

¹ **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**

⁸ **1 Abstract**

⁹ Introduction

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

¹⁷ Methods and Materials

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

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

³⁰ Results

³¹ The data demonstrated proteomic differences between the cohorts, with the results from the
³² iTRAQ approach supporting those of the label-free analysis. A total of 79 and 87 differentially
³³ abundant proteins across AIS and longitudinal groups were identified from the iTRAQ and label-
³⁴ free analyses, respectively. Alpha-2-macroglobulin (A2M), retinol binding protein 4 (RBP4), serum
³⁵ 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); @ha-
58 gen_acute_2015]

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

65 Biomarkers studies in SCI often investigate protein changes in cerebral spinal fluid (CSF) as the
66 closer proximity of this medium is thought to be more reflective of the parenchymal injury.(Brian
67 K. Kwon et al. 2019; Hulme et al. 2017) Whilst this makes CSF potentially more informative for
68 elucidating the pathology of SCI, the repeated use of CSF for routine analysis presents challenges
69 in clinical care due to the risk and expense associated with the invasiveness of the collection
70 procedure. In contrast, systemic biomarkers measurable in the blood represent a source of
71 information that can be accessed and interpreted both a lower cost and risk. Studies of traumatic
72 brain injury have demonstrated that protein markers identified in CSF are also detectable in both
73 plasma and serum.(Wang et al. 2018) More recently, circulating white blood cell populations
74 have also been identified as potential SCI injury biomarkers, with a 2021 study showing that
75 elevated levels of neutrophils were associated with no AIS grade conversion, while conversely
76 an increase in lymphocytes during the first week post-SCI were associated with an AIS grade
77 improvement.[@jogia_peripheral_2021]

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

81 Further, changes in inflammatory marker levels detected in acute SCI patients were found to
82 be mirrored in donor-matched blood and CSF, albeit at lower absolute concentrations systemi-
83 cally.(Brian K. Kwon et al. 2010)

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

89 **3 Methods and Materials**

Table 1. Patient demographics. ± denotes interquartile range

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

90 **3.1 Patients**

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

96 **3.2 Plasma collection and storage**

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

102 **3.3 Sample preparation and analysis using iTRAQ proteomics**

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

106 A total of 100mg of plasma protein was taken from each sample and pooled equally to form a
107 patient test group. For example, the AIS C improver group was pooled from 10 separate patient
108 samples, 10mg of protein per patient.

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

123 **3.3.0.1 iTraq mass spectrometry analysis** The samples were analysed at the BSRC St. An-
124 drews University Mass Spectrometry and Proteomics Facility using methods previously described.

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

128 **SECTION TO BE REWRITTEN**

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

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

133 **3.3.1 Sample preparation and analysis using label-free proteomics**

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

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

142 **3.3.1.1 Label free mass spectrometry analysis** Tryptic peptides were subjected to LC-MC/MC
143 via a 2-h gradient on a NanoAcuity™ ultraperformance LC (Waters, Manchester, UK) connected

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

146 **REWRITE IN BRIEF**

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

157 **3.3.2 iTraq OpenMS analysis**

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

167 The MSFQPlusAdapter was used to run the search. For the -fixed_modifications "Methylthio (C)"
168 and "iTRAQ4plex (N-term)" were passed due to the alkylating agent used in sample preparation
169 and to account for the N-terminus modifications made by iTRAQ tags. "Oxidation (M)" was passed
170 to -variable_modifications to reflect the likely occurrence of methionine oxidation. To reflect the
171 instrument the following flags were also set: -precursor_mass_tolerance 20 -enzyme Trypsin/P
172 -protocol iTRAQ -instrument high_res.

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

177 IsobaricAnalyzer with -type itraq4plex was used with the merged .mzML files to assign protein-
178 peptide identifications to features or consensus features with IDMapper. The files for each run
179 output by IDMapper were then merged with FileMerger. Bayesian score estimation and protein
180 inference was performed with Epifany and the following flags: -greedy_group_resolution
181 remove_proteins_wo_evidence -algorithm:keep_best_PSM_only false Decoys were removed
182 and 0.05 FDR filtering was done via IDFfilter with -score:protgroup 0.05 -remove_decoys.
183 Finally, IDConflictResolver was used to resolve ambiguous annotations of features with peptide
184 identifications, before quantification with ProteinQuantifier.

185 **3.3.3 Label free OpenMS analysis**

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

189 FileConverter tool via the open-source ThermoRawFileParser.(Röst et al. 2016; Hulstaert et al.
190 2020) Unless otherwise stated, default arguments were used throughout.

191 The decoy database generated in the prior section (iTRAQ OpenMS analysis) was also re-used. The
192 CometAdapter was used to run the search.(Eng, Jahan, and Hoopmann 2013) Fixed modifications
193 were set to "Carbamidomethyl (C)" and "Oxidation (M)" was set as a variable modification. To reflect
194 the instrument the following flags were also set: -precursor_mass_tolerance 20 -isotope_error
195 0/1.

196 To annotate the identified peptides with proteins the PeptideIndexer tool was used. PeptideIndexer
197 and PSMFeatureExtractor were used for annotation. For peptide level score estimation and fil-
198 tering PercolatorAdapter was used with the following flags: -score_type q-value -enzyme
199 trypsin. IDFFilter was used to filter to a peptide score of 0.01 with -score:pep 0.01 followed
200 by IDScoreSwitcher with the following flags: -new_score "MS:1001493" -new_score_orientation
201 lower_better -new_score_type "pep" -old_score "q-value". The ProteomicsLFQ was used for
202 subsequent processing with the flags: -proteinFDR 0.05 -targeted_only true. The -out_msstats
203 flag was also used to produce quantitative data for downstream statistical analysis with the R
204 package MSstats.(Choi et al. 2014)

205 **3.3.4 Network and pathway analysis**

206 Protein interation networks were created using the Bioconductor package STRINGdb which pro-
207 vides an R interface to STRING version 11.(Szklarczyk et al. 2019) Instantiation of the STRINGdb
208 reference class was done with species and score_threshold set to 9606, for *Homo sapiens*, and
209 400 respectively. Clustering of networks with STRINGdb used the "fastgreedy" algorithm from the
210 iGraph package.

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

214 **3.3.5 Enzyme-linked immunosorbent assays**

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

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

229 **4 Results**

230 **4.1 Results**

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

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

237 **4.1.1 Comparing OpenMS and ProteinPilot**

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

²⁴² **4.1.2 iTRAQ analyses**

²⁴³ **4.1.3 Differential protein abundances**

²⁴⁴ AIS C improvers had 18 more abundant proteins and 49 less abundant proteins at the acute phase
²⁴⁵ relative to non-improvers. Similarly, at the subacute phase, AIS C improvers had 34 more abun-
²⁴⁶ dant proteins and 34 less abundant proteins relative to non-improvers. The AIS A group had 56
²⁴⁷ more abundant and 9 less abundant proteins respectively relative to non-improvers. Acutely, AIS
²⁴⁸ C improvers relative to AIS A and D had 21 and 53 more abundant and 46 and 12 less abundant
²⁴⁹ proteins. Please see the appendix for a full list of protein changes.

²⁵⁰ **4.1.4 Heatmaps**

²⁵¹ The majority of the pathways associated with the proteins identified by these iTRAQ experiments
²⁵² are related to the complement cascade and platelet activity (Figure 1, 2, S1, S2, S3, S4, S5, S6, S7, S8).
²⁵³ There are also several pathways implicated in metabolic processes, particularly with apolipopro-
²⁵⁴ teins and retinoids.

Acute AIS C Improvers VS non-Improvers

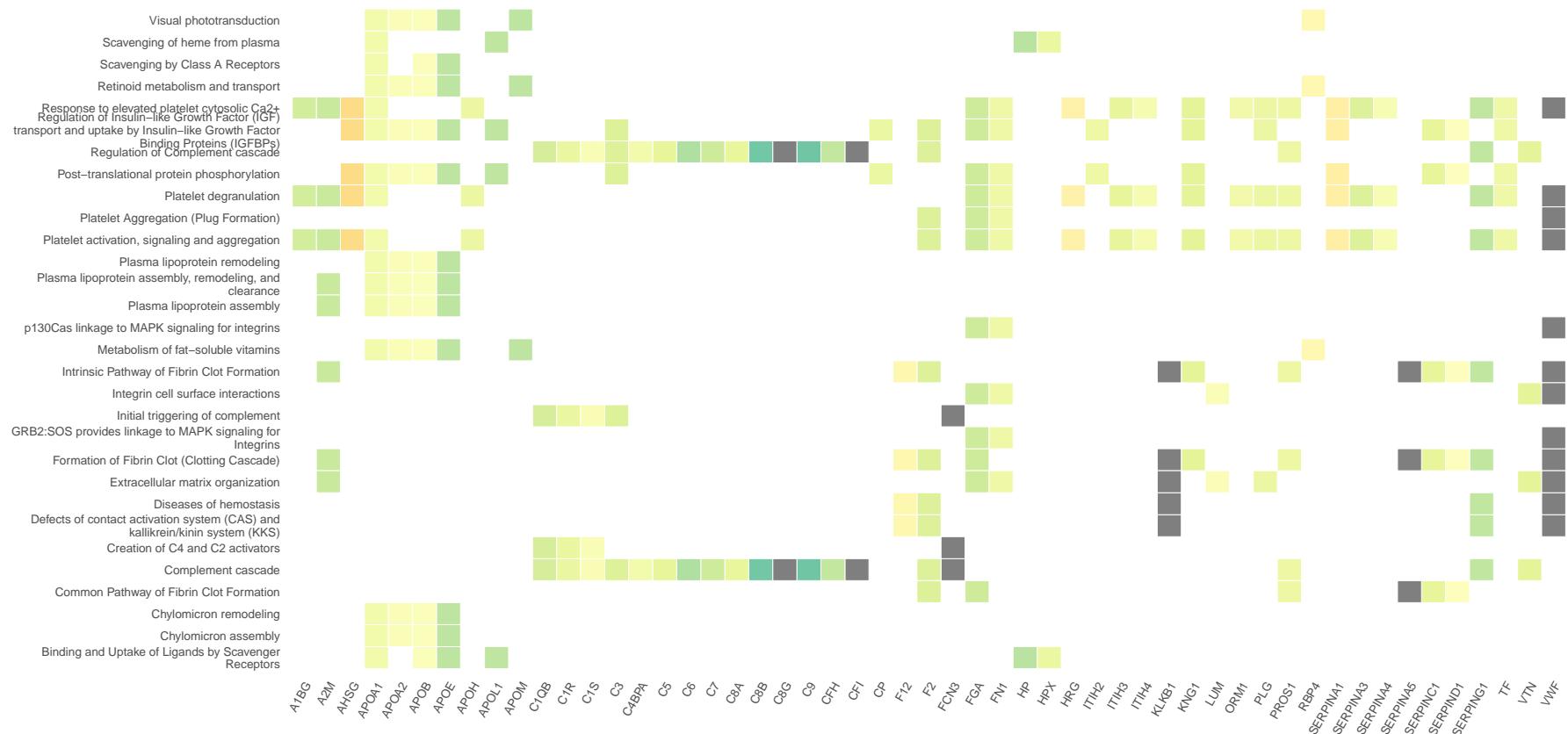


Figure 1. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

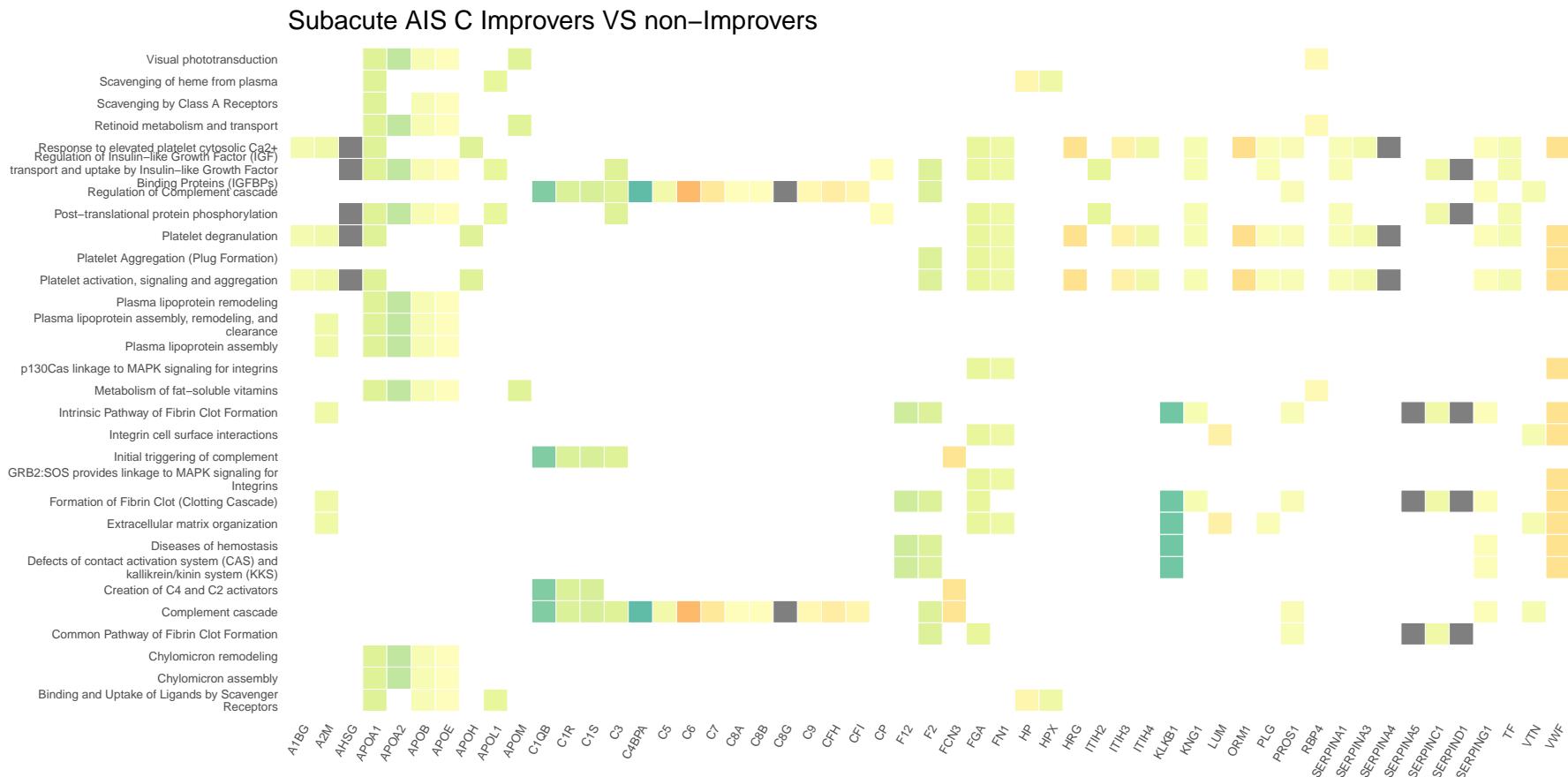


Figure 2. Heatmap denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

²⁵⁵ 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).

²⁵⁷ Please see appendix section 5.6 for additional plots.

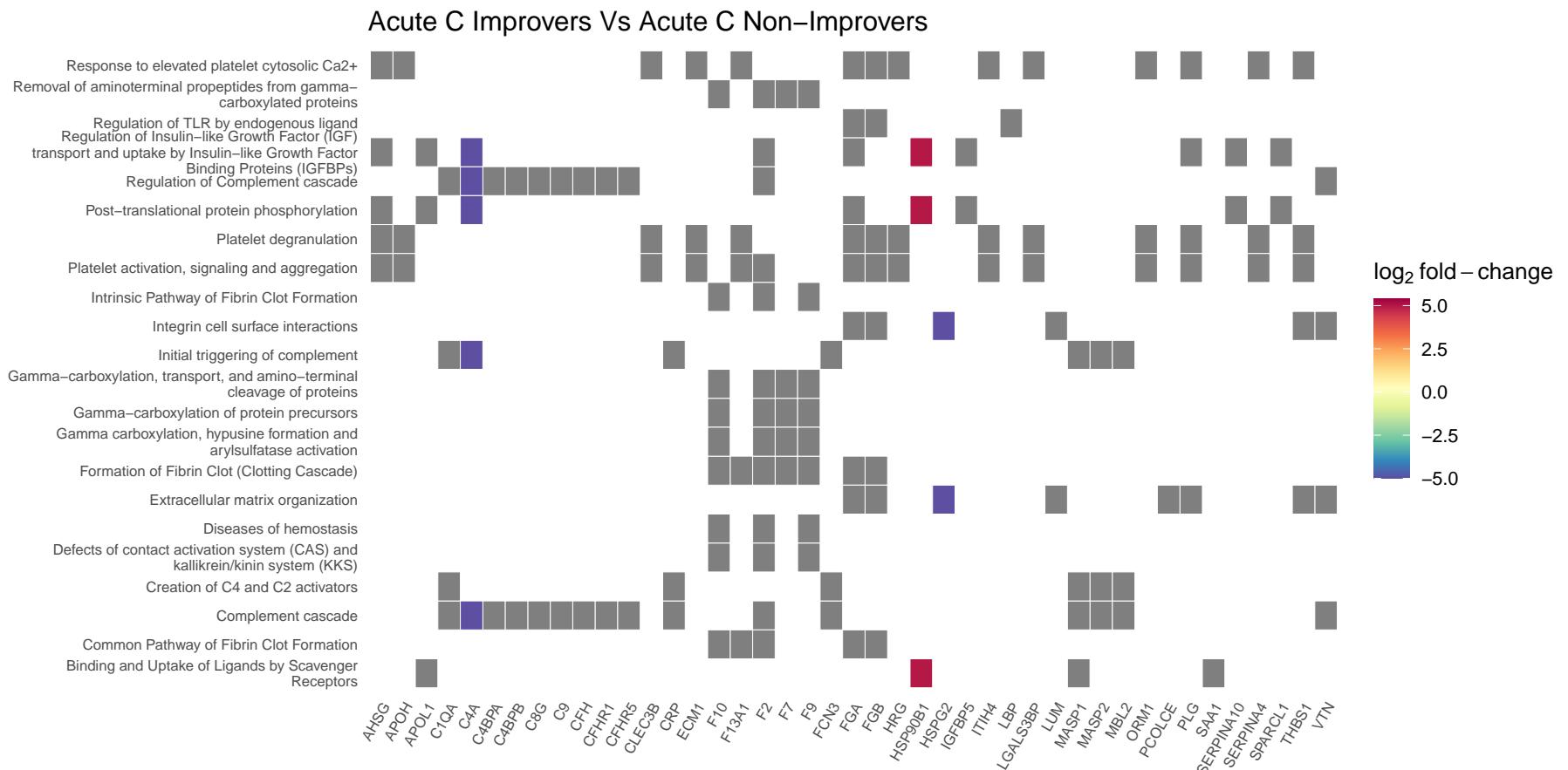


Figure 3. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins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

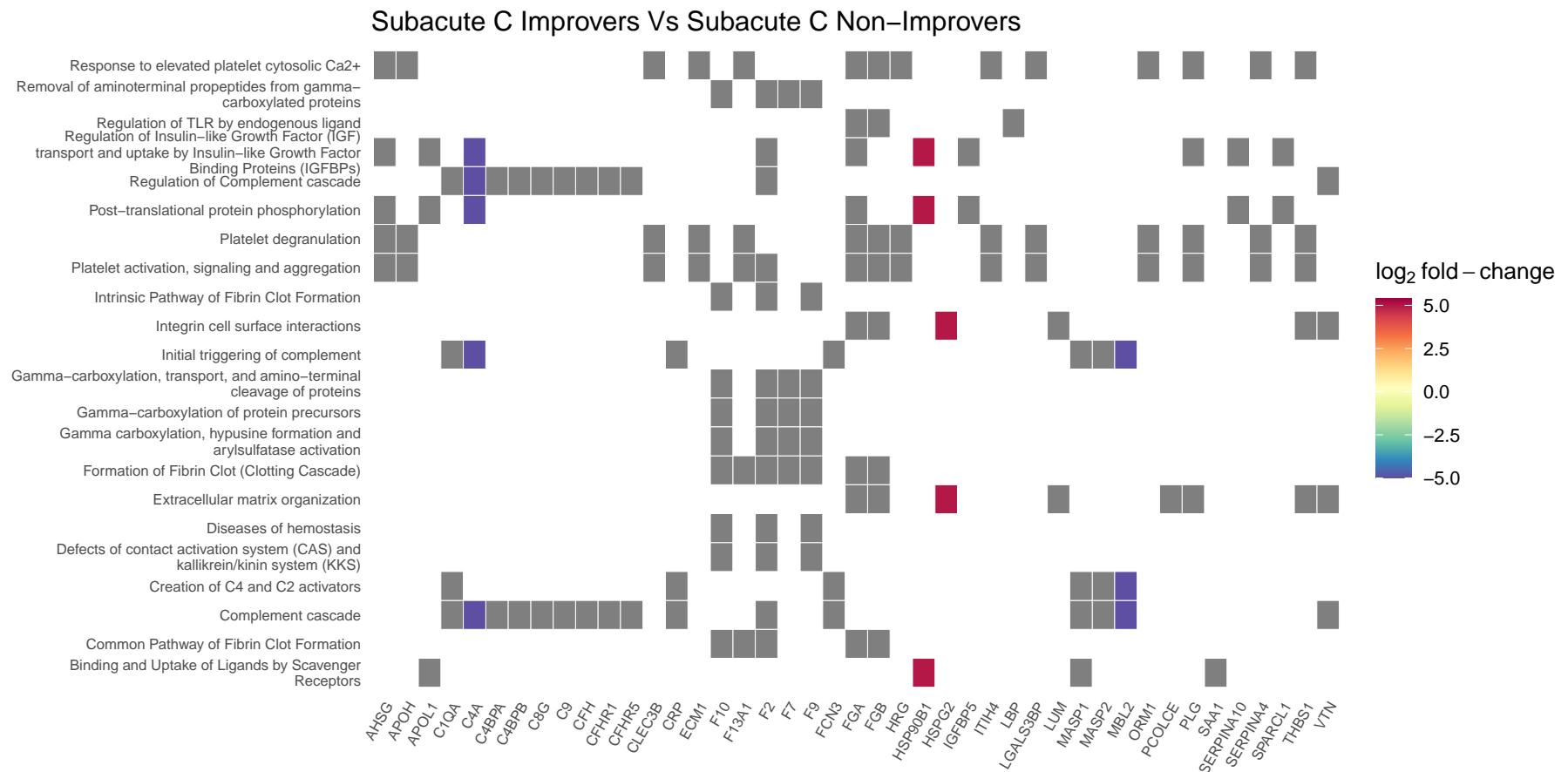


Figure 4. Heatmap denoting the \log_2 fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins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

258 **4.1.5 Network analysis of Differentially Abundant Proteins between AIS C improvers and**
259 **non-improvers**

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

Acute AIS C Improvers VS non-Improvers

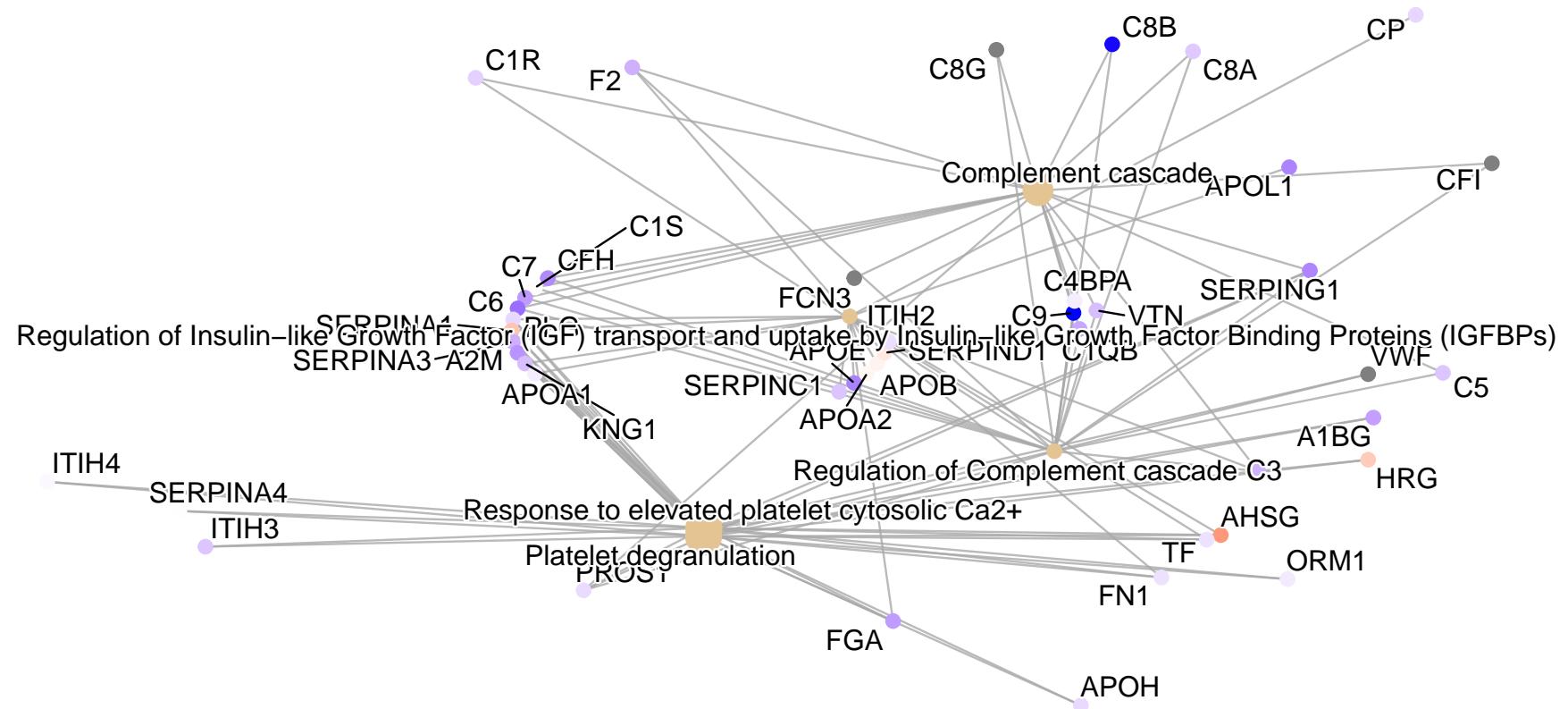


Figure 5. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute AIS C Improvers VS non-Improvers

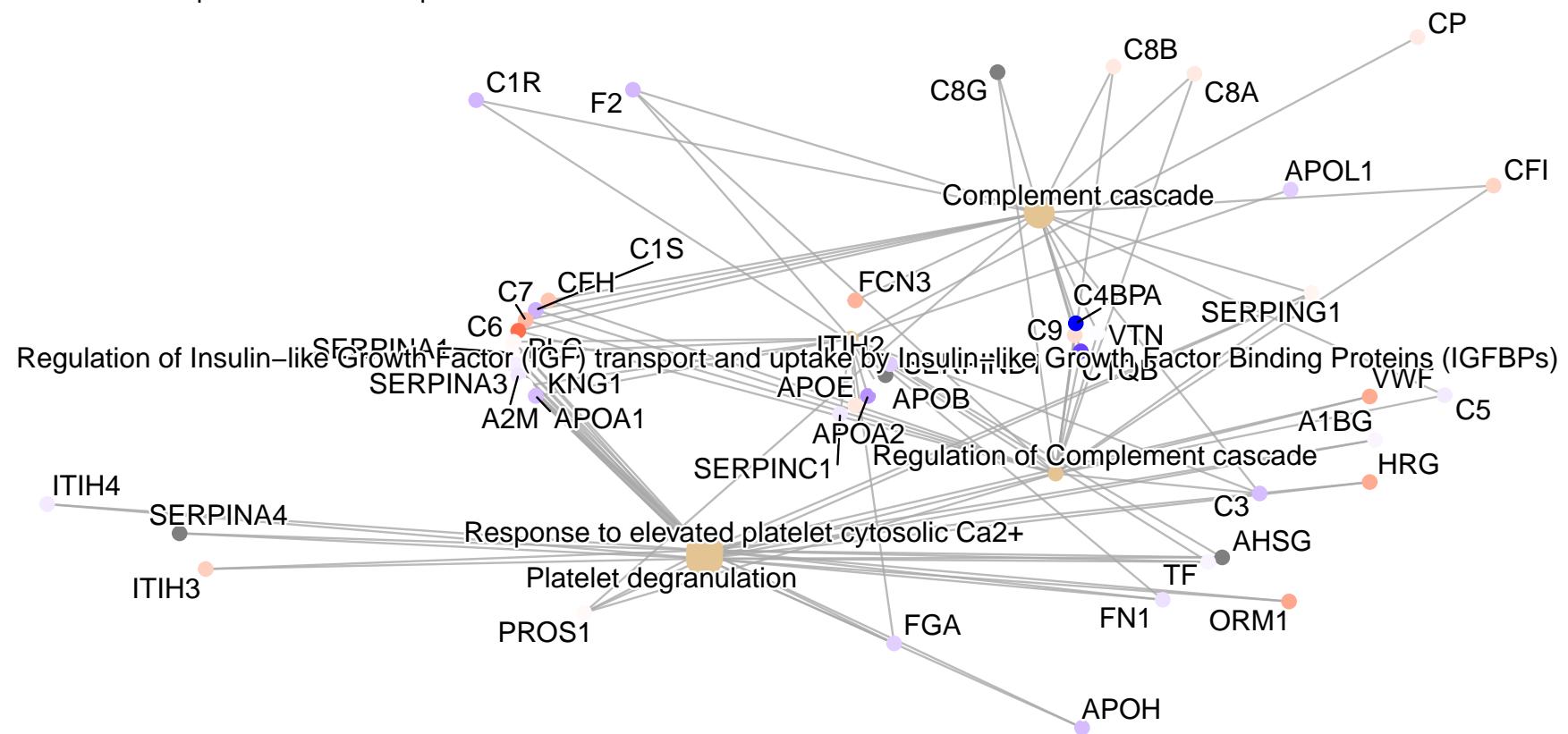


Figure 6. Network plot denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

- ²⁶⁴ Similarly to the heatmaps and the iTRAQ data, network plots derived using the label-free data
²⁶⁵ highlight the majority of differential proteins are associated with the complement cascade and
²⁶⁶ pathways linked to platelets (Figures 7, 8, S24, S25, S26, S27, S28, S29, S30).
- ²⁶⁷ Please see appendix section 5.7 for additional plots.

