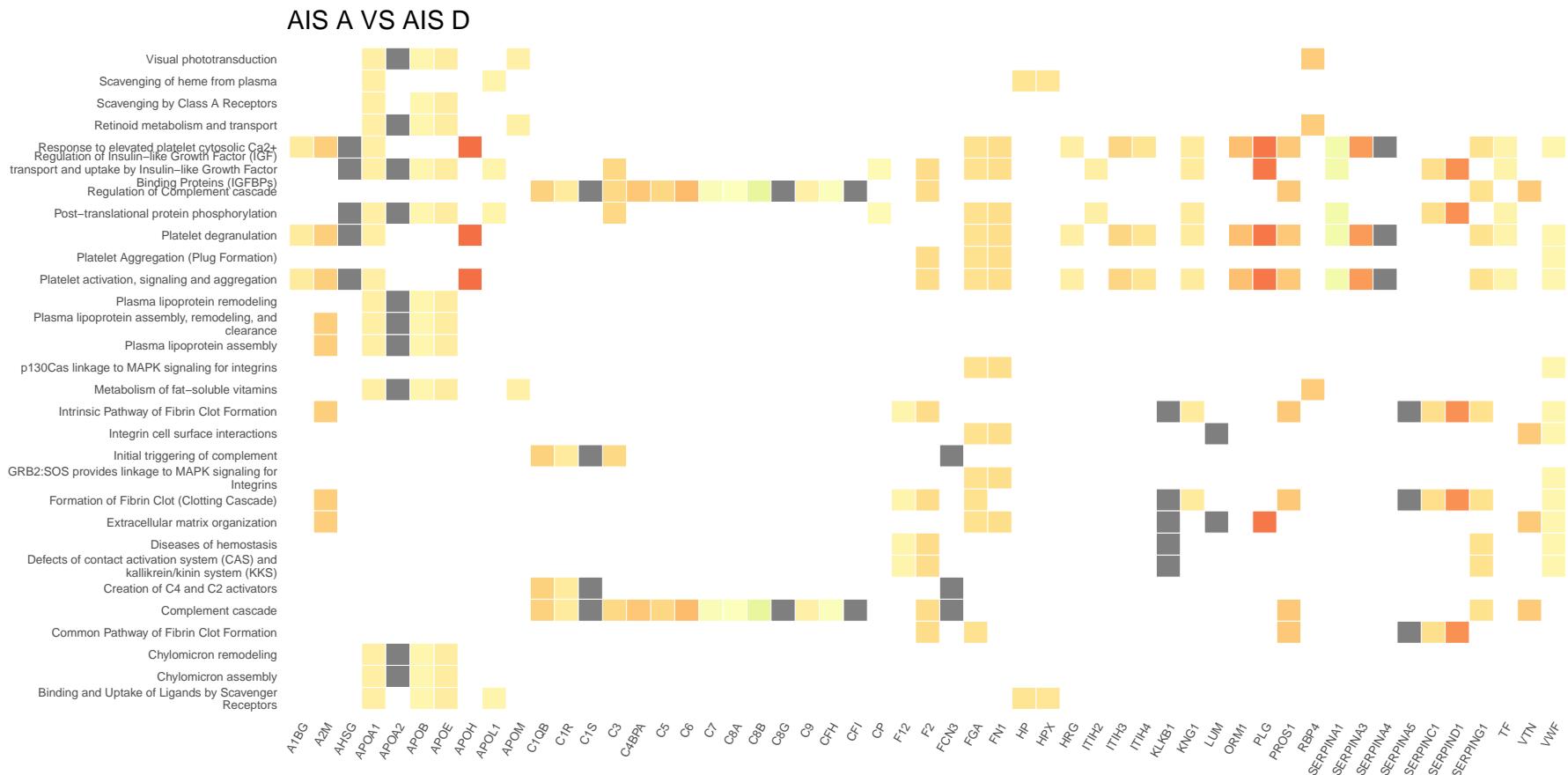


**Figure S2.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

### Acute AIS C Improvers VS non-Improvers Run 2



**Figure S3.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.



**Figure S4.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

### Acute AIS C Improvers VS AIS D



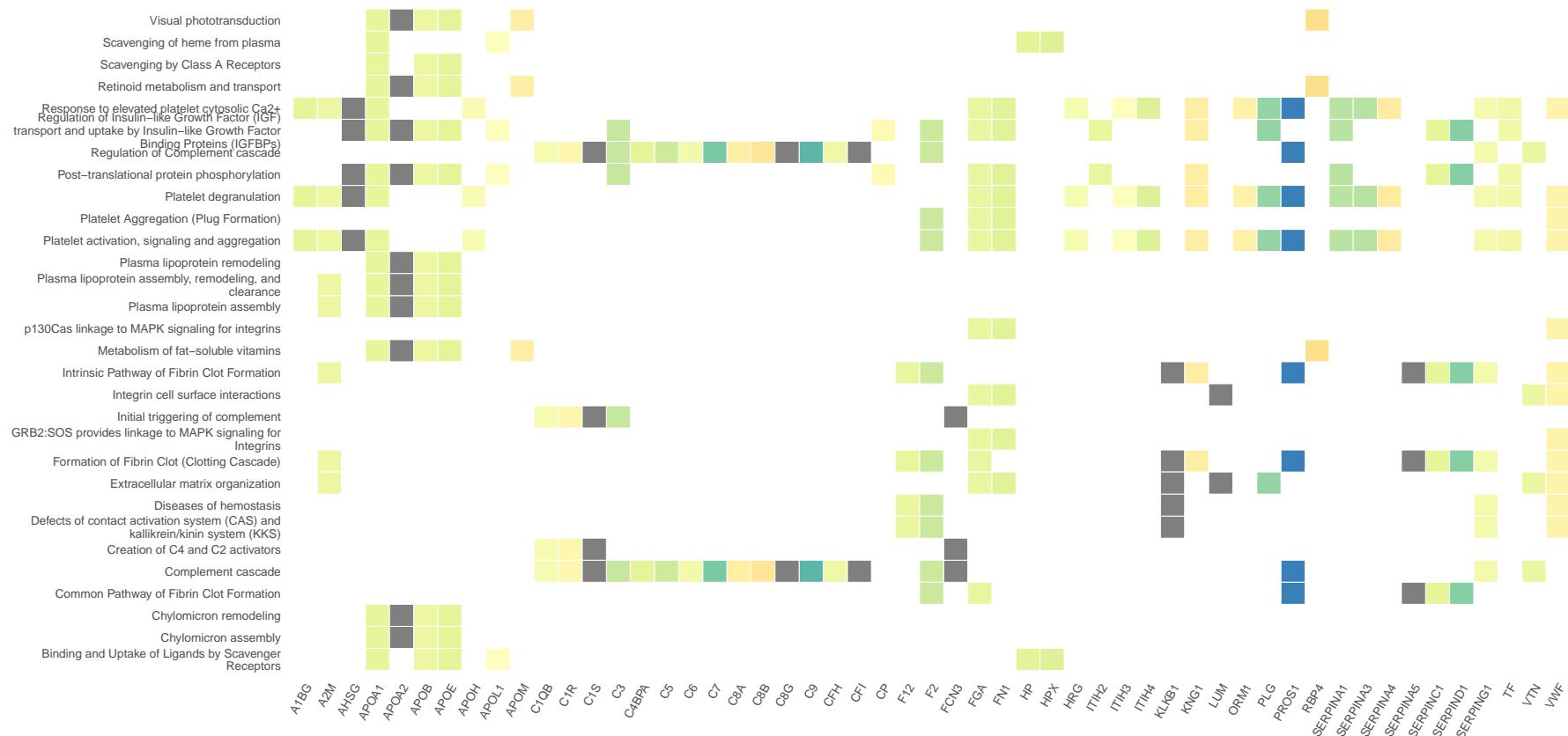
**Figure S5.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

### Acute AIS C Improvers VS AIS A



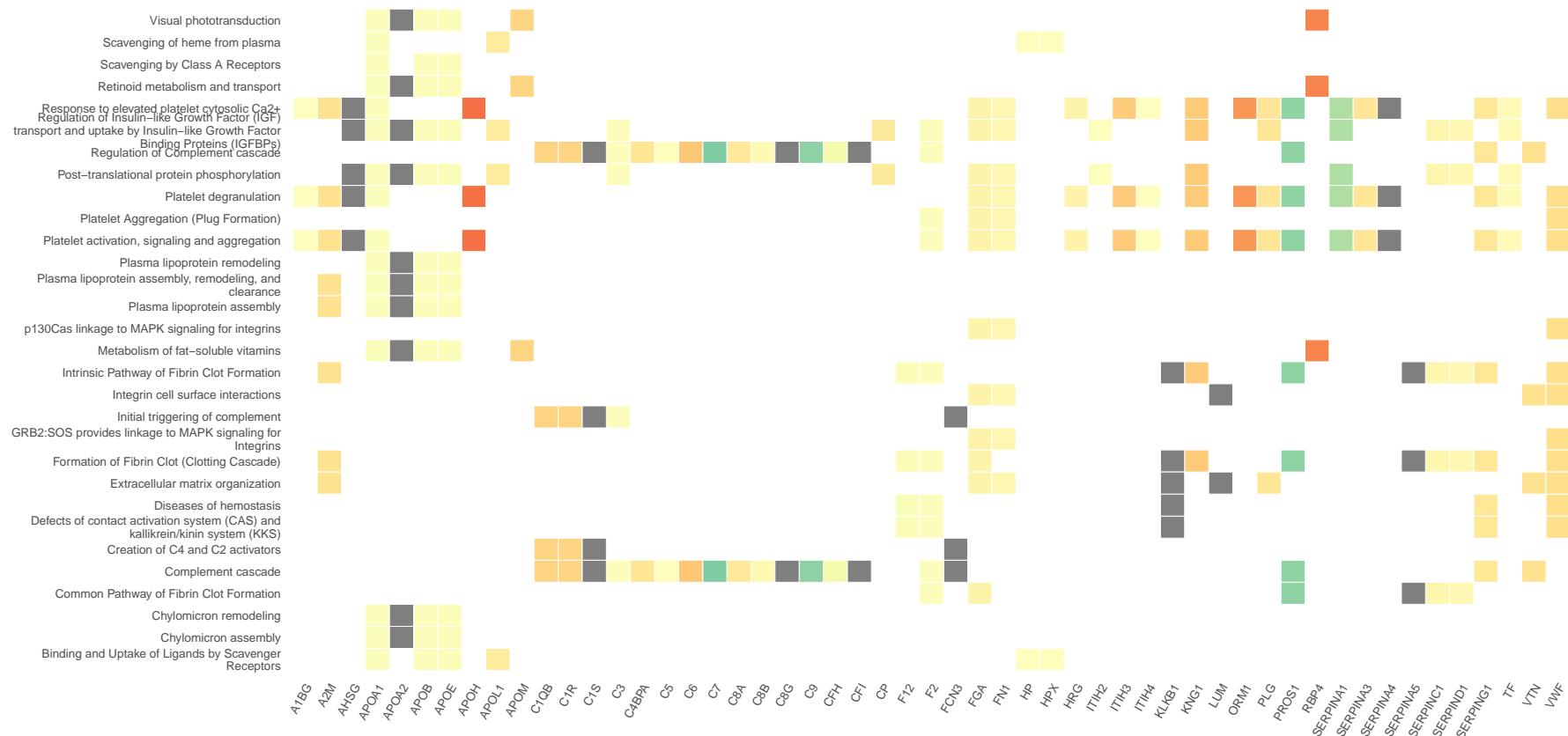
**Figure S6.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

### Acute AIS C non-Improvers VS AIS A



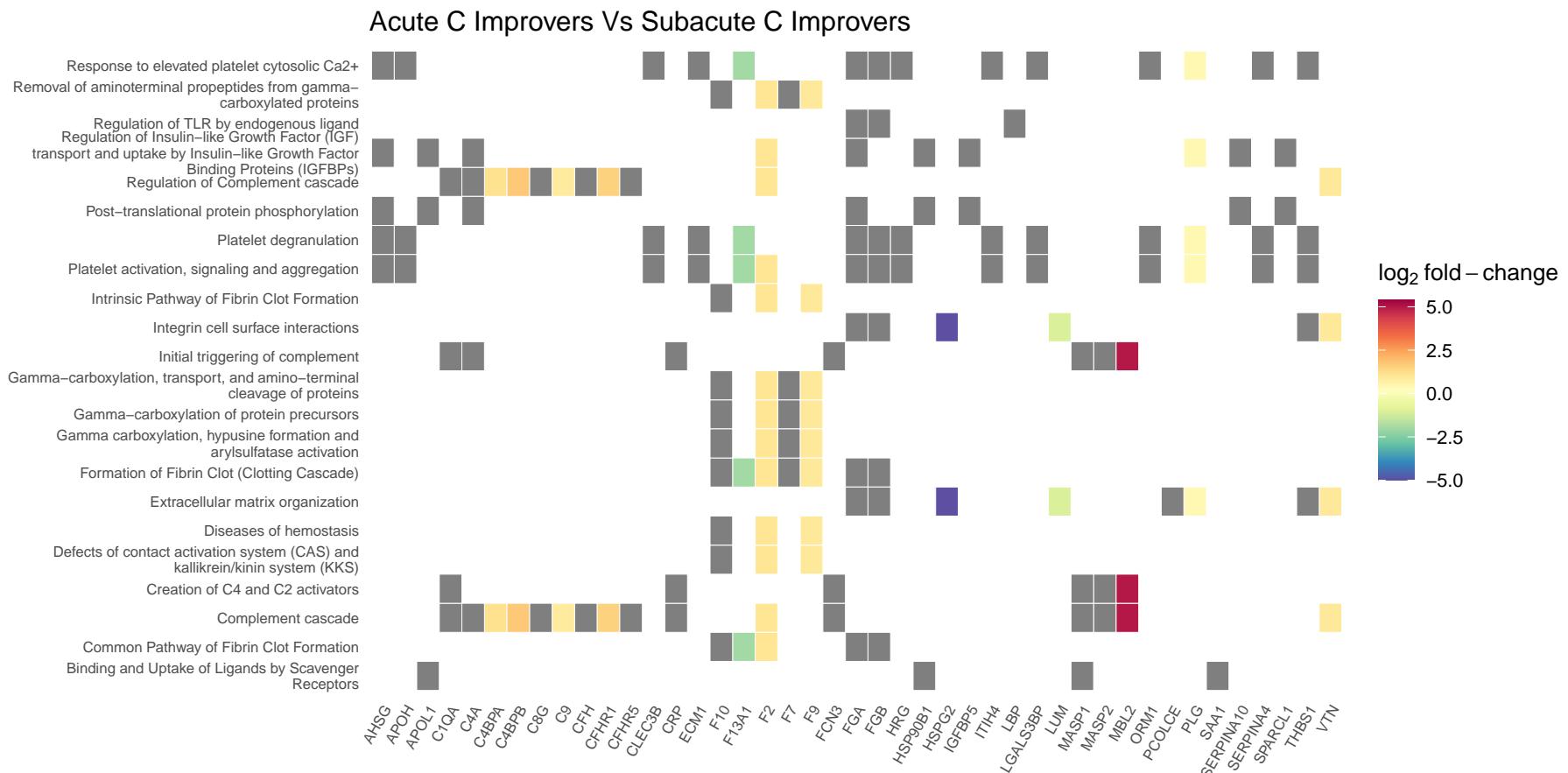
**Figure S7.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