Acute C Improvers Vs Acute C Non-Improvers

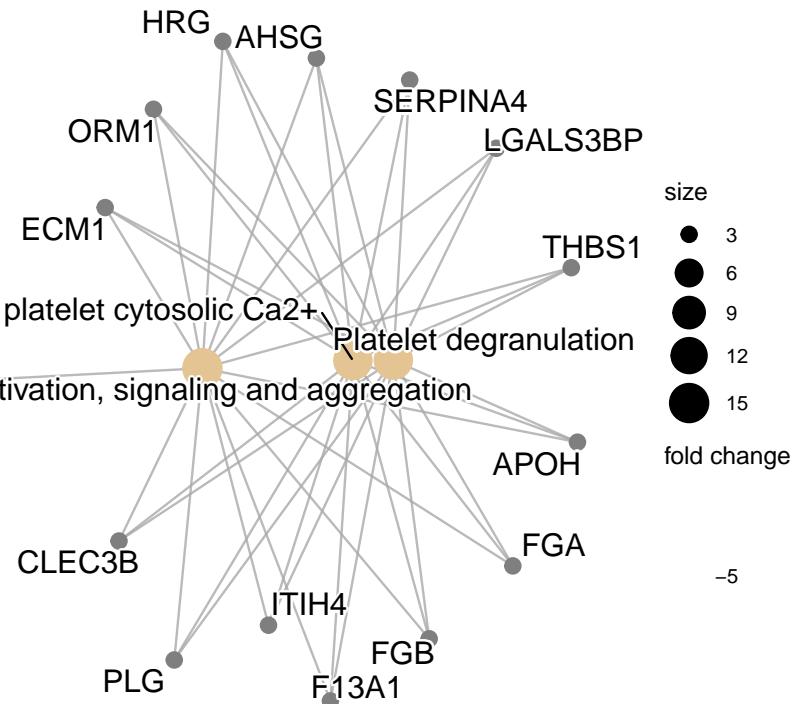
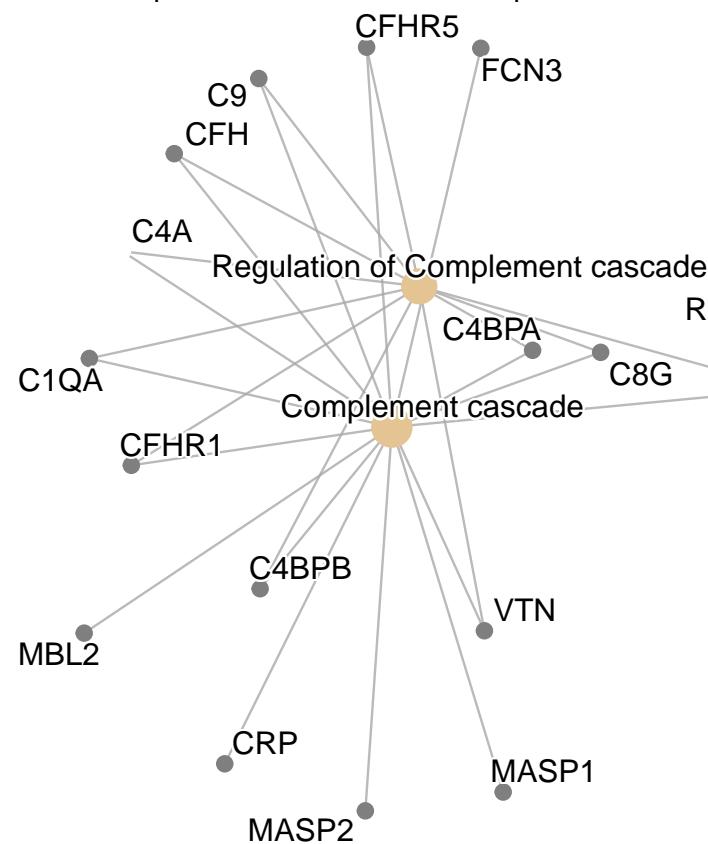


Figure 7. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute C Improvers Vs Subacute C Non-Improvers

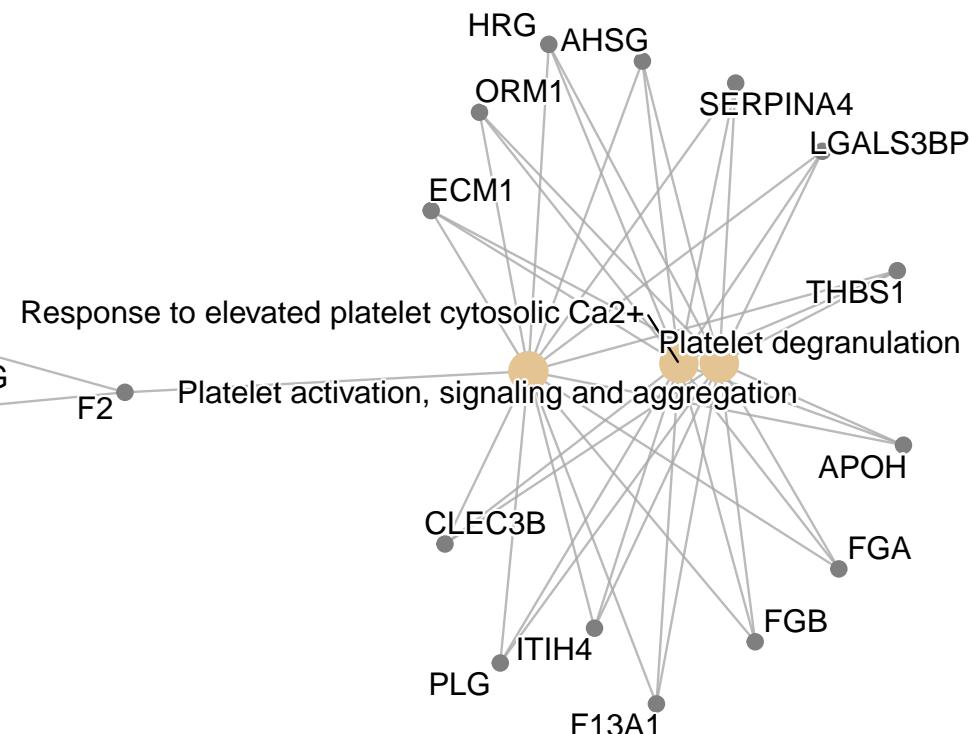
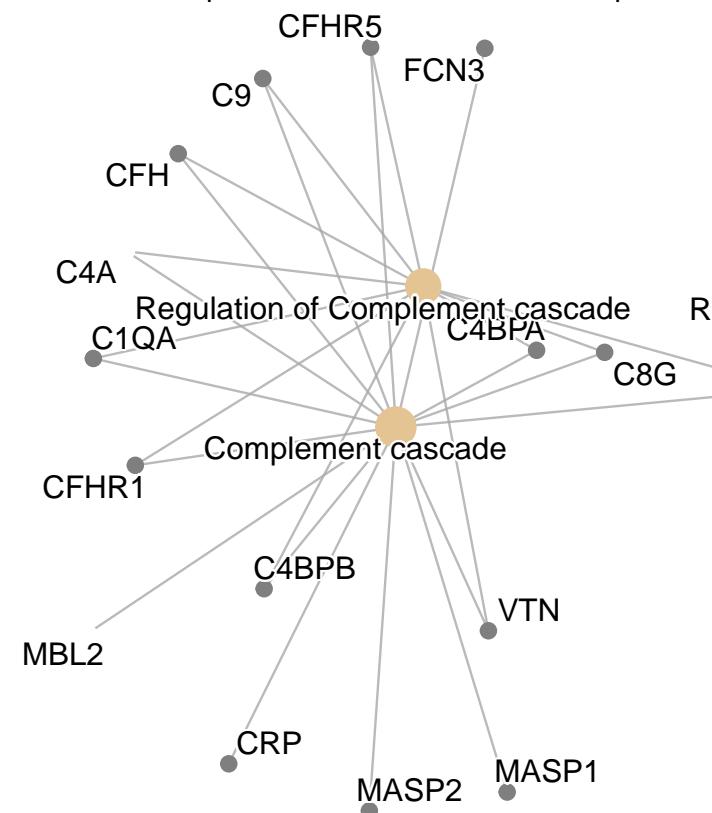


Figure 8. Network plot denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

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).

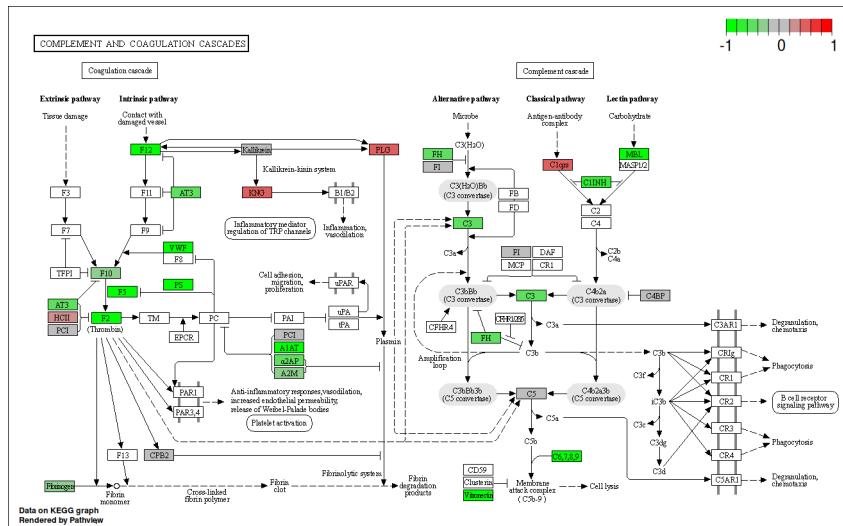


Figure 9. KEGG complement cascade pathway annotated with log₂ fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

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).

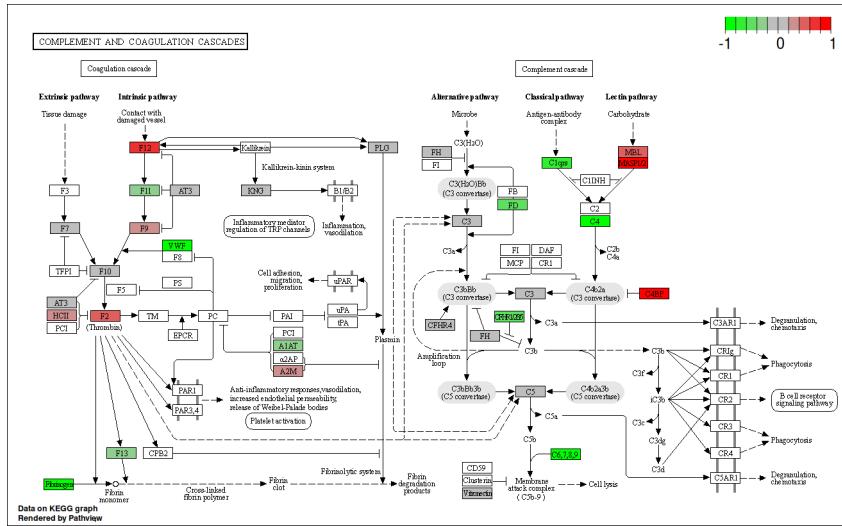


Figure 10. KEGG complement cascade pathway annotated with \log_2 fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

279 4.1.7 Validation of Proteomic Data using ELISA

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

283 A significant difference was found between AIS C non-improvers at 2-weeks and AIS D for SAA1,
 284 with outliers in AIS C non-improvers at 2-weeks, and both AIS C improvers and non-improvers at
 285 3-months post-injury (Figure 11). For ApoA1 plasma abundance estimated via Quantikine® ELISAs,
 286 statistically significant differences were found between AIS C improvers at 2-weeks and both AIS C
 287 improvers and non-improvers at 3-months, AIS C 3-month improvers and AIS A and D, and AIS C
 288 3-month non-improvers and AIS A and D (Figure 11). A statistically significant difference was also
 289 found between AIS C improvers and non-improvers at 2-weeks post-injury for RBP4 (Figure 11).

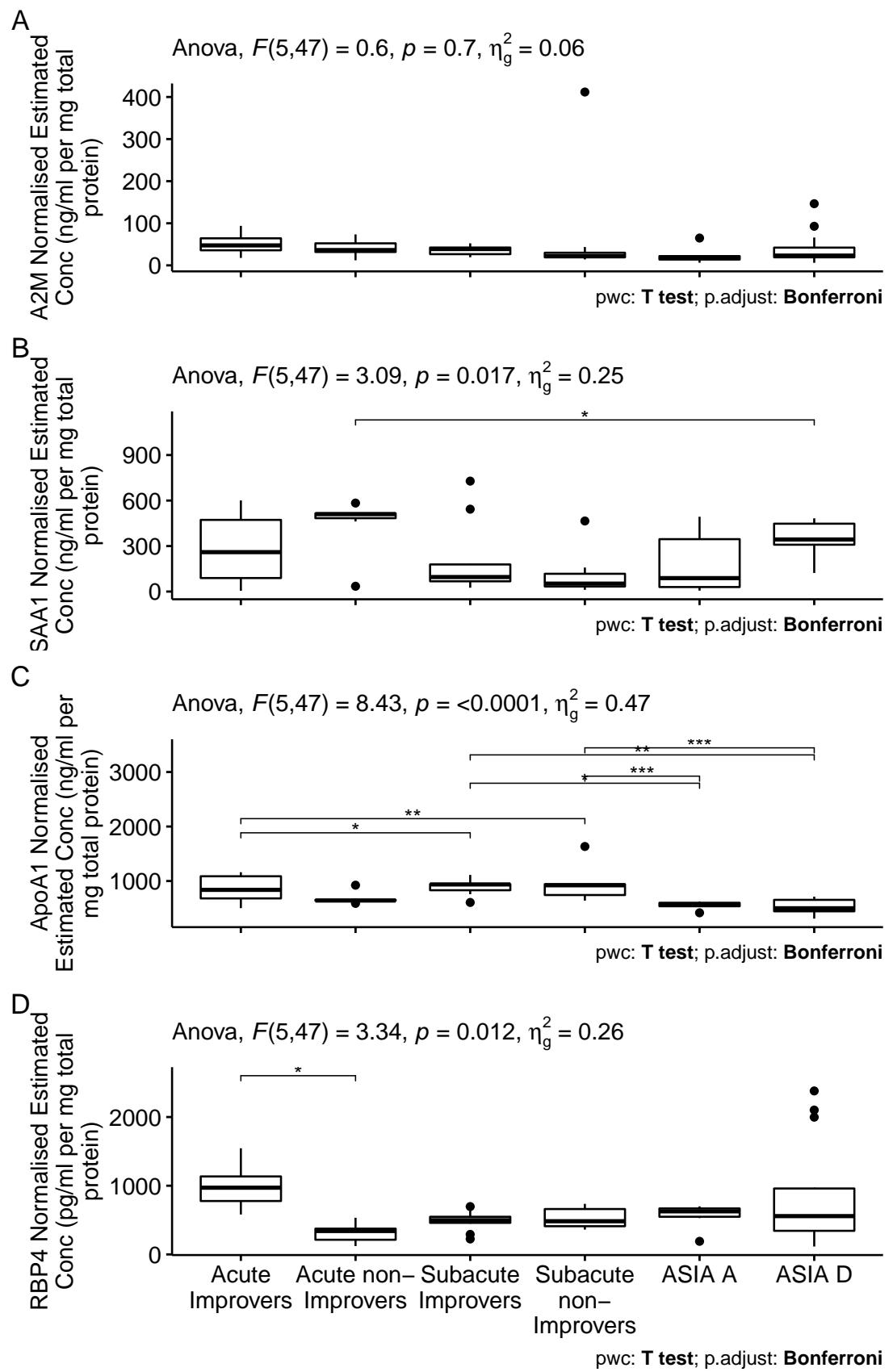


Figure 11. Normalised estimated concentration of α -2-macroglobulin (A), serum amyloid A1 (B), apolipoprotein A1 (C) and retinol binding protein 4 (D). Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled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

290 **4.1.8 STRINGdb plots**

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

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

297 **4.1.9 Volcano plots**

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

304 **4.1.10 Comparing iTRAQ and label-free proteins**

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

307 **5 Discussion**

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

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

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

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

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

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

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

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

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

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

380 An *in vitro* primary neurons and microglia co-culture study reported PRX-2 activating microglia via
381 TLR-4, potentially leading to neuronal apoptosis.(Y. Lu et al. 2018) A mouse study found over-
382 expression of PRX-2 attenuated oxidative stress and neuronal apoptosis following subarachnoid
383 haemorrhage.(Y. Lu et al. 2019) Over-expression of PRX-2 is speculated to protect again ischaemic
384 neuronal injury by modulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase
385 (ASK) 1 signalling complex.(Gan et al. 2012) Several molecular chaperones can interact with ASK1,
386 including thioredoxin and TNF receptor-associated factor 6.(Matsuzawa et al. 2005) The disso-
387 ciation of the thioredoxin-ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free
388 radicals, whereupon its antioxidant activity is reduced. This inactivation can be reversed by the
389 thioredoxin-thioredoxin reductase system, whereby oxidised PRX-2 can regain its activity by re-
390 ducing thioredoxin, leading to the dissociation of the thioredoxin-ASK1 complex.(Rhee and Woo
391 2011) Additionally, oxidised PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding
392 domain on ASK1.(S. Y. Kim, Kim, and Lee 2008)

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

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

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

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

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

435 **5.1 THE FOLLOW IS COPIED FROM THESIS**

436 **5.2 iTRAQ discussion**

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

441 **5.2.1 ProteinPilot and OpenMS**

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

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

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

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

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

473 **5.2.2 Proteins identified**

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

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

485 Alpha macroglobulins are an integral part of innate immunity and thus are evolutionarily con-
486 served.(Buresova et al. 2009) Alpha macroglobulins have significant primary sequence homology
487 with complement components C3, C4 and C5. The A2M-proteinase complex is cleared from circu-
488 lation primarily by receptors on hepatocytes.(Bond, Cianciolo, and Pizzo 2007; Travis and Salvesen
489 1983) The mammalian receptor for proteinase-reacted A2M is a low-density lipoprotein receptor
490 related protein.(Fujiyoshi et al. 2011; Larios and Marzolo 2012; Wyatt and Wilson 2013)

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

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

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

517 **5.2.2.2 Apolipoproteins** We found ApoA1, ApoA2, ApoH, ApoL1 and ApoM to be less abundant
518 in AIC improvers at both time points, whereas ApoA4 was more abundant at both time points (Ta-

bles S1 and S2). ApoA1 is the main protein component of high-density lipoproteins (HDL). Plasma HDL include two main apolipoproteins, these being ApoA1 and ApoA2 (~70% and ~20% of total HDL protein content respectively), but some HDL particles can also contain small amounts of other apolipoproteins, including ApoA4, ApoA5, ApoC, ApoD, ApoE, ApoJ and ApoL. The primary function of HDL in plasma is the transport of cholesterol, which can have dietary origins, but also be produced endogenously in the liver.

5.2.2.2.1 HDL Activity HDLs have serve a wide range of functions, including contributing to anti-inflammatory activity. They can limit chemokine secretion from multiple cells types including endothelial cells and monocytes.(Cockerill Gillian W. et al. 1995; Vorst et al. 2013; Bursill Christina A. et al. 2010) Rats injected with ApoA1 showed significant reduction in expression of CCR2 and CX₃CR1, the receptors for chemokines of the same name, which play a role in leukocyte migration. (Bursill Christina A. et al. 2010)

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

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

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

Prior *in vivo* rodent studies have demonstrated up-regulation of ApoE following SCI and TBI, though ApoE is not observed in neurons of rodents under normal neuropathology, and they only posses a single ApoE allele.(Iwata et al. 2005; Seitz et al. 2003; Mahley, Weisgraber, and Huang 2006) A separate rodent study reported ApoE levels decreased for the first 3 days post-injury, and then increased peak expression at 7 days post-injury, a similar pattern to our results.(X. Yang et al. 2018) Furthermore, mouse studies have demonstrated replacement of ApoE in neurons with human ApoE4 have impaired neurite outgrowth compared to replacement with ApoE2 or ApoE3, suggesting ApoE4 interferes with neuroplasticity.(Seitz et al. 2003; White et al. 2001) The underlying mech-

566 anism/s by which ApoE and its alleles effect neuroplasticity is not currently known, but proposals
567 have been made. One possibility is reduced lipid transport from astrocytes to neurons, potentially
568 impeding the membrane generation required to support axon growth or dendrite sprouting.
569 ApoE has anti-oxidant properties, so others have suggested impaired anti-oxidant activity may contribute.
570 ApoE4 has been found to be both secreted less than ApoE2 or ApoE3, and to have inferior
571 anti-oxidant abilities, lending some credence to this idea.(Mishra and Brinton 2018; Miyata and
572 Smith 1996) Knowing this, whilst ApoE may make for a useful biomarker for SCI, it will be important
573 that particular variants of ApoE a given patient has could be just as important, if not more so,
574 than simple abundance.

575 **5.2.2.3 Serum Amyloid A1** SAA1 was less abundant in AIS C improvers at 2-weeks relative to
576 non-improvers, but more abundance in plasma at 3-months (Table S1. SAA1 was also more abundant
577 in AIS A relative to less severe injuries, and in AIS Cs relative to Ds (Table S1. SAA1 is a major
578 acute-phase protein mainly produced in the liver by hepatocytes in response to infection, tissue
579 injury and malignancy.(L. Sun and Ye 2016) SAA1 is a precursor of amyloid A (AA), the aberrant
580 deposition of which leads to inflammatory amyloidosis.(Tape et al. 1988) There are 5 known SAA1
581 variants, though currently, no indication of substantial functional differences have been identified.(J. Lu et al. 2014) However, some alleles have been linked to disease, including increased amyloidogenesis and tumour suppression.[van der Hilst et al. (2008); lung_saa1_2015]

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

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

603 Macrophages are a major source of SAA in inflammatory tissues, and elevated SAA production has
604 been observed in rheumatoid arthritis, Crohn's disease, Type 2 diabetes and atherosclerosis.(Marzi
605 et al. 2013; Dong et al. 2011; Vallon et al. 2001; C, F, and B 1997; Meek, Urieli-Shoval, and Benditt
606 1994) SAA binding to HDL was reported to increase affinity for macrophages whilst decreasing
607 affinity for hepatocytes.(R. Kisilevsky and Subrahmanyam 1992) This change is thought to favour
608 the removal of cholesterol from site of inflammation.(R. Kisilevsky 1991) SAA inhibits the binding of
609 the scavenger receptor SR-BI and cholesterol efflux is enhanced in a SR-BI-dependent manner.(Cai
610 et al. 2005; van der Westhuyzen et al. 2005) It has been suggested that the SR-BI-mediated re-
611 uptake of cholesterol underpins the role of SAA in cholesterol recycling during tissue repair, where
612 a great deal of cholesterol is required.(Robert Kisilevsky and Manley 2012)

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

623 **5.2.2.4 Retinol-binding protein 4 (RBP4)** In plasma within 2-weeks post-injury, RBP4 was less
624 abundant in AIS C improvers relative to AIS D and A, and more abundant in AIS C non-improvers
625 again, relative to AIS D and A (Table S1). Similarly, AIS A plasma had more RBP4 compared to AIS
626 D, and AIS C improvers were also more abundant in RBP4 compared to non-improvers at both
627 2-weeks and 3-months post-injury (Table S1).

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

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

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

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

657 **5.2.3 Metabolism and SCI**

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

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

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

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

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

699 **5.2.4 Microbiome & SCI**

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

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

715 5.2.5 Drivers of liver steatosis

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

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

736 5.2.6 Chronic liver inflammation in SCI

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

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

753 **5.2.7 Longitudinal metabolic health**

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

766 **5.2.8 Validation of results**

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

778 **5.2.9 Conclusion**

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

792 **5.3 thesis label-free discussion**

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

798 **5.3.1 Proteins identified**

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

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

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

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

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

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

853 The following hours and days post-injury are characterised by a substantive complement sys-
854 tem activation and sequential leukocyte migration from the periphery into the injured neural
855 parenchyma.(Brennan et al. 2015; S. L. Peterson and Anderson 2014; Qiao et al. 2006) Curiously,
856 though the breakdown of the BSCB would presumably allow unrestricted access of circulating
857 leukocytes into the injured cord segment, recruitment of these cells remains a highly controlled
858 process.(Beck et al. 2010; Brennan et al. 2019) A mouse study reported lymphocytes, which
859 account for approximately 80% of circulating leukocytes, only enter the cord in substantial
860 numbers at least several weeks to months post-injury.(Beck et al. 2010) Early infiltrate is instead
861 largely comprised of myeloid cells, predominantly neutrophils, which are a minority of circulating
862 cells but are the swiftest peripheral responders to SCI, with studies detecting them at the lesion
863 site within 4 hours of injury.(Wright et al. 2010) Neutrophil numbers have been reported to peak
864 at 1 day post-trauma, but also to remain at the site for a minimum of 42 days post-injury.(Okada
865 2016; Kigerl, McGaughy, and Popovich 2006)

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

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

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

891 Circulating inflammatory monocytes are also recruited during the first days post-trauma. Adoptive
892 transfer experiments have shown recruitment to pick up at approximately 3 days post-injury,
893 and peak at 7 days.(Blomster et al. 2013) Whilst monocyte turnover at the lesion appears to be
894 high, infiltrating monocyte-derived macrophages remain at the site of weeks to months post-
895 trauma.(Blomster et al. 2013; Shechter et al. 2009) Interestingly, the timing of monocyte recruit-
896 ment appears to be delayed relative to non-neurological tissue injury. For instance, monocytes
897 are reported to be rapidly recruited to the heart following a myocardial infarction, as early as 1 day
898 post-injury, and their numbers return to baseline by roughly 16 days post-injury.(Nahrendorf et al.
899 2007)

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

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

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

935 Neuro-inflammation is less understood at the chronic phase of SCI, as most studies focus on the
936 first hours and days post-injury. By this stage, the glial scar has established a well-defined border
937 between the lesion core and the healthy tissue flanking it.(Sofroniew and Vinters 2010) Infiltrating
938 immune cells are largely restricted to within the lesion itself, as opposed to the surrounding spared
939 tissue. B and T cells, macrophages and neutrophils have all been detected here many months post-
940 trauma.(Beck et al. 2010; Ankeny, Guan, and Popovich 2009; Prüss et al. 2011) The chronic phase is
941 also marked by substantial metabolic dysfunction, characterised by reduced lipid metabolites and
942 increased oxidative stress, in addition to elevated pro-inflammatory mediators.(Dulin et al. 2013)

943 There are fewer studies that attempt to elucidate the underlying mechanisms driving this non-
944 resolving inflammatory response in the chronic phase of SCI. One study suggested communication
945 with infiltrating monocytes suppresses chronic microglial activation and inflammation after
946 SCI.(Greenhalgh et al. 2018) Interruption of this communication was linked to worsened function
947 outcomes, implying the initial microglial response to trauma may be beneficial, their pro-
948 tracted activation can eventually become detrimental.(Bellver-Landete et al. 2019; Greenhalgh et
949 al. 2018) Furthermore, a rodent model study of chronic SCI, found use of the anti-inflammatory
950 drug licoferone, applied daily for 1 month at 8 months post-injury, observed some improvement
951 to metabolic functions, but no benefit to locomotor function.(Dulin et al. 2013) To summarise, un-
952 derstanding of persistent inflammation during the chronic phase of SCI is lacking, and particularly
953 complicated by the plateaus in locomotive recovery that typically occurs well before the chronic
954 SCI phase is reached. Thus, there is a need for further studies to uncover the role of the various
955 immune cell populations with respect to ongoing neurological dysfunction and pathology during
956 the chronic phase of SCI.

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

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

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

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

997 **5.3.1.1.2 Speculative mechanisms of action for IVIG in SCI** As IVIG is made from pooled anti-
998 bodies taken from thousands of donors, it includes a vast repertoire of antibodies specific against
999 millions of unique antigens, allowing for a diverse variety of effects in differing disease contexts.
1000 Whilst there is extensive research of IVIG and autoimmune disorders, such as Guillain-Barré syn-
1001 drome, the immune pathology found in the acute phase of CNS injury is not typically considered
1002 to be driven by autoimmune processes.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et
1003 al. 1998) There may be some overlap in therapeutic mechanism, but it seems more likely any
1004 benefits are conferred through modulation of the innate rather than adaptive immune responses.
1005 The potential mechanisms of IVIG can be split between those mediated via the IgG constant (Fc)
1006 fragment, which binds the Fc receptors, and the F(ab')₂ fragment, which governs antigen recogni-
1007 tion.(Schwab and Nimmerjahn 2013) In the context of neurological diseases, mechanisms related
1008 to F(ab')₂ are thought to potentially bind and therefore neutralise cell surface receptors, comple-
1009 ment, cytokines and autoantibodies. By contrast, Fc-dependent mechanisms are speculated to in-
1010 clude regulation of Fc receptor expression, saturation of the neonatal Fc receptor, block activation
1011 of Fc receptors, and modulate T cells.(Schwab and Nimmerjahn 2013; Lünemann, Nimmerjahn,
1012 and Dalakas 2015; Dalakas 2014) Furthermore, models of neurological injury suggest both F(ab')₂
1013 and Fc-dependent signalling cascades could be involved in the modulation of several chemokines
1014 and cytokines.(Dalakas 2014)

1015 Modulation via the variable F(ab')₂ region

1016 Self-reactive antibodies have been found circulating in both chronic rodent SCI models and hu-
1017 man patients 1 year post-injury.(Ankeny, Guan, and Popovich 2009; Hayes et al. 2002) Whilst some
1018 studies have suggested potential relevance of naturally occurring autoantibodies (germline en-
1019 coded and produced by B1 cells) in acute SCI, it remains unclear whether IVIG treatment may have
1020 any impact on them.(Palmers et al. 2016; Narang et al. 2017) The impact or lack thereof of IVIG on
1021 chronic phase SCI autoimmunity also remains to be seen.

1022 A separate potential F(ab')₂-dependent mechanism involves the neutralisation of the cell death
1023 mediator Fas (AKA CD95). Studies of Lyell's syndrome, a disorder whereby active Fas ligand binds
1024 Fas present on keratinocytes, inducing apoptosis, reported IVIG therapy completely inhibited Fas
1025 ligand-induced cell death both *in vitro* and in human patients.(Viard et al. 1998; Altnauer et al.
1026 2003) Importantly, IVIG blocked Fas, as opposed to Fas ligand, in these studies, as this result was
1027 only observed with cells pre-treated with IVIG. Incubation of IVIG with soluble Fas ligand did not
1028 attenuate cell death, implying IVIG contains antibodies specific to Fas.(Viard et al. 1998; Altnauer
1029 et al. 2003) This modulatory effect of the Fas-Fas ligand pathway may have relevance in SCI, as a
1030 study using knock-out mice lacking Fas showed a reduction in both apoptosis at the lesion site and

1031 glial scarring, and improved motor function post-SCI.(Sobrido-Cameán and Barreiro-Iglesias 2018;
1032 W. R. Yu and Fehlings 2011) Neurons and glial cells from post-mortem human patients were found
1033 to be more Fas- and Fas ligand-positive, but this was limited to the acute phase of SCI, and not
1034 observed chronically, suggesting this pathway is more significant immediately post-injury.(W. R. Yu
1035 and Fehlings 2011) Therefore, acute IVIG treatment could act by attenuating secondary cell death
1036 by blocking Fas, thus disrupting this pathway.