### Acute AIS C non-Improvers VS AIS D

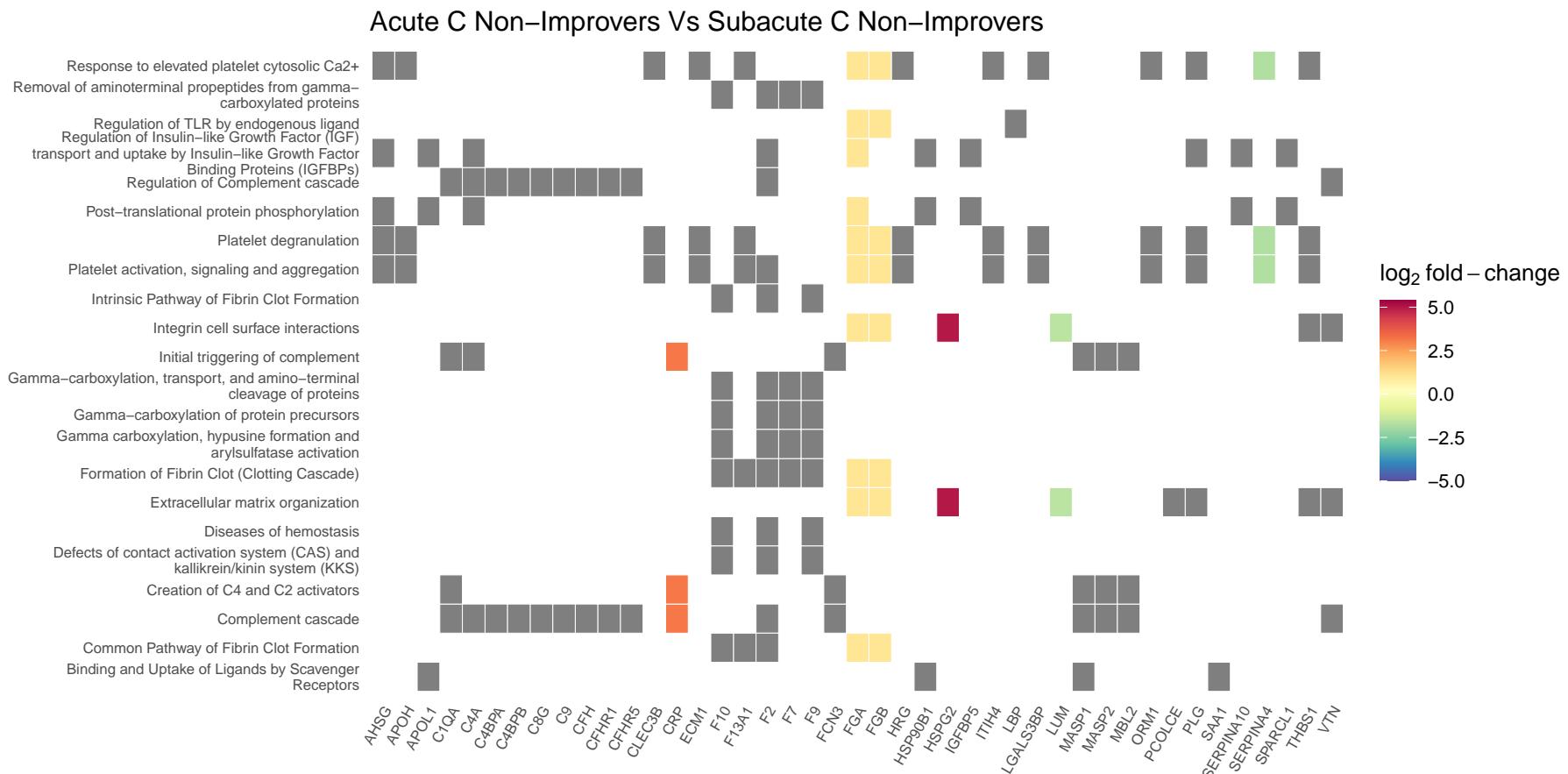


**Figure S8.** Heatmap denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

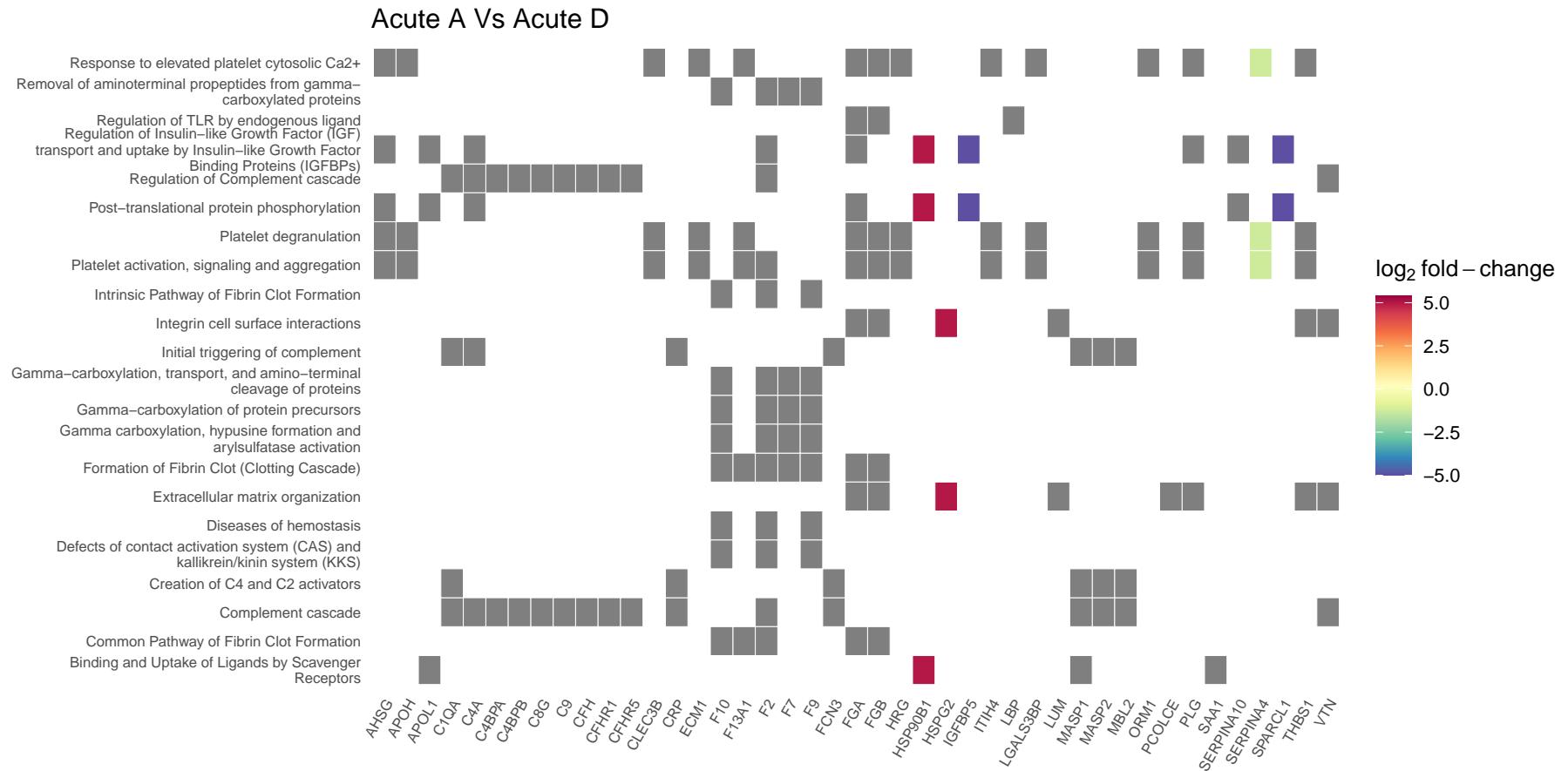




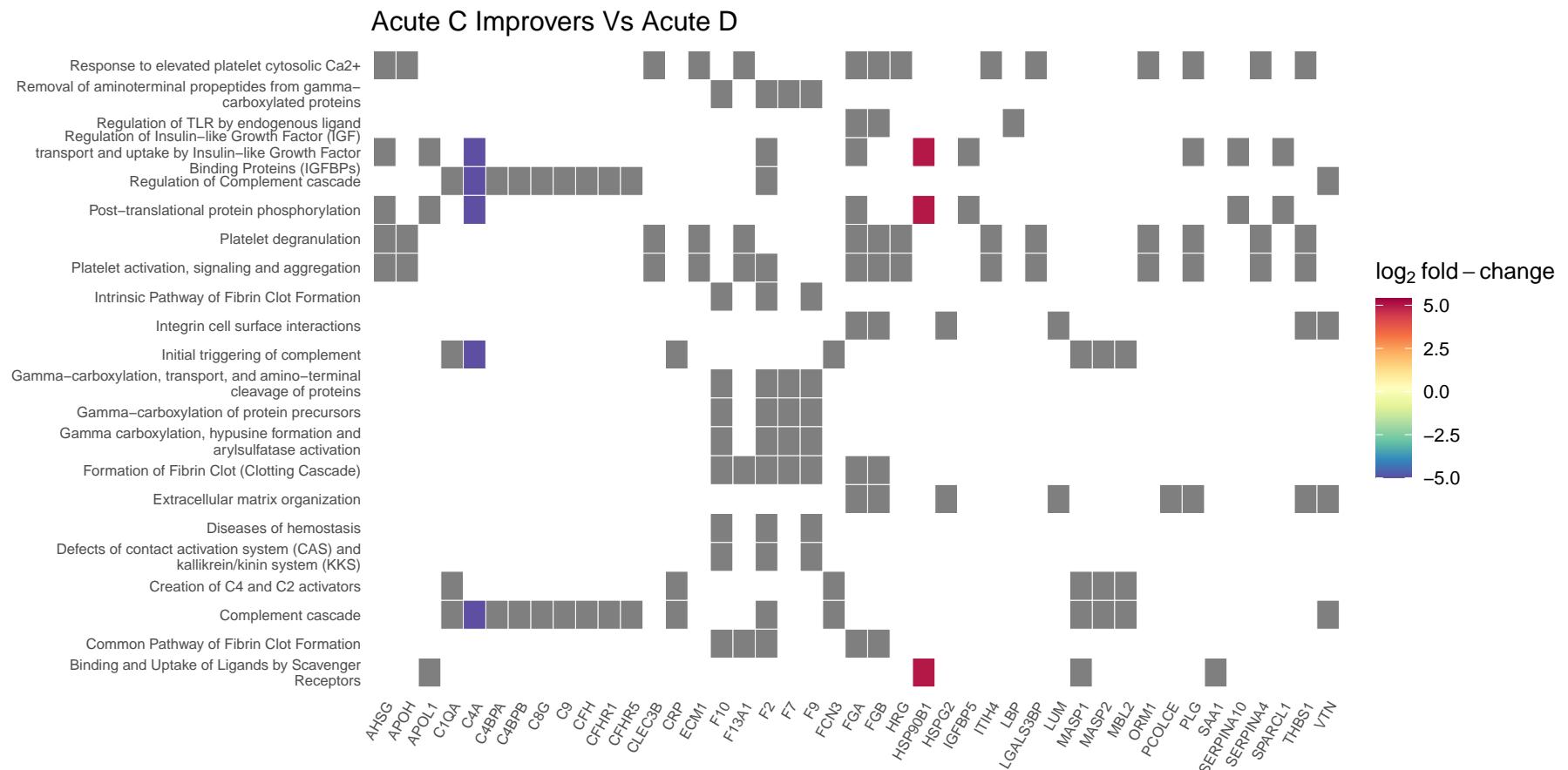
**Figure S9.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury. Grey blocks denote proteins not present in the comparison.



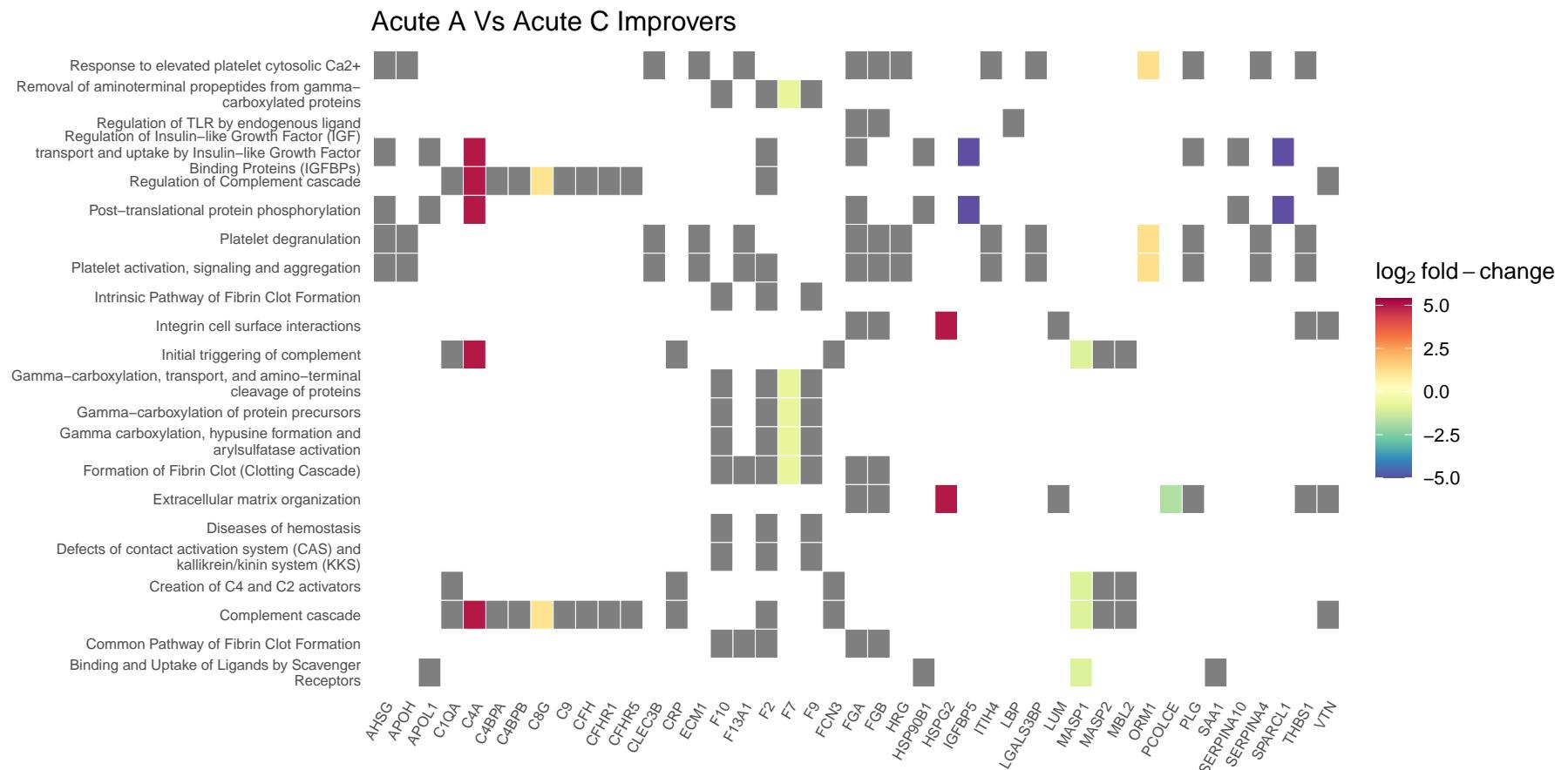
**Figure S10.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury. Grey blocks denote proteins not present in the comparison.



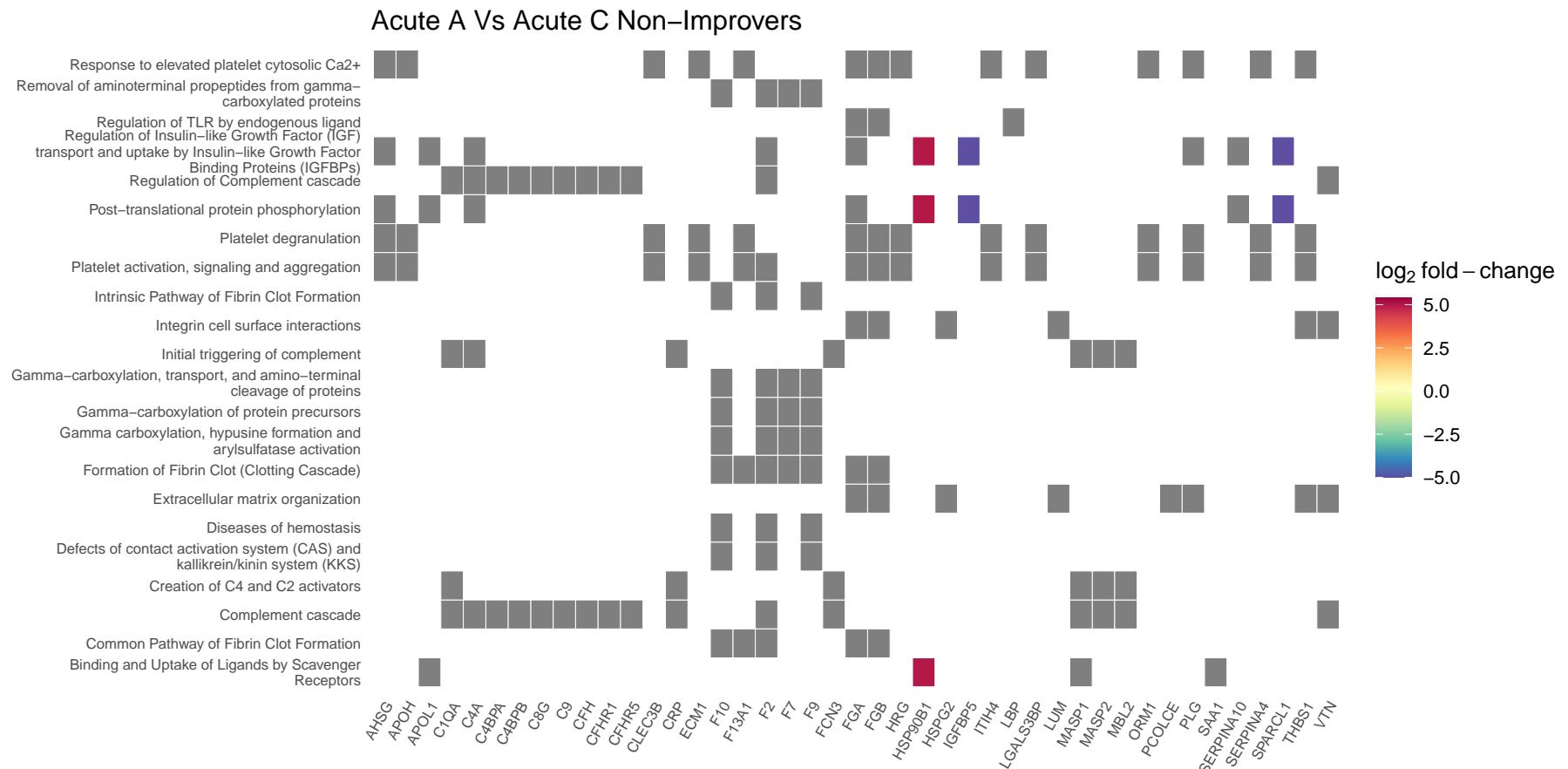
**Figure S11.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients. Grey blocks denote proteins not present in the comparison.



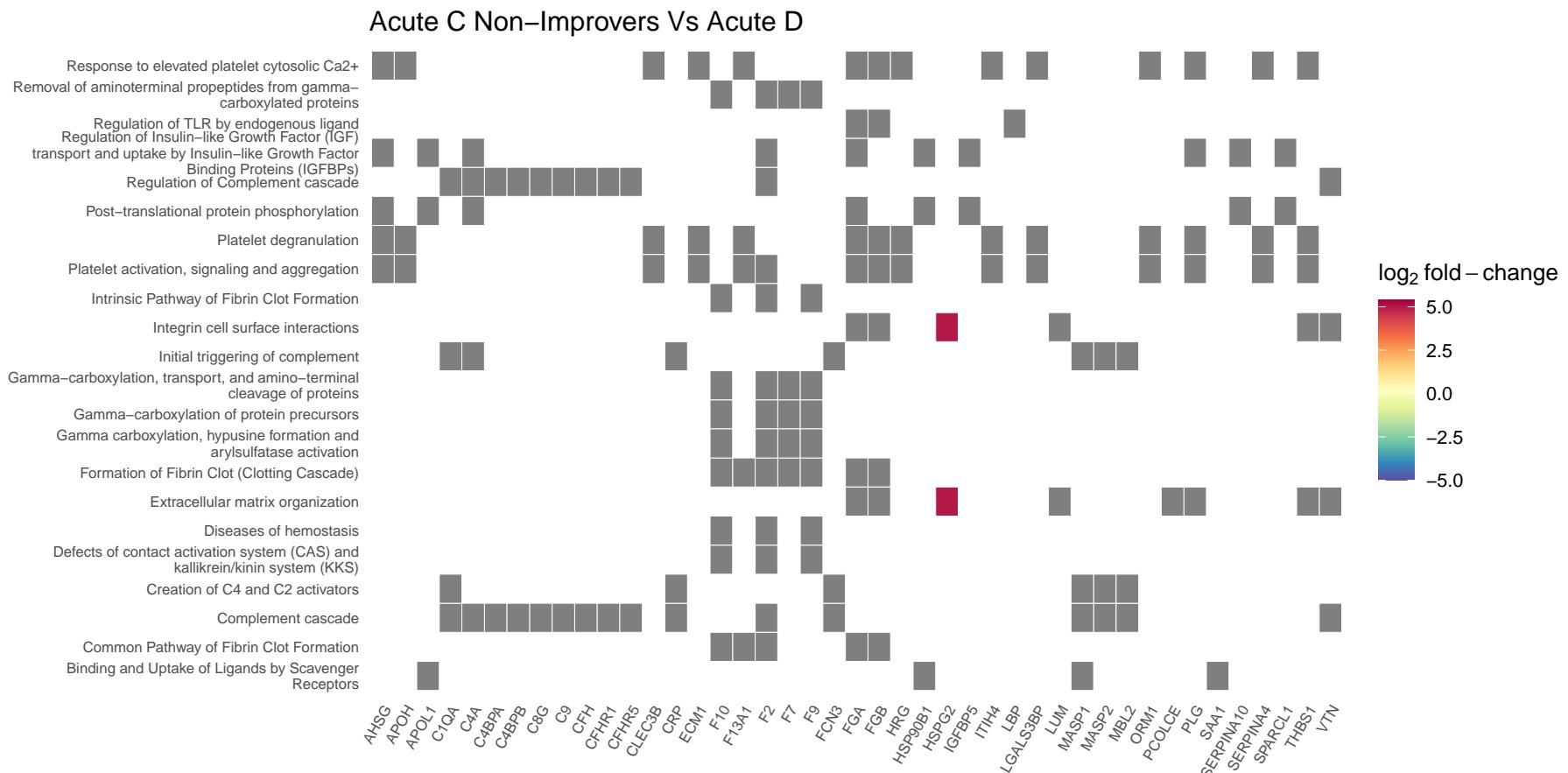
**Figure S12.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients. Grey blocks denote proteins not present in the comparison.



**Figure S13.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.



**Figure S14.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.

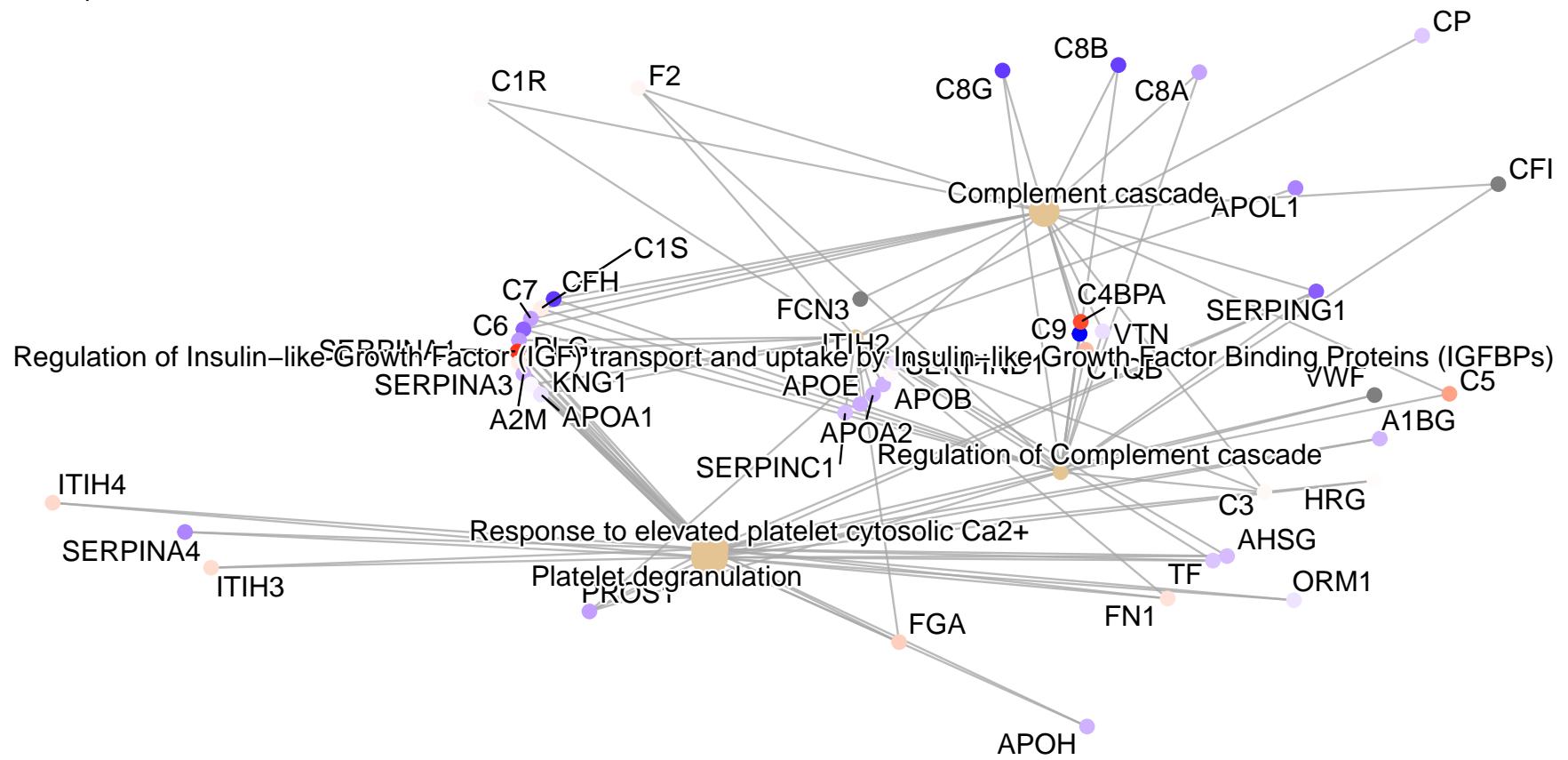


**Figure S15.** Heatmap denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients. Grey blocks denote proteins not present in the comparison.

1274 **5.7 Cnetplots**

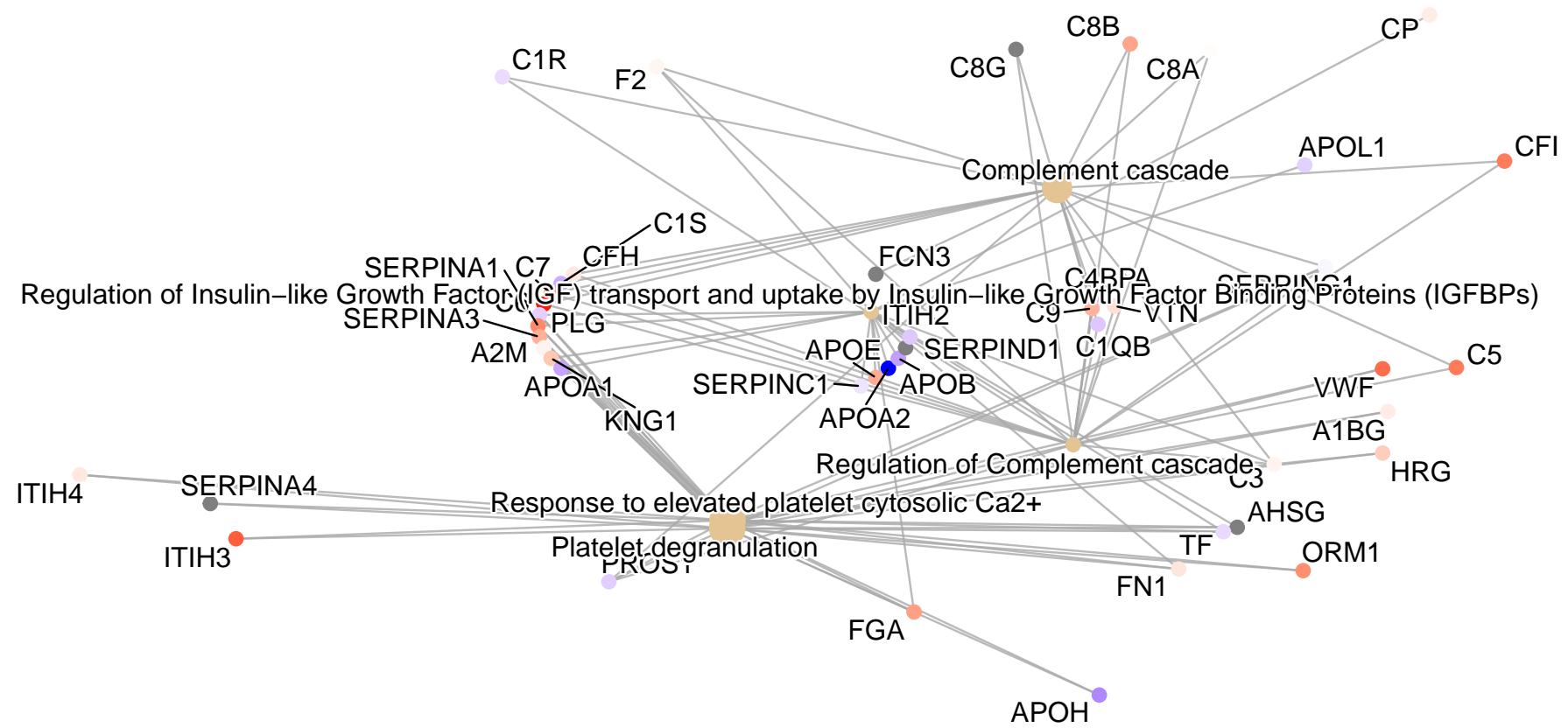
1275 **5.7.1 iTRAQ data**

AIS C Improvers acute vs subacute



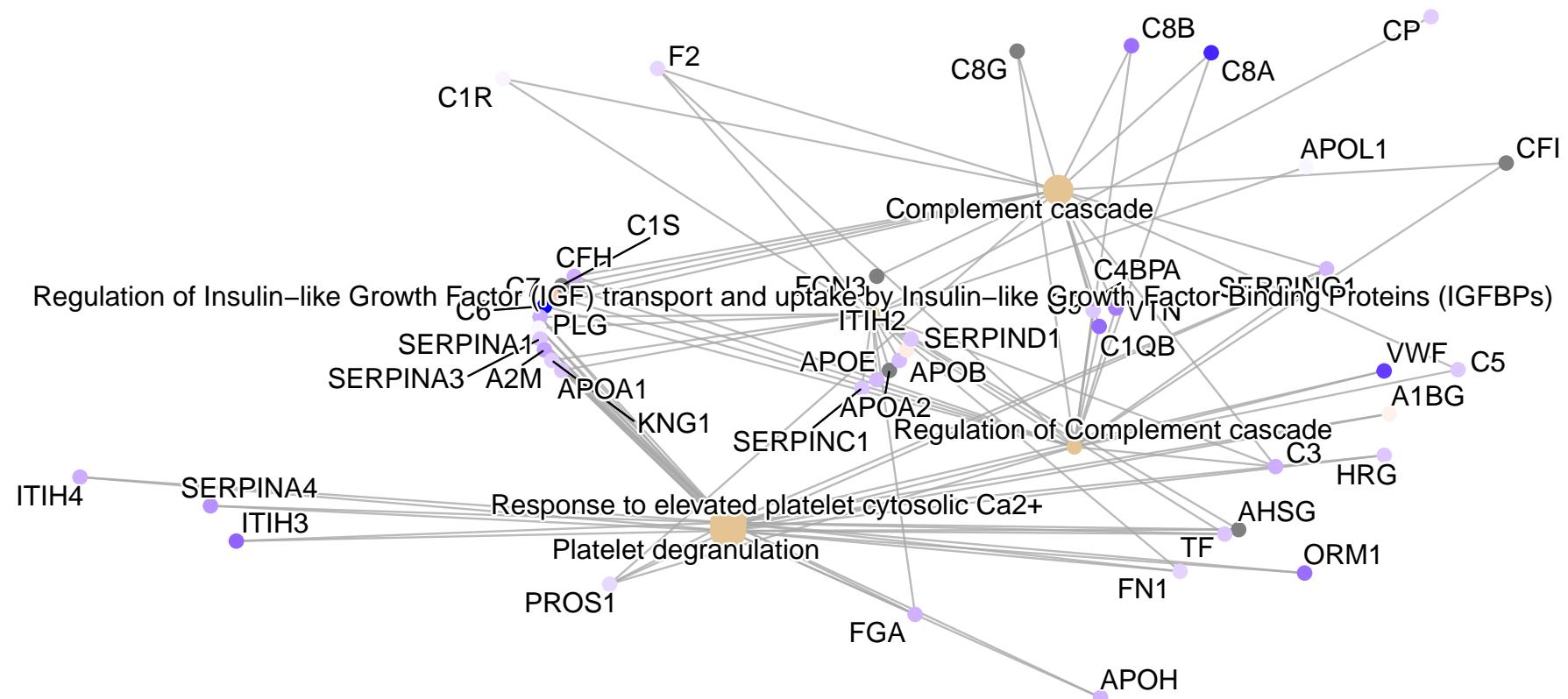
**Figure S16.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

## AIS C non-Improvers acute vs subacute



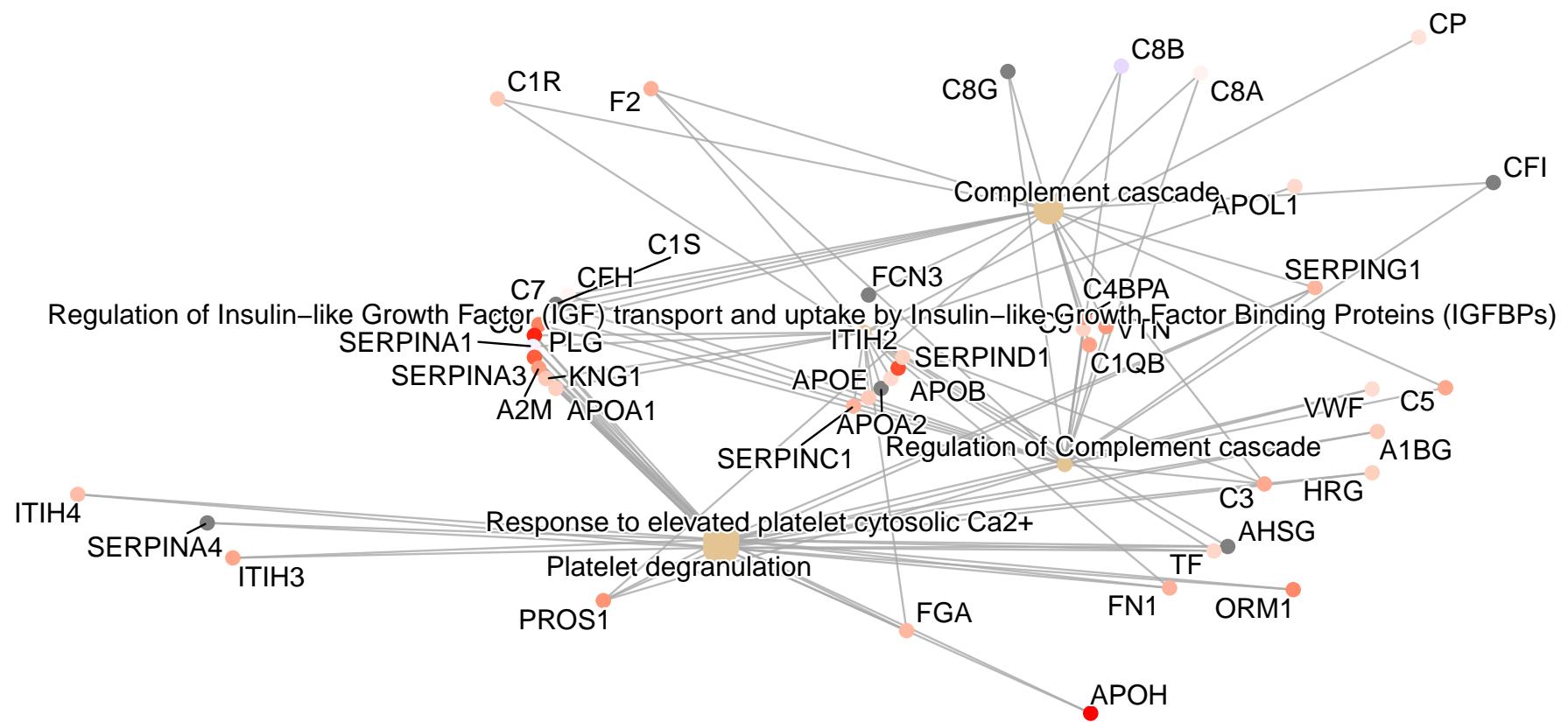
**Figure S17.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute AIS C Improvers VS non-Improvers Run 2



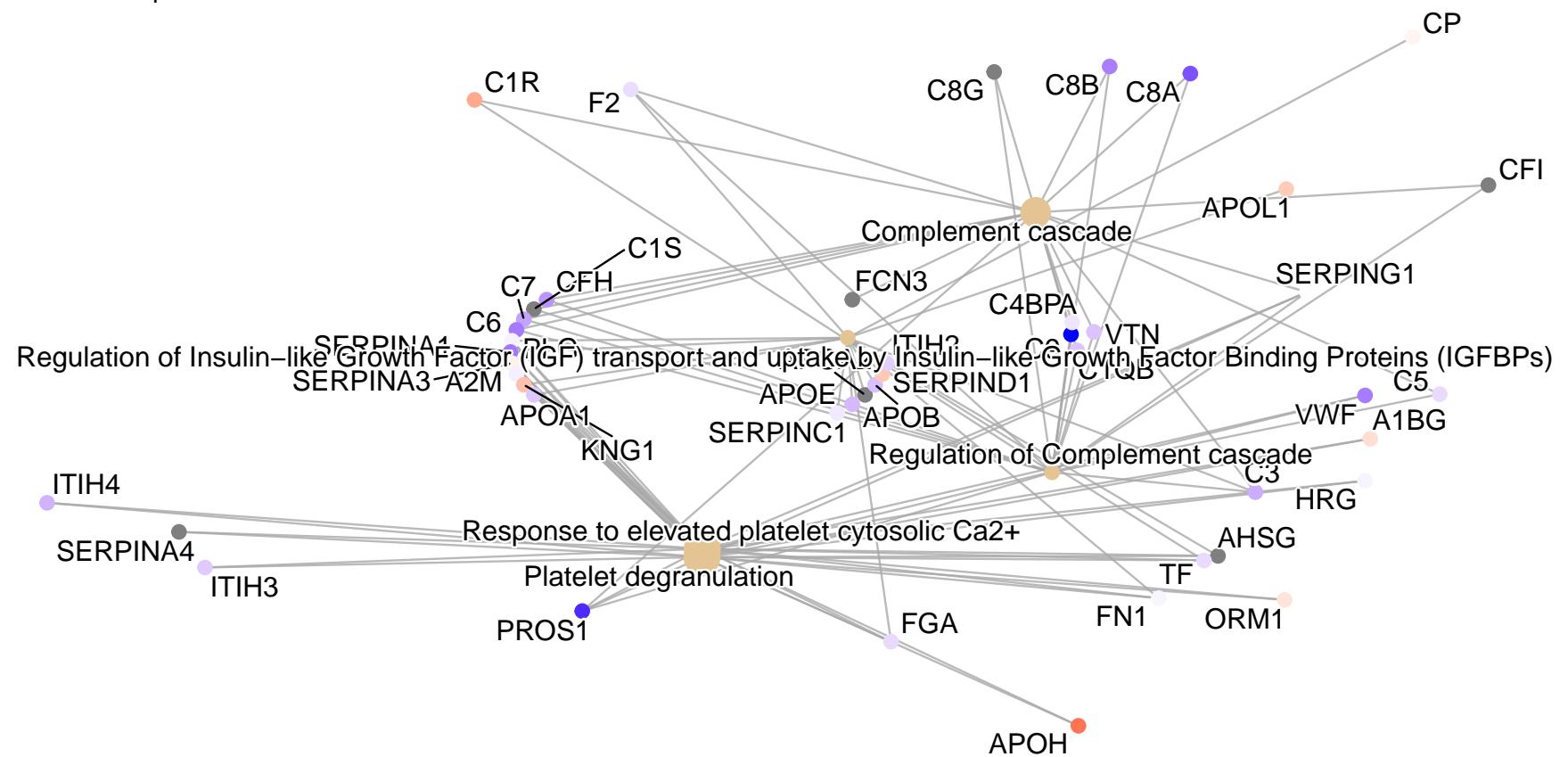
**Figure S18.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.

AIS A VS AIS D



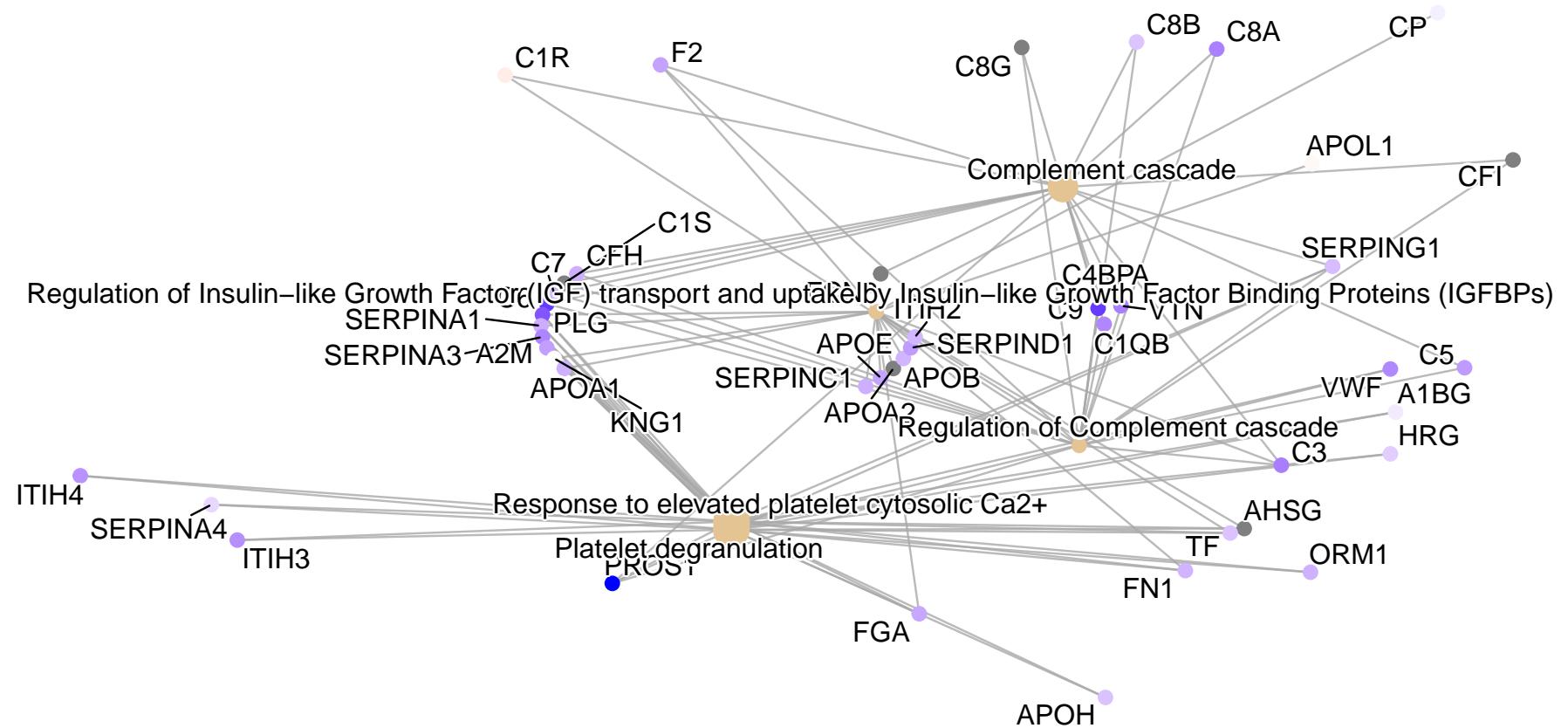
**Figure S19.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

### Acute AIS C Improvers VS AIS D



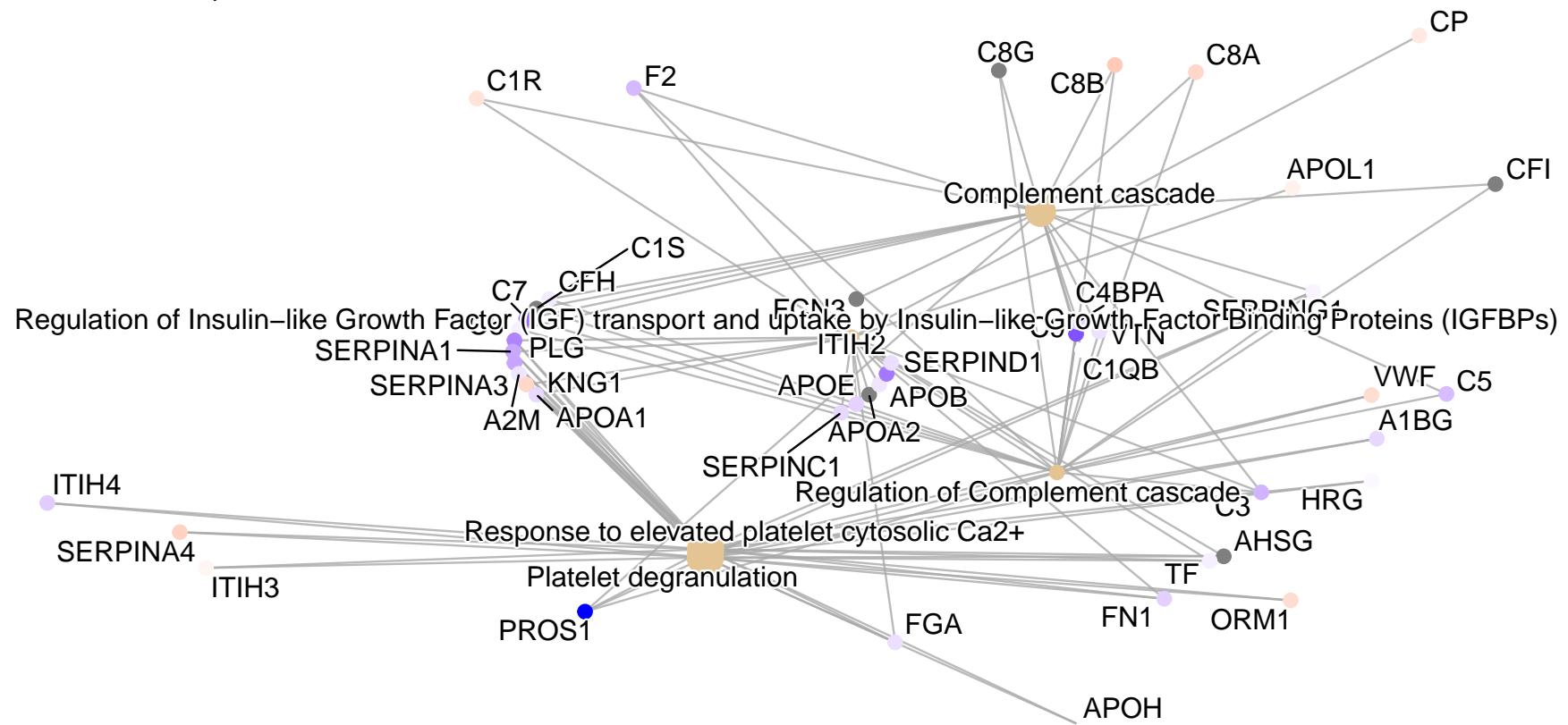
**Figure S20.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

## Acute AIS C Improvers VS AIS A



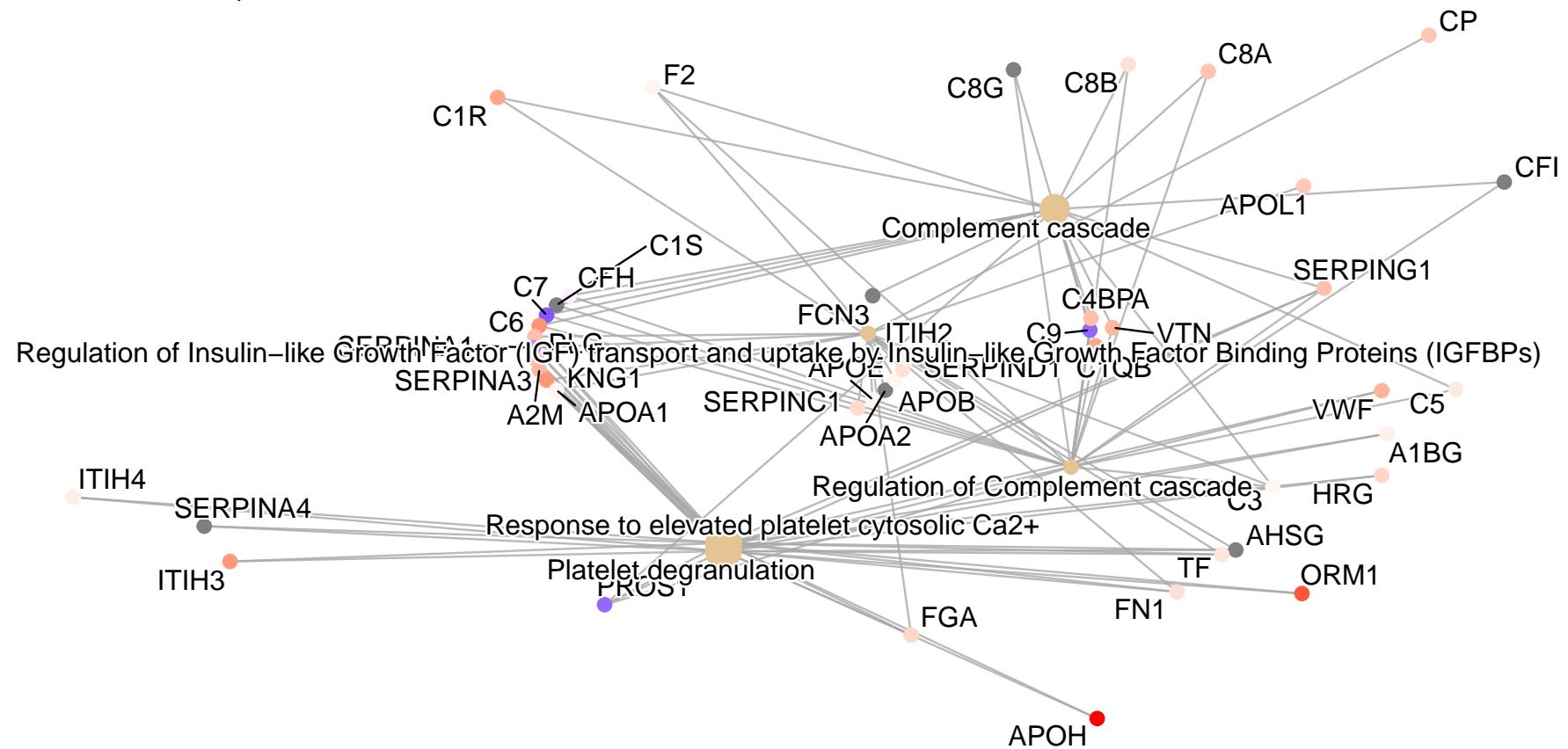
**Figure S21.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS A