1037 Conversely, agonistic anti-Fas antibodies have also been reported within IVIG prepara-
1038 tions.(Altnauer et al. 2003) Whilst it remains unknown how these agents may act in SCI,
1039 one could postulate a benefit if they induce apoptosis in circulating leukocytes, which could
1040 otherwise do harm.(Schneider et al. 2017) Supporting this, papers have found reductions in poly-
1041 morphonuclear cell populations within the lesion at 1 day post-injury in rodent models.(Nguyen
1042 et al. 2012; Chio et al. 2019; Gok et al. 2009) However, IVIG-induced apoptosis has only been
1043 observed in human leukocytes, not in rodents, casting doubt on this idea.(Altnauer et al. 2003;
1044 Schneider et al. 2017) Alternatively, the reduced recruitment could be a result of IVIG regulating
1045 the expression of adhesion molecules or molecules involved in leukocyte trafficking. A feline
1046 ischaemia-reperfusion injury model study found IVIG to down-regulate expression of integrins
1047 on leukocyte cell surfaces, inhibiting adhesion and subsequent extravasation of the cells into the
1048 damaged site.(Gill et al. 2005) Again however, these findings are contradicted by an experimental
1049 stroke study where IVIG was found to increase leukocyte and platelet trafficking to the injury,
1050 leading to formation of aggregates within cerebral vasculature.(Lapointe et al. 2004)

1051 Finally, $F(ab')_2$ may act by complement scavenging. Both *in vitro* and *in vivo* studies have found the
1052 non-antigen-binding regions of $F(ab')_2$ can bind and neutralise the complement activation prod-
1053 ucts C3a and C5a, thus preventing complement-mediated tissue damage.(Milan Basta et al. 2003;
1054 M. Basta et al. 1989) Multiple studies utilising various models of CNS injury have reported IVIG
1055 attenuating complement.(Brennan et al. 2016; Arumugam et al. 2007) Specifically in SCI, IVIG was
1056 found to reduce levels of the complement activation products C3b and C5a within the damaged
1057 cord.(Brennan et al. 2016) Similarly, an experimental stroke study reported IVIG reducing C3b lev-
1058 els in the infarct area.(Arumugam et al. 2007) Interestingly, whilst this study found IgG able to
1059 bind mouse C3b, supporting the hypothetical neutralisation of complement activation products,
1060 they also found IVIG able to attenuate oxygen deprivation-induced production of C3 itself in pri-
1061 mary neuron cultures. This seems to suggest IVIG is able to scavenge both secreted complement
1062 activation products, and their local production.(Arumugam et al. 2007)

1063 Modulation via the constant Fc region

1064 With respect to the Fc region, this portion normally binds to $Fc\gamma$ receptors ($Fc\gamma$ Rs), which are
1065 present on most leukocytes and resident CNS cells. Many $Fc\gamma$ Rs act as activating receptors, such as
1066 inducing phagocytosis in response to opsonised targets, or as an inhibitory receptor that dampens
1067 effector cell responses.(Schwab and Nimmerjahn 2013) A given cell's response to an immunoglobu-
1068 lin isotype is determined by the combination of which $Fc\gamma$ Rs are expressed by said cell. Myeloid cell
1069 all express some combination of these activating $Fc\gamma$ Rs, as do some innate lymphoid cells which
1070 do not express more classical antigen receptors, such as natural killer cells, whereas T and B cells
1071 do not.(Perussia et al. 1989) The inhibitory $Fc\gamma$ RIIb receptor is also expressed on myeloid cells, in
1072 addition to B cells, but not natural killer cells or resting T cells.(Bruhns and Jönsson 2015) Whilst
1073 there is debate over the expression and function of $Fc\gamma$ Rs in neurons, *in vitro* work with neuronal
1074 cultures has detected mRNA for all $Fc\gamma$ Rs.(Thom et al. 2017) Astrocytes, microglia and oligoden-
1075 drocyte precursors have also been found to express $Fc\gamma$ R, and up-regulate them under some disease
1076 states.(Thom et al. 2017)

1077 Studies utilising just the Fc fragment have been found to be equally effective as normal IVIG in
1078 several non-neurological autoimmune diseases, including nephrotoxic nephritis, ITP and K/BxN

1079 arthritis models, suggesting Fc γ Rs play a key role in the mechanism of IVIG.(Samuelsson, Towers,
1080 and Ravetch 2001; I. K. Campbell et al. 2014; Kaneko et al. 2006) With respect to CNS injury, some
1081 evidence suggesting a role of Fc γ Rs comes from a mouse study with animals lacking the common
1082 γ -chain, and thus no functional Fc γ Rs, which were found to be protected from experimental stroke
1083 and SCI.(Ankeny, Guan, and Popovich 2009; Komine-Kobayashi et al. 2004)

1084 Within the context of antibody-mediated autoimmune disorders, high-does IVIG may saturate Fc
1085 receptor and reduce the half-life of pathogenic endogenous IgG.(Schwab and Nimmerjahn 2013)

1086 **5.3.1.1.3 Immunoglobulins** Several immunoglobulin components were identified here, includ-
1087 ing 3 λ variable precursors (3-19, 3-10 and 2-18), 3 heavy variable precursors (3-15, 1-69 and 1-24)
1088 and 2 heavy constant gamma regions (2 and 4). For the λ variable precursors, acute AIS C improvers
1089 the precursors 3-19 and 3-10 were detected, whereas 3-10 and 2-18 were detected in acute C non-
1090 improvers. That acute C non-improvers expressed the 2-18 precursor whilst the improvers did
1091 not, suggests potential as a biomarker of poorer functional outcomes. It is difficult to comment
1092 on the biological mechanisms that may be at play here from this data, but one could infer that
1093 it is indicative of either a more robust, or a more maladaptive, immune response to the trauma.
1094 Given that the injuries are of the same severity by AIS grade, the latter seems more likely, though
1095 again, further research is needed to highlight the precise nature of this difference. Interestingly,
1096 whilst the acute C improvers do not express precursor 2-18, both the subacute C improvers and
1097 non-improvers, and subacute As do, whereas acute or subacute Ds do not, seemingly implying this
1098 precursor is also indicative of more severe injury in the latter phases of SCI.

1099 In addition of acute C improvers, subacute As and acute Ds also express the 3-19 precursor, with
1100 subacute As possessing the greatest abundance. Again, this would seem to suggest this marker
1101 is indicative of positive outcomes or less severe injury in the acute phase, but may be more detri-
1102 mental in the latter phases. The final λ precursor, 3-10, is present in acute As, subacute As and
1103 both subacute C groups as well as the aforementioned acute C improvers. The curious absence
1104 of 3-10 in both AIS D groups and C non-improvers groups suggests the marker is implicated in a
1105 more beneficial response, but perhaps this is limited to more severe injuries.

1106 With respect to the immunoglobulin heavy variable precursors, 3-15 was present in all groups
1107 except acute As and acute C non-improvers, though there was insufficient power to confidently
1108 compare the fold change of groups expressing 3-15. Another heavy variable precursor, 1-69, was
1109 expressed in subacute As, both acute and subacute C improvers, and both acute and subacute
1110 Ds. The final heavy variable precursor, 1-24, was found in all groups except acute C improvers and
1111 non-improvers.

1112 For the two immunoglobulin heavy constant γ s, 4 was significant in acute C improvers and non-
1113 improvers, relative to subacute As, whereas γ 2 was only significant in acute C improvers relative to
1114 subacute Ds. Both acute C improvers and non-improvers had a lower abundance of γ 4 relative to
1115 subacute As (-2.2 and -2.7 respectively), whilst γ 2 had a -1.8 fold change between acute C improvers
1116 and subacute Ds.

1117 **5.3.2 Conclusion**

1118 Much like the iTRAQ experiments (5.2.9), the majority of proteins identified are functionally asso-
1119 ciated with the complement cascade. Unlike the iTRAQ however, many of the proteins were only
1120 detected in one group of the pairwise comparisons, suggesting greater suitability as biomarkers.
1121 PRX-2, a protein associated with oxidative stress, is of particular interest, both as a biomarker for
1122 improvement in acute AIS C patients, but also mechanistically in relation to functional recovery.
1123 Furthermore, several immunoglobulins were identified as differentially abundant, though further
1124 *in vitro/vivo* work is needed to elucidate the pathophysiological relevance of each precursor. The

¹¹²⁵ λ 2-18 and 3-10 precursors are of particular relevance to acute and subacute AIS C improvement
¹¹²⁶ respectively, and both are of interest longitudinally in AIS As, with 2-18 potentially being linked to
¹¹²⁷ severity of injury.

¹¹²⁸ The small number of statistically significant proteins speaks to the variability of human samples,
¹¹²⁹ and is likely exacerbated by the inconstant timing of sample collection relative to injury. Post-hoc
¹¹³⁰ power analysis of the data reveals that to identify a 2.5 fold change with an FDR of 0.5 and a power
¹¹³¹ of 0.9, 14 biological replicates would be needed, in contrast to the 7-11 replicates used across
¹¹³² groups here. Thus, a repeat of this experiment with a larger sample size will likely reveal many
¹¹³³ more proteins of potential interest. Furthermore, a metabolomic analysis with a similar sample
¹¹³⁴ size would greatly compliment this work, particularly with regards to investigating further links to
¹¹³⁵ the liver.

1136 **Supplementary material**

1137 **5.4 Session Information**

```
1138 ##          -
1139 ## platform      aarch64-apple-darwin20
1140 ## arch         aarch64
1141 ## os           darwin20
1142 ## system       aarch64, darwin20
1143 ## status
1144 ## major        4
1145 ## minor        1.3
1146 ## year         2022
1147 ## month        03
1148 ## day          10
1149 ## svn rev      81868
1150 ## language     R
1151 ## version.string R version 4.1.3 (2022-03-10)
1152 ## nickname     One Push-Up

1153 Packages Used

1154 package

1155 version

1156 date

1157 base

1158 4.1.3

1159 2022-03-18

1160 MSstats

1161 4.2.0

1162 2021-05-31

1163 STRINGdb

1164 2.6.5

1165 2020-01-10

1166 ReactomePA

1167 1.38.0

1168 2021-10-26

1169 rlang

1170 1.0.2

1171 2022-03-04

1172 bookdown

1173 0.26
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1175 lime
1176 0.5.2
1177 2021-02-24
1178 RColorBrewer
1179 1.1.3
1180 2022-04-03
1181 ggVennDiagram
1182 1.2.0
1183 2021-10-19
1184 DiagrammeR
1185 1.0.9
1186 2022-03-04
1187 lubridate
1188 1.8.0
1189 2021-10-03
1190 patchwork
1191 1.1.1
1192 2020-12-15
1193 cowplot
1194 1.1.1
1195 2020-12-15
1196 BiocManager
1197 1.30.18
1198 2022-05-18
1199 data.table
1200 1.14.2
1201 2021-09-23
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1203 0.6.1
1204 2021-05-14
1205 psych
1206 2.2.5
1207 2022-05-01
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1208 Hmisc
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1211 Formula
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1213 2020-10-16
1214 survival
1215 3.3.1
1216 2022-02-20
1217 lattice
1218 0.20.45
1219 2021-09-18
1220 bibtex
1221 0.4.2.3
1222 2020-09-19
1223 captioner
1224 2.2.3
1225 2015-07-15
1226 kableExtra
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1229 knitr
1230 1.39
1231 2022-04-26
1232 rmarkdown
1233 2.14
1234 2022-04-25
1235 magrittr
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1237 2022-03-29
1238 janitor
1239 2.1.0
1240 2021-01-04
1241 readxl
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1250 dplyr
1251  1.0.9
1252  2022-04-27
1253 purrr
1254  0.3.4
1255  2020-04-16
1256 readr
1257  2.1.2
1258  2022-01-30
1259 tidyverse
1260  1.2.0
1261  2022-01-27
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1263  3.1.7
1264  2022-04-26
1265 ggplot2
1266  3.3.6
1267  2022-04-27
1268 tidyverse
1269  1.3.1
1270  2021-04-15
```

1271 **5.5 Fold changes**

Table S1. OpenMS log₂ 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 log₂ 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 log₂ 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	-4.365158	-5.248075	NA	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

¹²⁷² **5.6 Heatmaps**

¹²⁷³ **5.6.1 iTRAQ data**

AIS C Improvers acute vs subacute

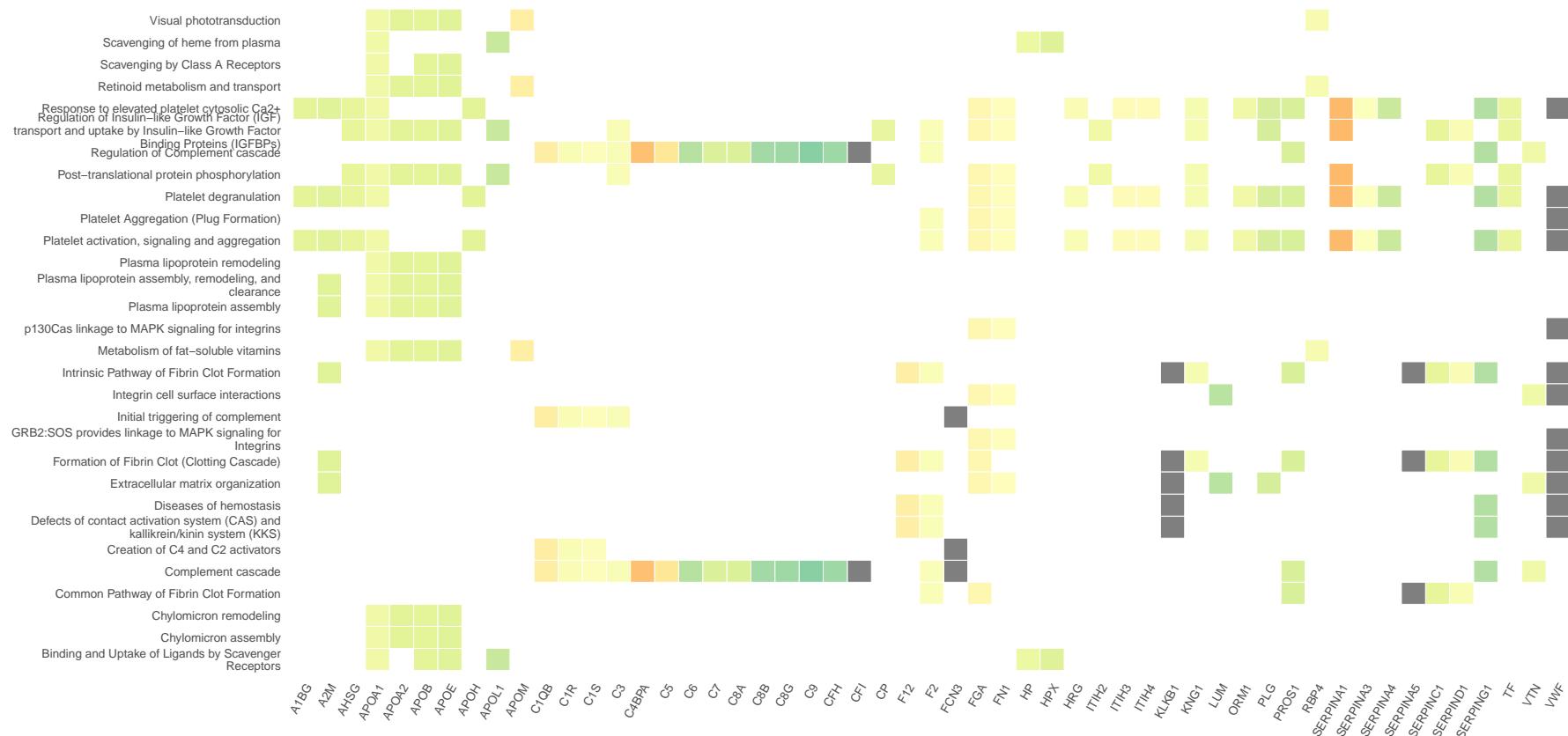


Figure S1. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

AIS C non-Improvers acute vs subacute

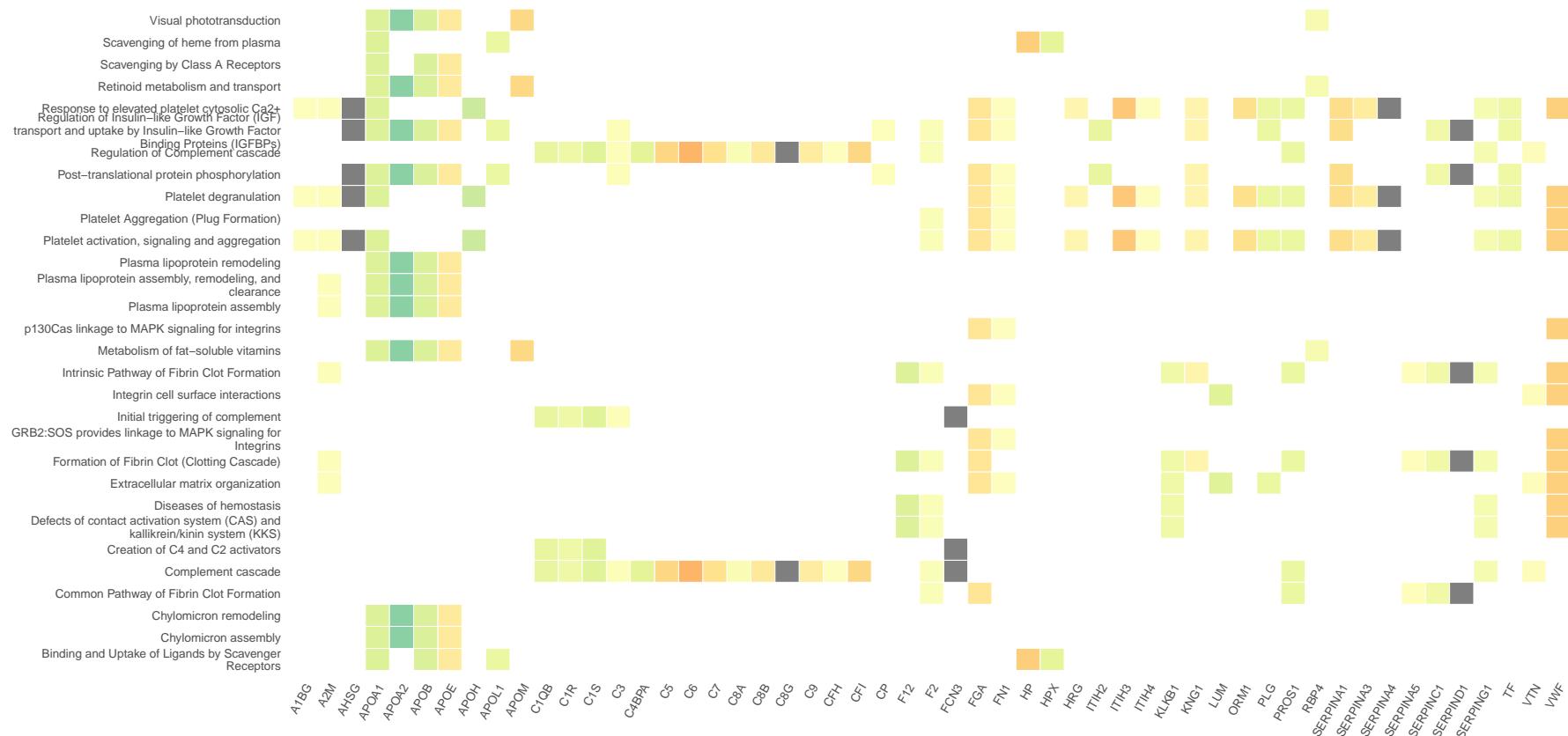


Figure S2. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute AIS C Improvers VS non-Improvers Run 2



Figure S3. Heatmap denoting the log₂ 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.

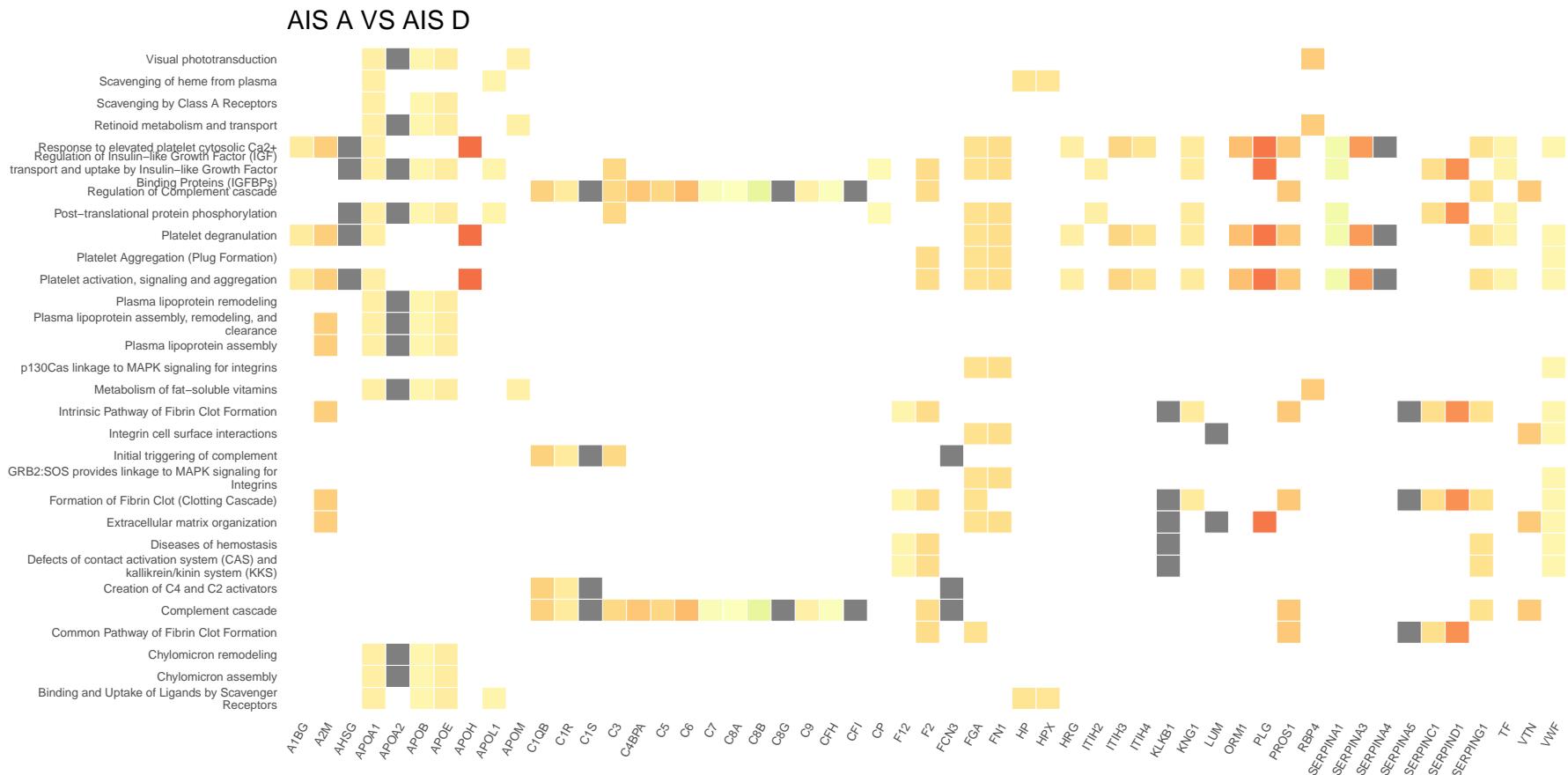


Figure S4. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Acute AIS C Improvers VS AIS D



Figure S5. Heatmap denoting the log₂ 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 log₂ 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

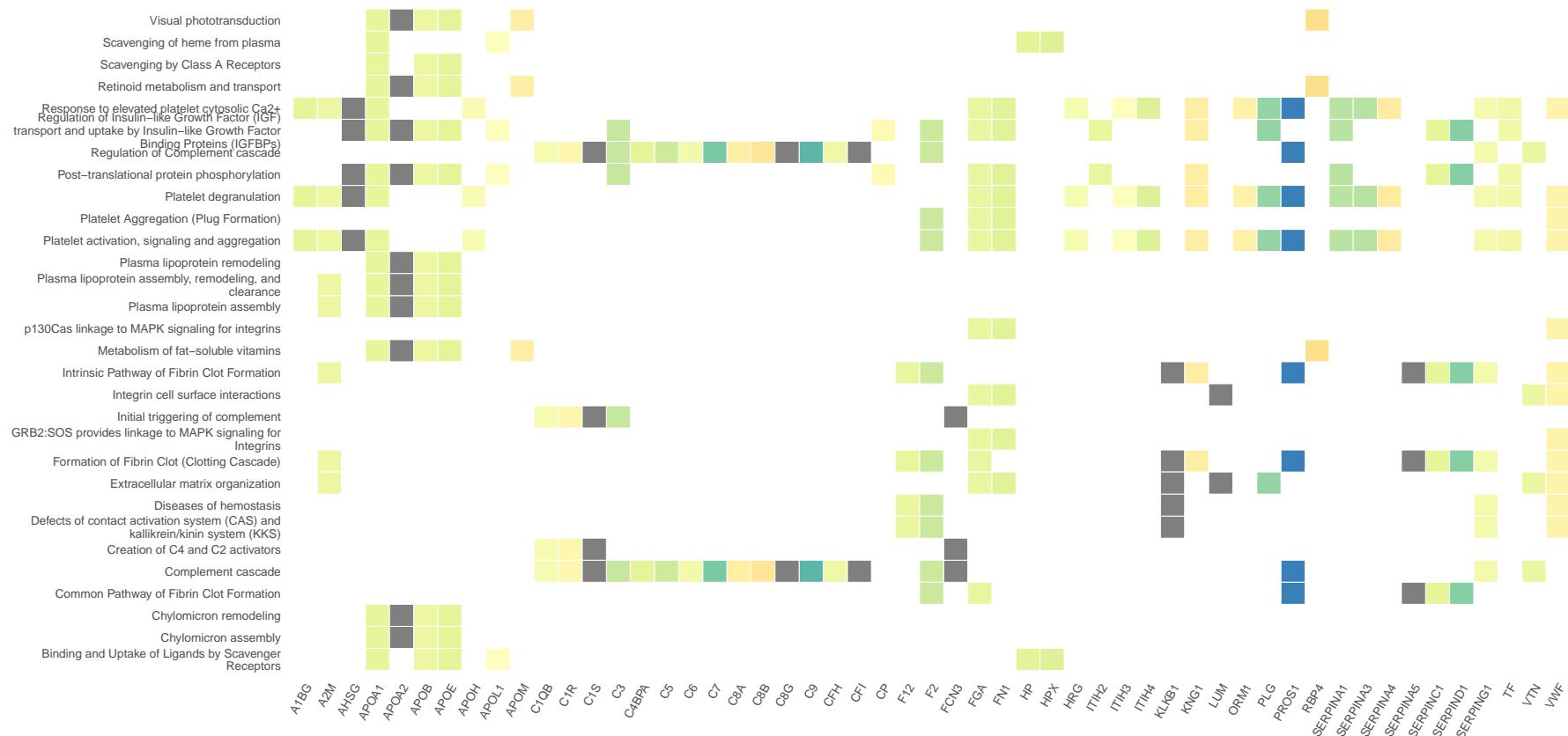


Figure S7. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D

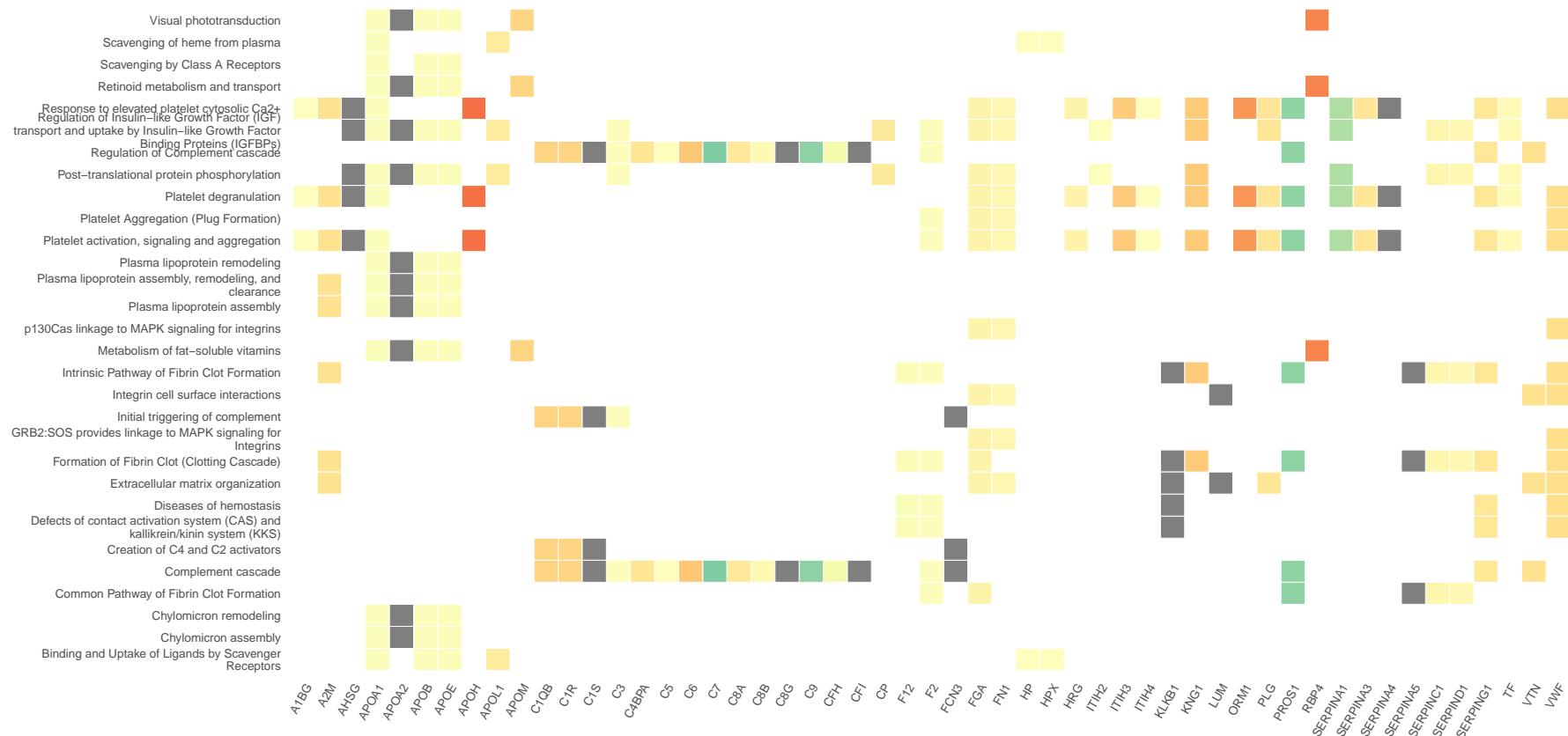


Figure S8. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

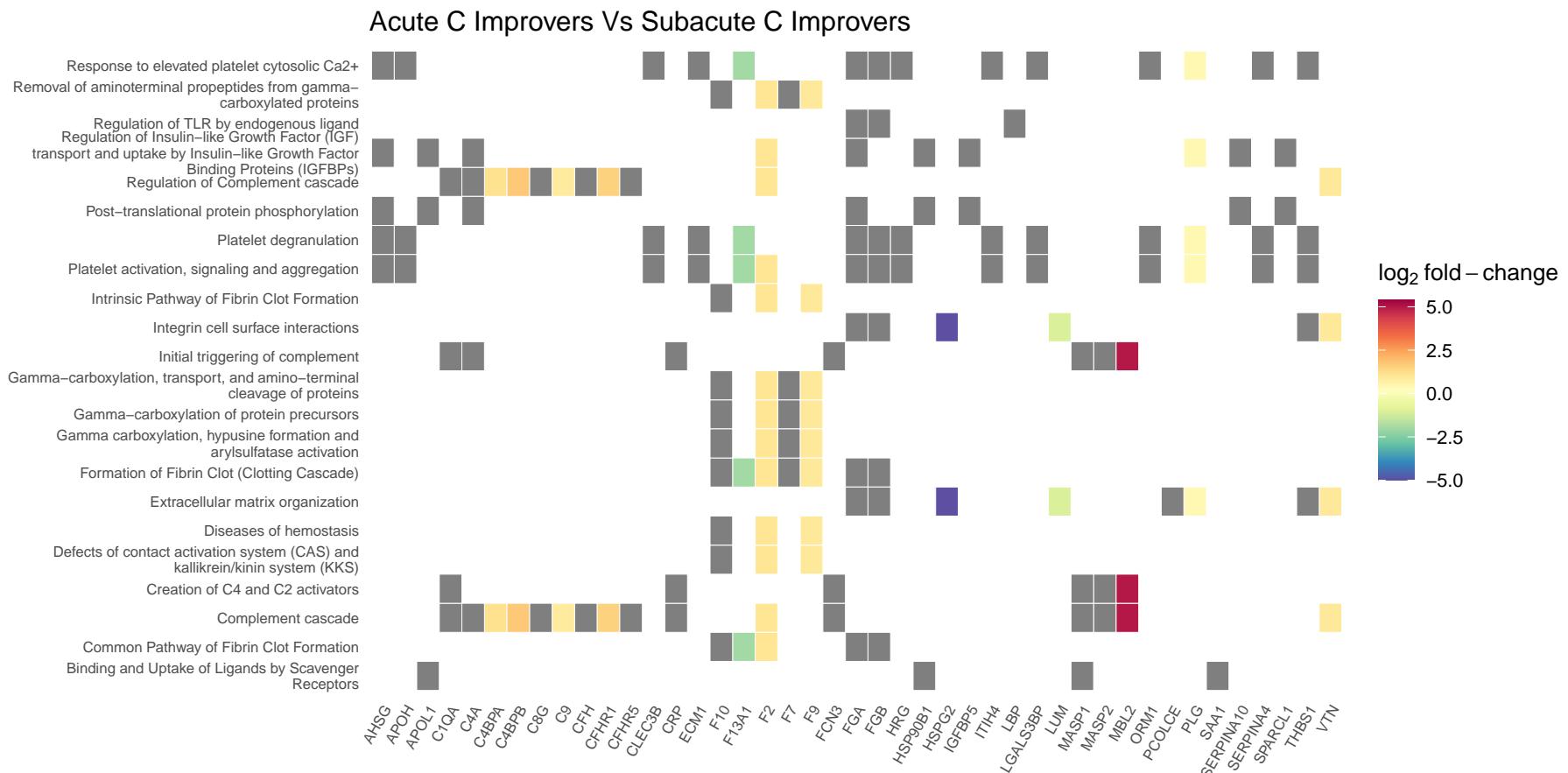


Figure S9. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.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