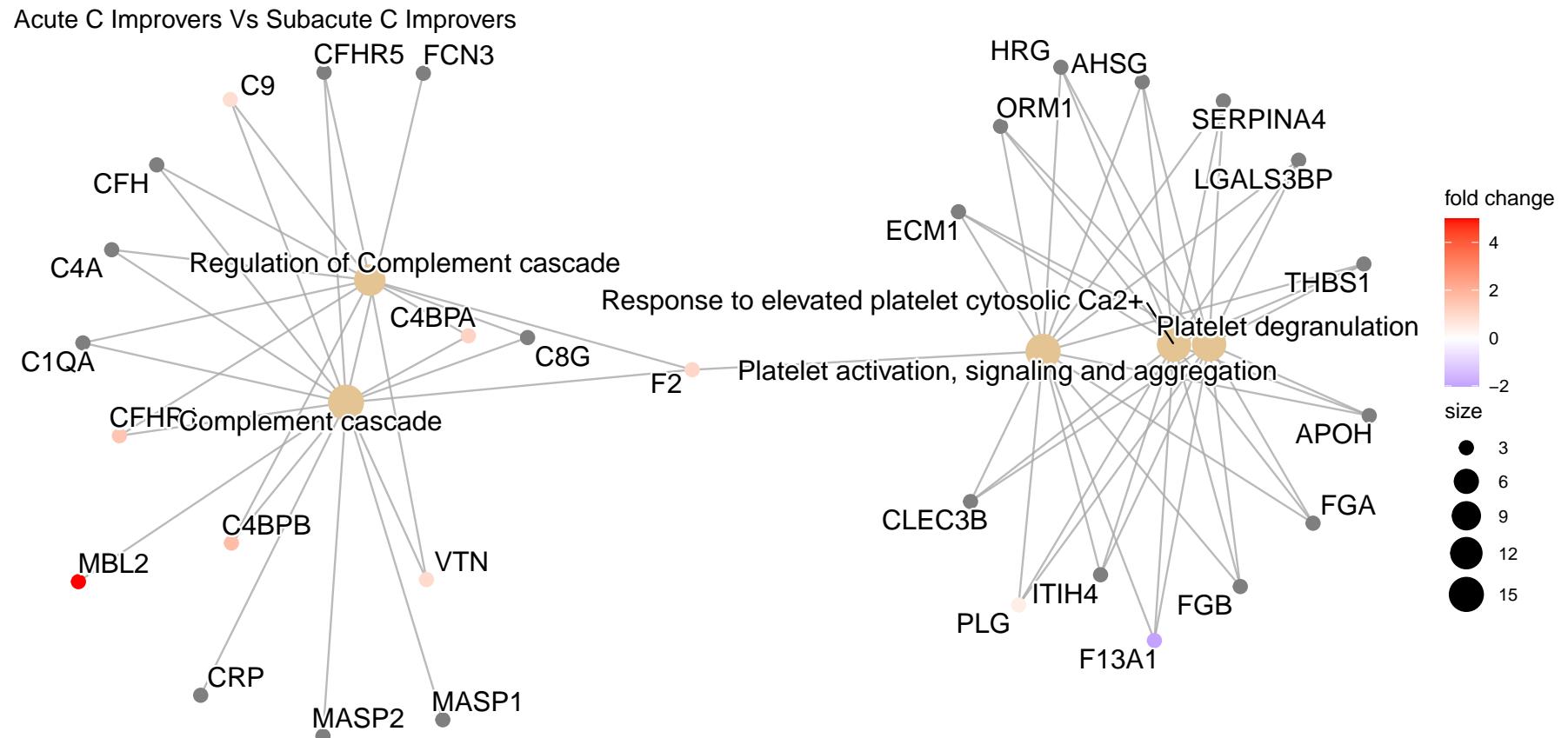
**Figure S22.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D



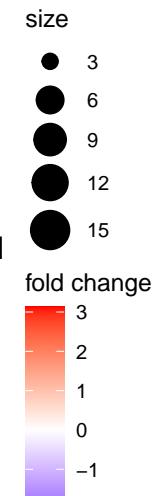
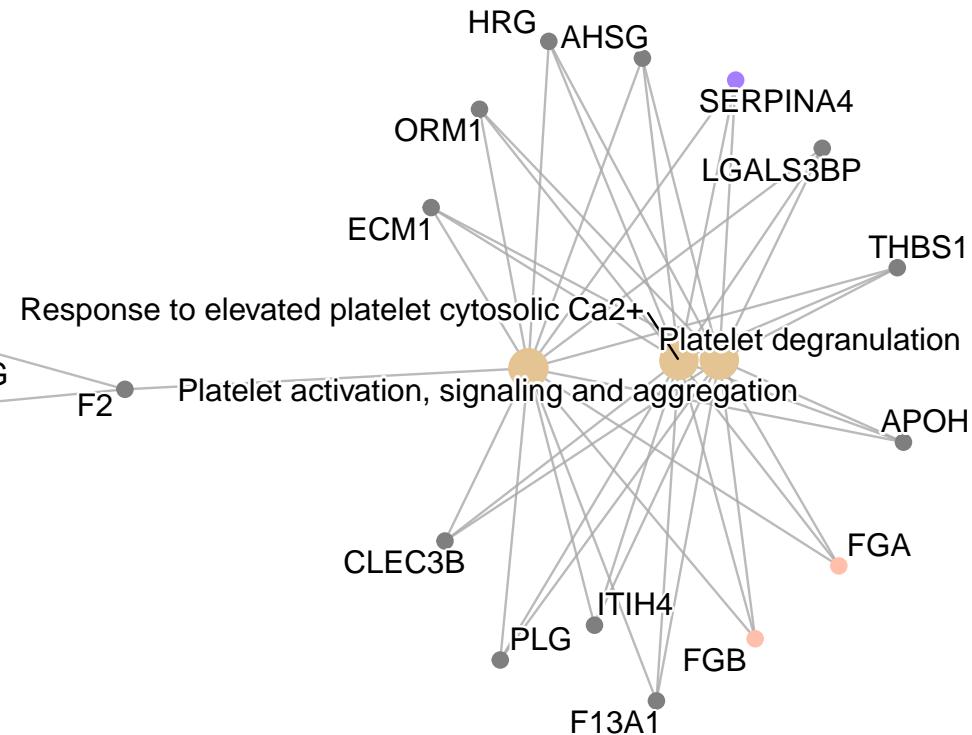
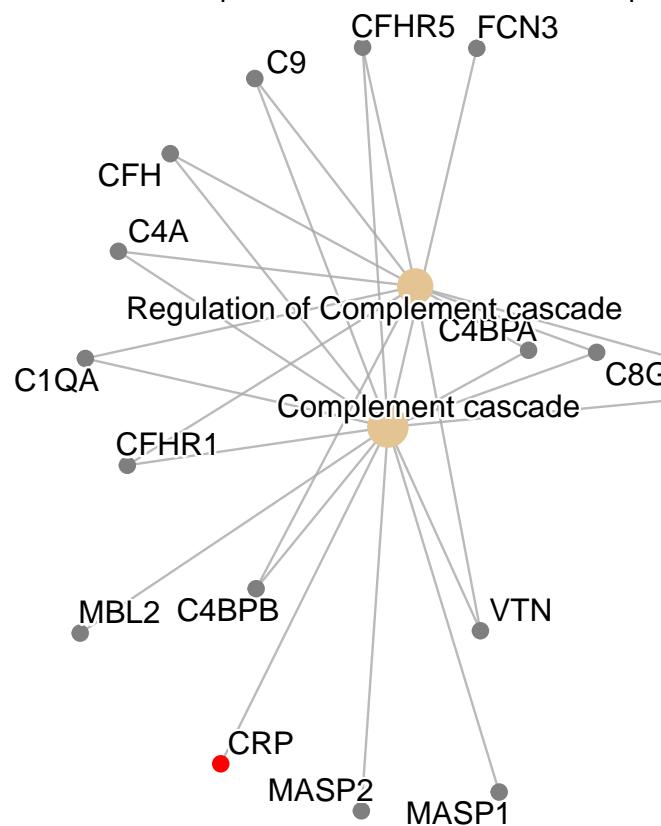
**Figure S23.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.





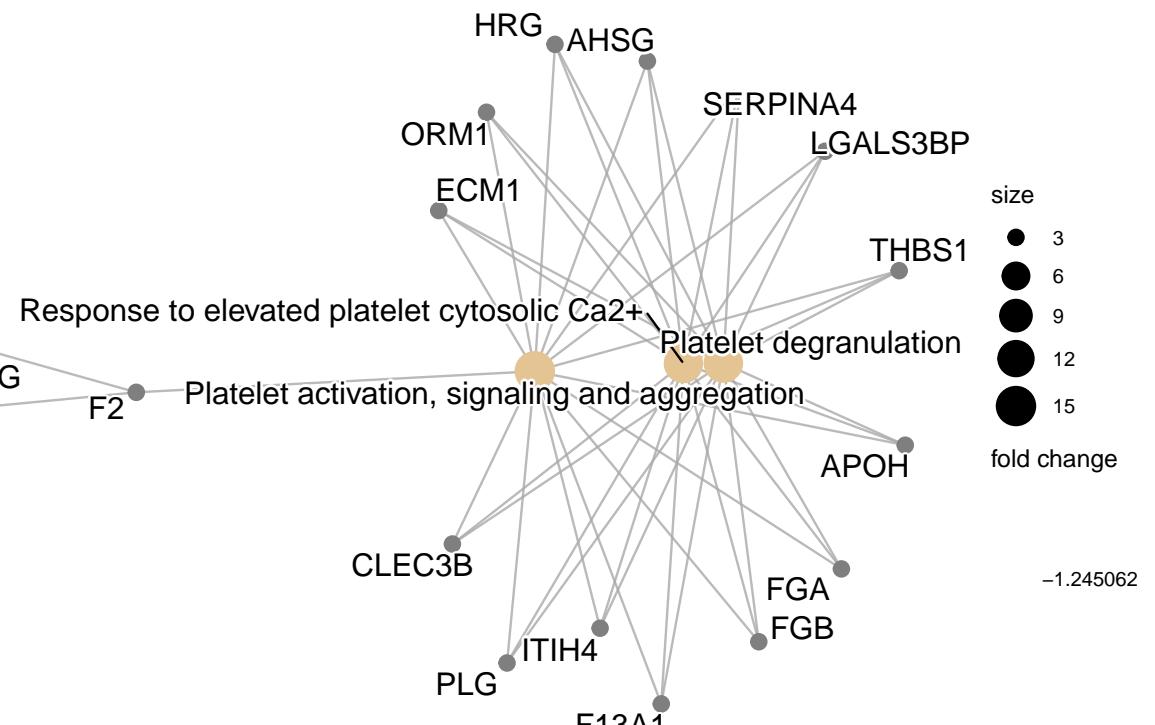
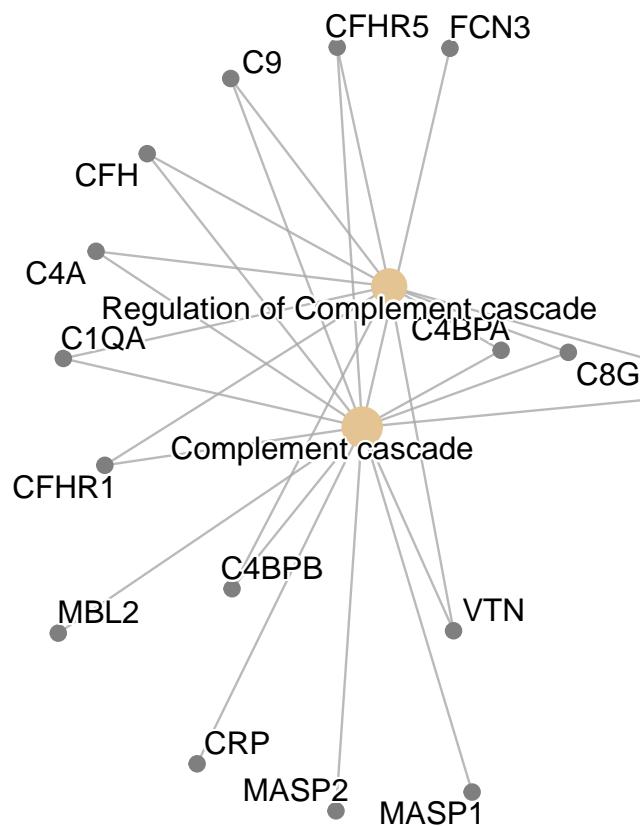
**Figure S24.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute C Non-Improvers Vs Subacute C Non-Improvers

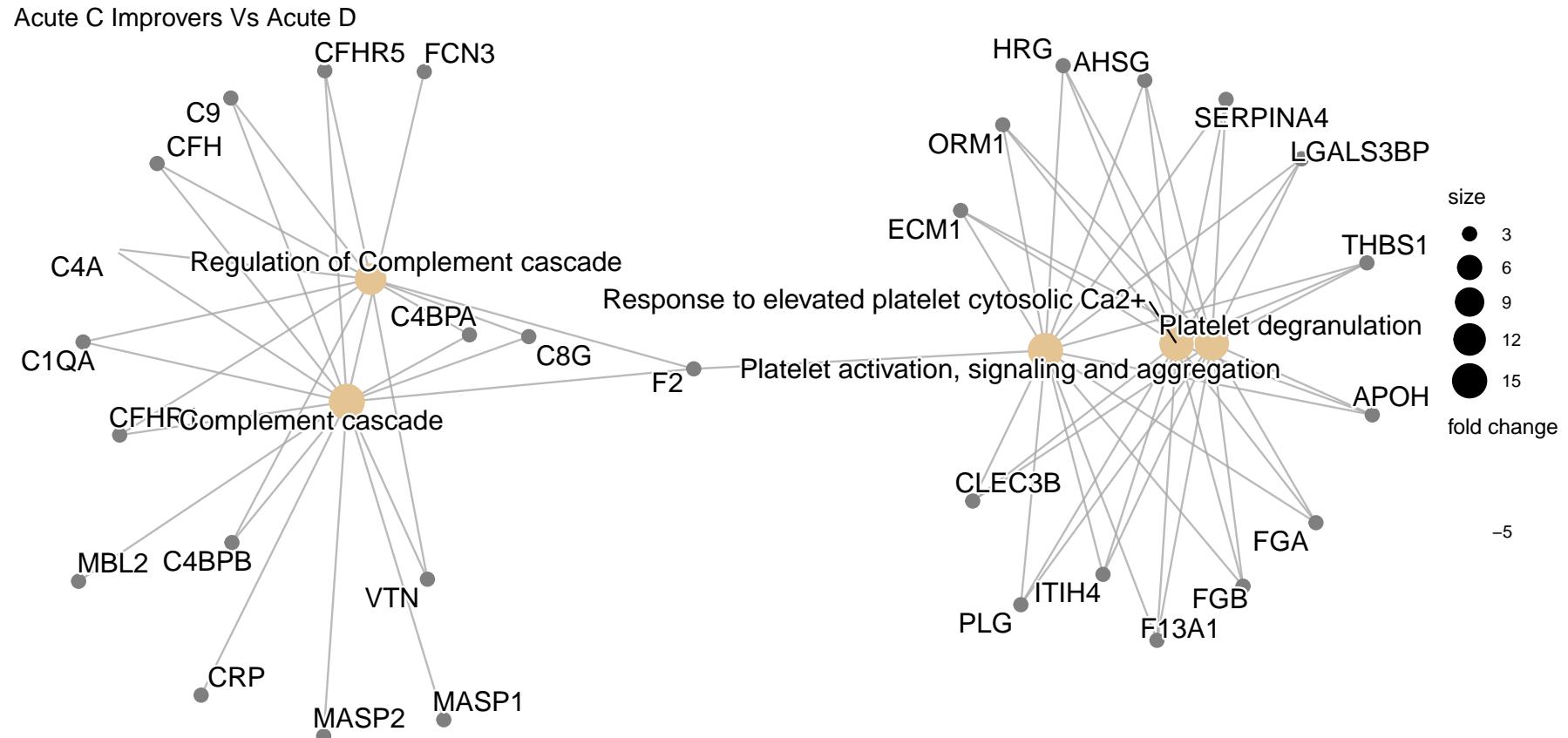


**Figure S25.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute A Vs Acute D

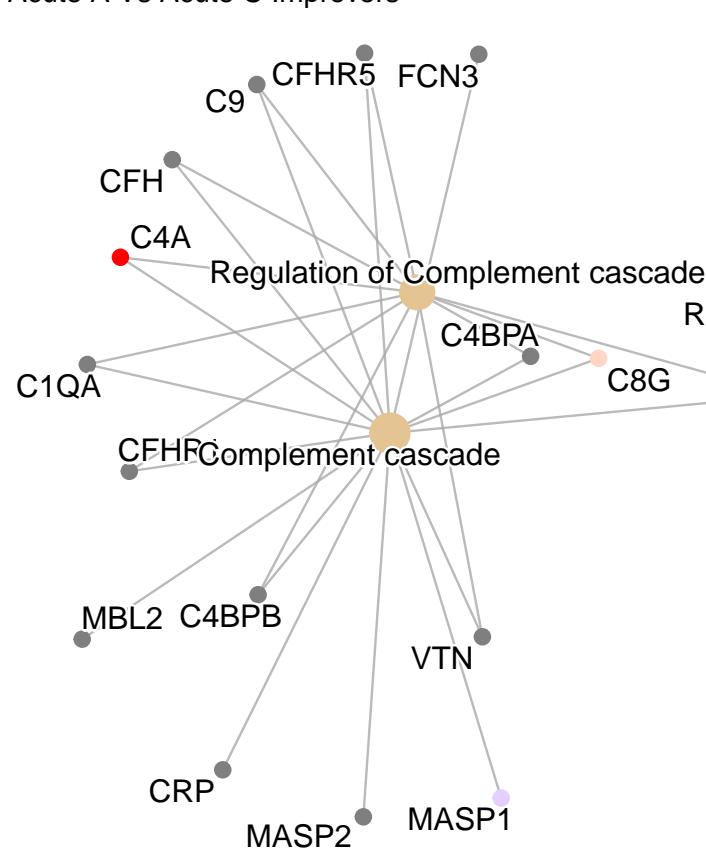


**Figure S26.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

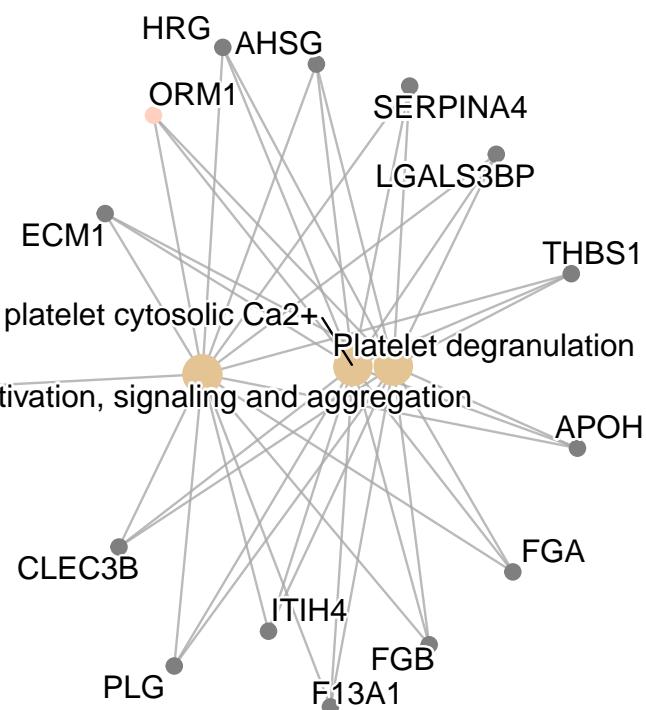


**Figure S27.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

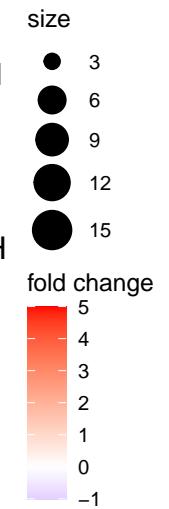
Acute A Vs Acute C Improvers



Regulation of Complement cascade  
Complement cascade

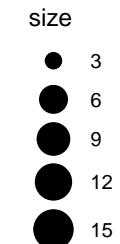
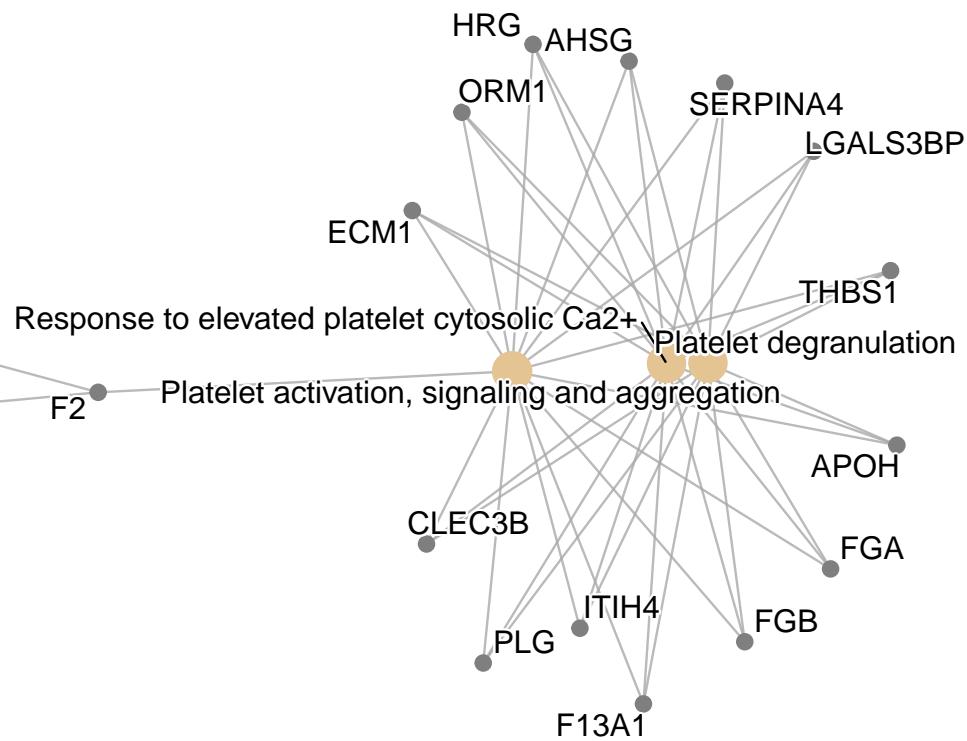
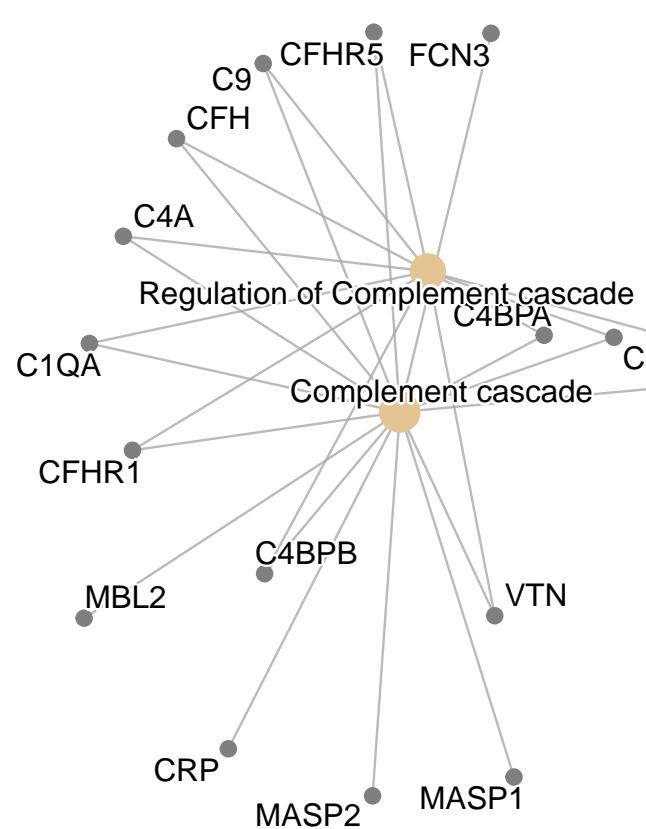


Response to elevated platelet cytosolic Ca<sup>2+</sup>  
Platelet degranulation  
Platelet activation, signaling and aggregation

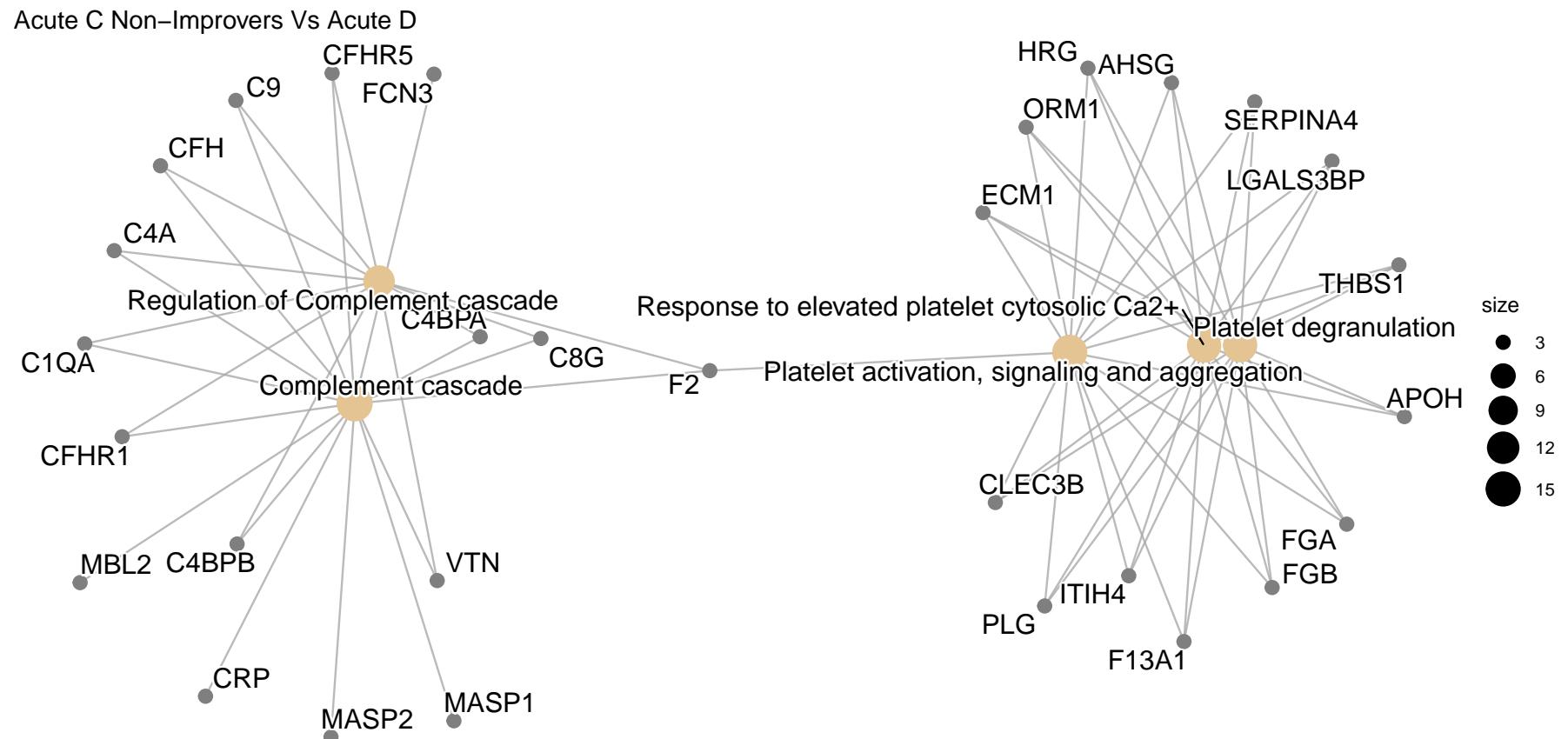


**Figure S28.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute A Vs Acute C Non-Improvers



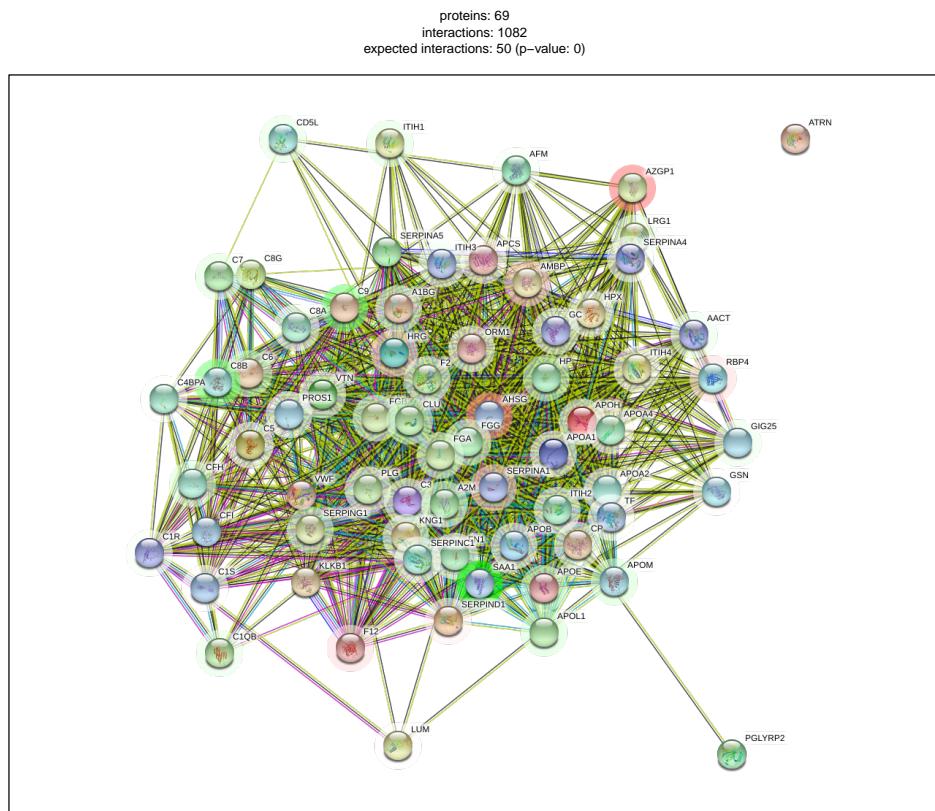
**Figure S29.** Network plot denoting the log<sub>2</sub> fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.



**Figure S30.** Network plot denoting the  $\log_2$  fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

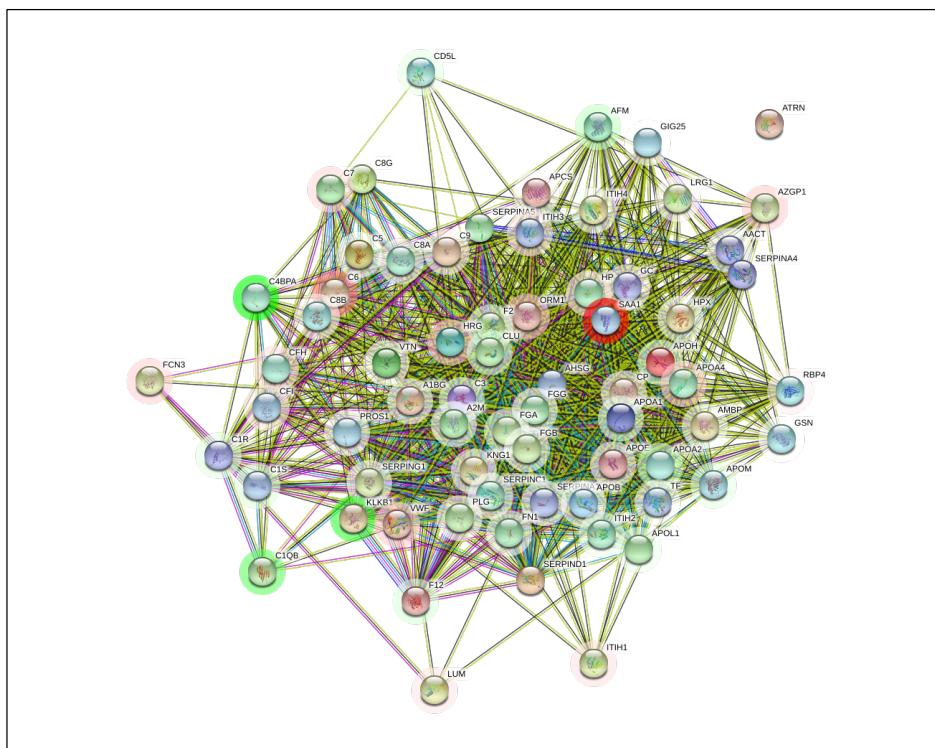
## 1277 5.8 STRINGdb network plots

## 1278 5.8.1 iTRAQ data



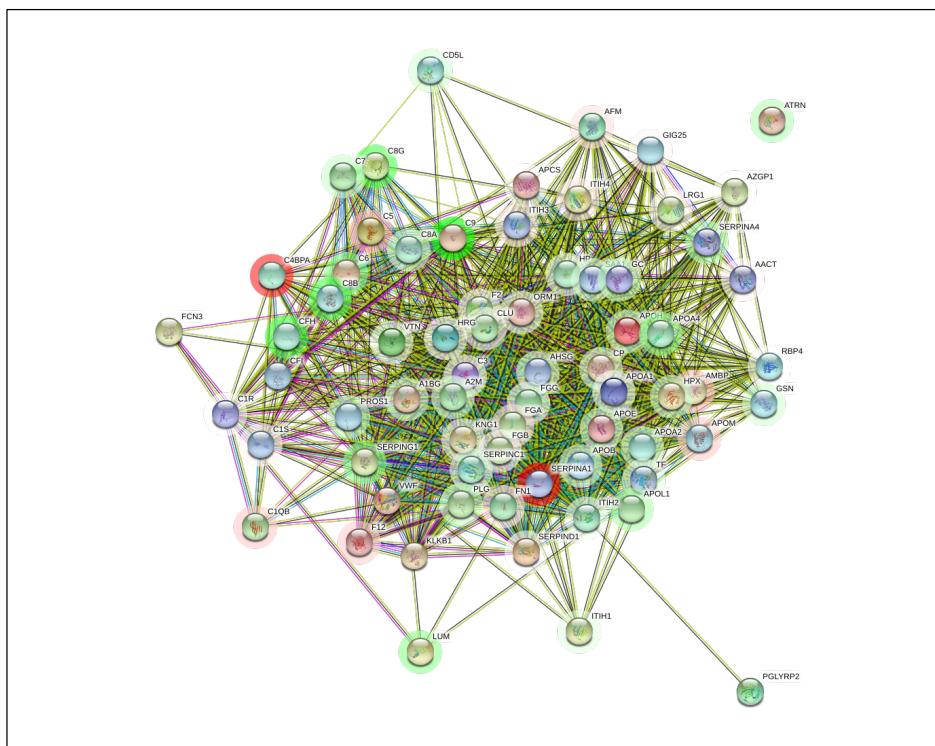
**Figure S31.** The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood; Others are from gene co-expression; text-mining and protein homology.

proteins: 69  
interactions: 1085  
expected interactions: 50 (p-value: 0)



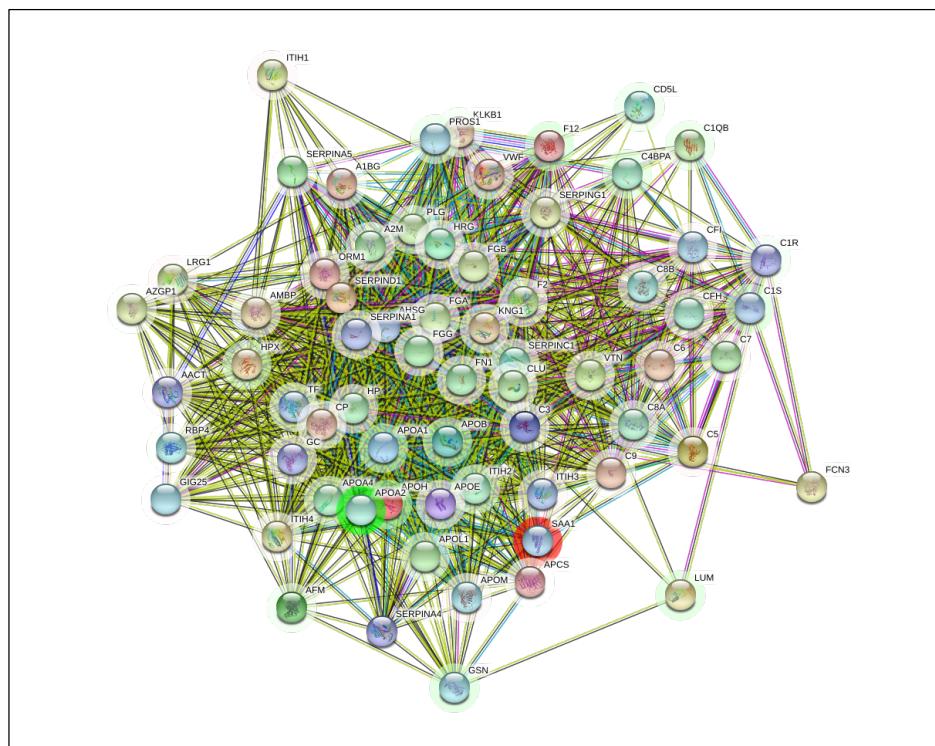
**Figure S32.** Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

proteins: 69  
interactions: 1064  
expected interactions: 50 (p-value: 0)

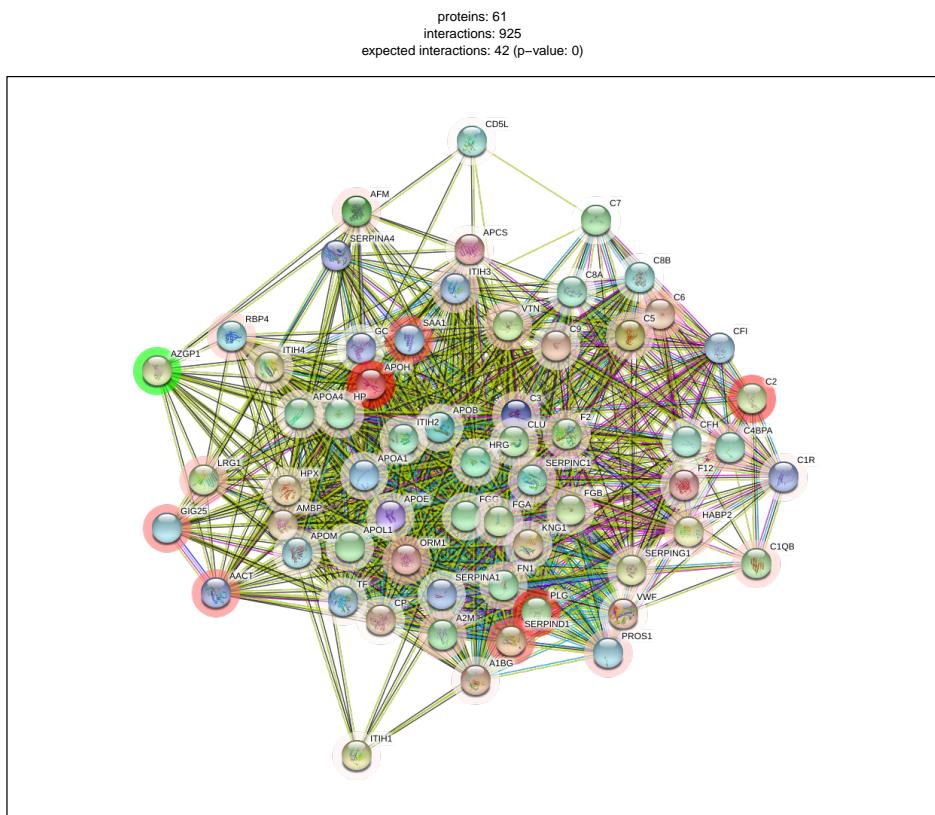


**Figure S33.** Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

proteins: 67  
interactions: 1071  
expected interactions: 49 (p-value: 0)

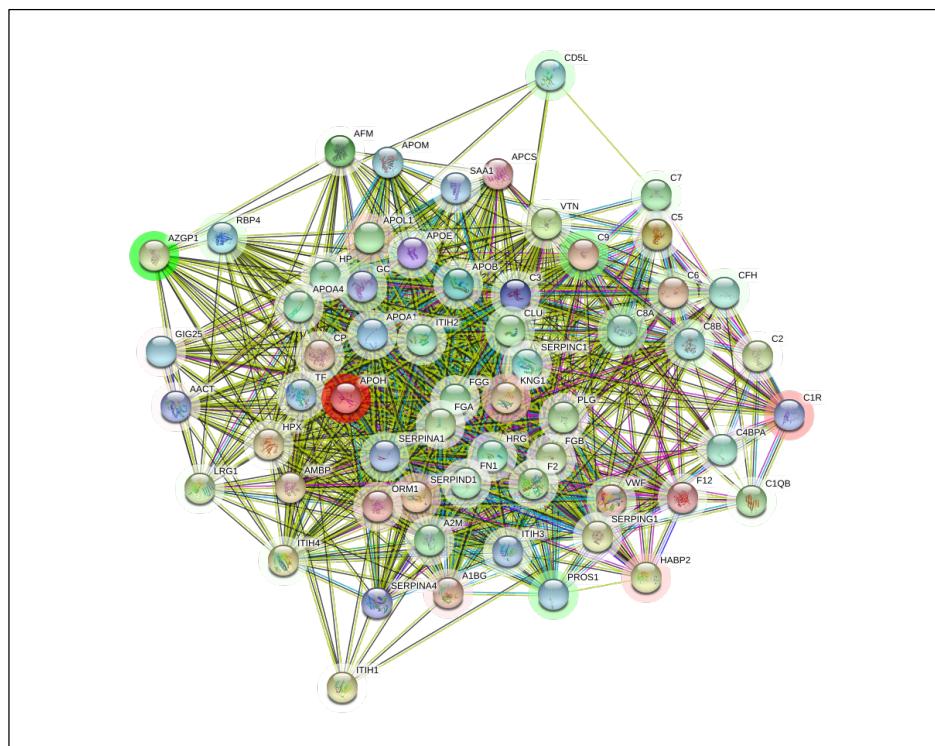


**Figure S34.** Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



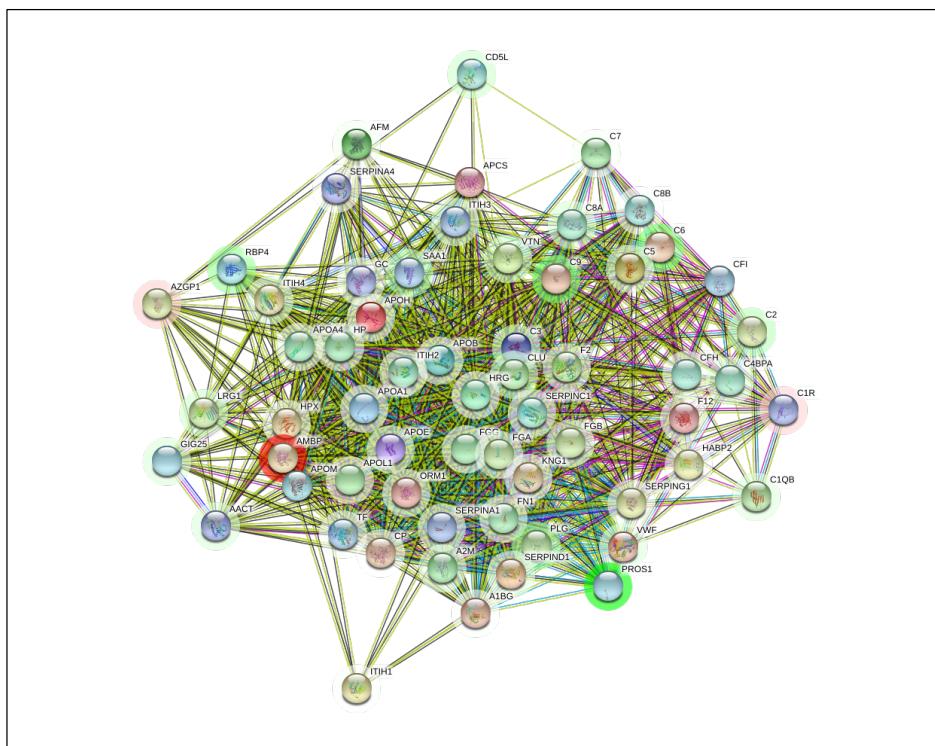
**Figure S35.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

proteins: 60  
interactions: 903  
expected interactions: 41 (p-value: 0)