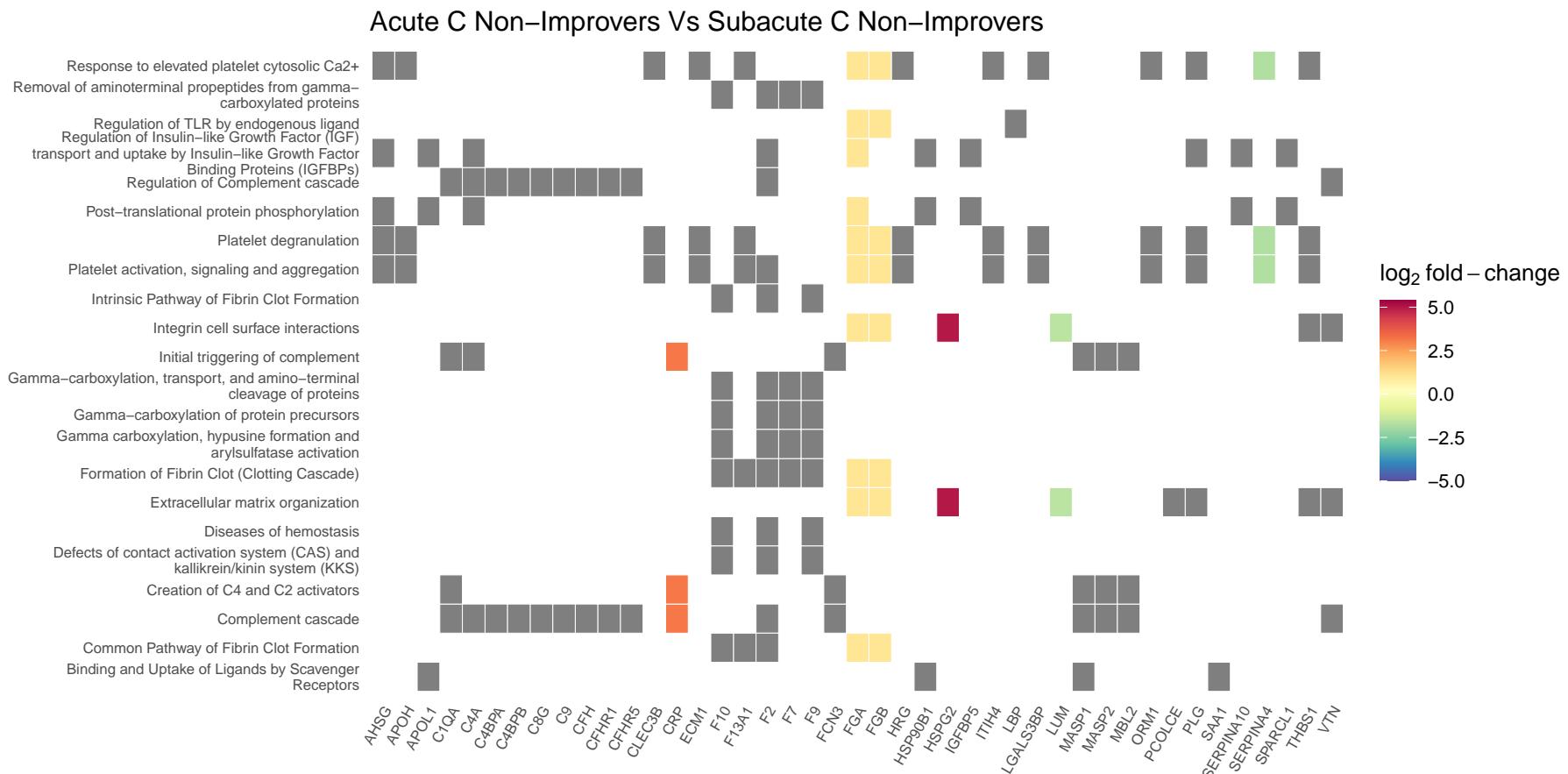


Figure S10. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.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

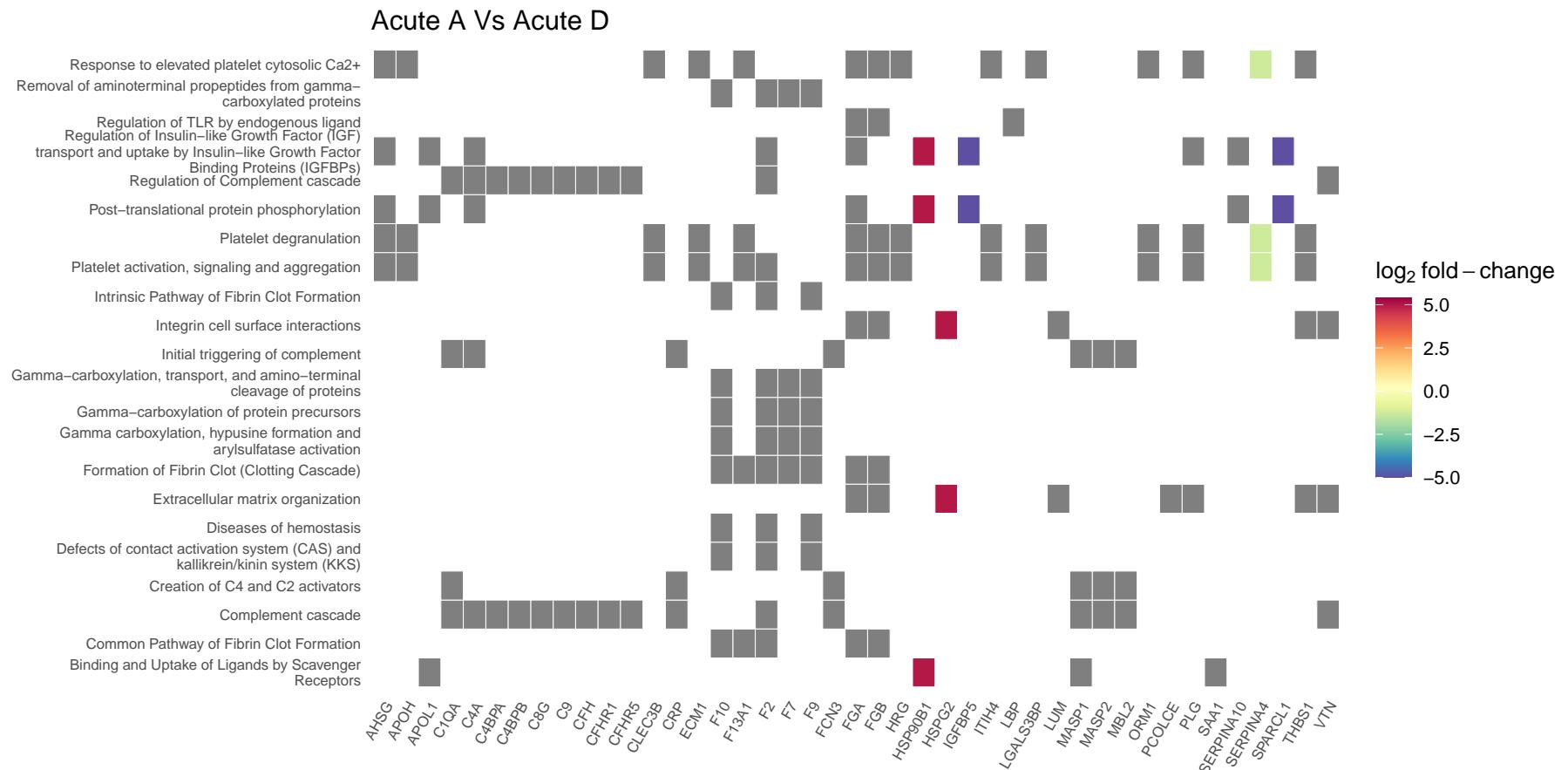


Figure S11. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients. Grey blocks denote proteins not present in the comparison.

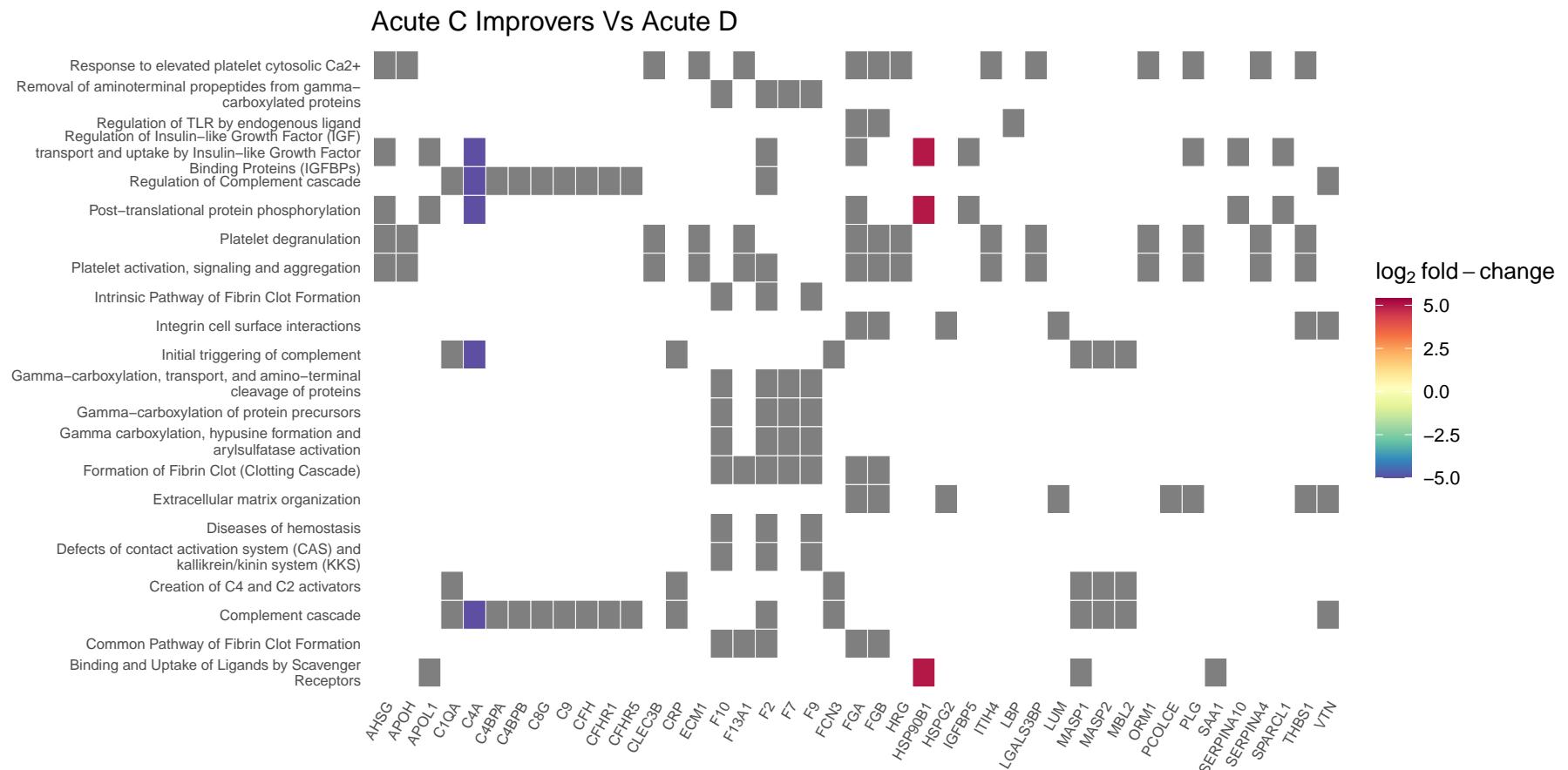


Figure S12. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients. Grey blocks denote proteins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

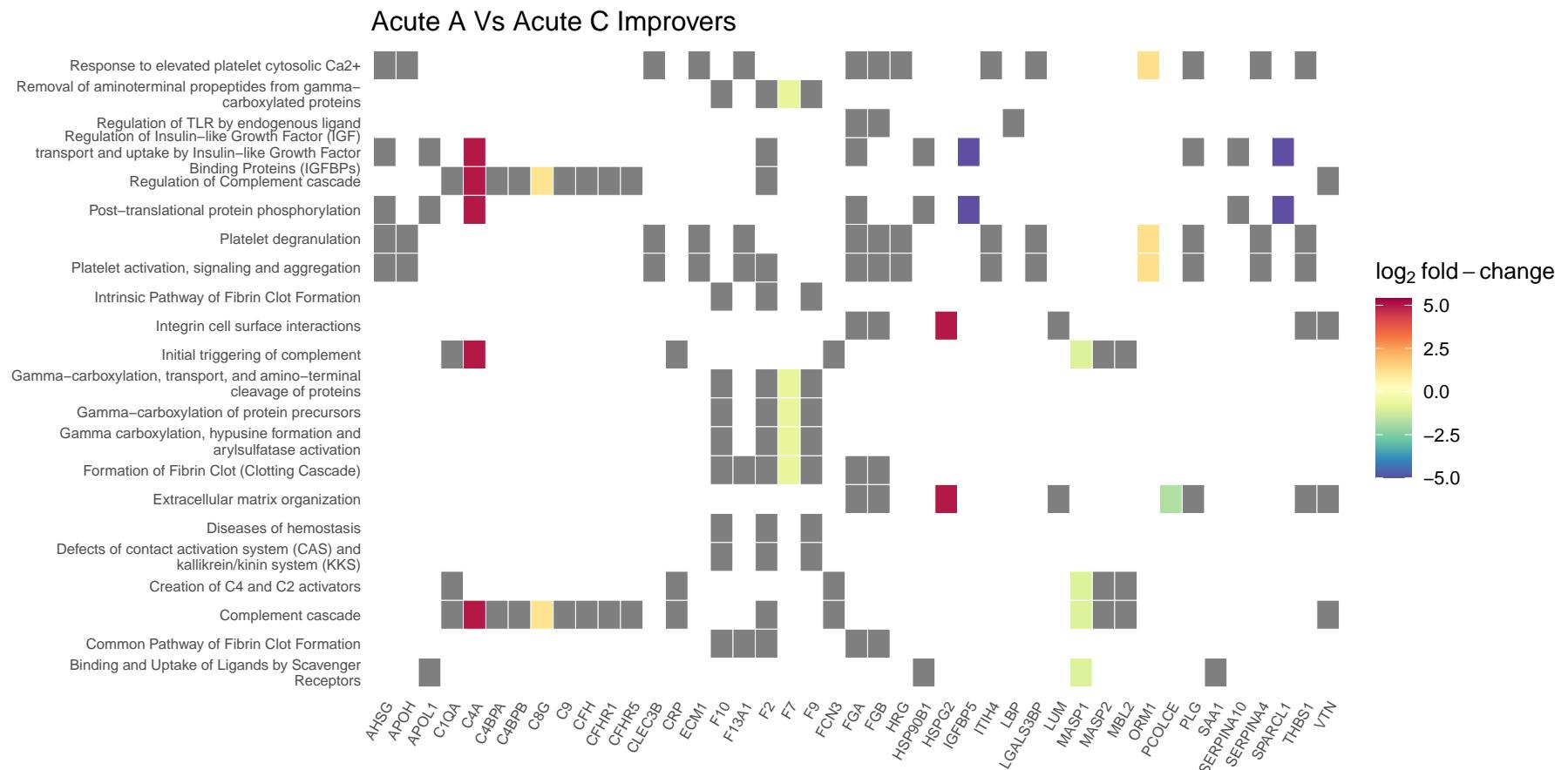


Figure S13. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients. Grey blocks denote proteins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

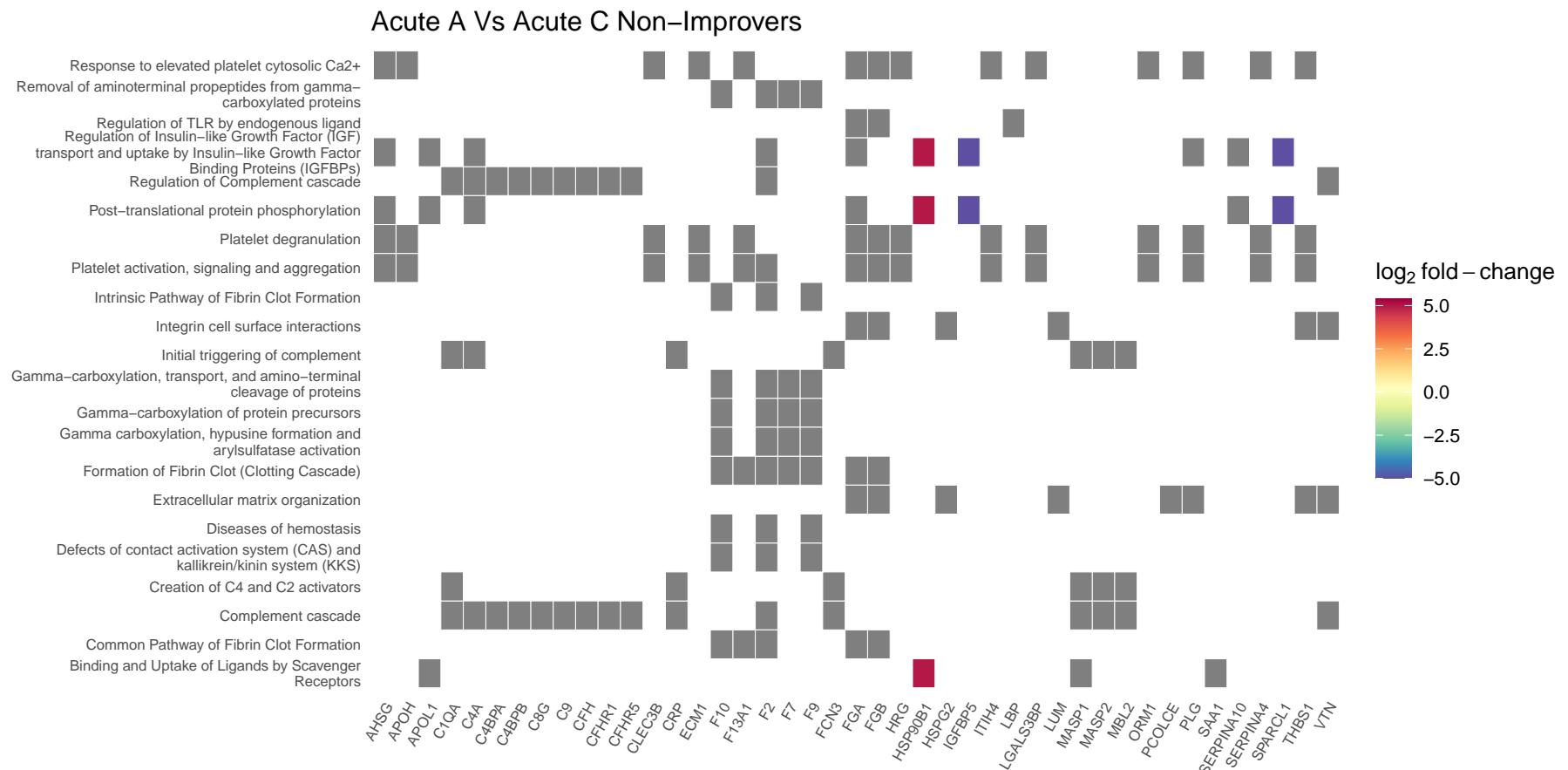


Figure S14. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients. Grey blocks denote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

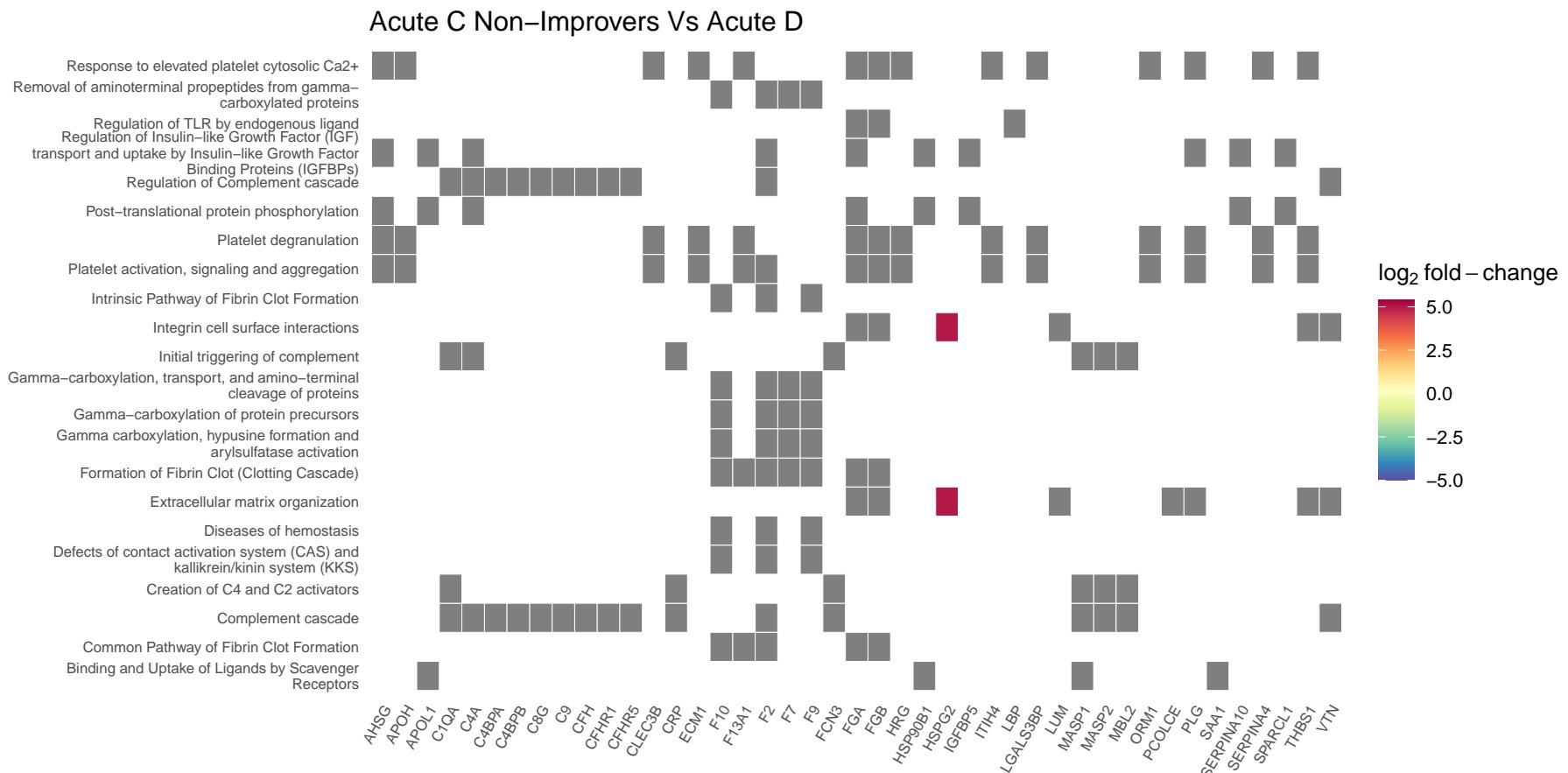


Figure S15. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients. Grey blocks denote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

1275 **5.7 Cnetplots**

1276 **5.7.1 iTRAQ data**

AIS C Improvers acute vs subacute

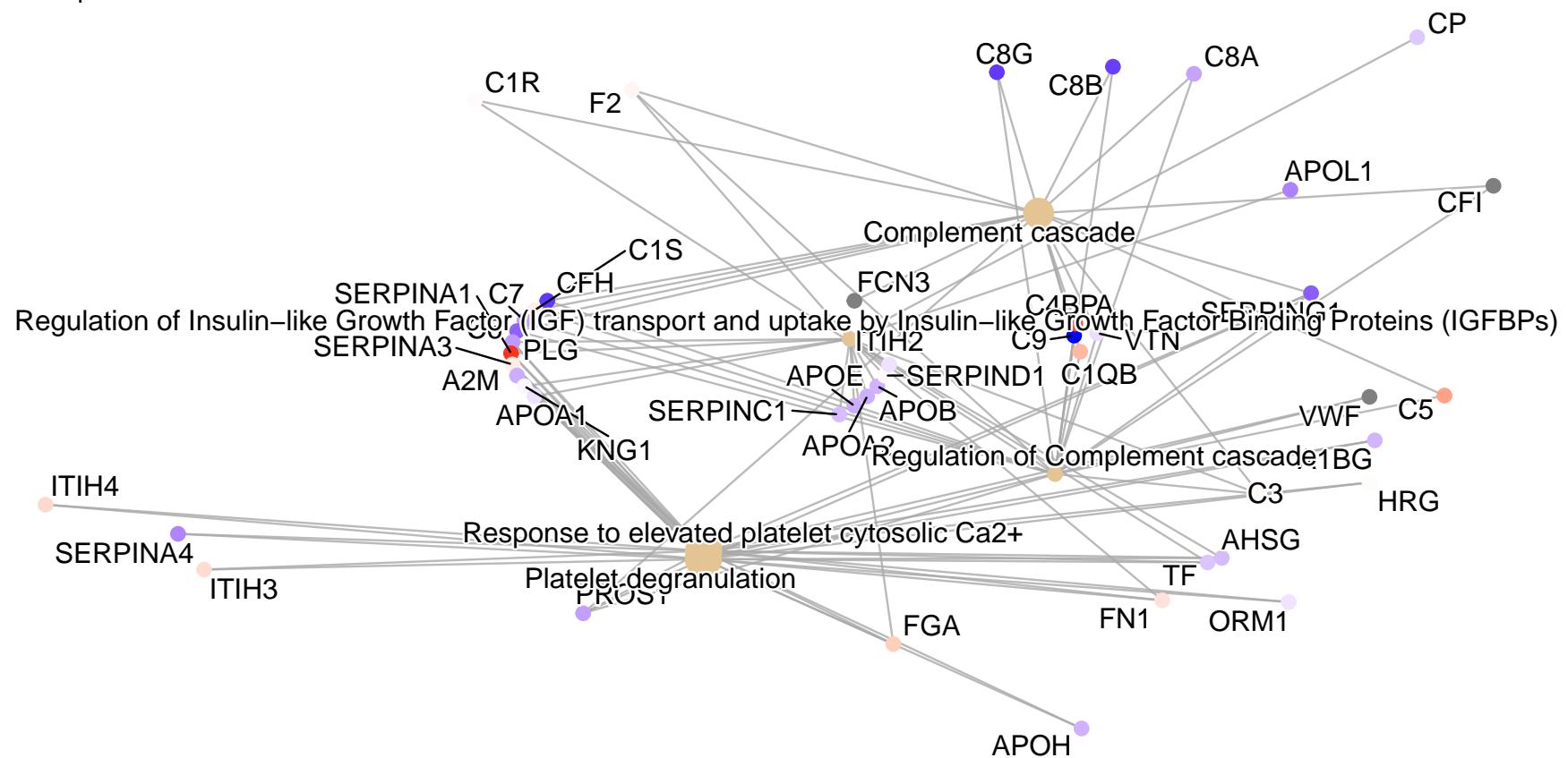


Figure S16. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

AIS C non-Improvers acute vs subacute

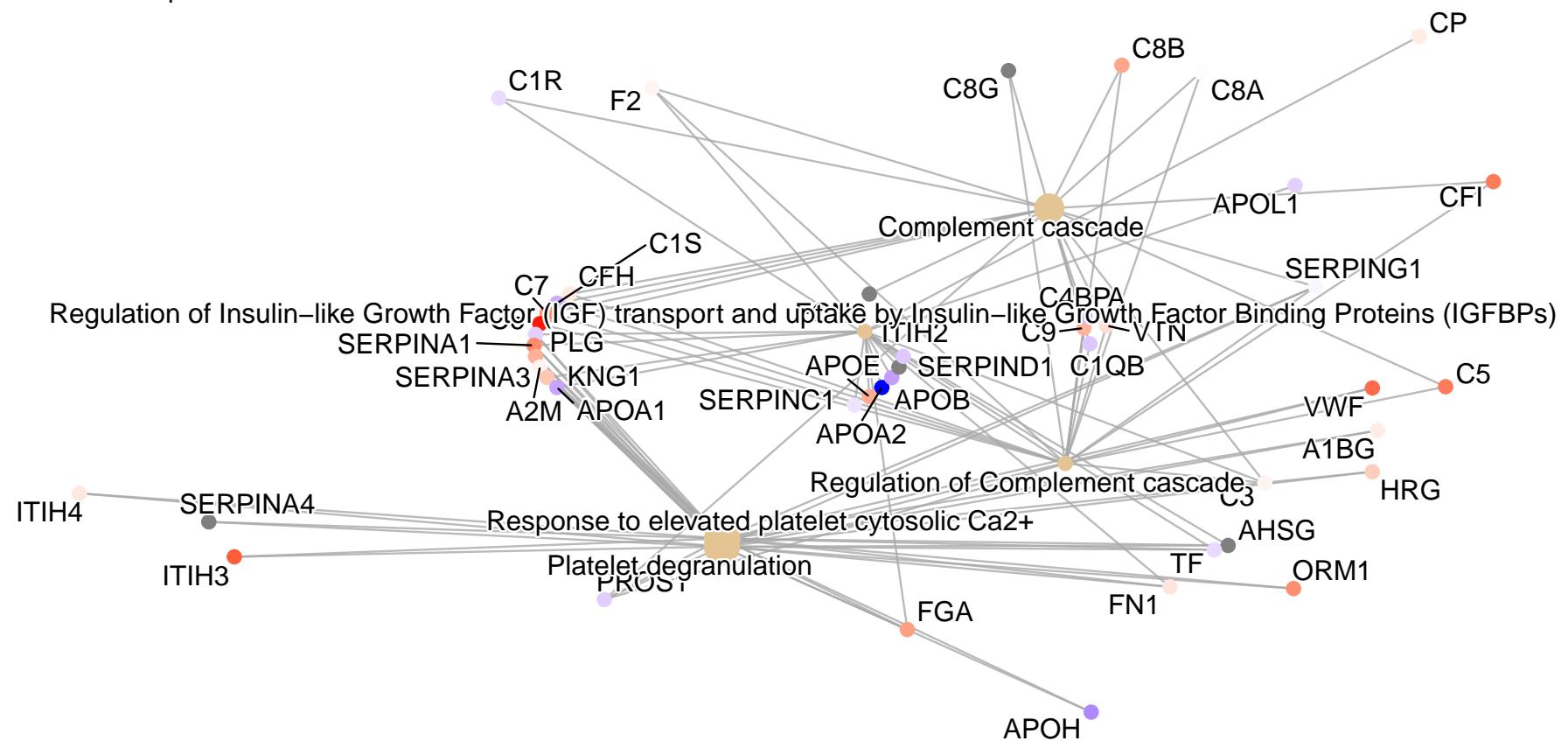


Figure S17. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months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

Acute AIS C Improvers VS non-Improvers Run 2

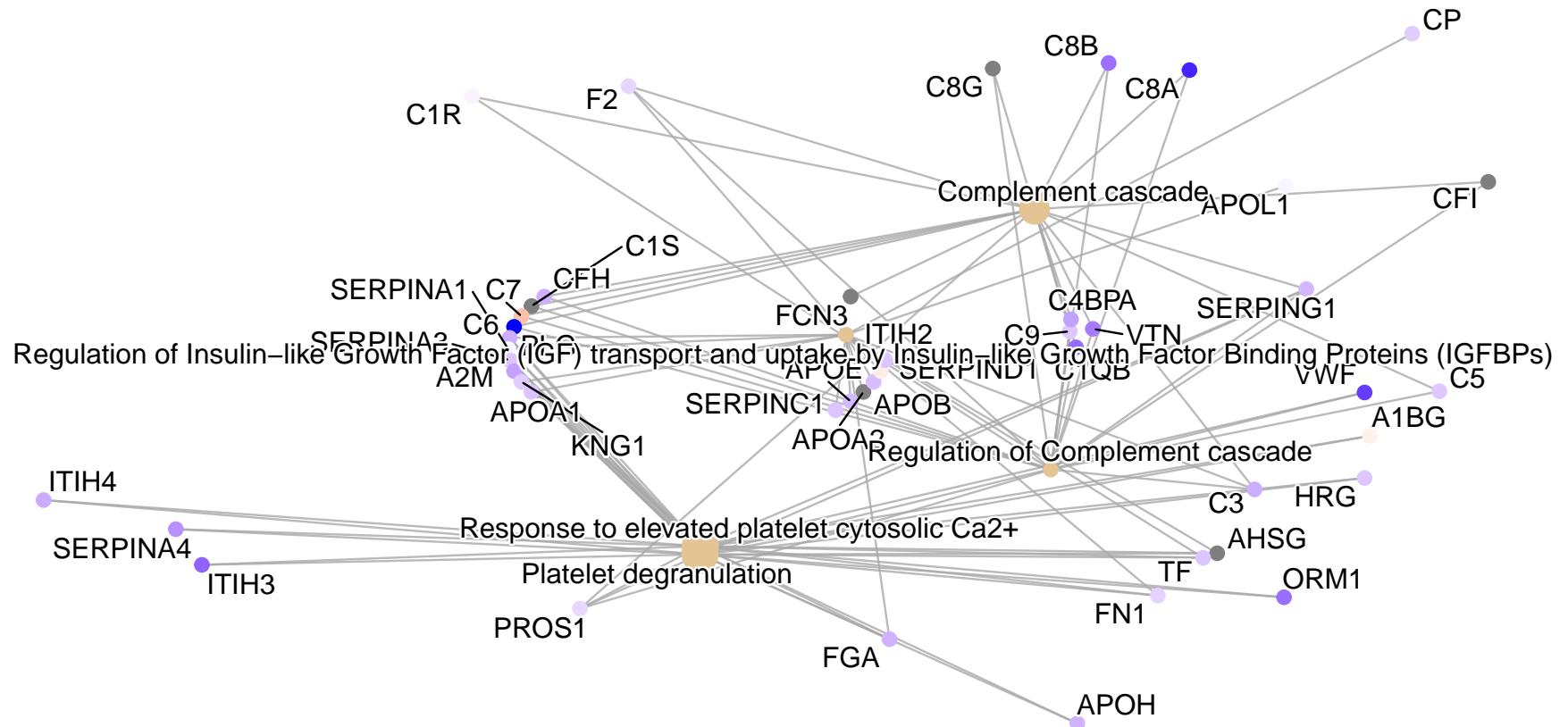


Figure S18. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.

AIS A VS AIS D

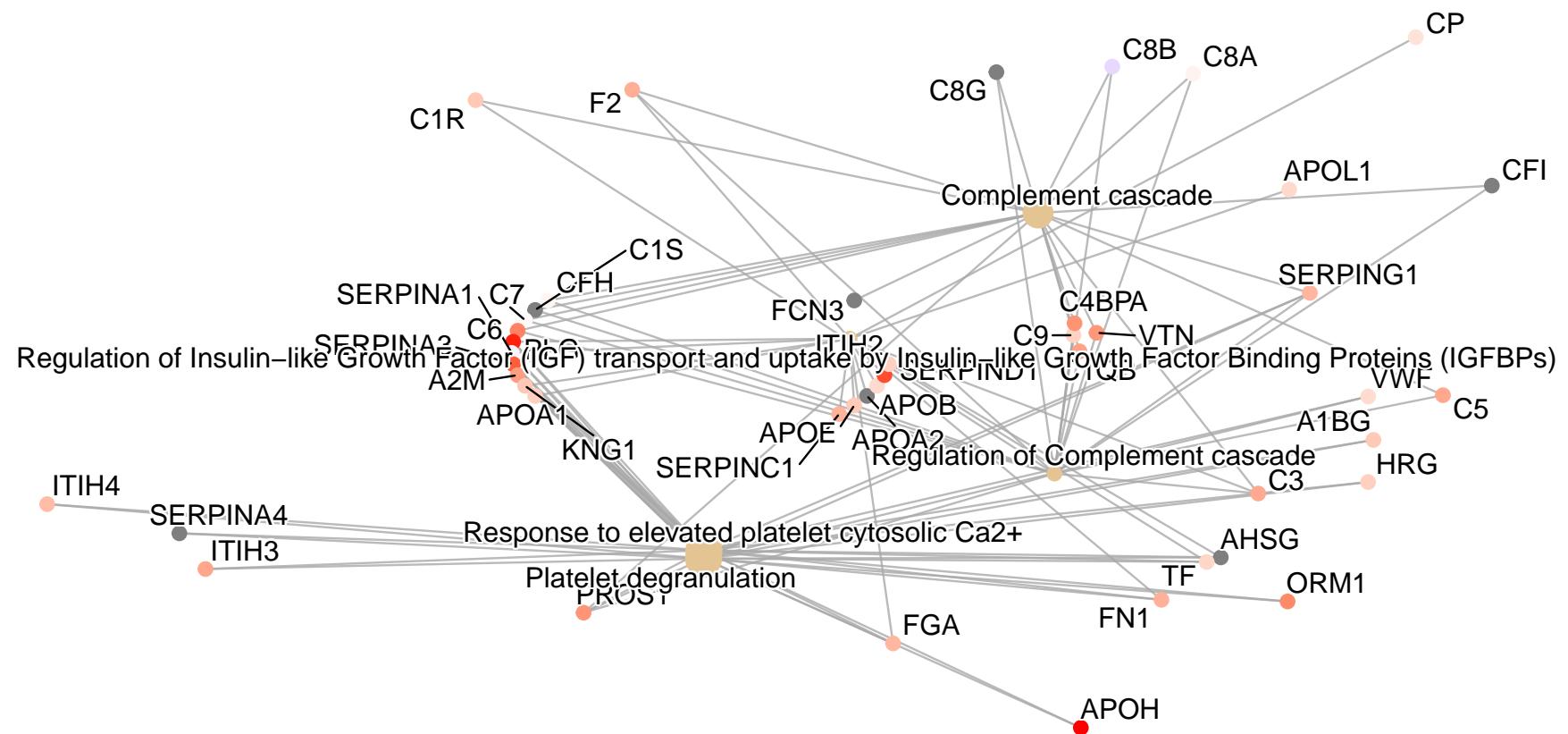


Figure S19. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Acute AIS C Improvers VS AIS D

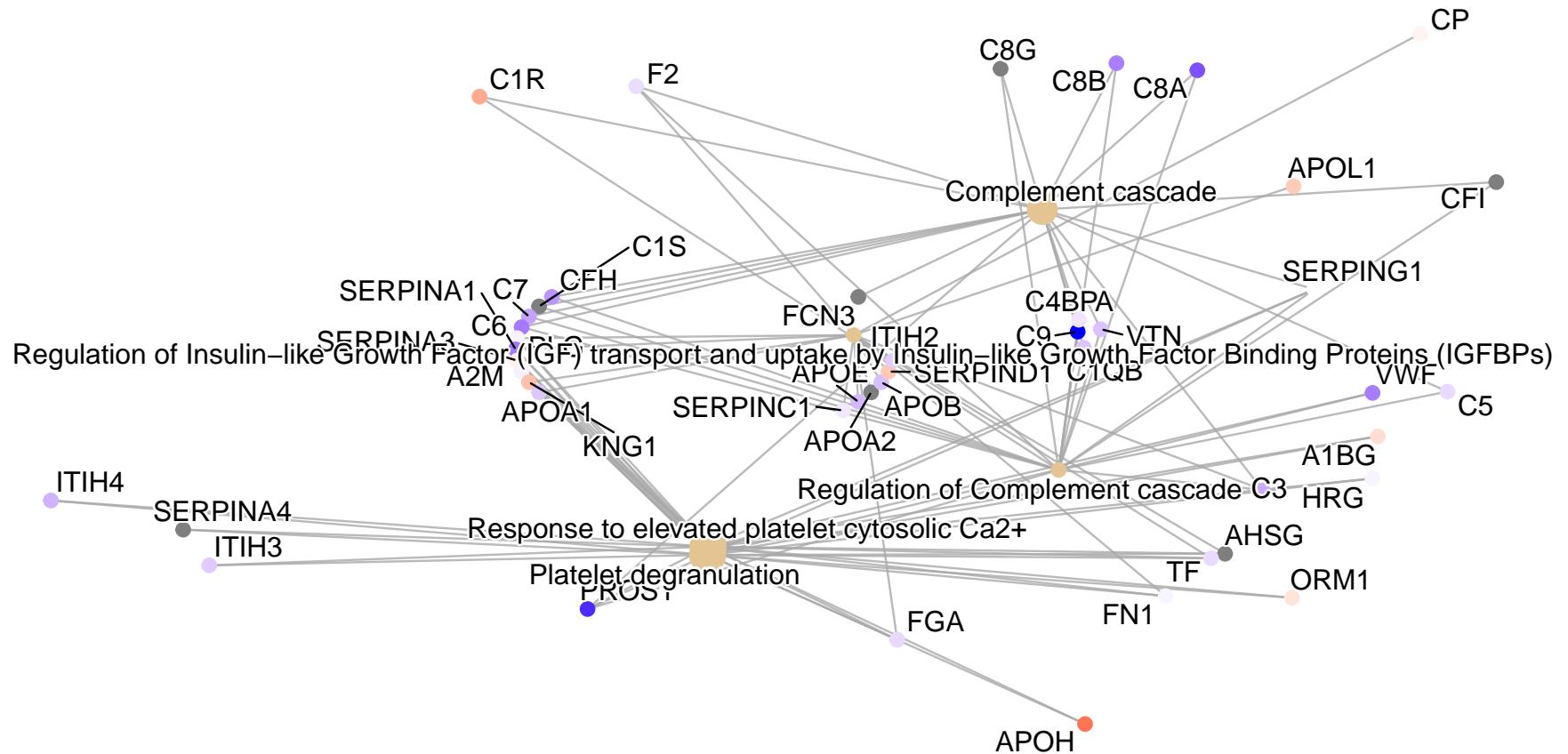


Figure S20. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Acute AIS C Improvers VS AIS A

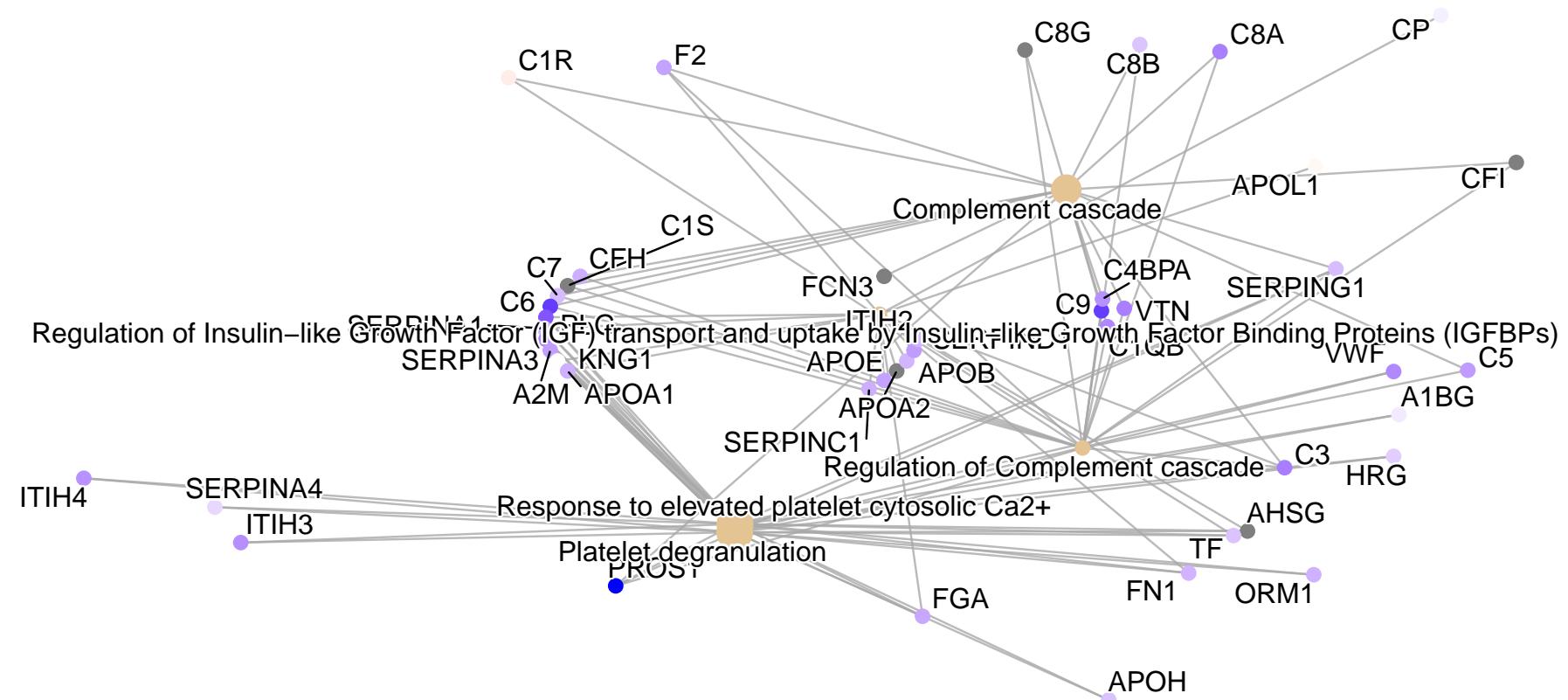


Figure S21. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS A

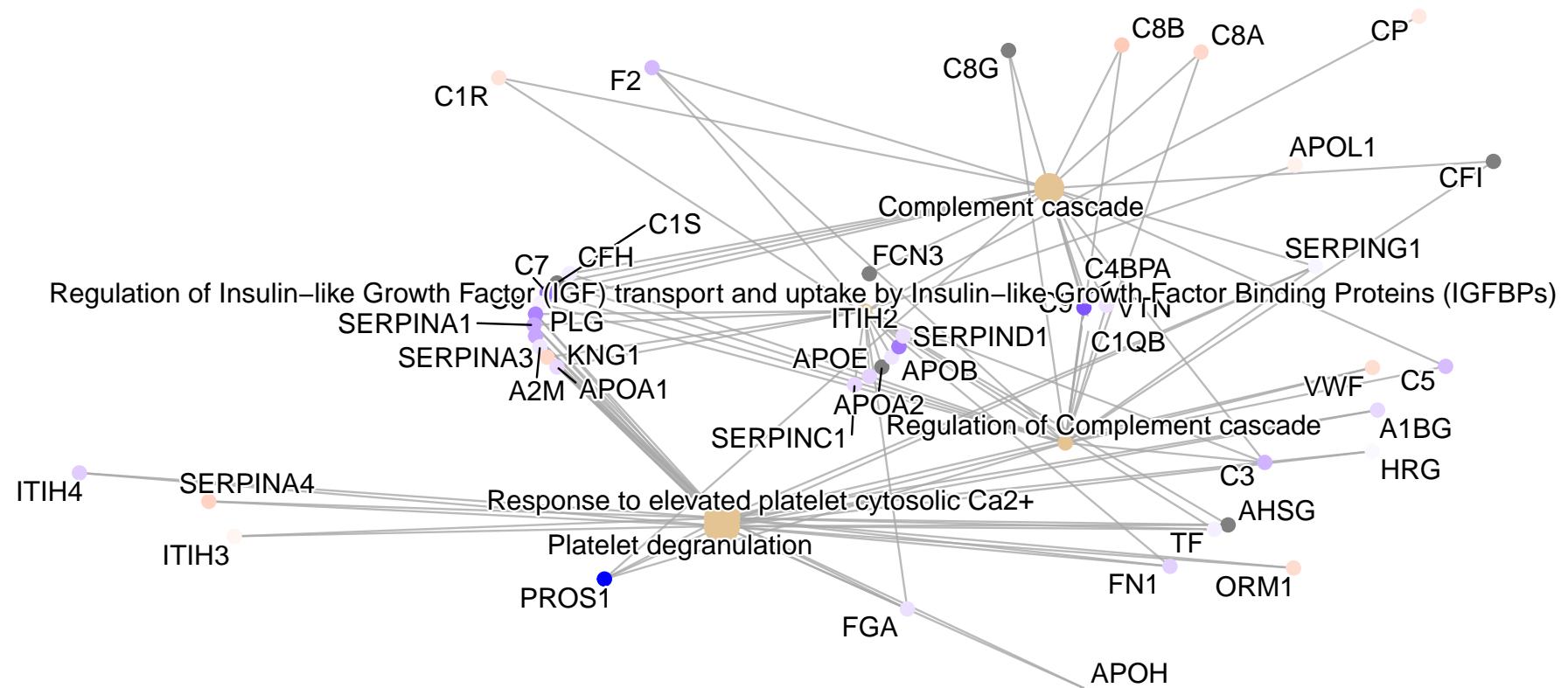


Figure S22. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D

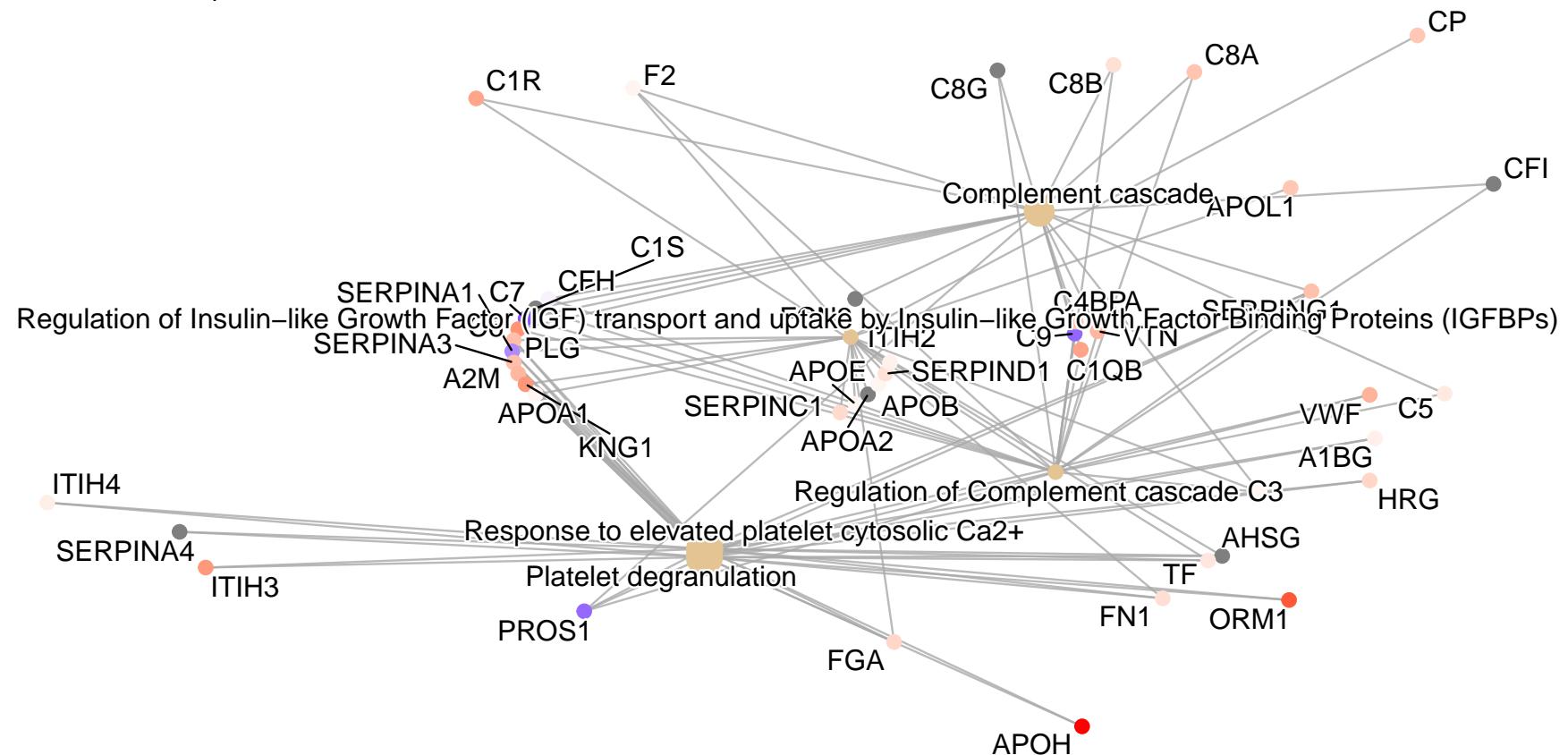


Figure S23. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

Acute C Improvers Vs Subacute C Improvers

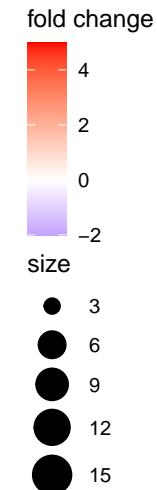
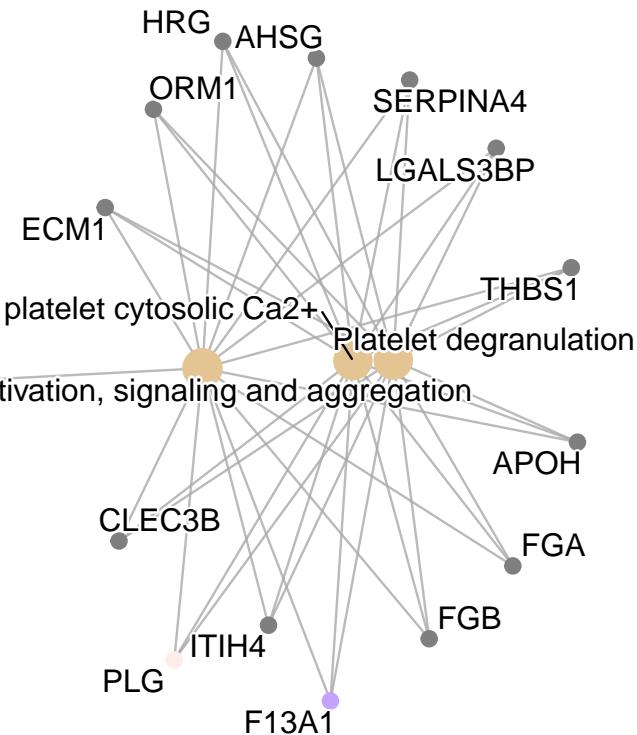
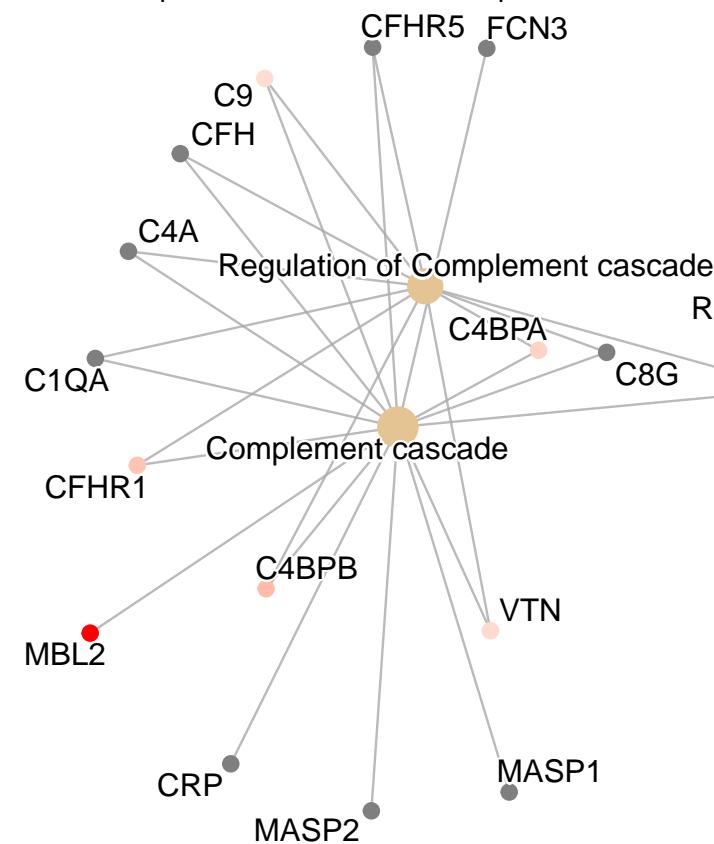


Figure S24. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute C Non-Improvers Vs Subacute C Non-Improvers

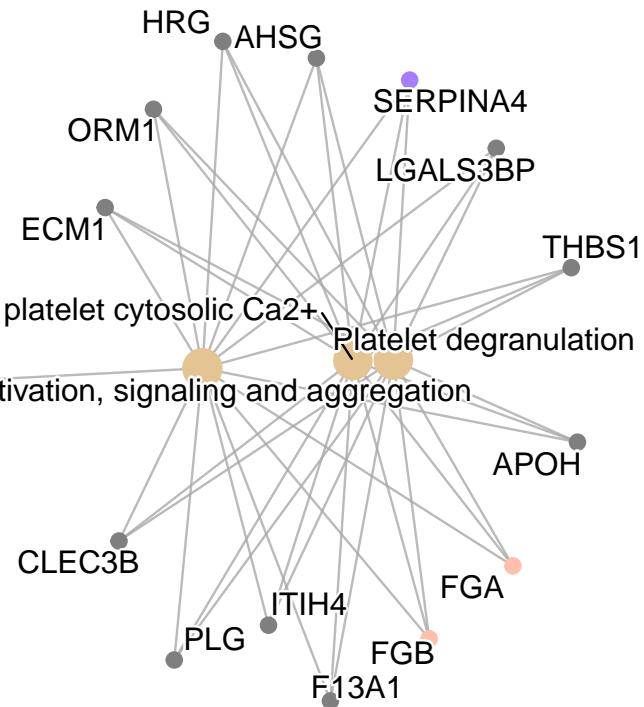
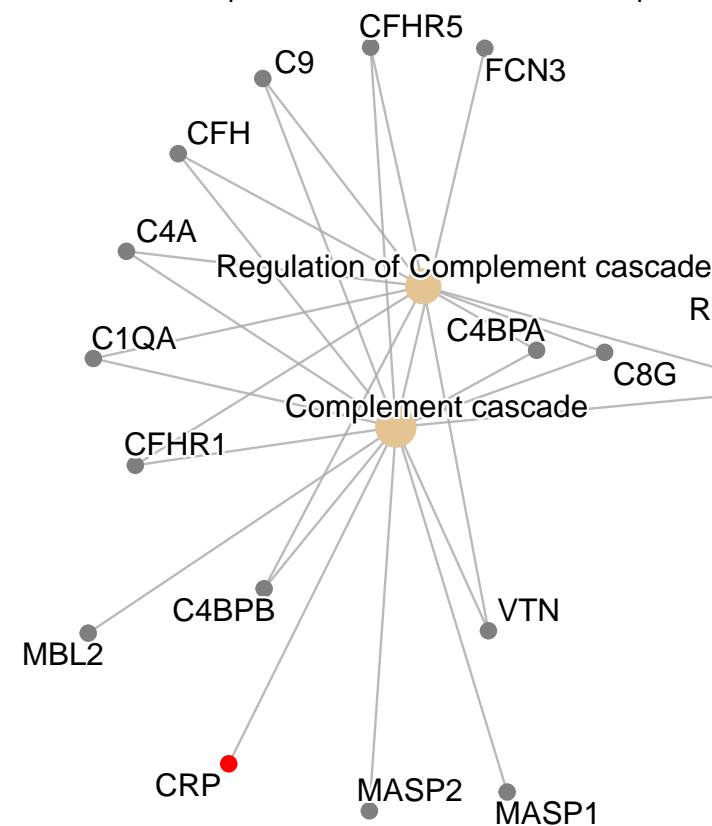
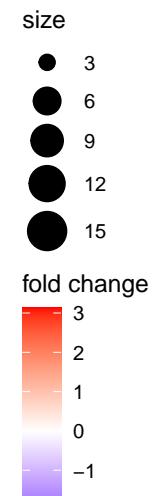


Figure S25. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.