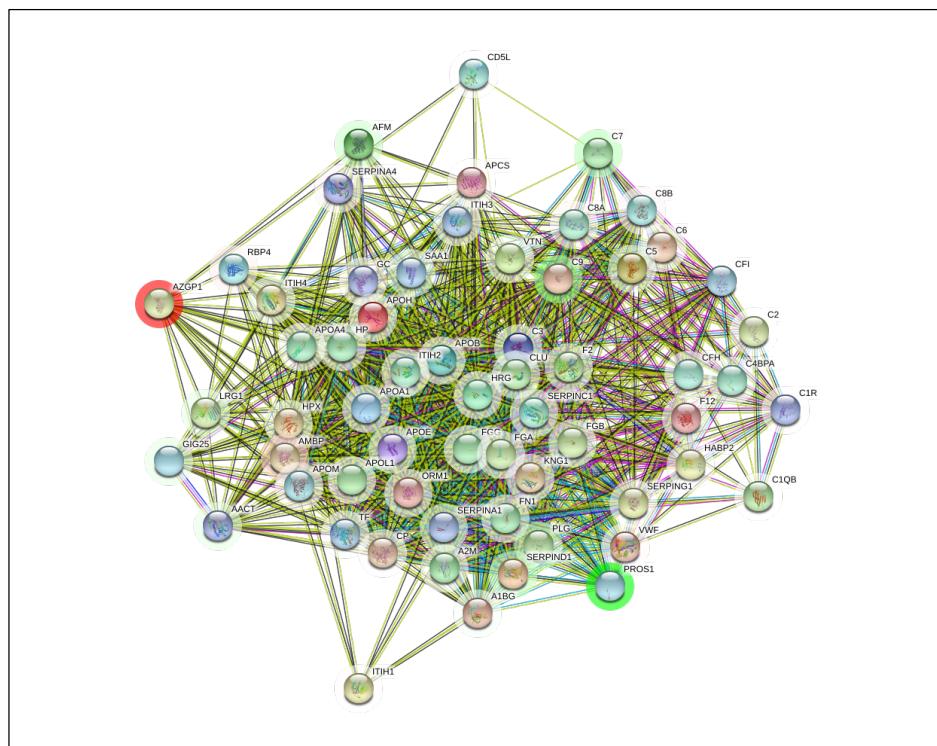
**Figure S36.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases  and those that are experimentally determined . Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining  and protein homology .

proteins: 61  
interactions: 925  
expected interactions: 42 (p-value: 0)



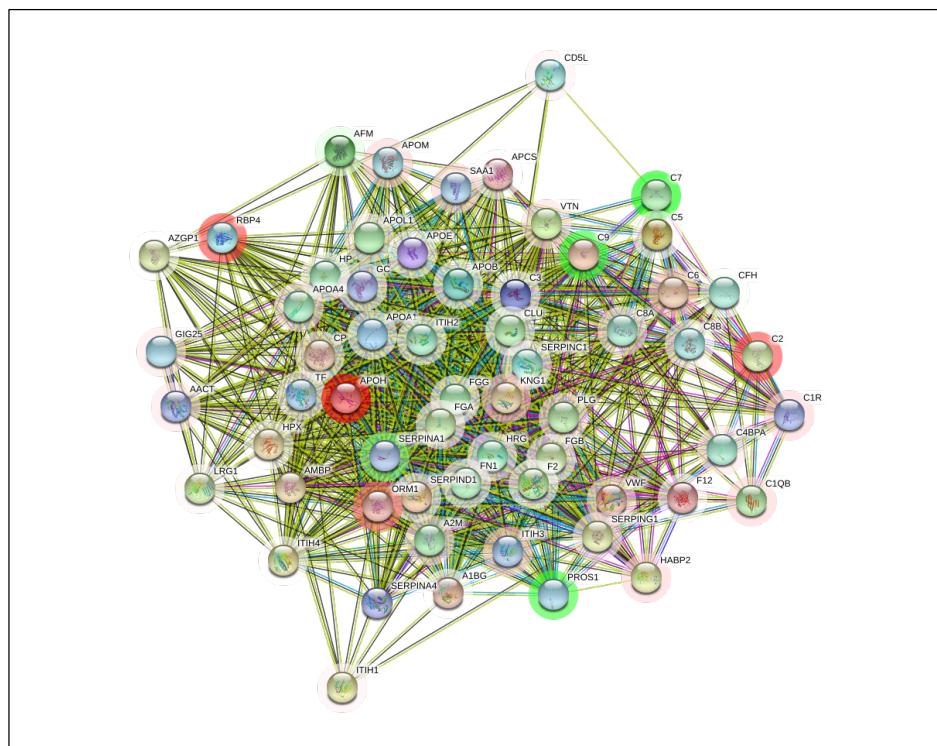
**Figure S37.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

proteins: 61  
interactions: 925  
expected interactions: 42 (p-value: 0)



**Figure S38.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

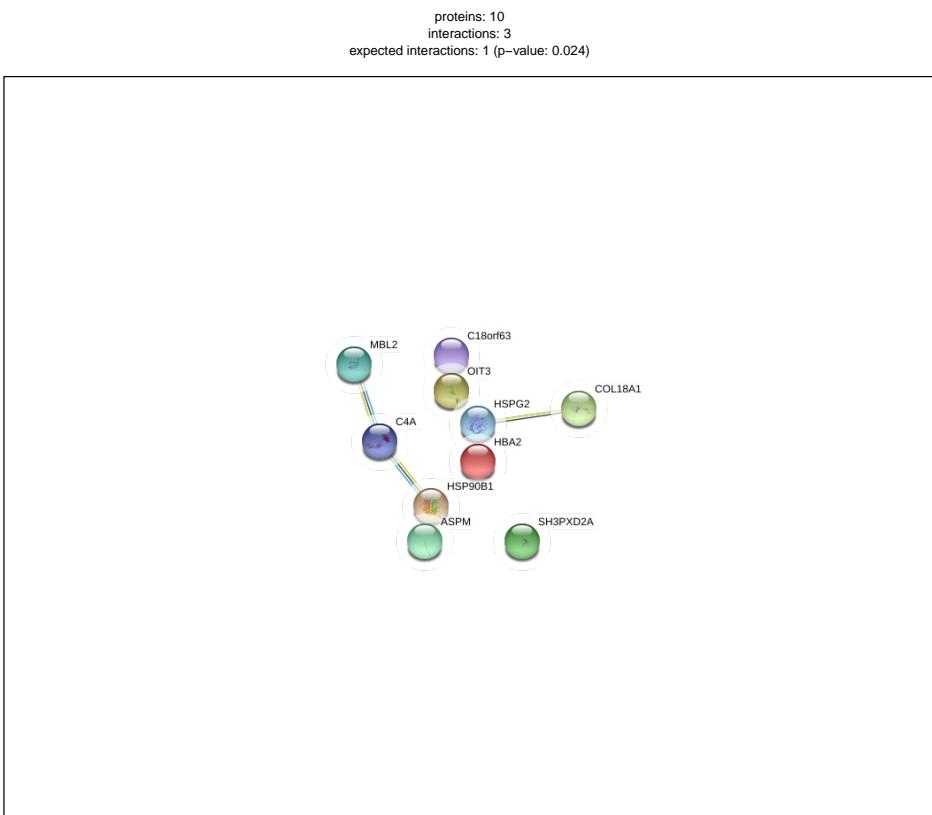
proteins: 60  
interactions: 903  
expected interactions: 41 (p-value: 0)



**Figure S39.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

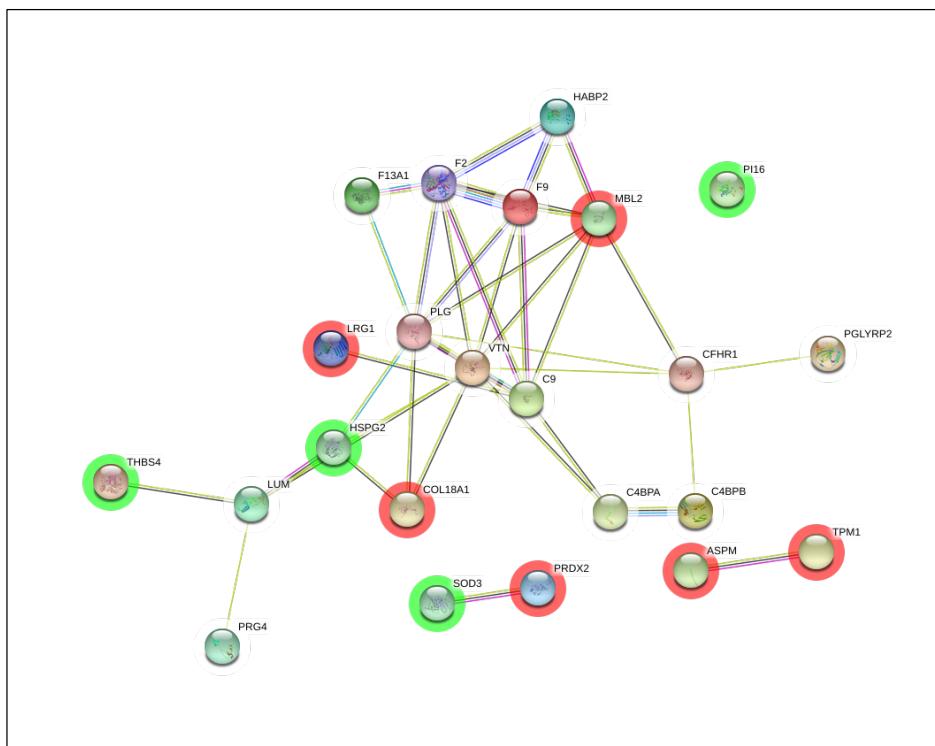


**Figure S40.** The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red indicates greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

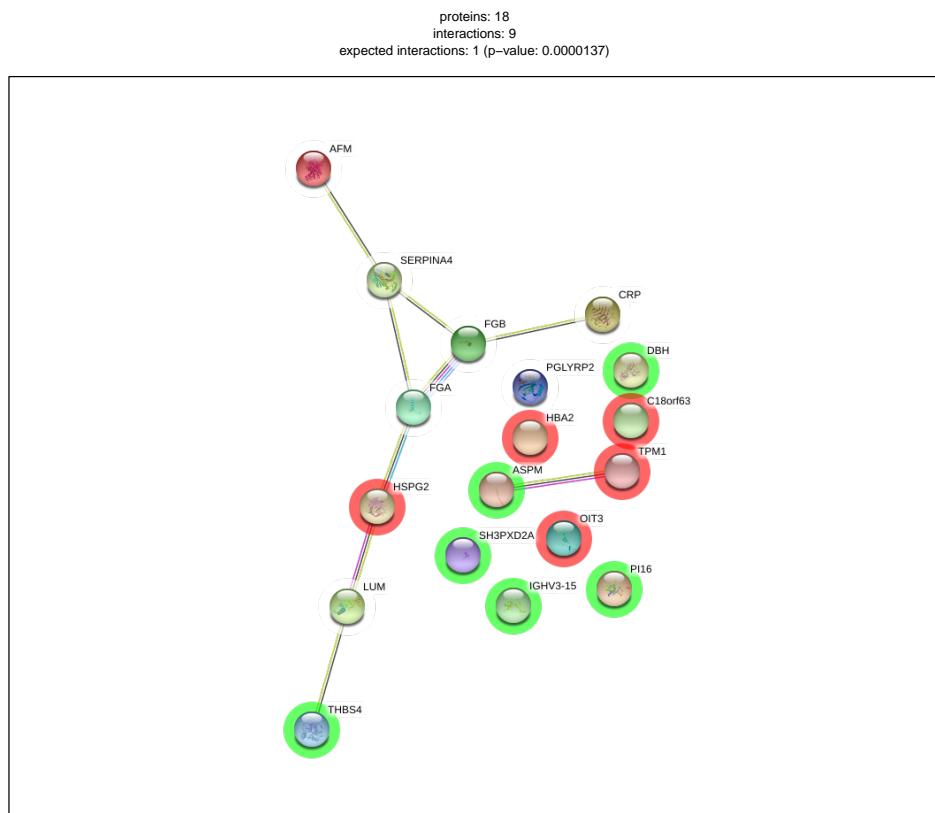


**Figure S41.** Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

proteins: 23  
interactions: 40  
expected interactions: 3 (p-value: 0)

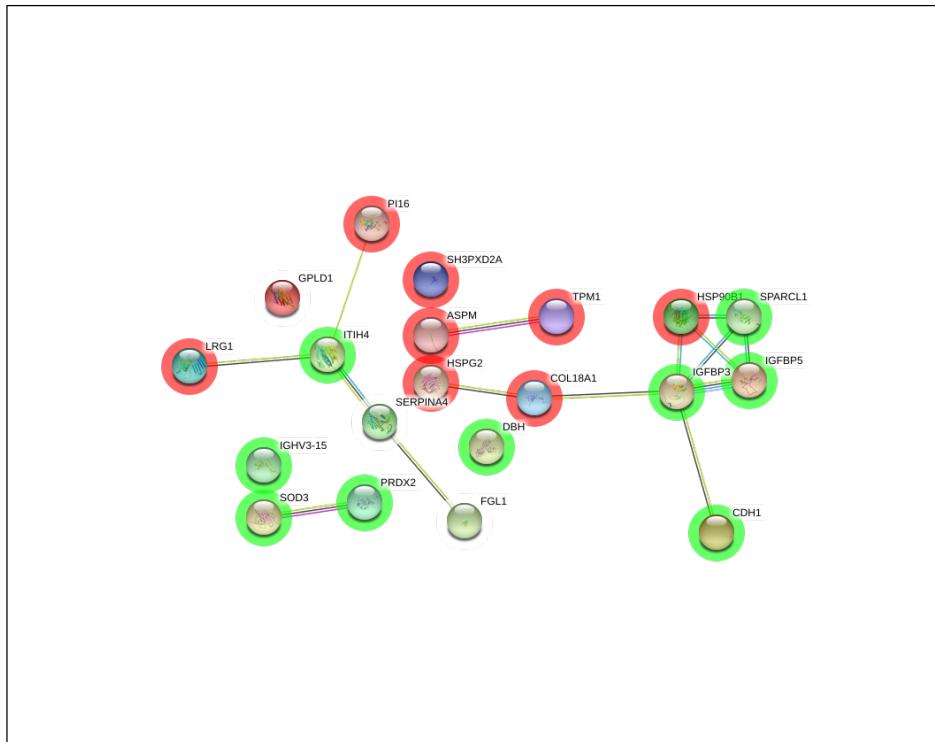


**Figure S42.** Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurrence , gene fusions , gene neighbourhood . Others are from gene co-expression , text-mining and protein homology .

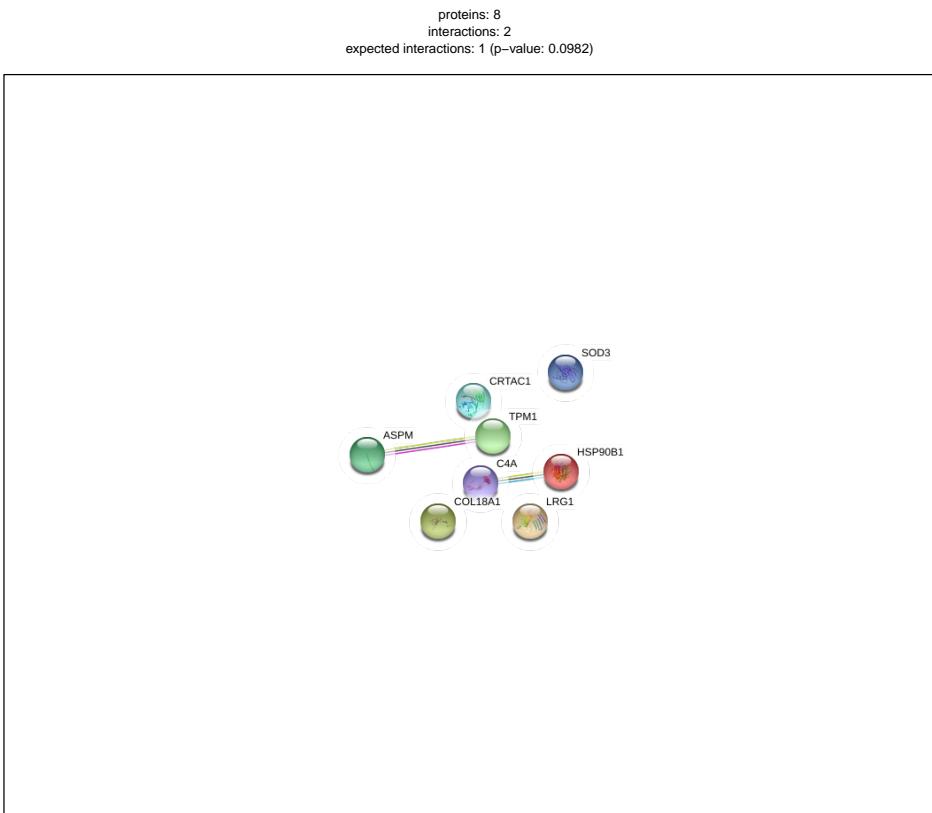


**Figure S43.** Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

proteins: 20  
interactions: 15  
expected interactions: 3 (p-value: 0.00000243)