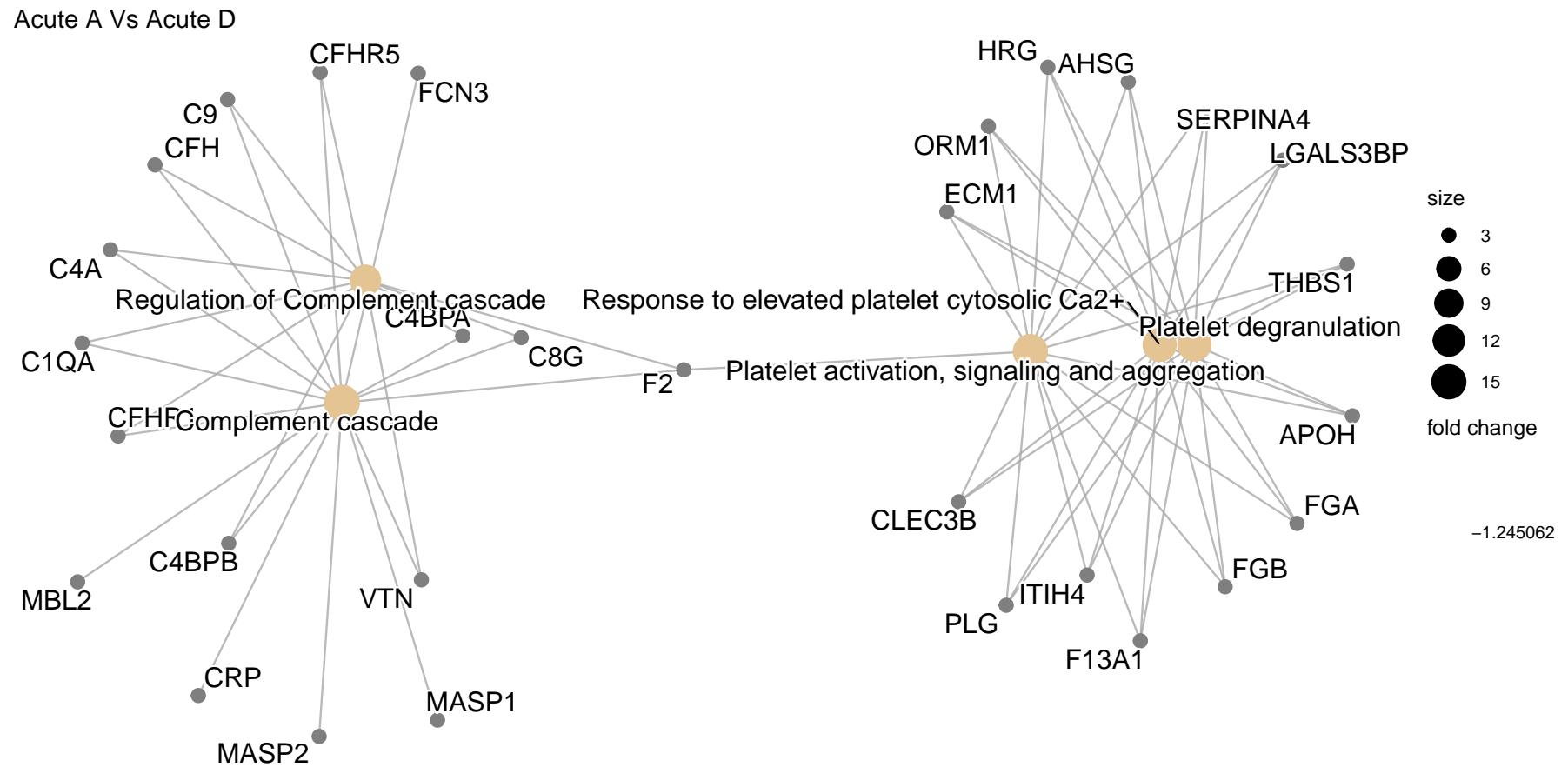


Figure S26. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

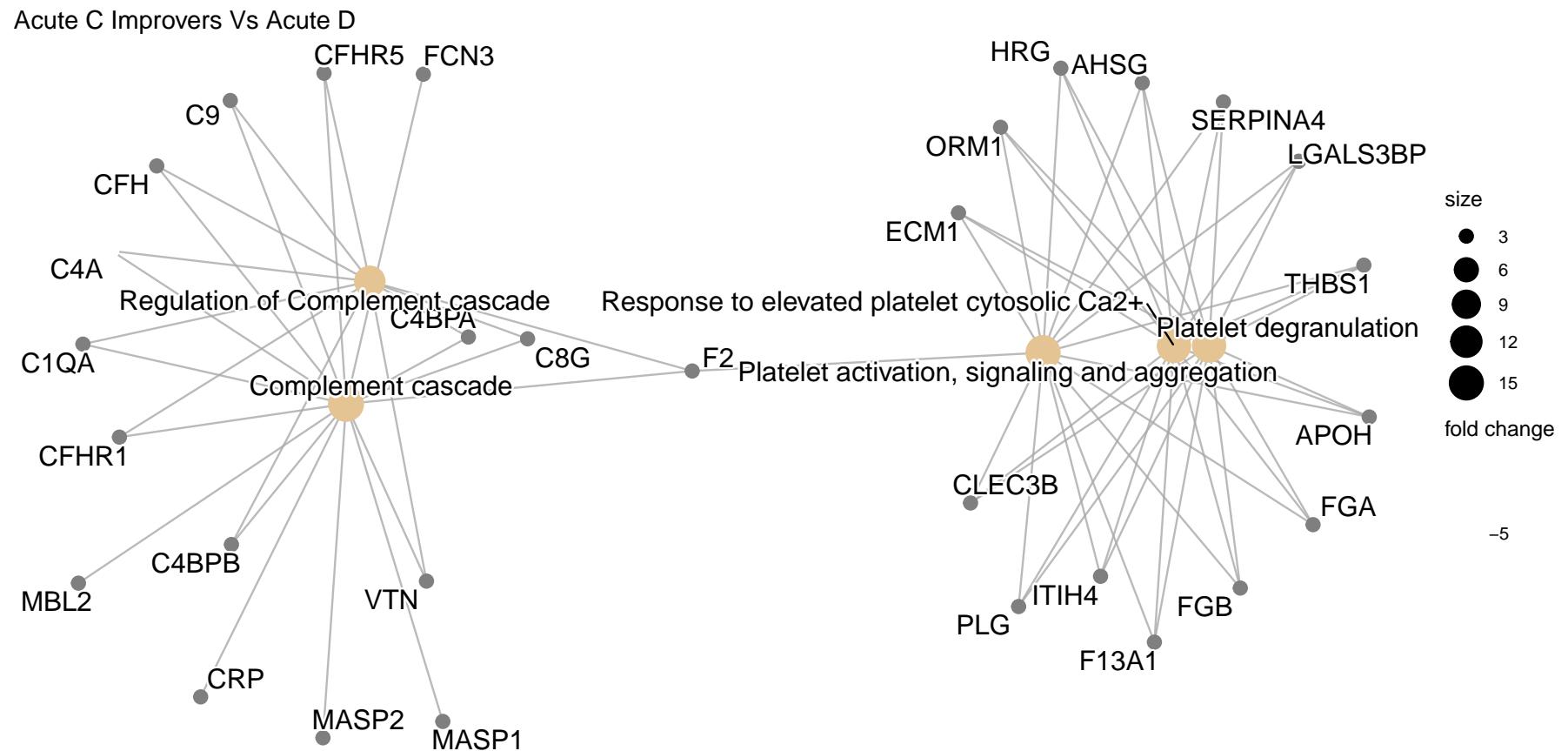


Figure S27. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Acute A Vs Acute C Improvers

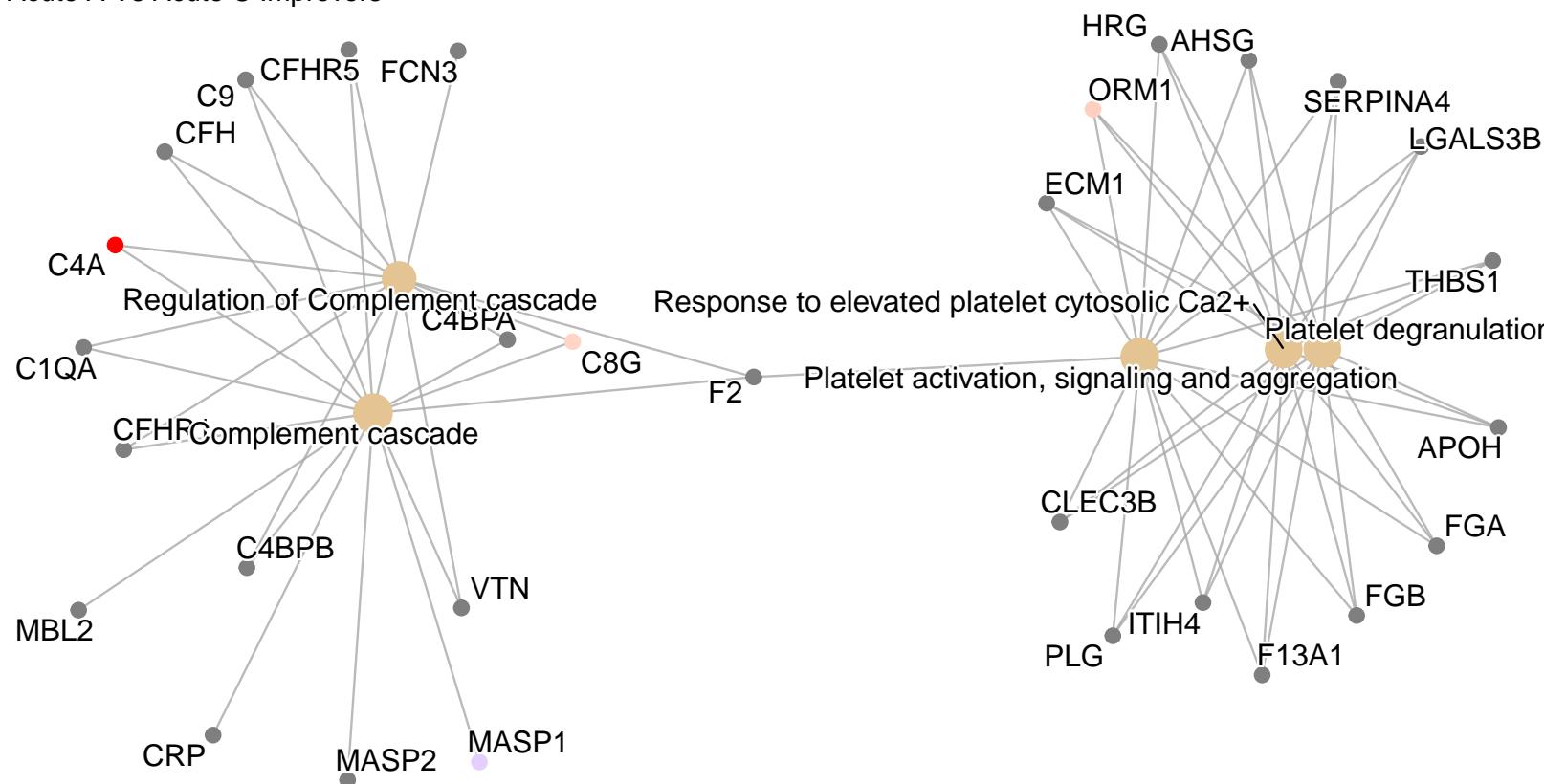
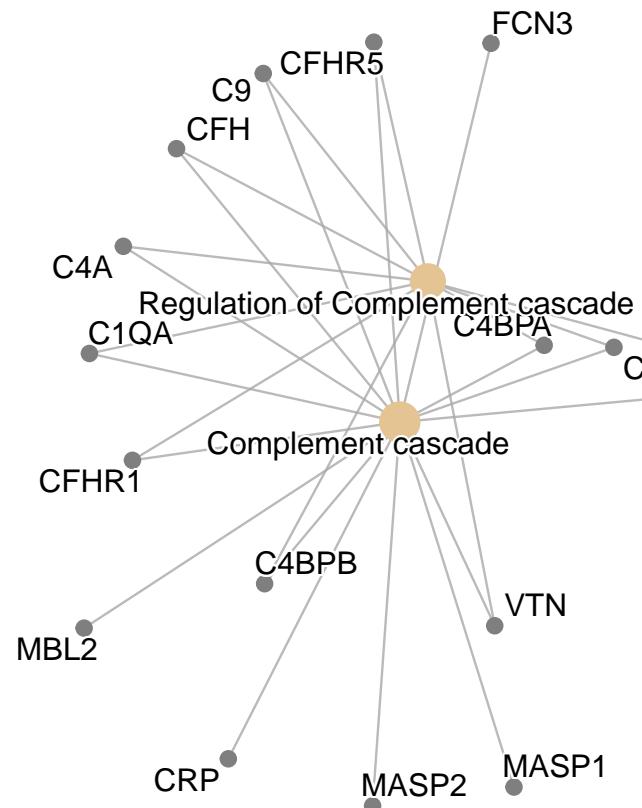


Figure S28. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute A Vs Acute C Non-Improvers



Response to elevated platelet cytosolic Ca²⁺, Platelet activation, signaling and aggregation, Platelet degranulation

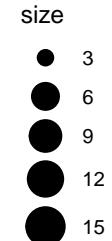
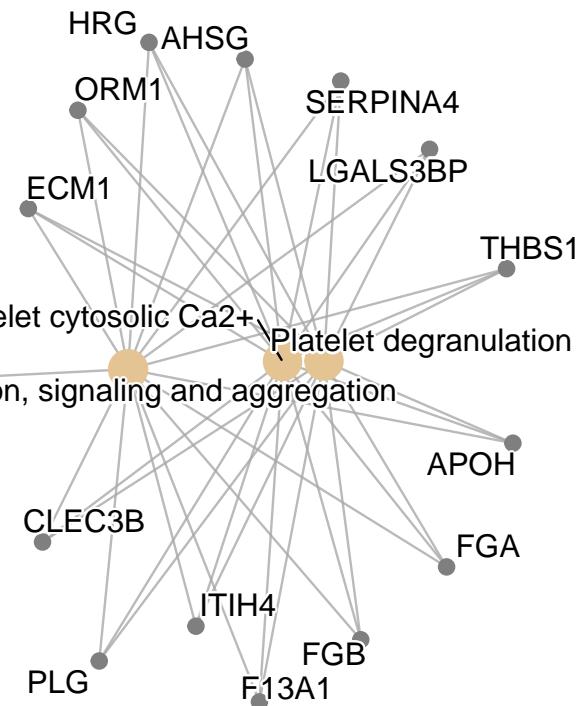


Figure S29. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute C Non-Improvers Vs Acute D

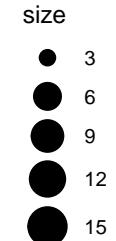
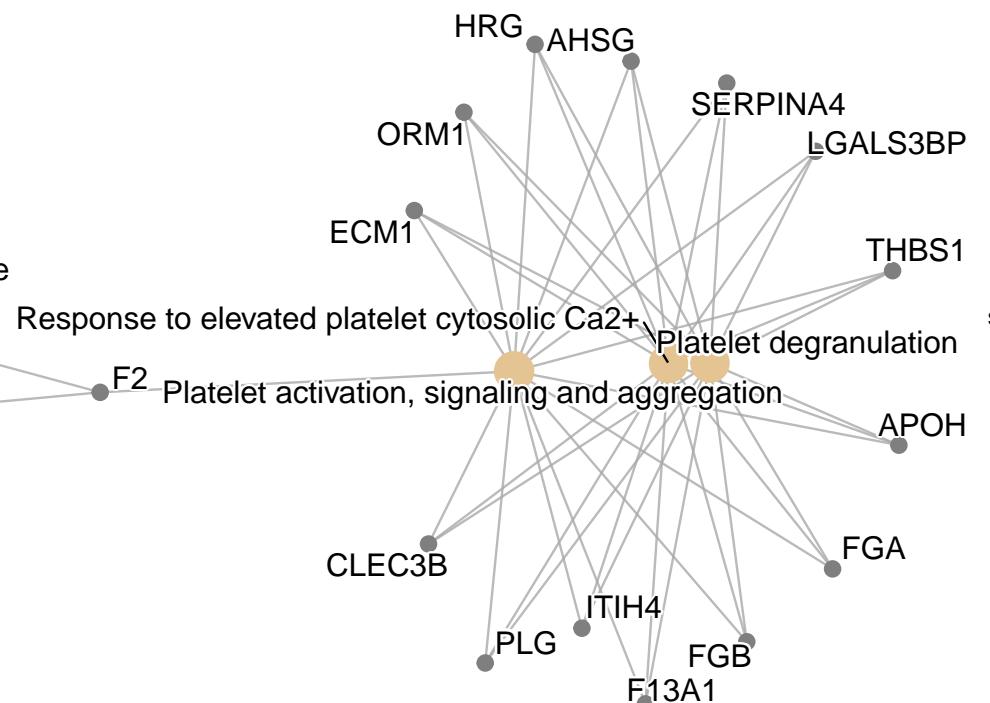
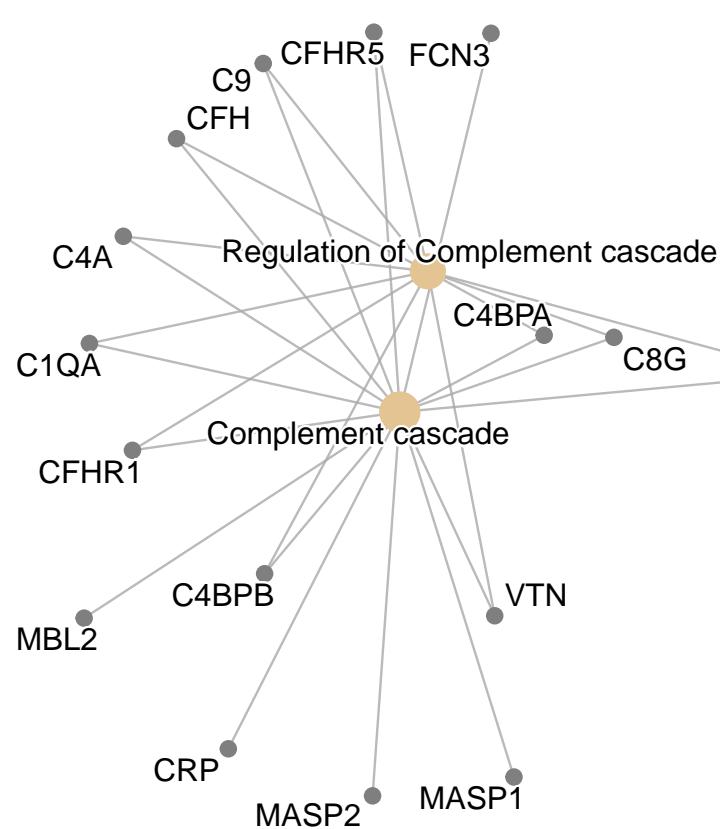


Figure S30. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

1278 5.8 STRINGdb network plots

1279 5.8.1 iTRAQ data

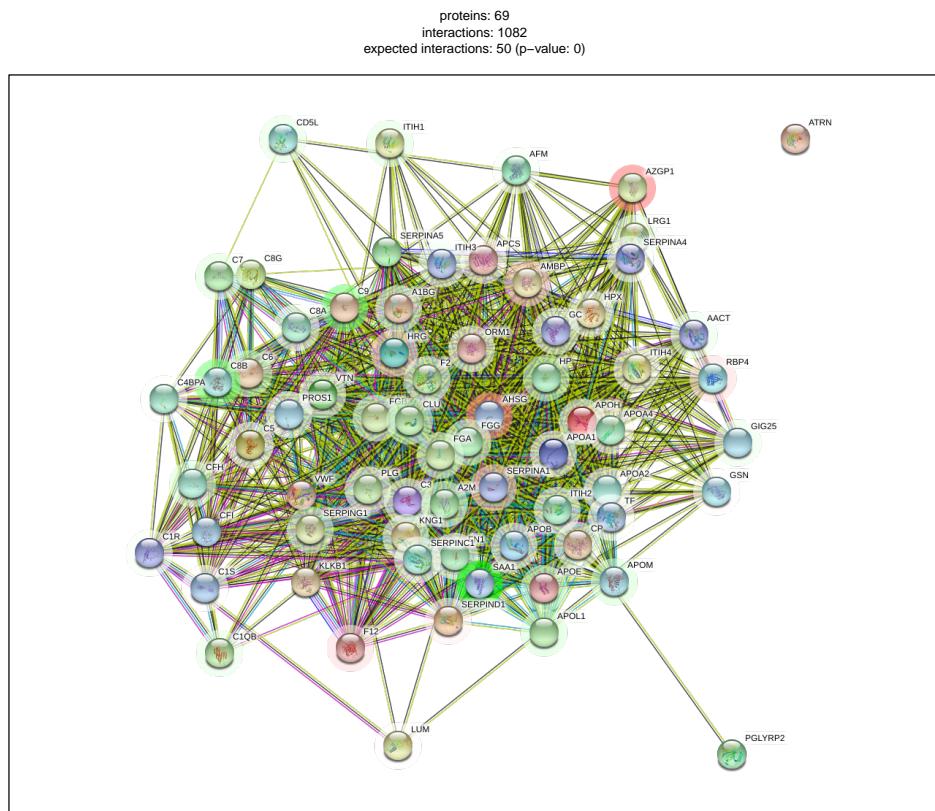


Figure S31. The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates 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)

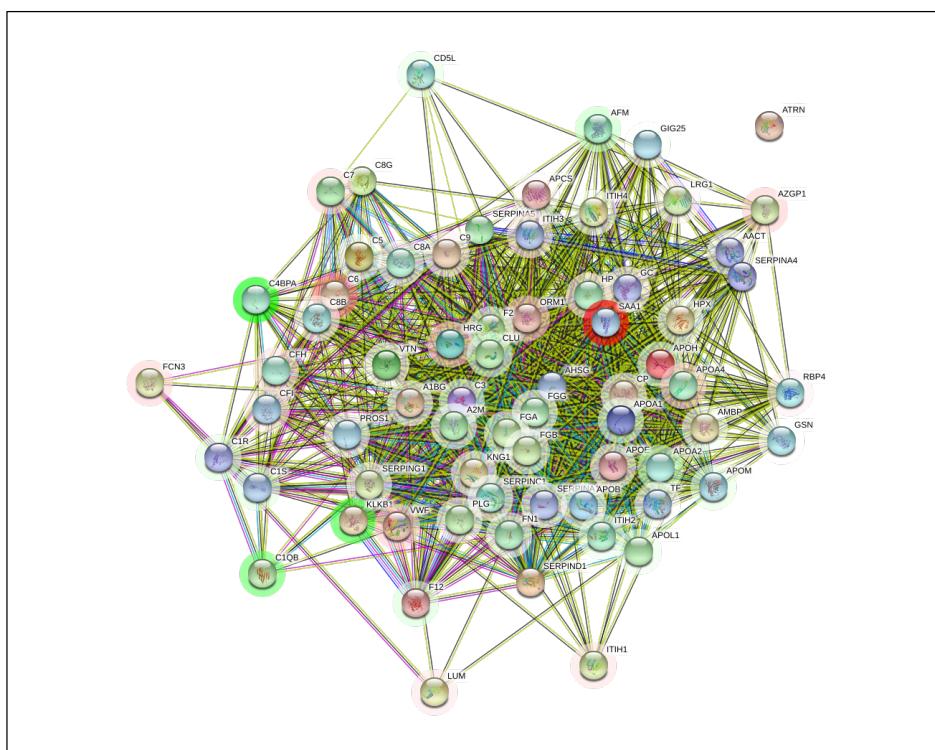


Figure S32. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there 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)

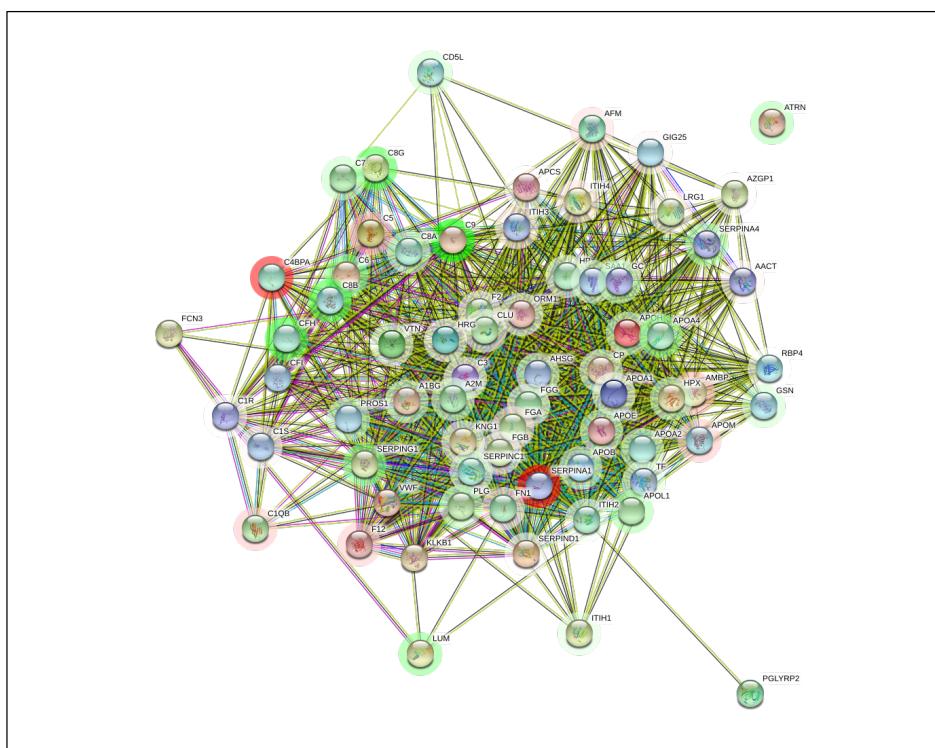


Figure S33. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. 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)

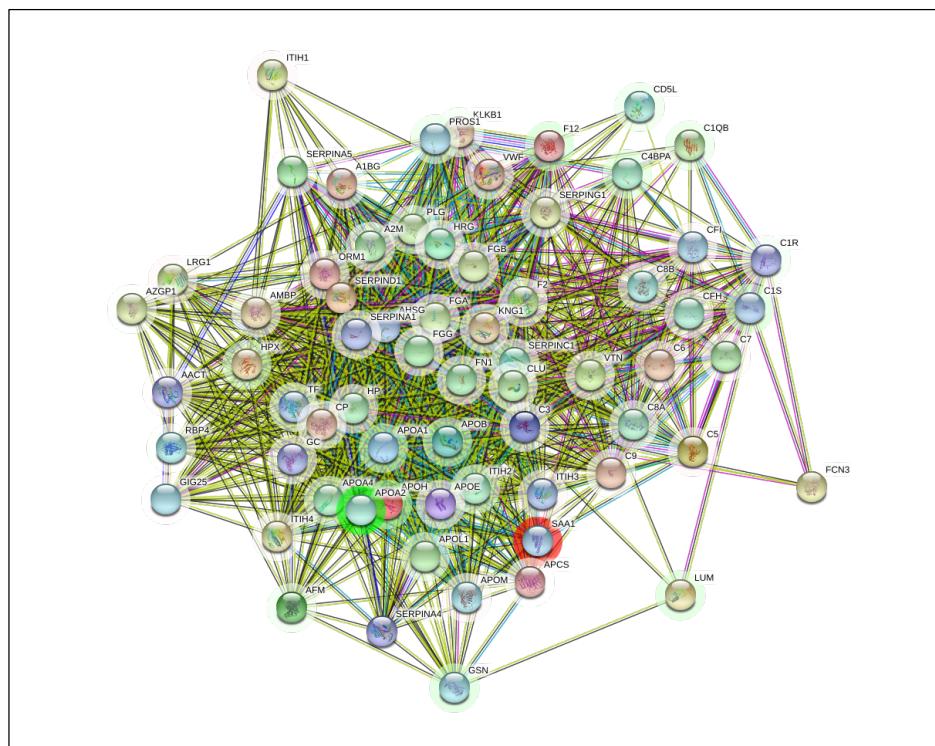


Figure S34. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater 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.