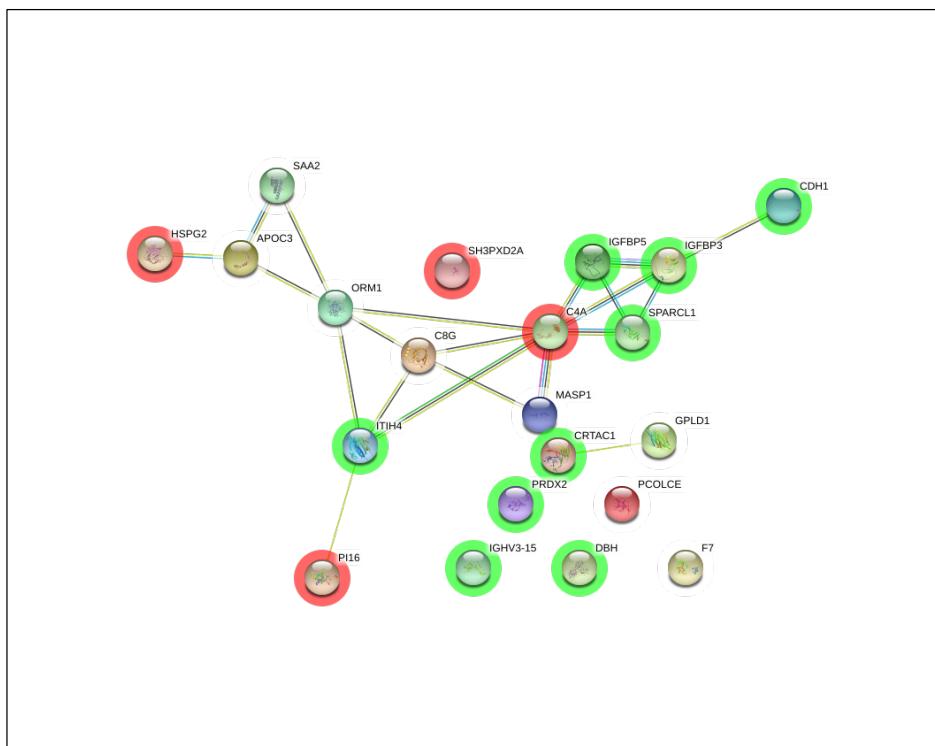


**Figure S44.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

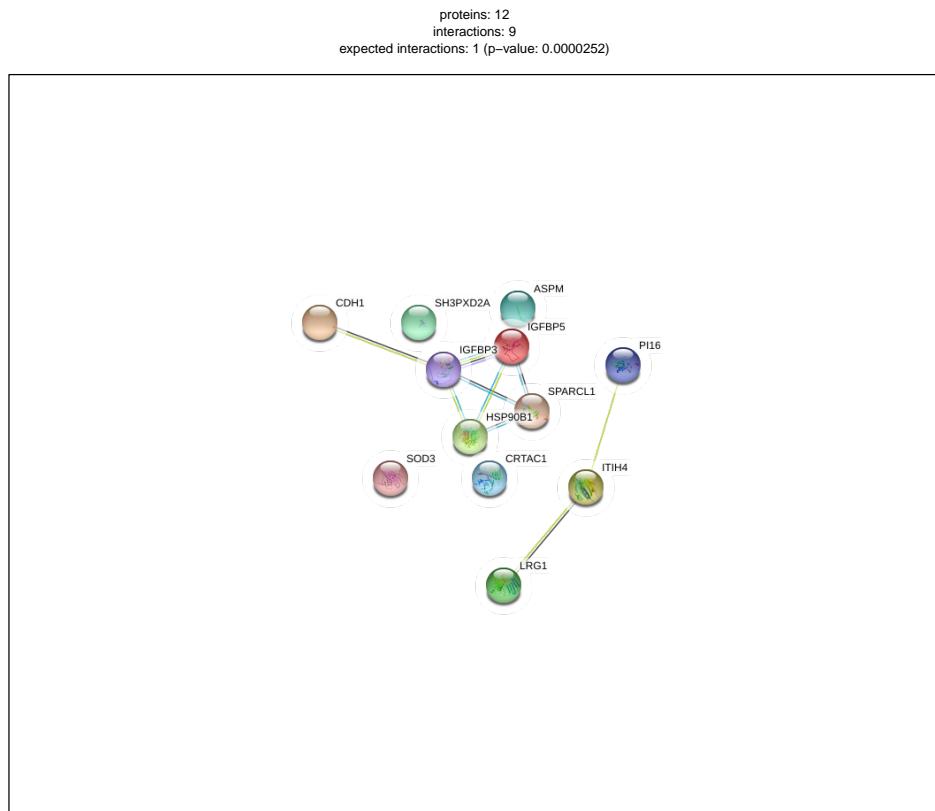


**Figure S45.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

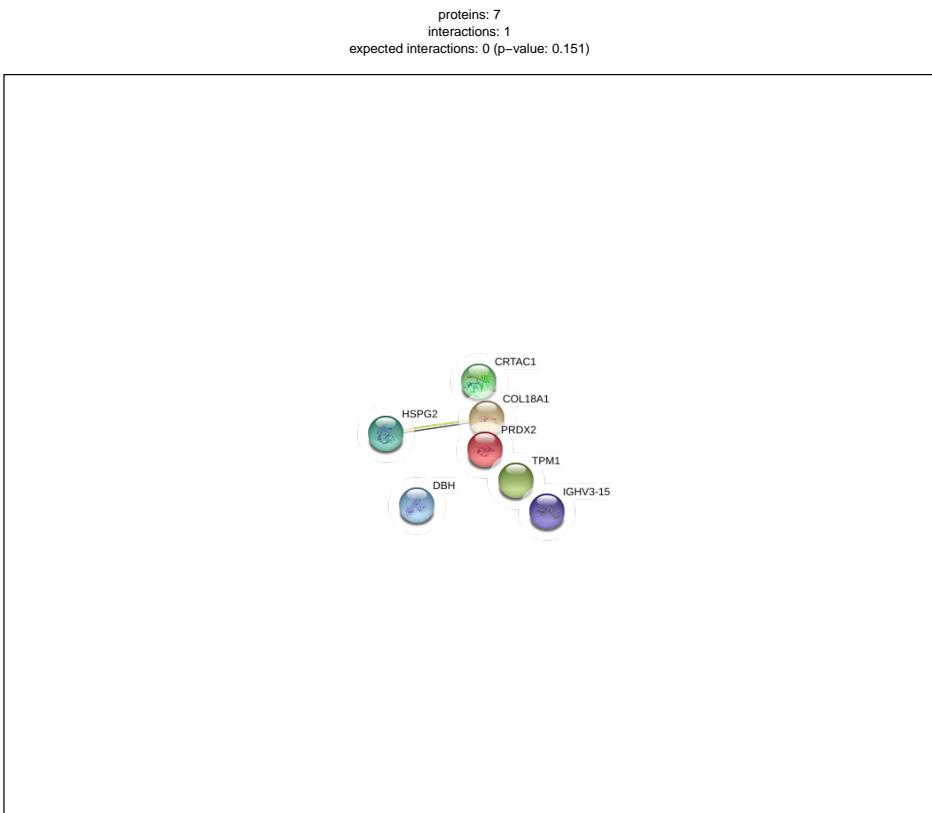
proteins: 21  
interactions: 21  
expected interactions: 2 (p-value: 1.64e-13)



**Figure S46.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

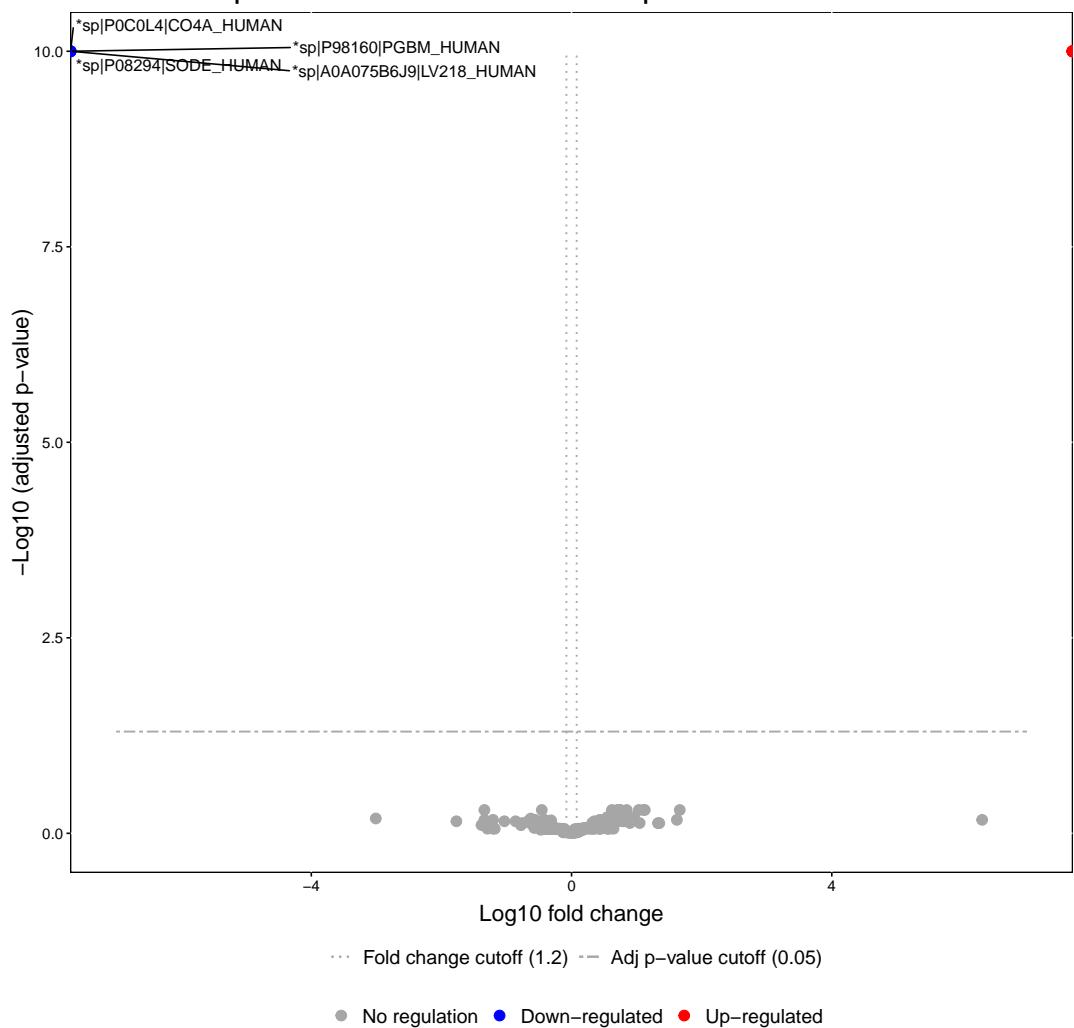


**Figure S47.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .



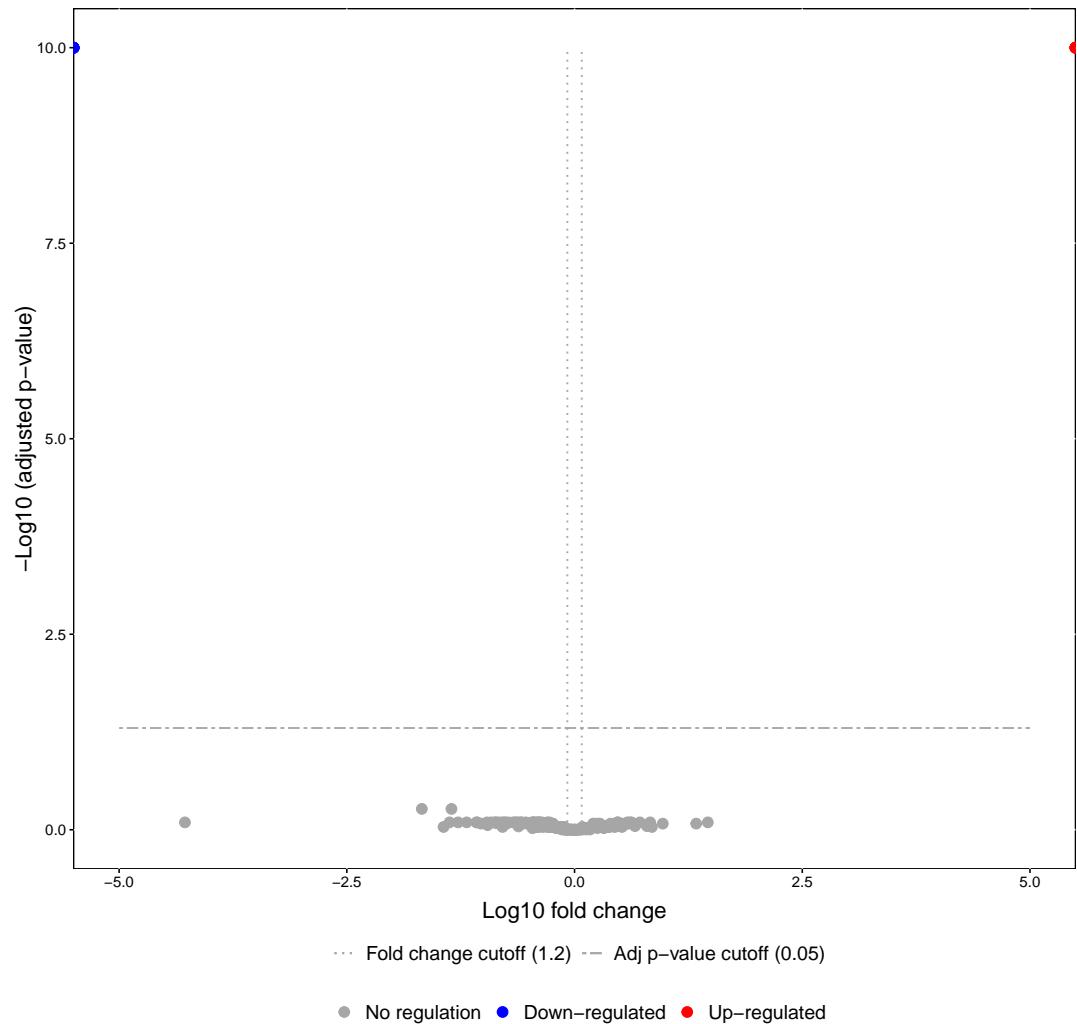
**Figure S48.** Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurrence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

### Acute C Improvers Vs Acute C Non-Improvers



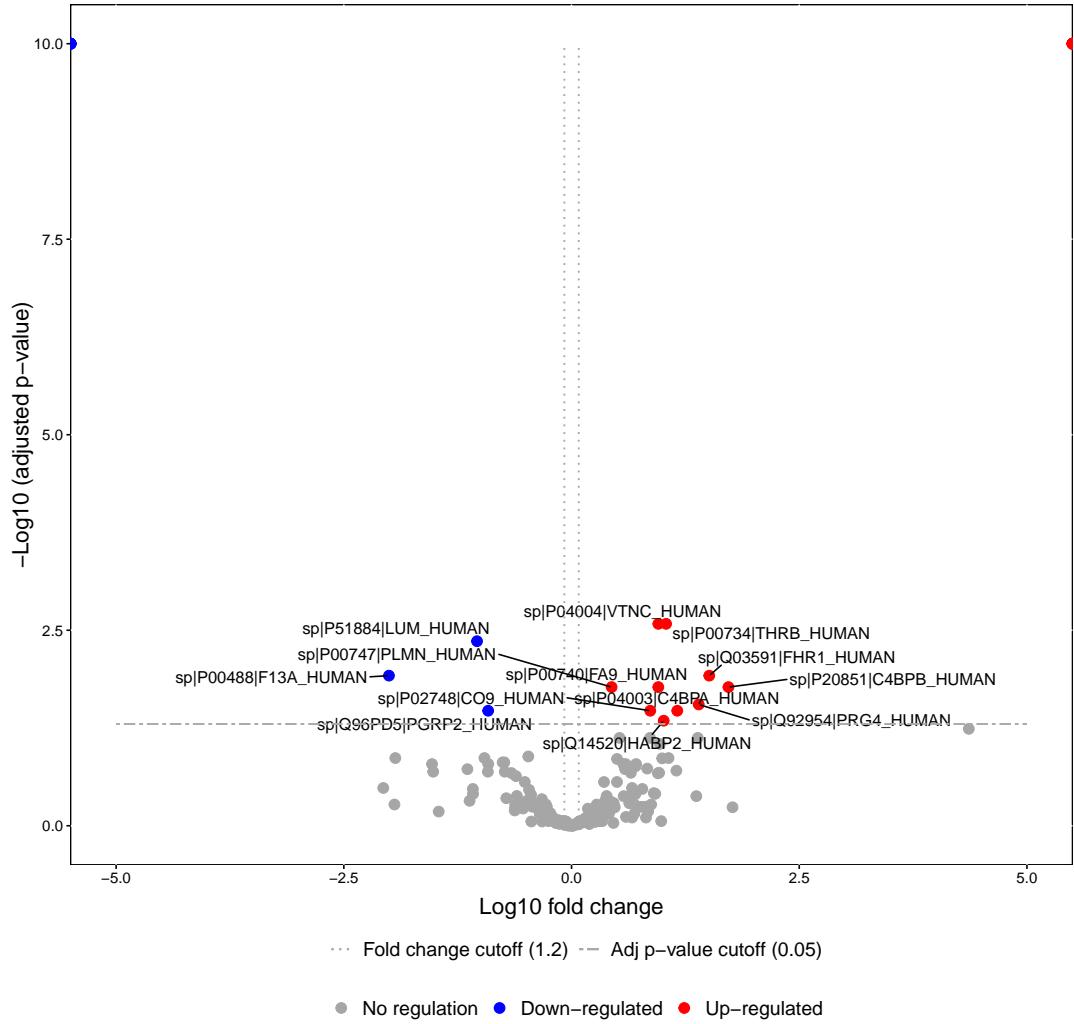
**Figure S49.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond  $\pm 1.2$  and an adjusted p-value less than 0.05 are labelled.

### Subacute C Improvers Vs Subacute C Non-Improvers



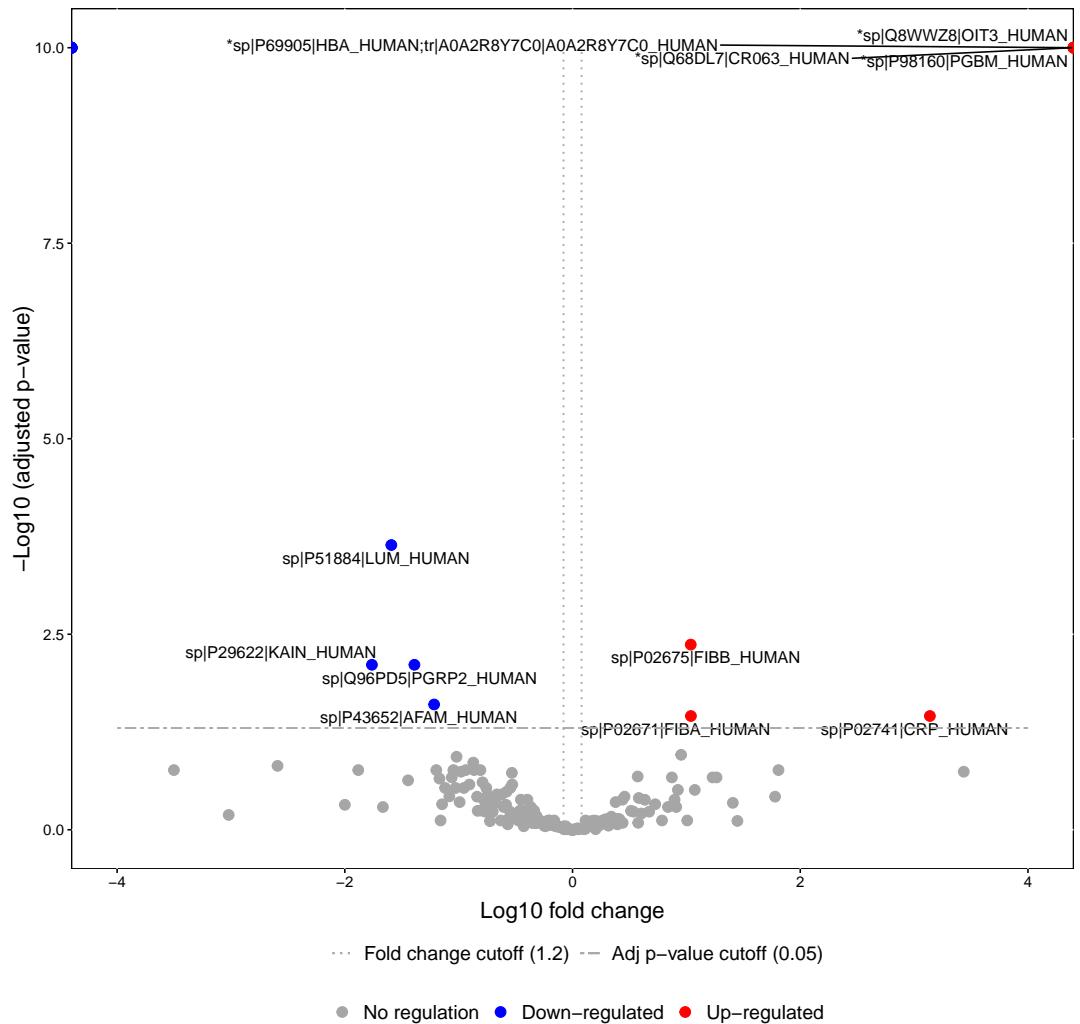
**Figure S50.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 3-months post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond  $\pm 1.2$  and an adjusted p-value less than 0.05 are labelled.

### Acute C Improvers Vs Subacute C Improvers



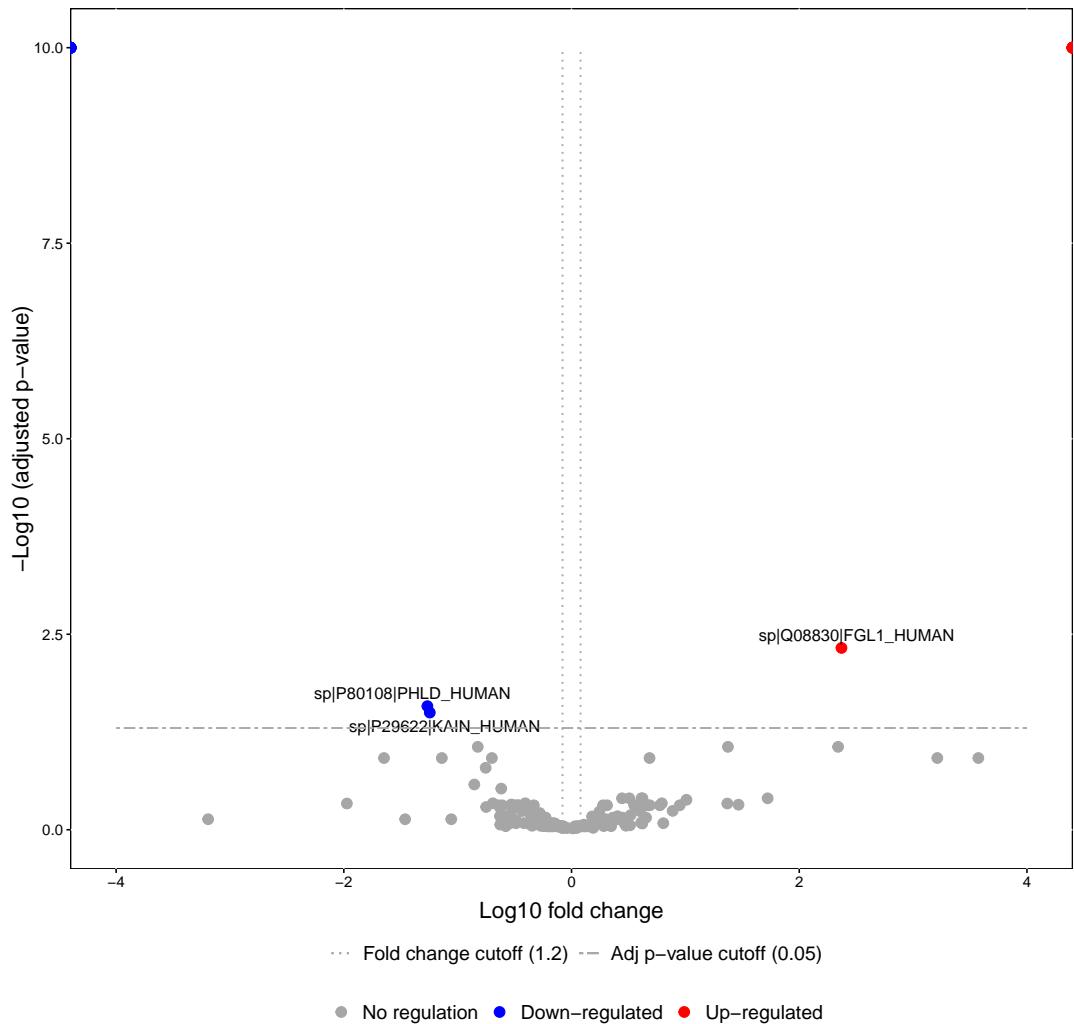
**Figure S51.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