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

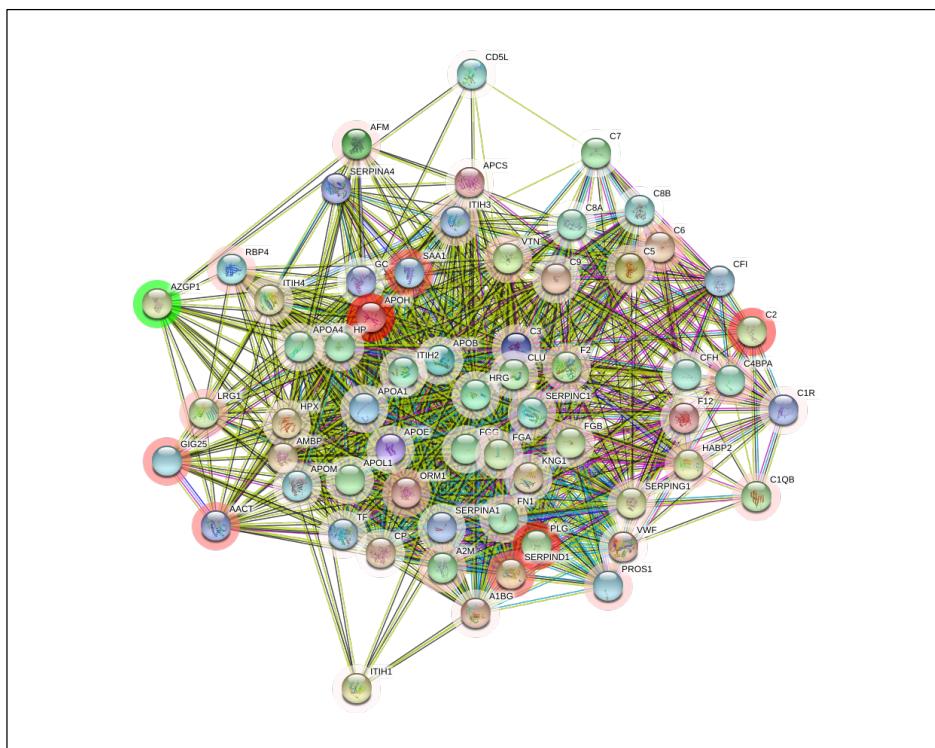


Figure S35. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; 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)

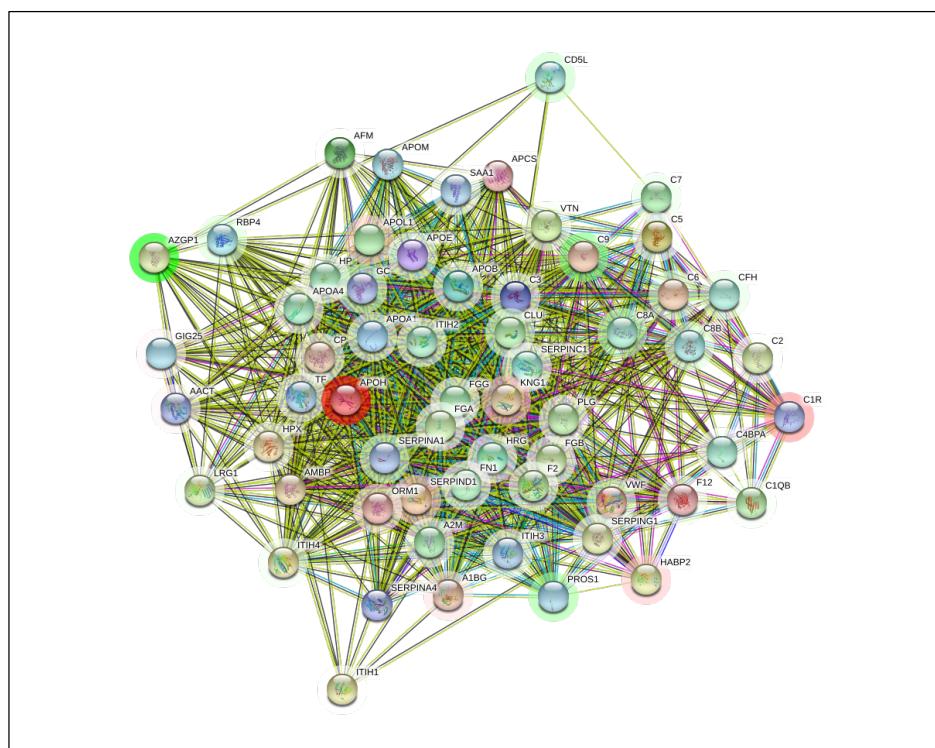


Figure S36. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; 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)

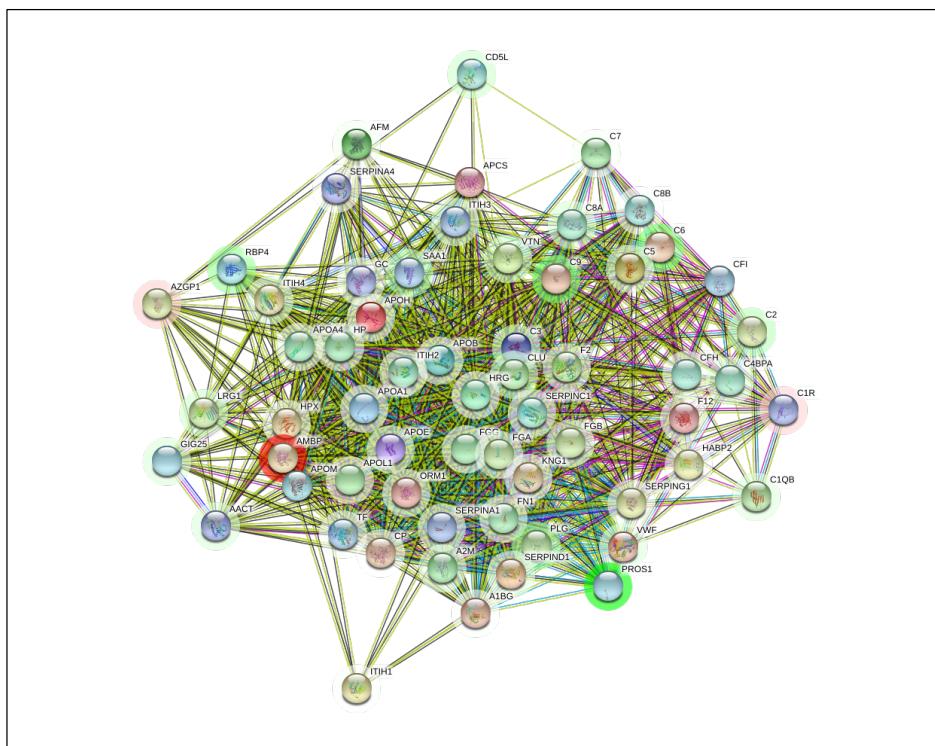


Figure S37. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; 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)

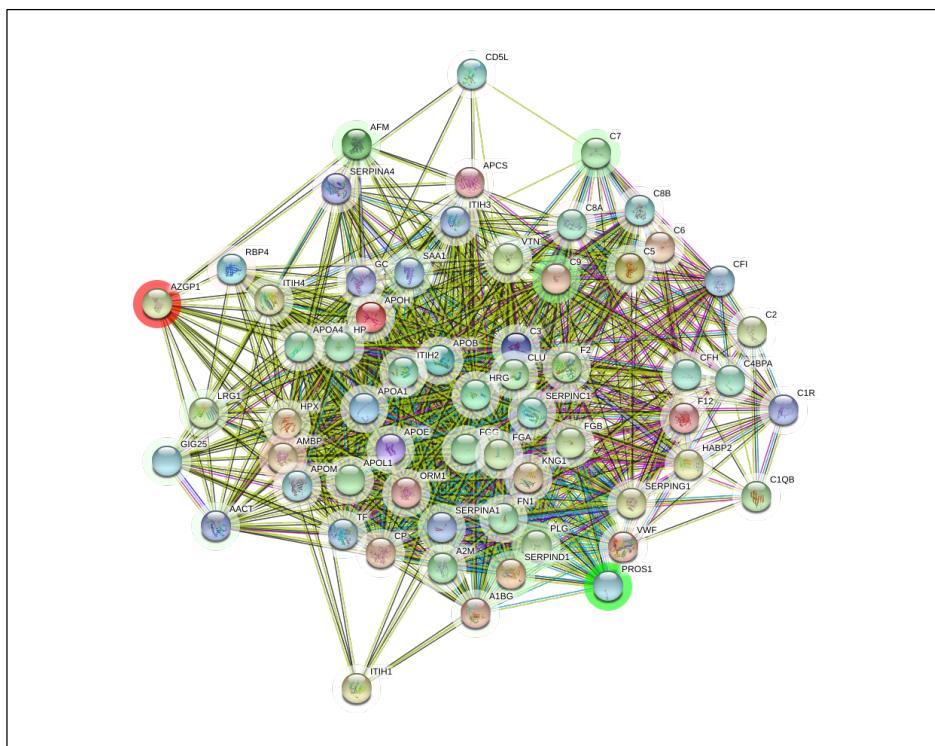


Figure S38. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein 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)

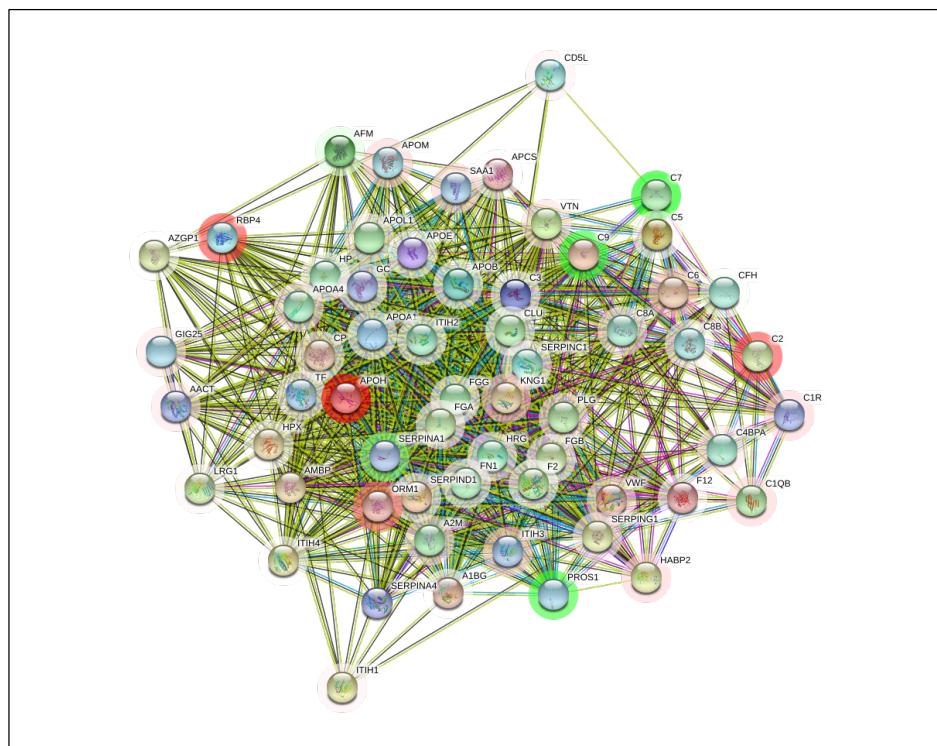


Figure S39. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein 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; gene co-expression; text-mining and protein homology.

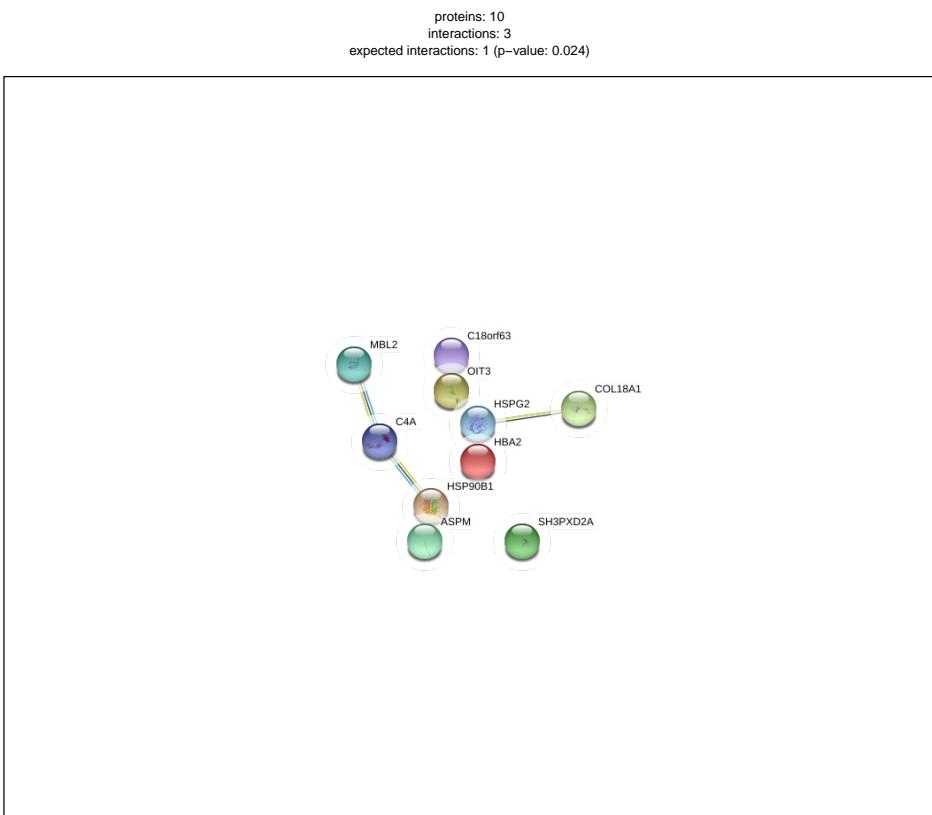


Figure S41. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there 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)

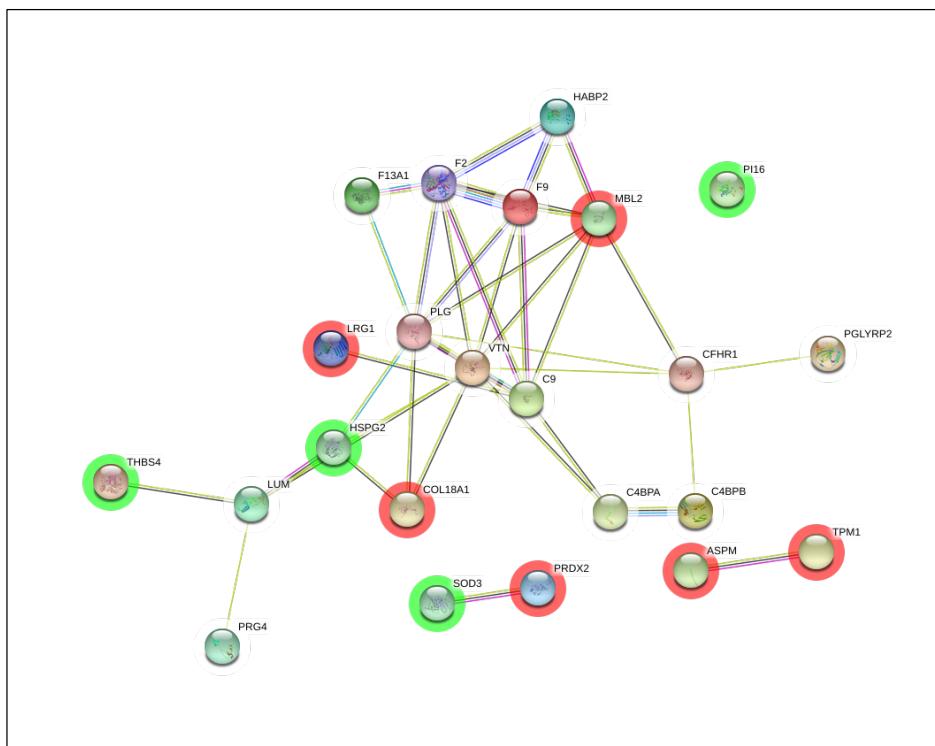


Figure S42. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. 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 .

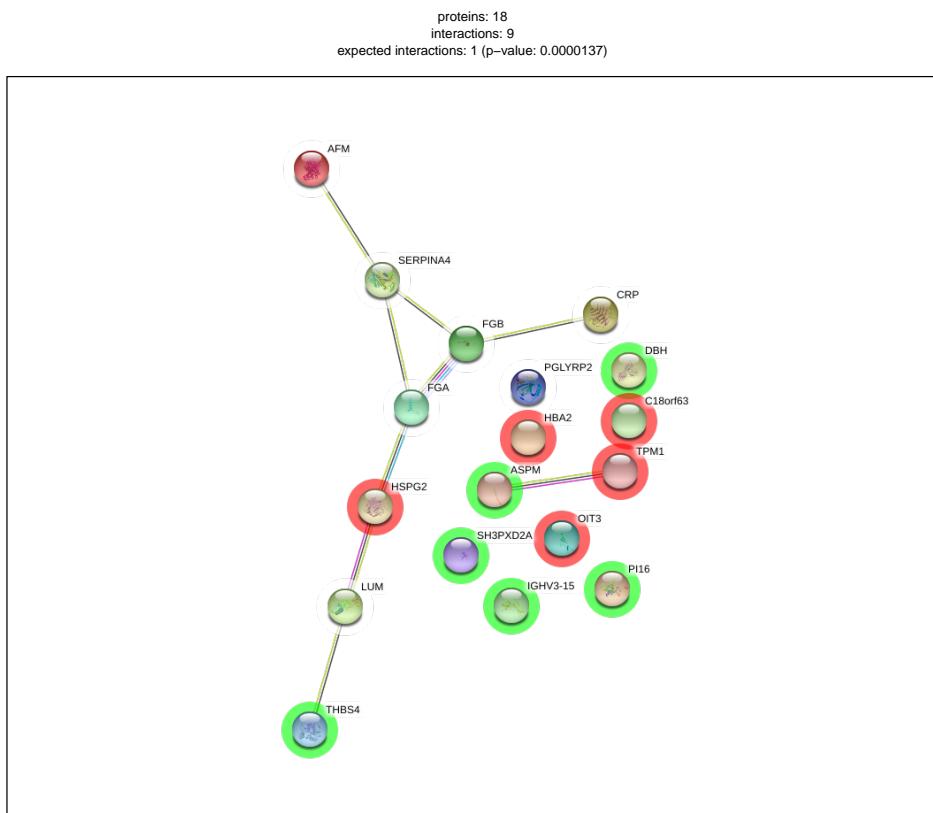


Figure S43. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater 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)

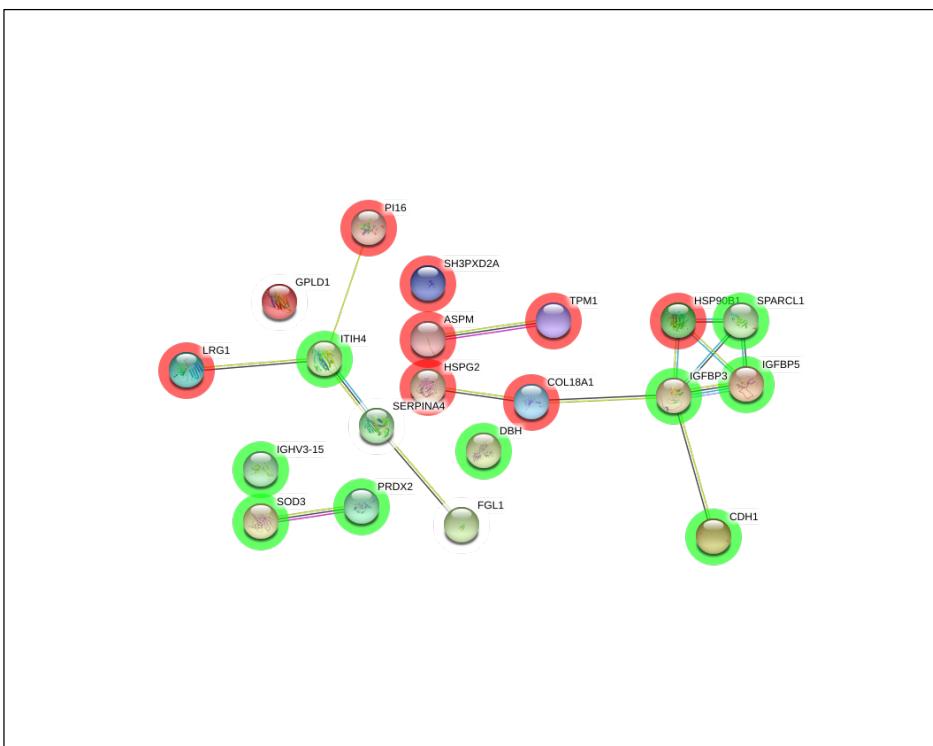


Figure S44. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; 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)

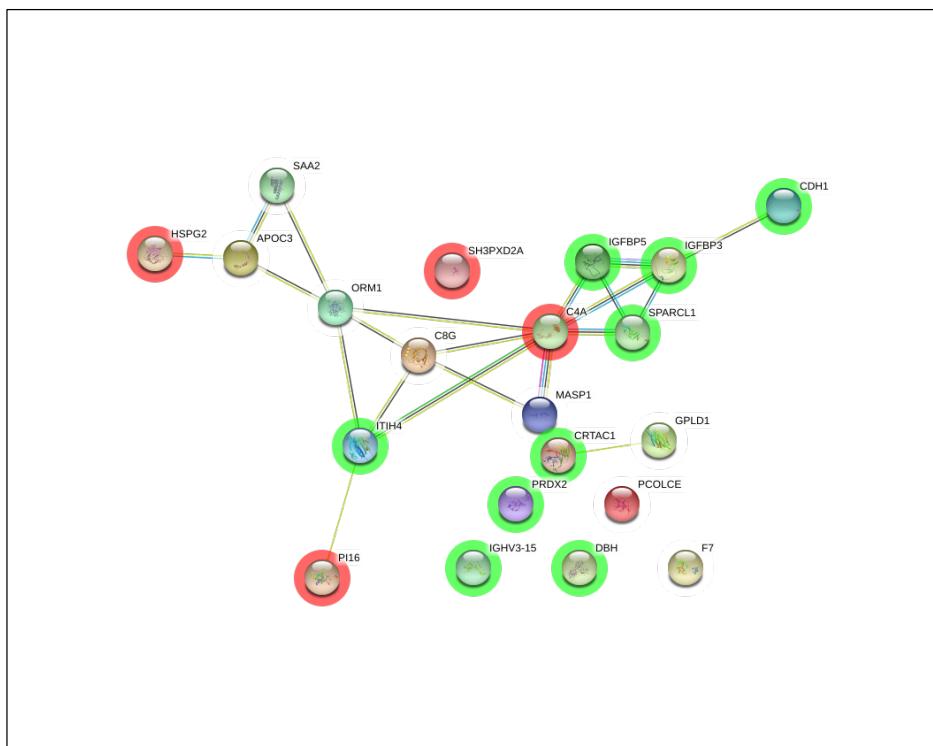


Figure S46. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; 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 .

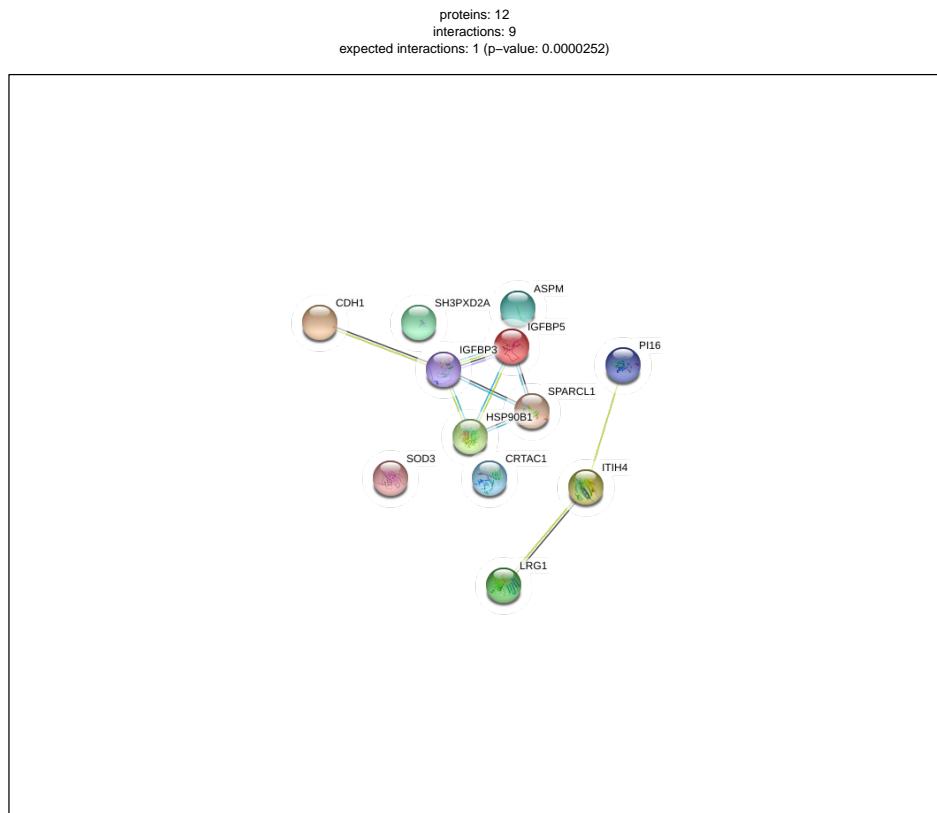


Figure S47. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein 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 .

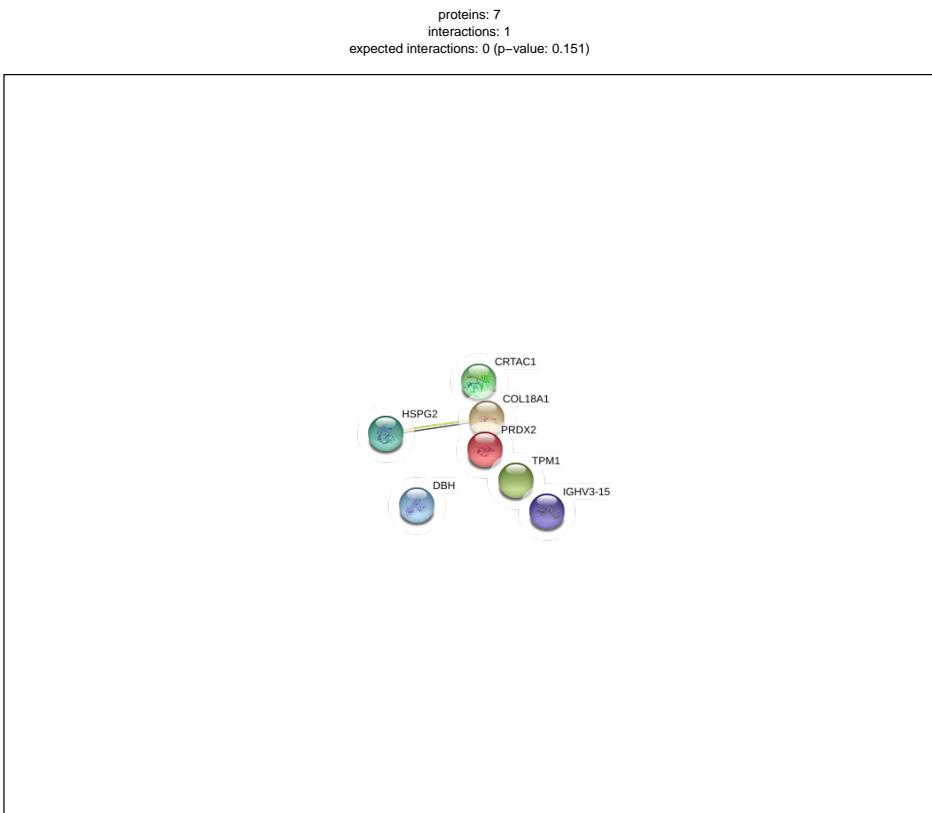


Figure S48. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein 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

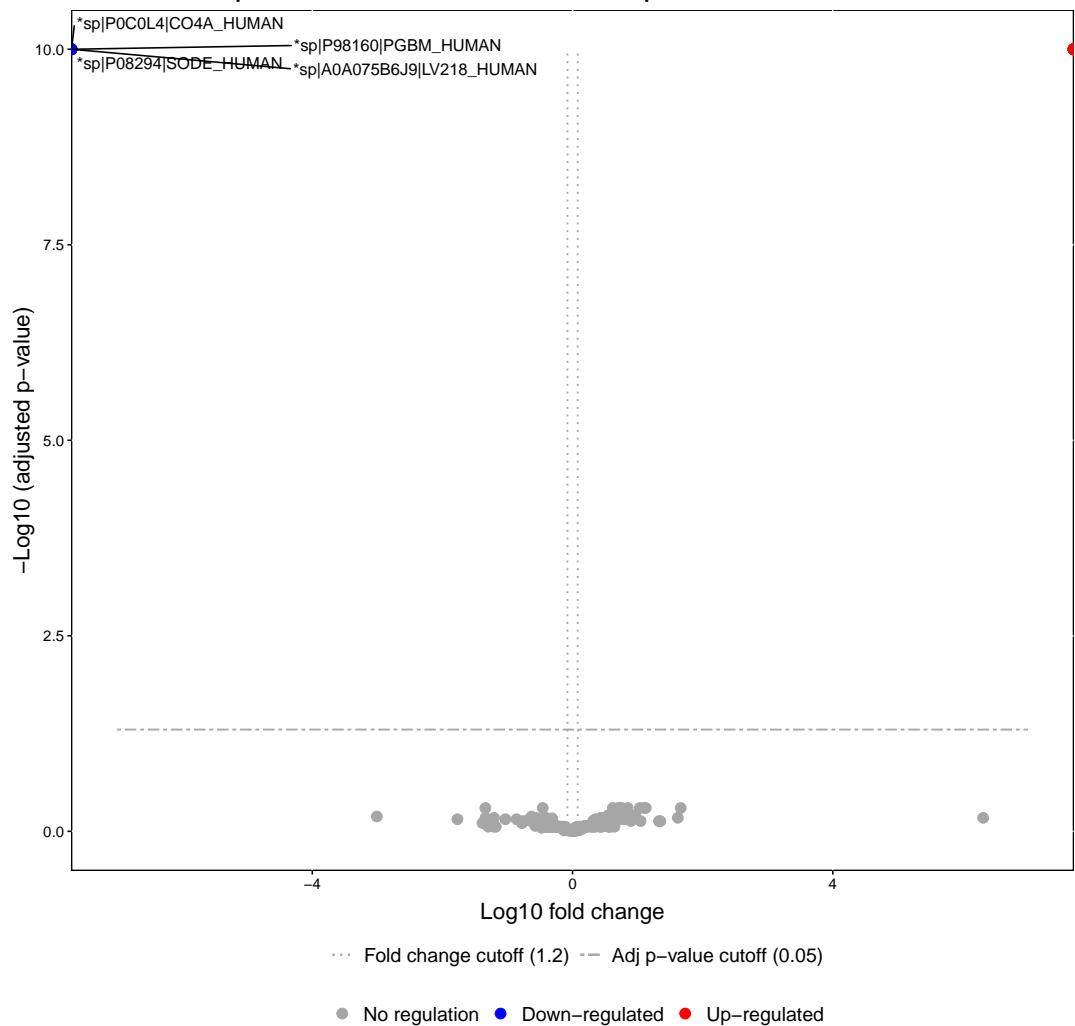


Figure S49. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Subacute C Improvers Vs Subacute C Non-Improvers

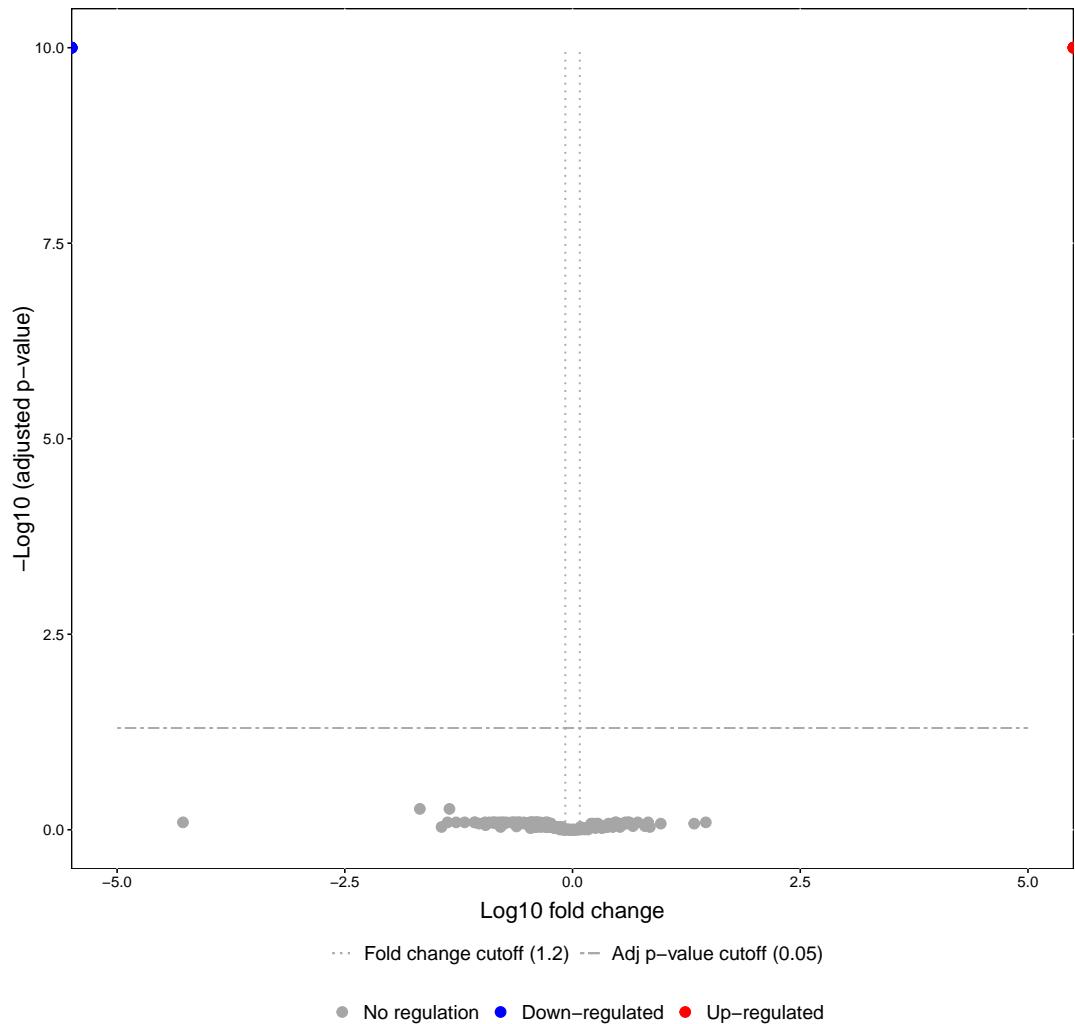


Figure S50. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 3-months post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Subacute C Improvers

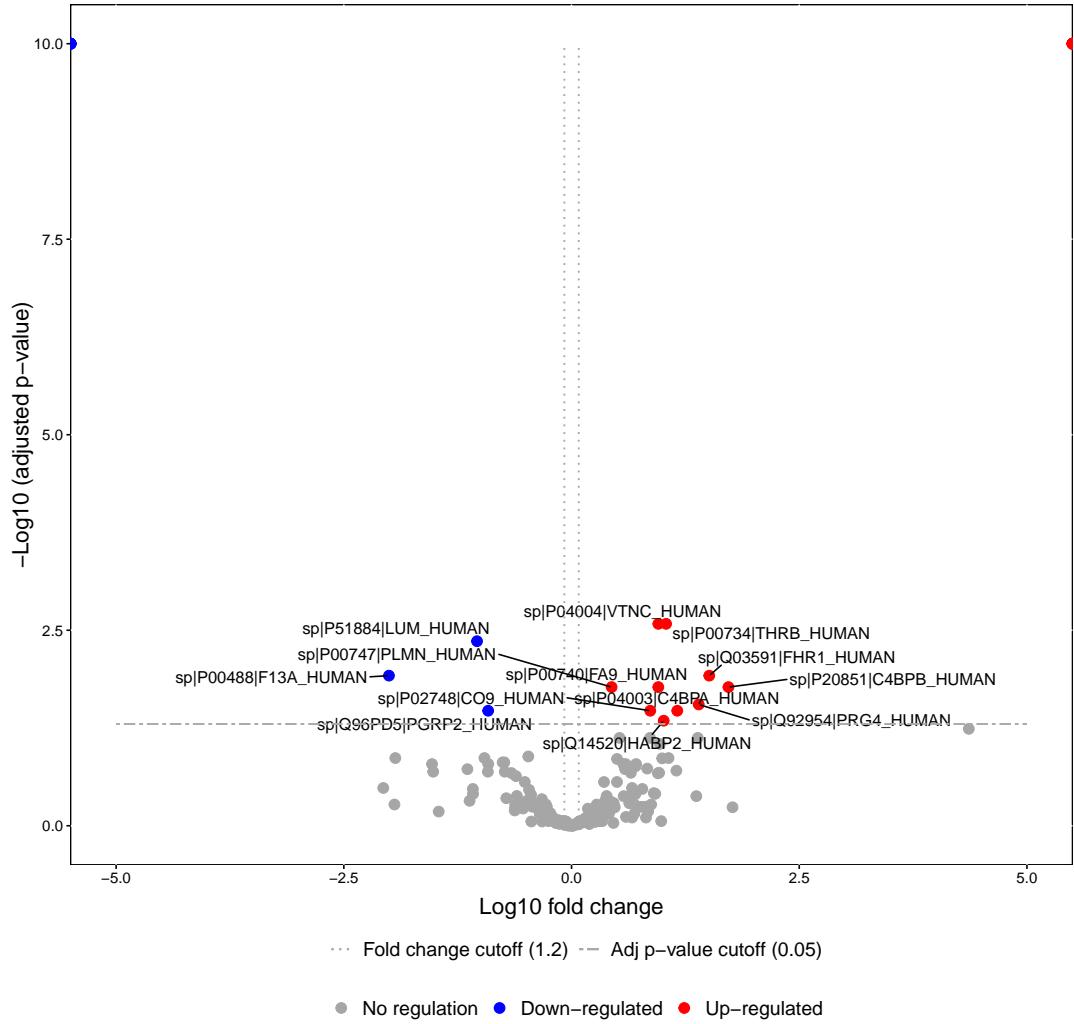


Figure S51. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Non-Improvers Vs Subacute C Non-Improvers

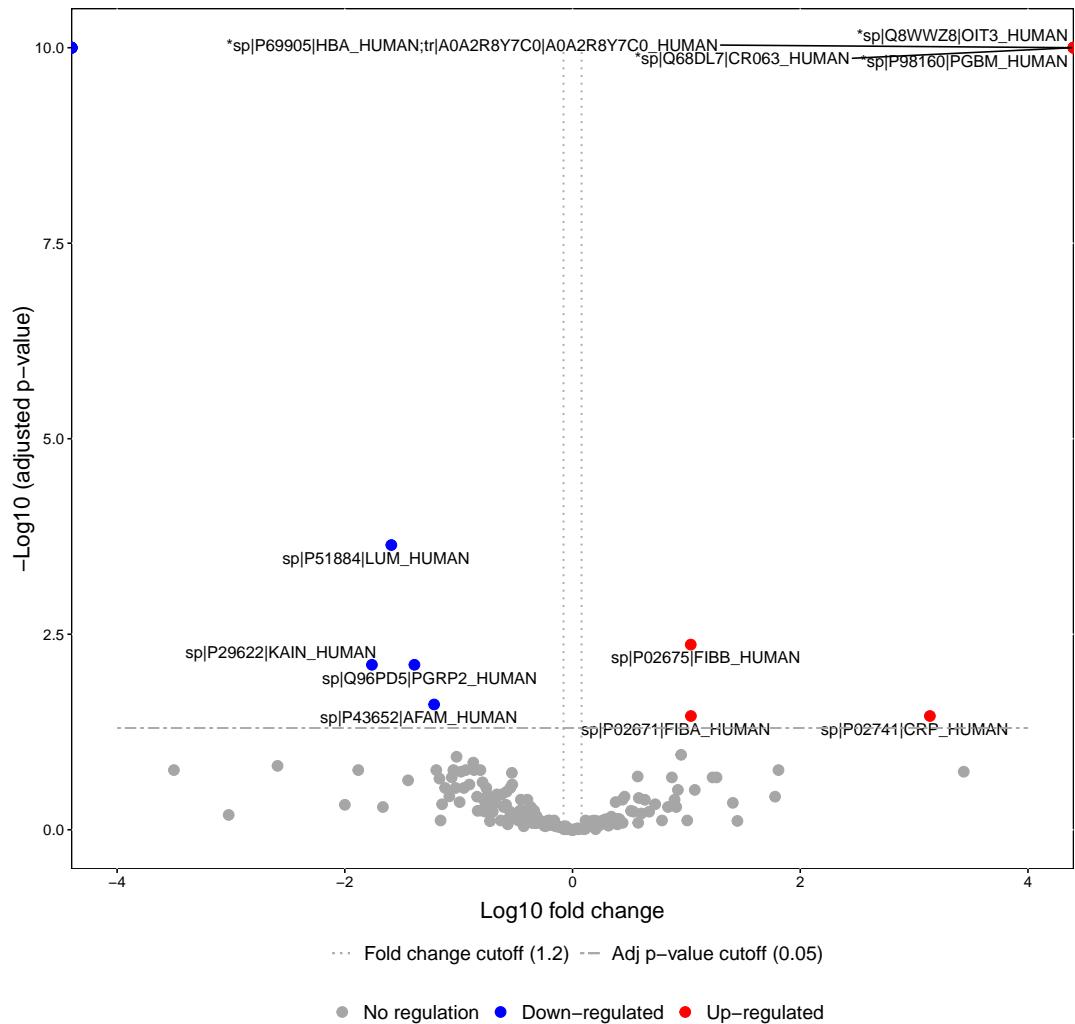


Figure S52. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute D

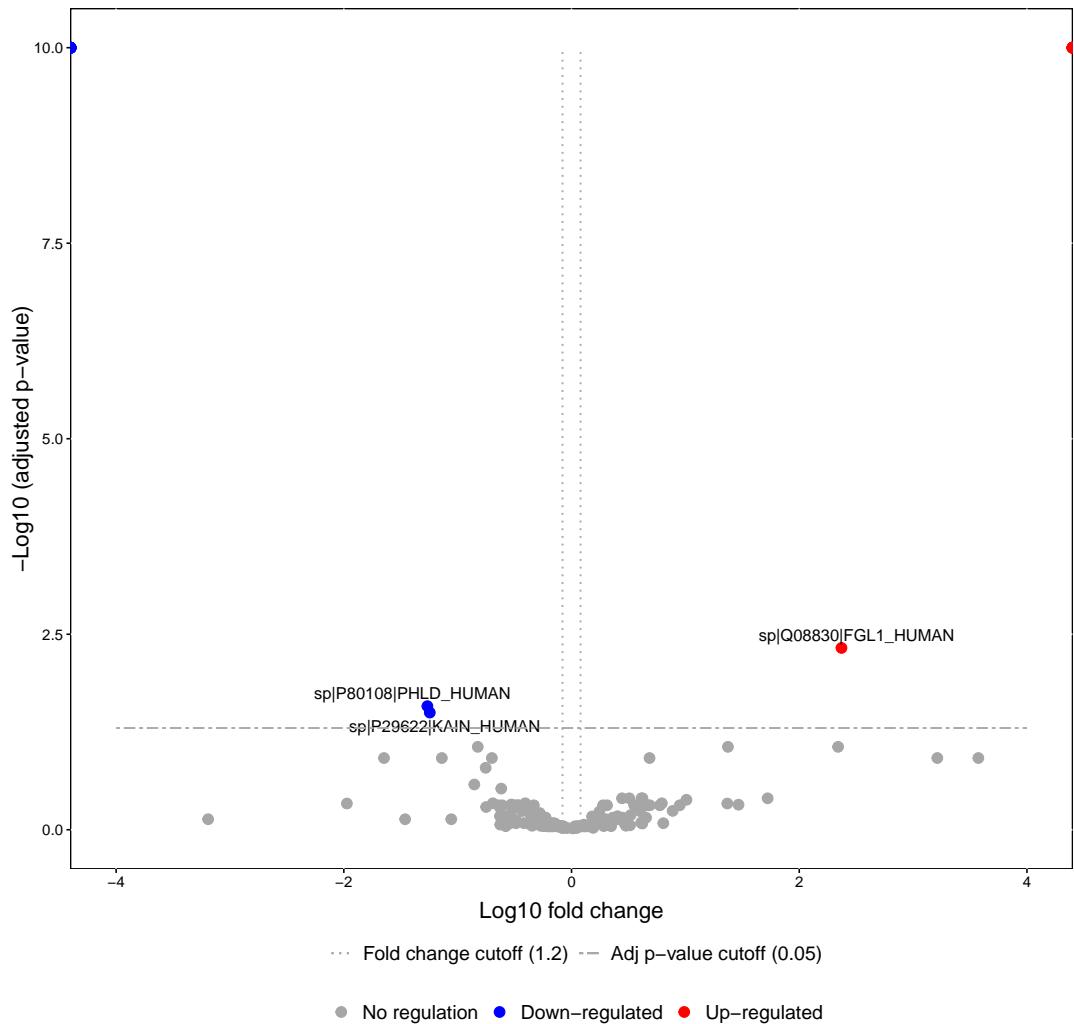


Figure S53. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Subacute A

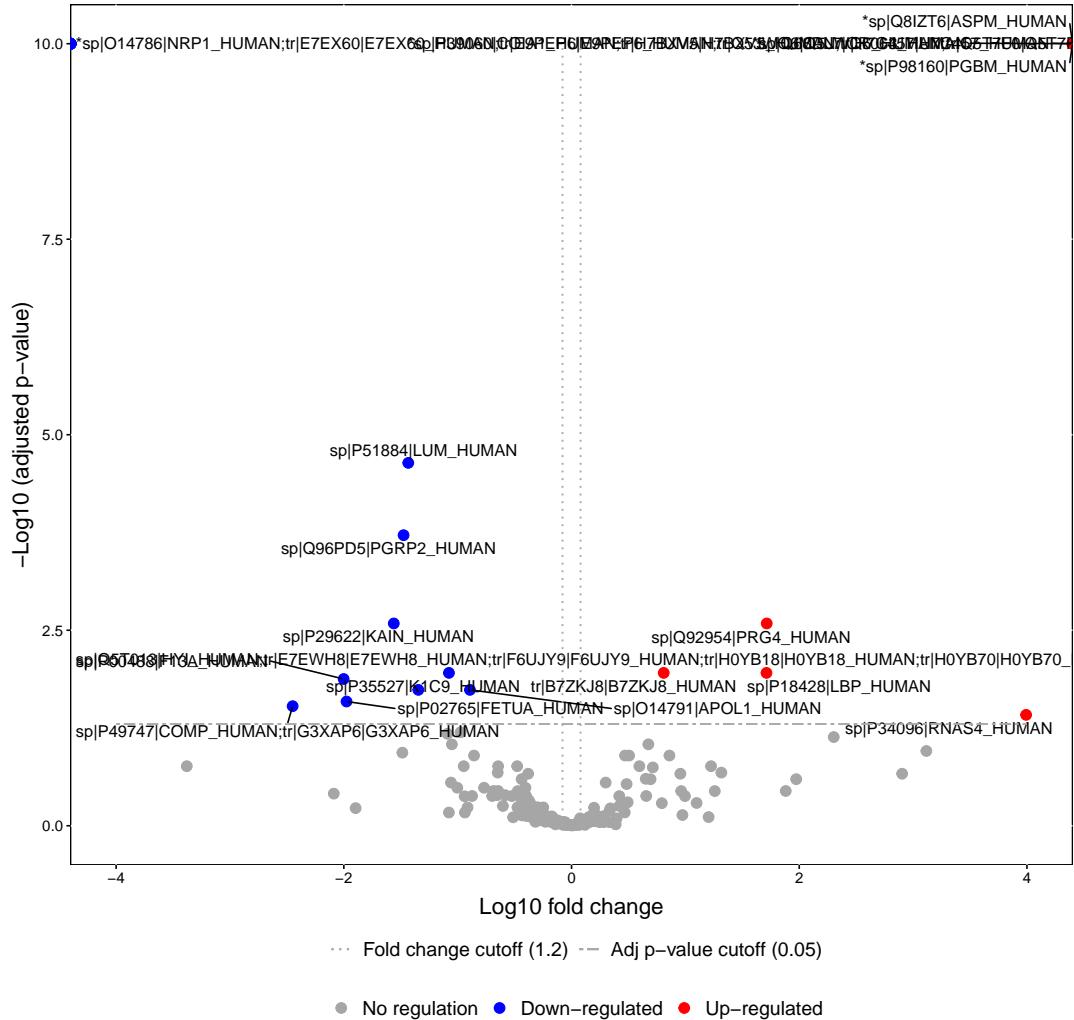


Figure S54. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS A patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute D Vs Subacute D

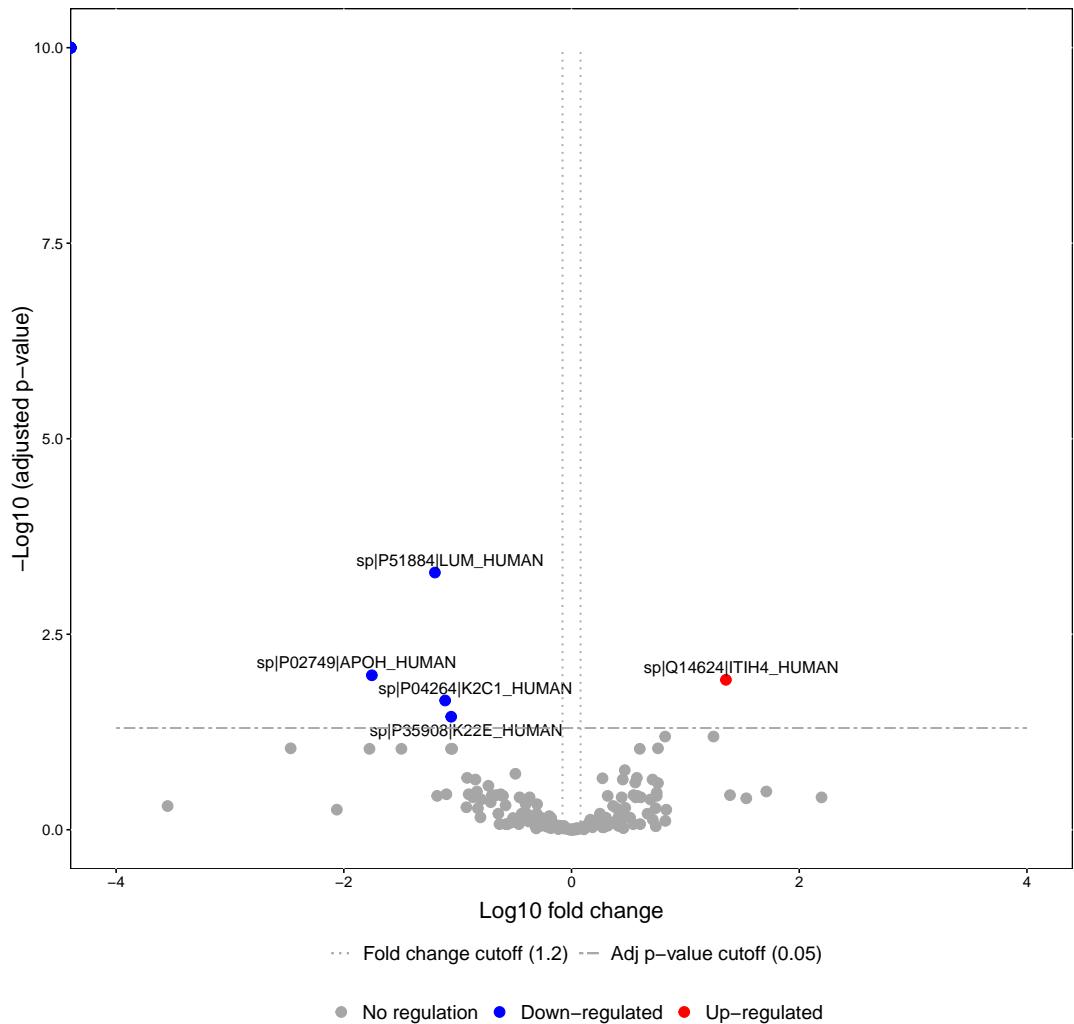


Figure S55. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS D patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Acute D

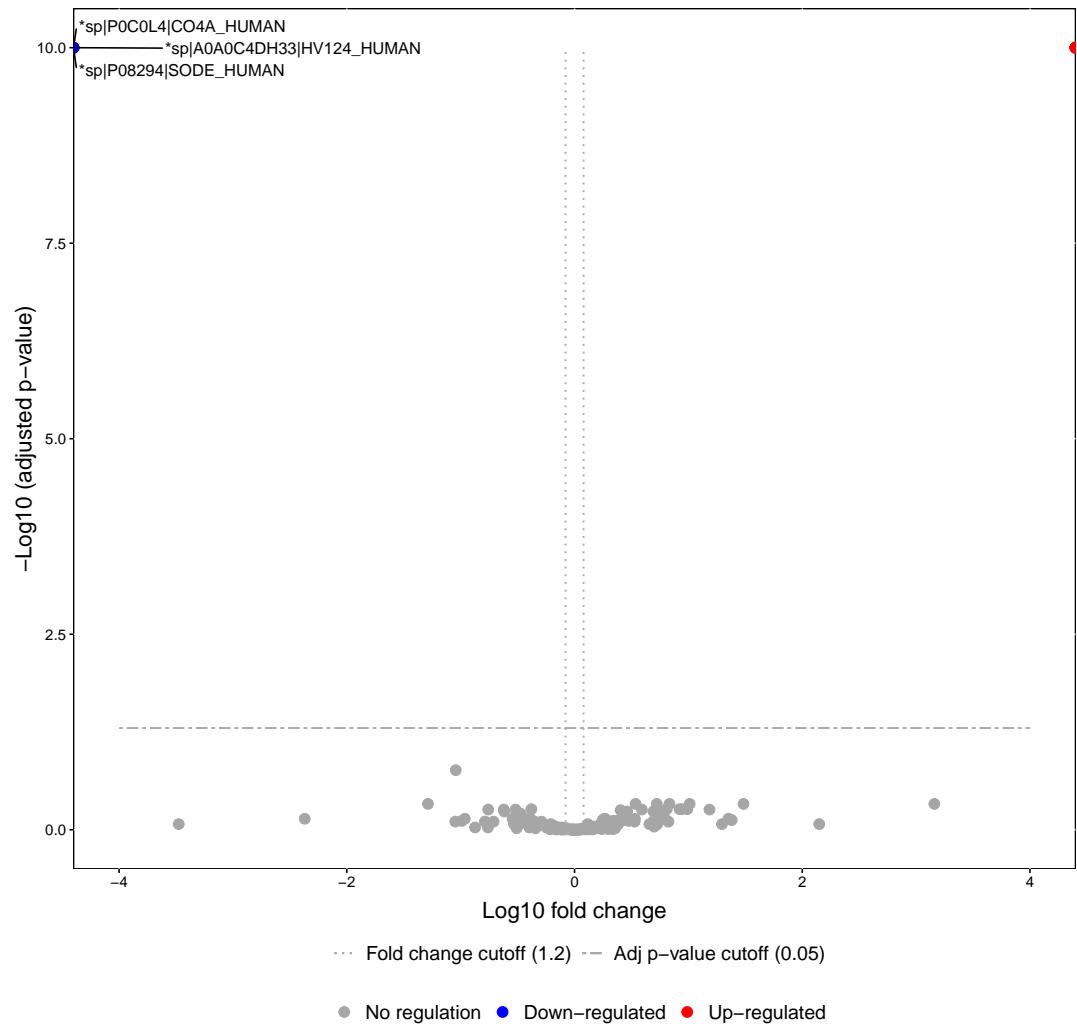


Figure S56. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute C Improvers

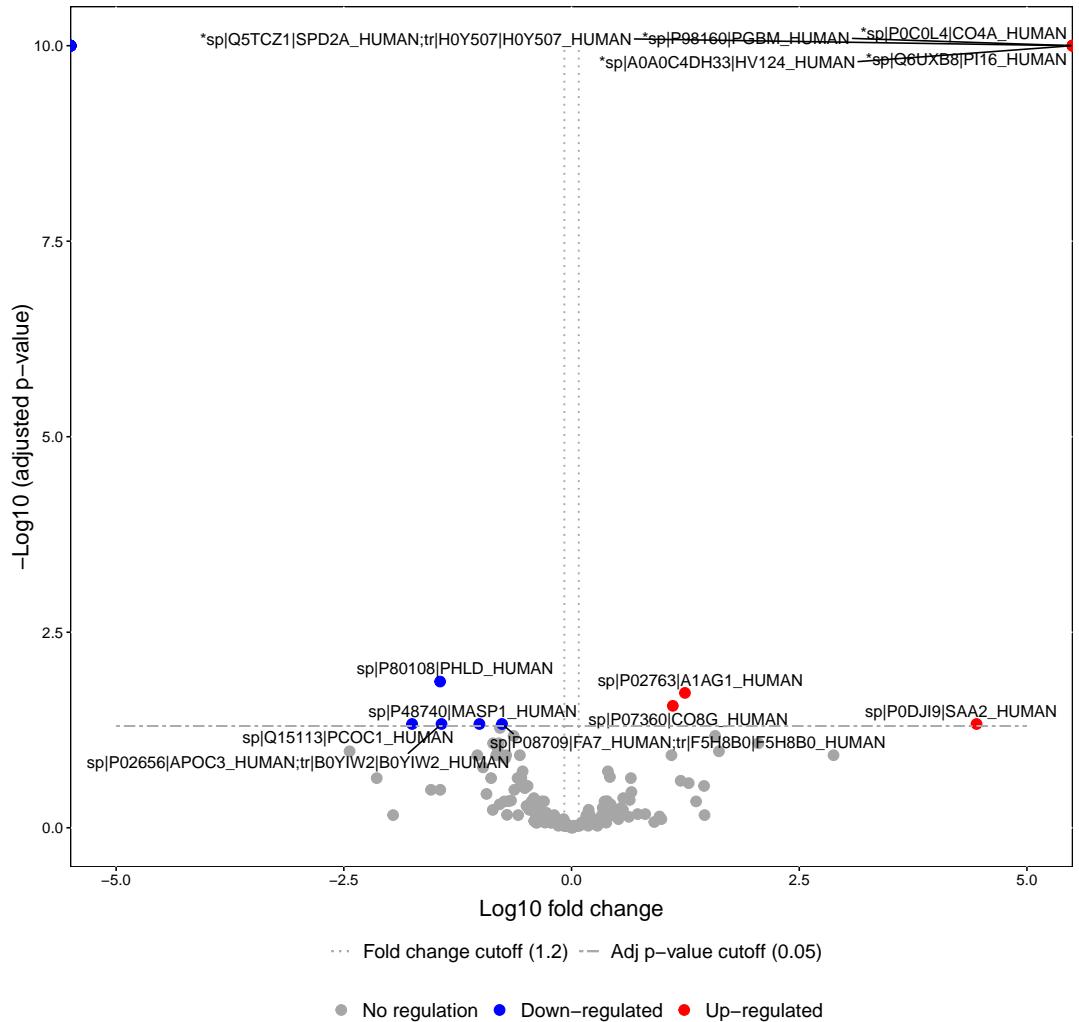


Figure S57. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute C Non-Improvers

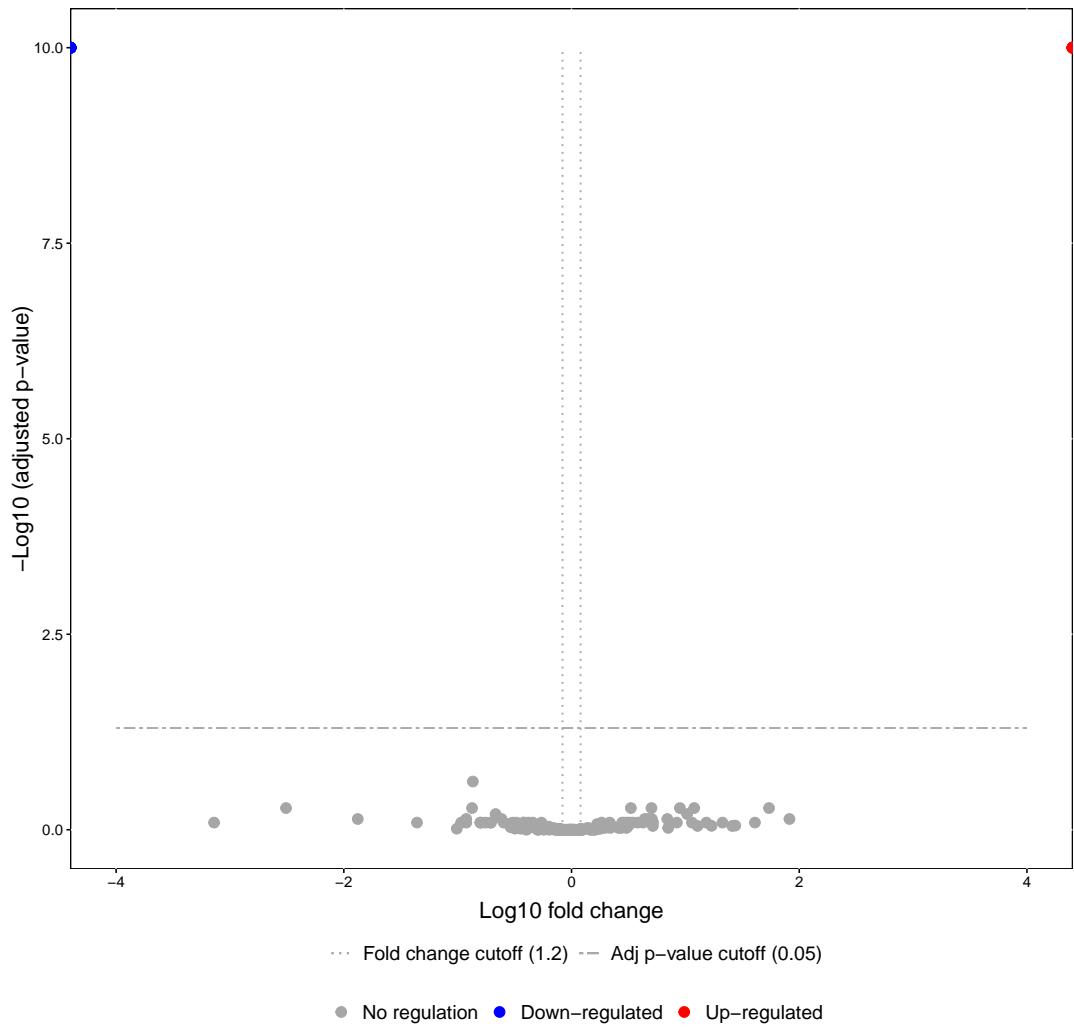


Figure S58. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

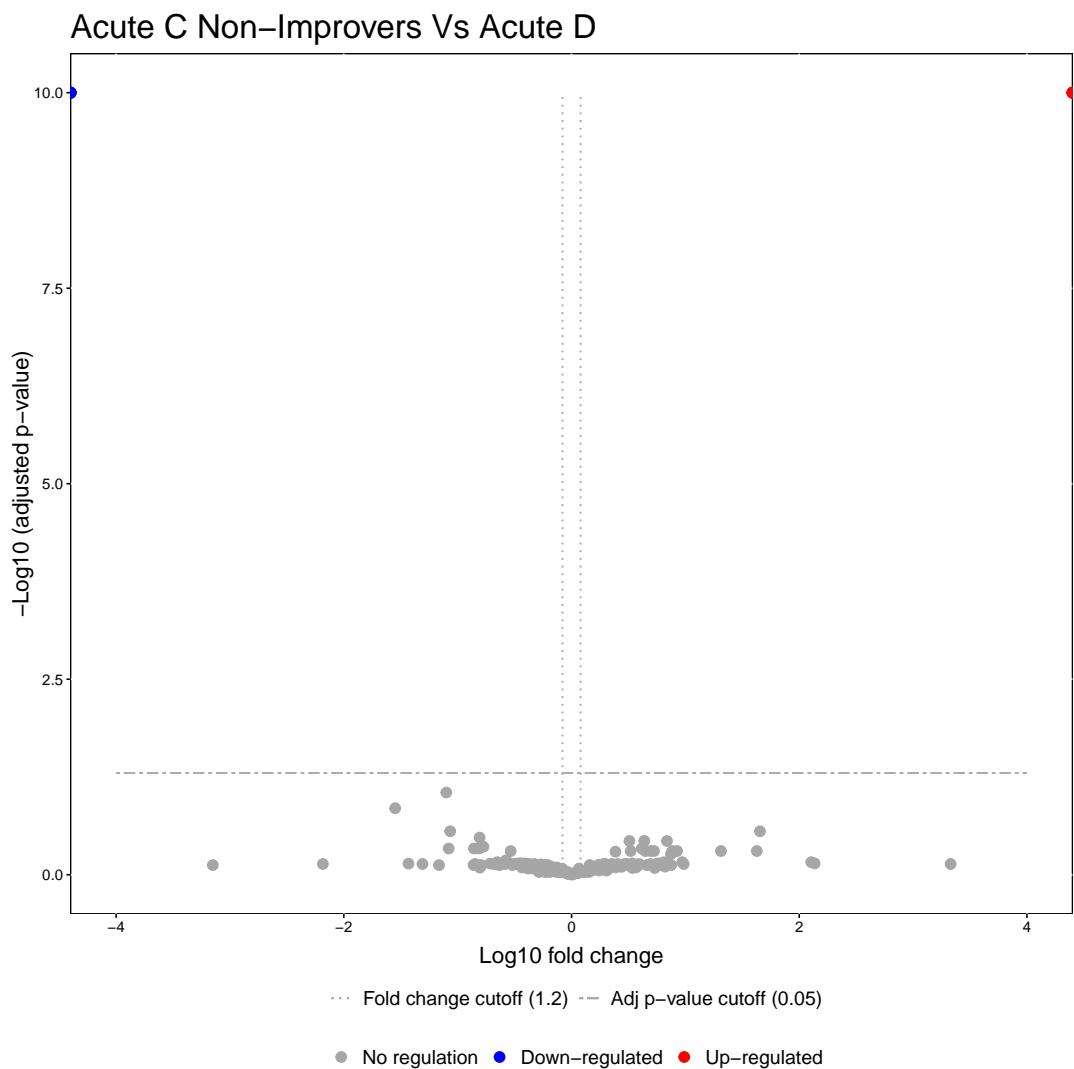


Figure S59. Volcano plot of log₁₀ fold change and log₁₀ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who did not experience an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

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