### Acute C Non-Improvers Vs Subacute C Non-Improvers



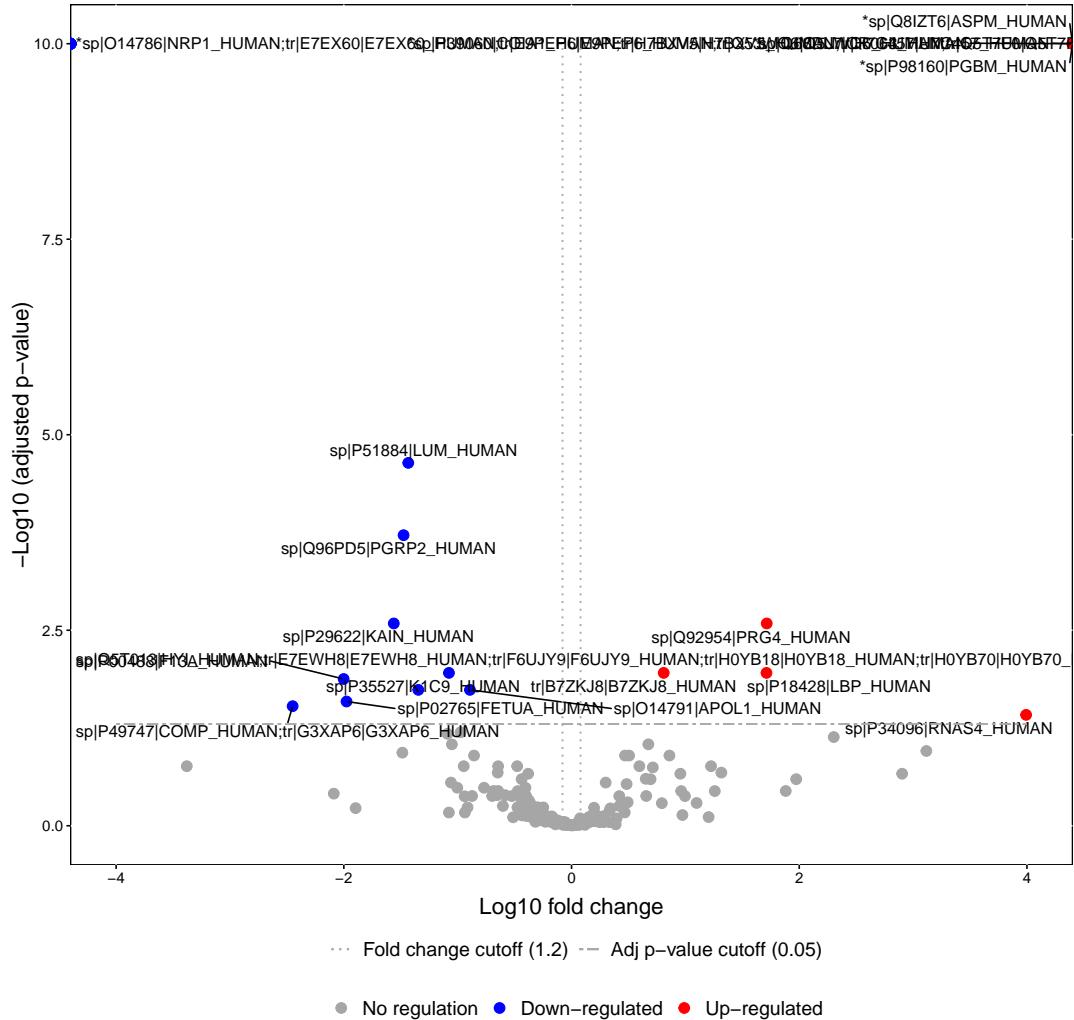
**Figure S52.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond  $>1.2$  and an adjusted p-value less than 0.05 are labelled.

### Acute A Vs Acute D



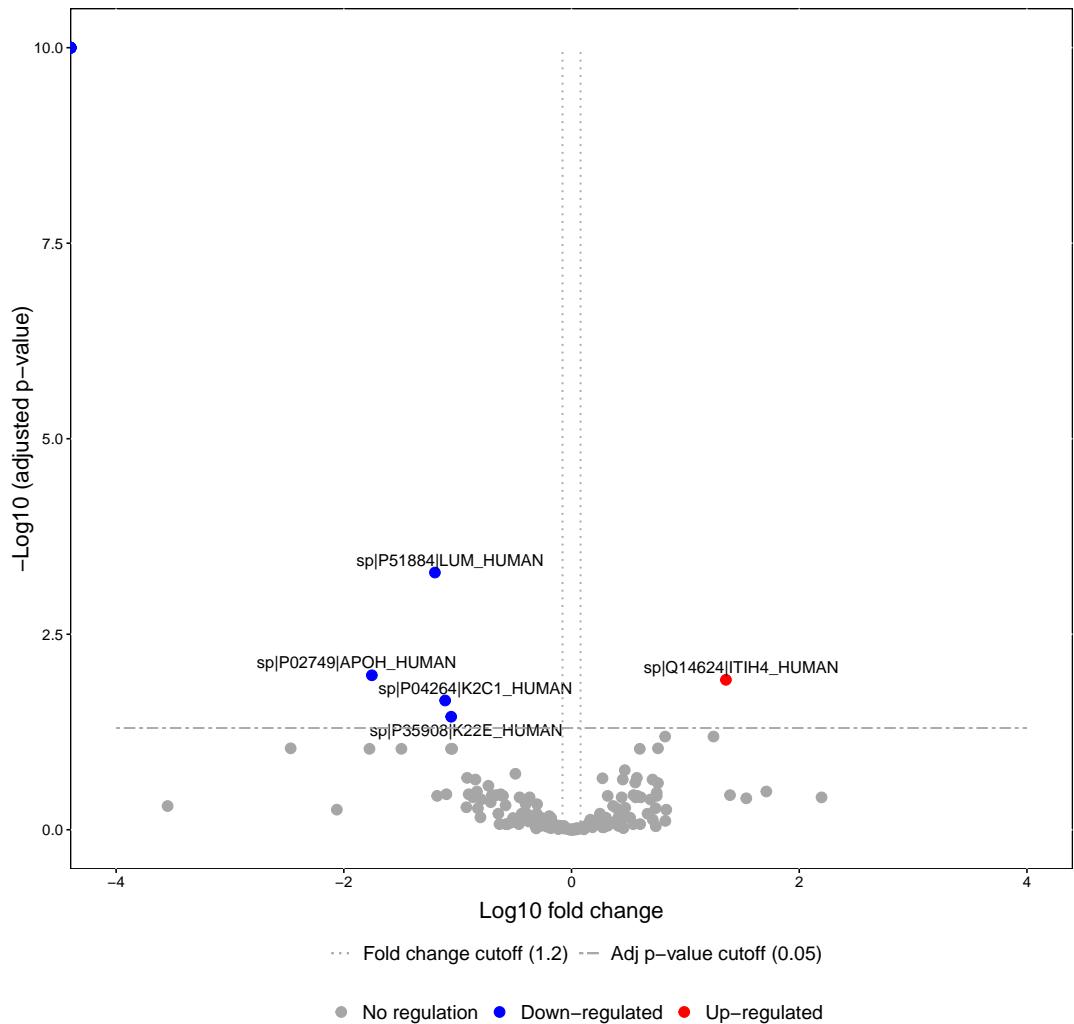
**Figure S53.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

### Acute A Vs Subacute A



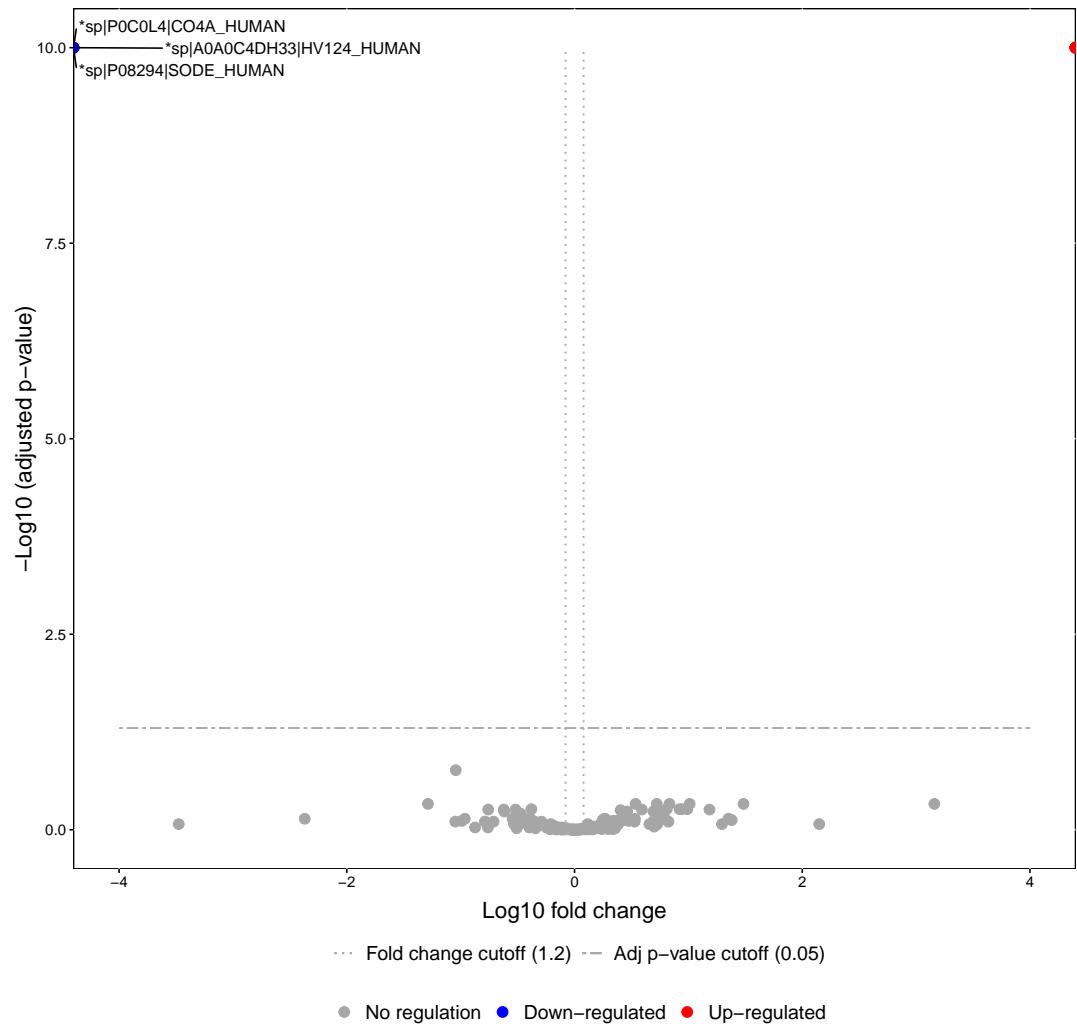
**Figure S54.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS A patients. Proteins with a fold changes beyond  $>1.2$  and an adjusted p-value less than 0.05 are labelled.

### Acute D Vs Subacute D



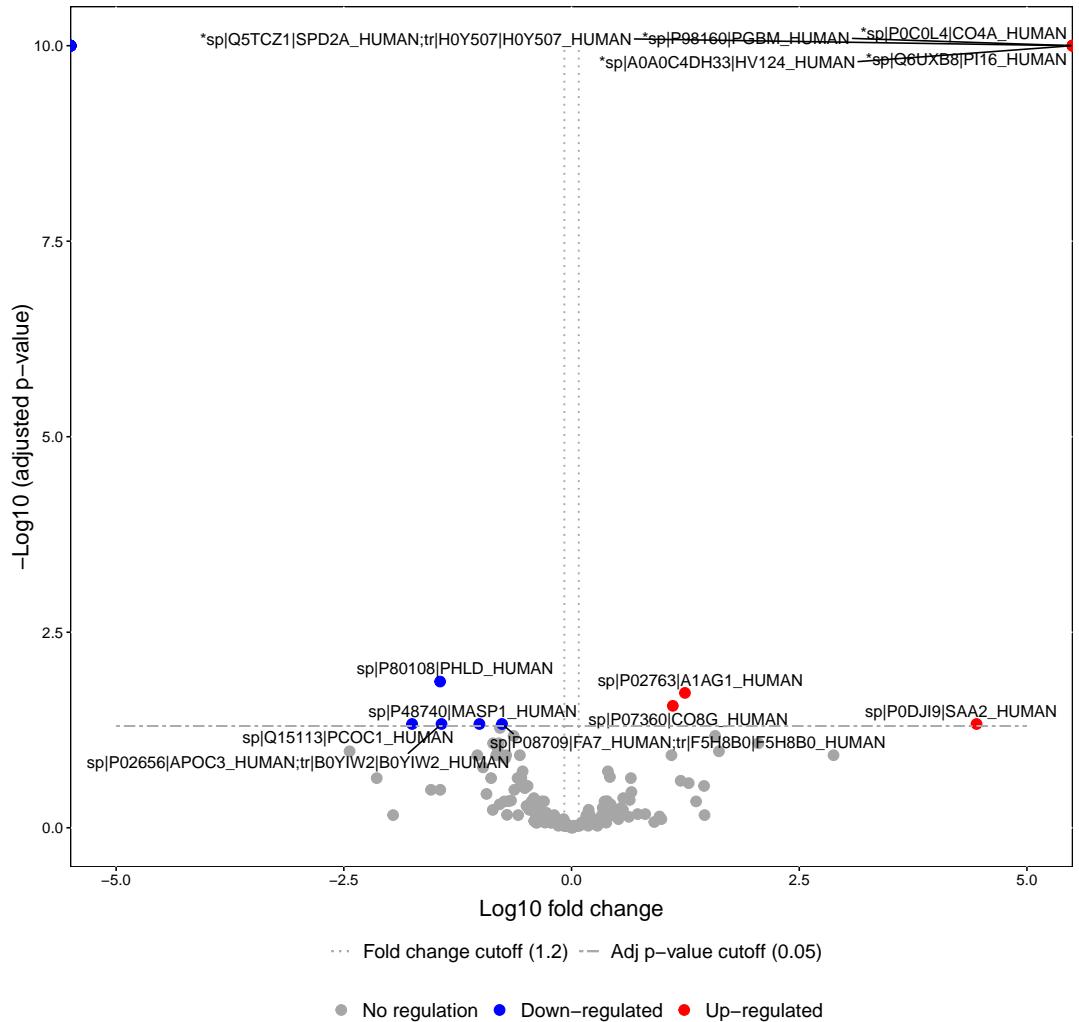
**Figure S55.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS D patients. Proteins with a fold changes beyond  $>1.2$  and an adjusted p-value less than 0.05 are labelled.

### Acute C Improvers Vs Acute D



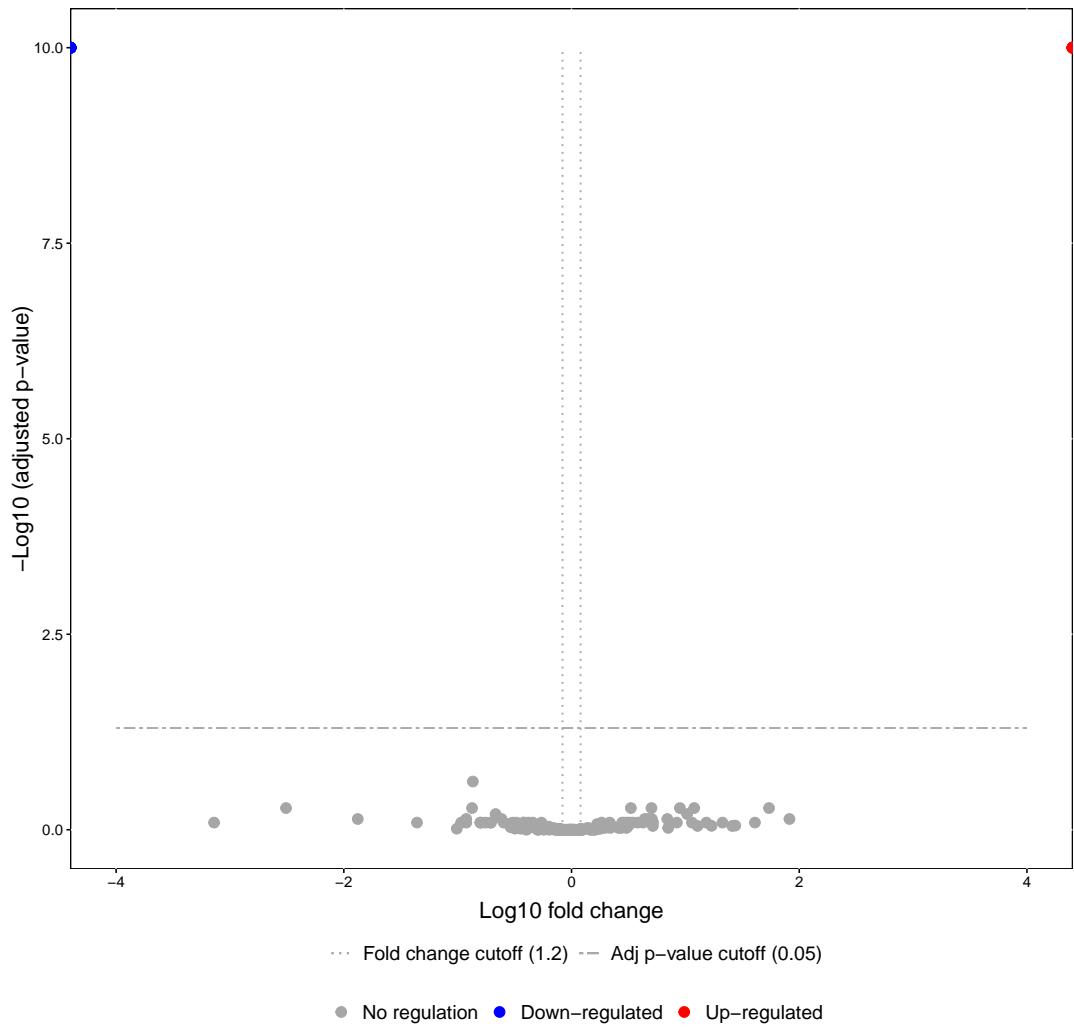
**Figure S56.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

### Acute A Vs Acute C Improvers



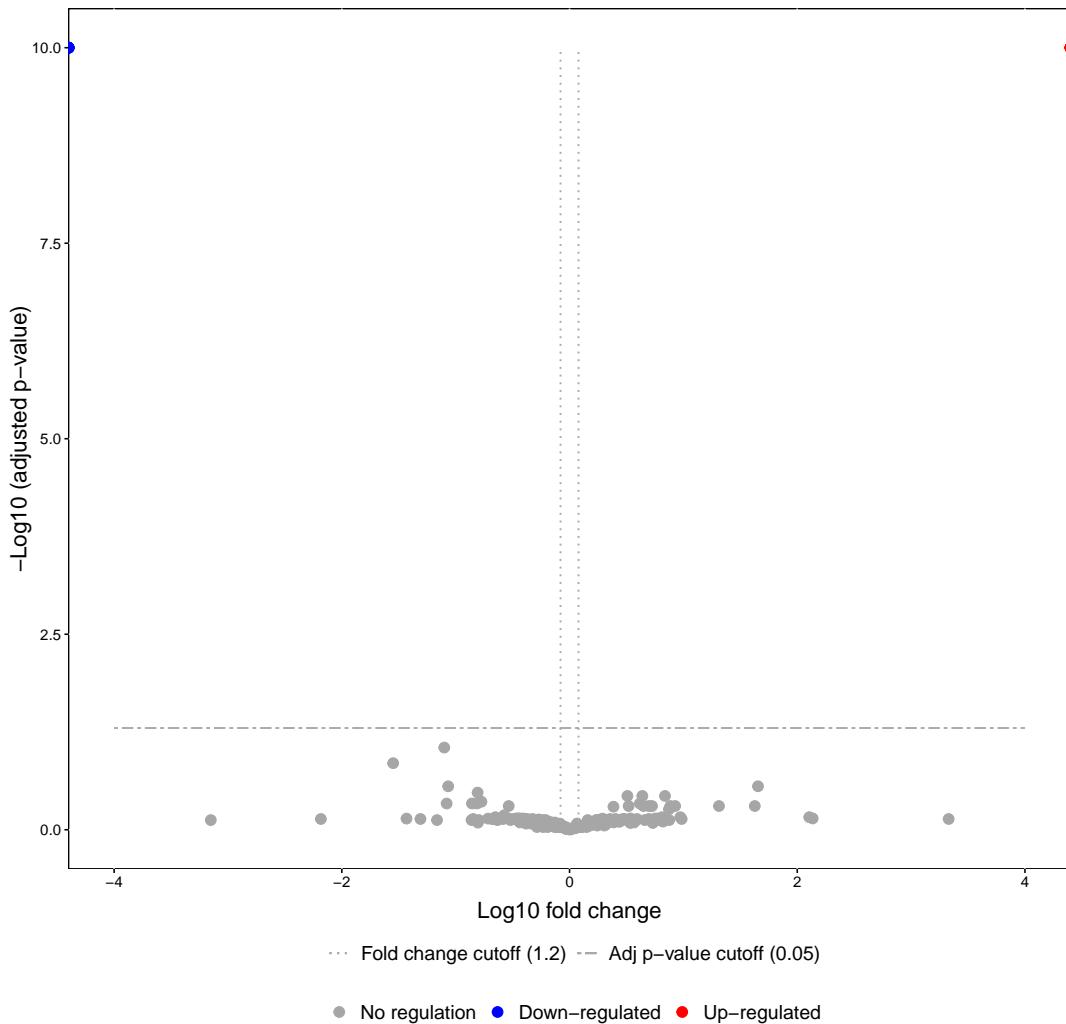
**Figure S57.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

### Acute A Vs Acute C Non-Improvers



**Figure S58.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

## Acute C Non-Improvers Vs Acute D



**Figure S59.** Volcano plot of  $\log_{10}$  fold change and  $\log_{10}$  adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who did not experience an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond  $>1.2$  and an adjusted p-value less than 0.05 are labelled.

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