

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)

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

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

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

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

⁸¹ cally.(Brian K. Kwon et al. 2010)

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

⁸⁷ 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	-

⁸⁸ 3.1 Patients

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

⁹⁴ 3.2 Plasma collection and storage

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

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

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

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

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

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

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

126 **SECTION TO BE REWRITTEN**

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

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

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

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

136 To reduce the dynamic range of proteins, ProteoMiner™ beads (BioRad, Hemel Hempstead, UK)
137 were used.(boschetti_proteominer_2008?) Total protein was quantitated with a Pierce™ 660nm
138 Protein Assay (Thermo Fisher Scientific, Hemel Hempstead, UK), whereupon 5 mg of total protein
139 was applied to ProteoMiner™ beads, and processed as described previously.(Stoscheck 1987)

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

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

144 **REWRITE IN BRIEF**

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

155 **3.3.2 iTraq OpenMS analysis**

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

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

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

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

183 **3.3.3 Label free OpenMS analysis**

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

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

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

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

203 3.3.4 Network and pathway analysis

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

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

212 3.3.5 Enzyme-linked immunosorbent assays

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

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

227 **4 Results**

228 **4.1 Results**

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

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

235 **4.1.1 Comparing OpenMS and ProteinPilot**

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

240 **4.1.2 iTRAQ analyses**

241 **4.1.3 Differential protein abundances**

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

248 **4.1.4 Heatmaps**

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

Acute AIS C Improvers VS non-Improvers



Figure 1. Heatmap denoting the 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.

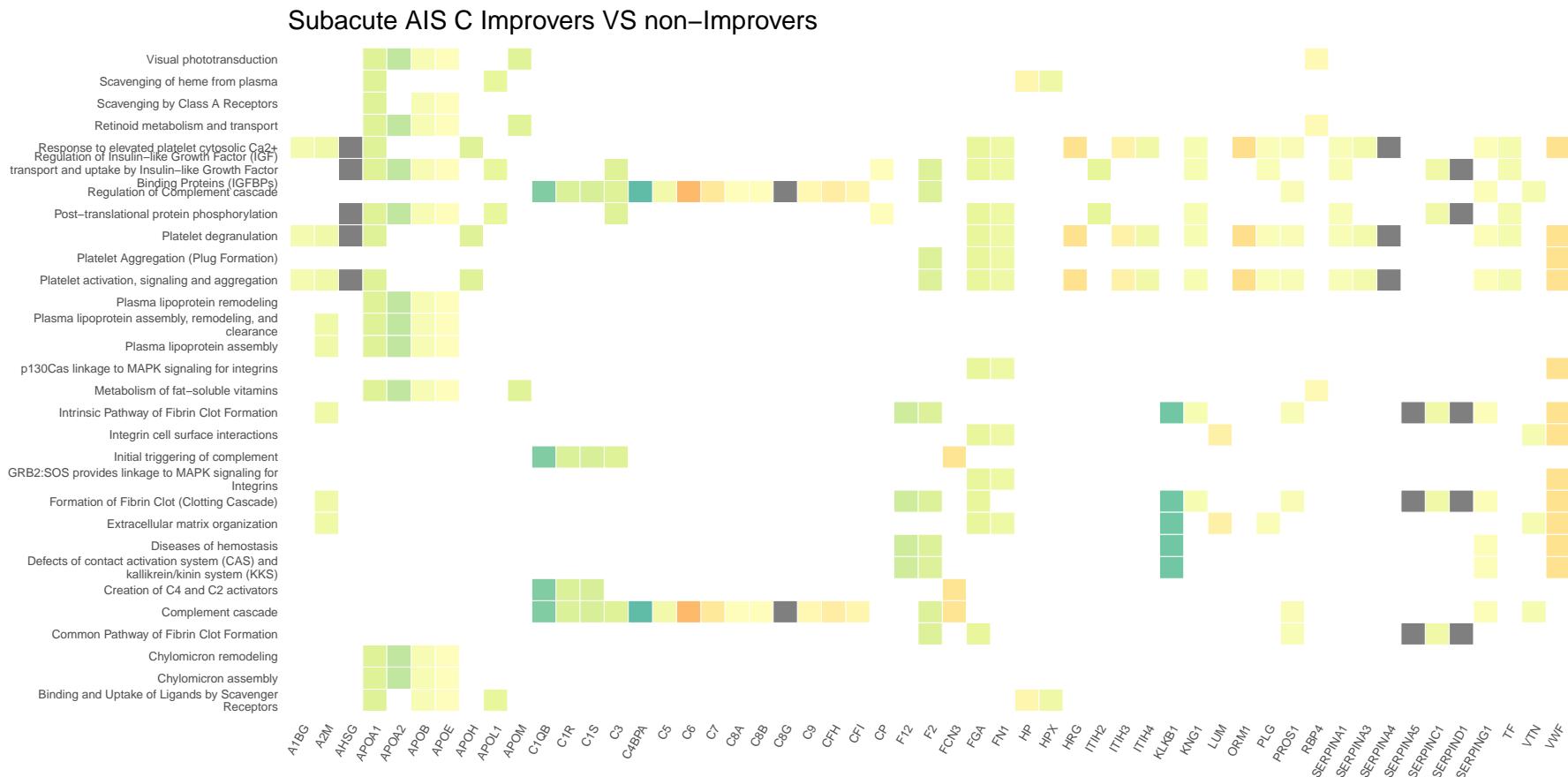


Figure 2. Heatmap denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

²⁵³ 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).

²⁵⁴ Please see appendix section 5.6 for additional plots.

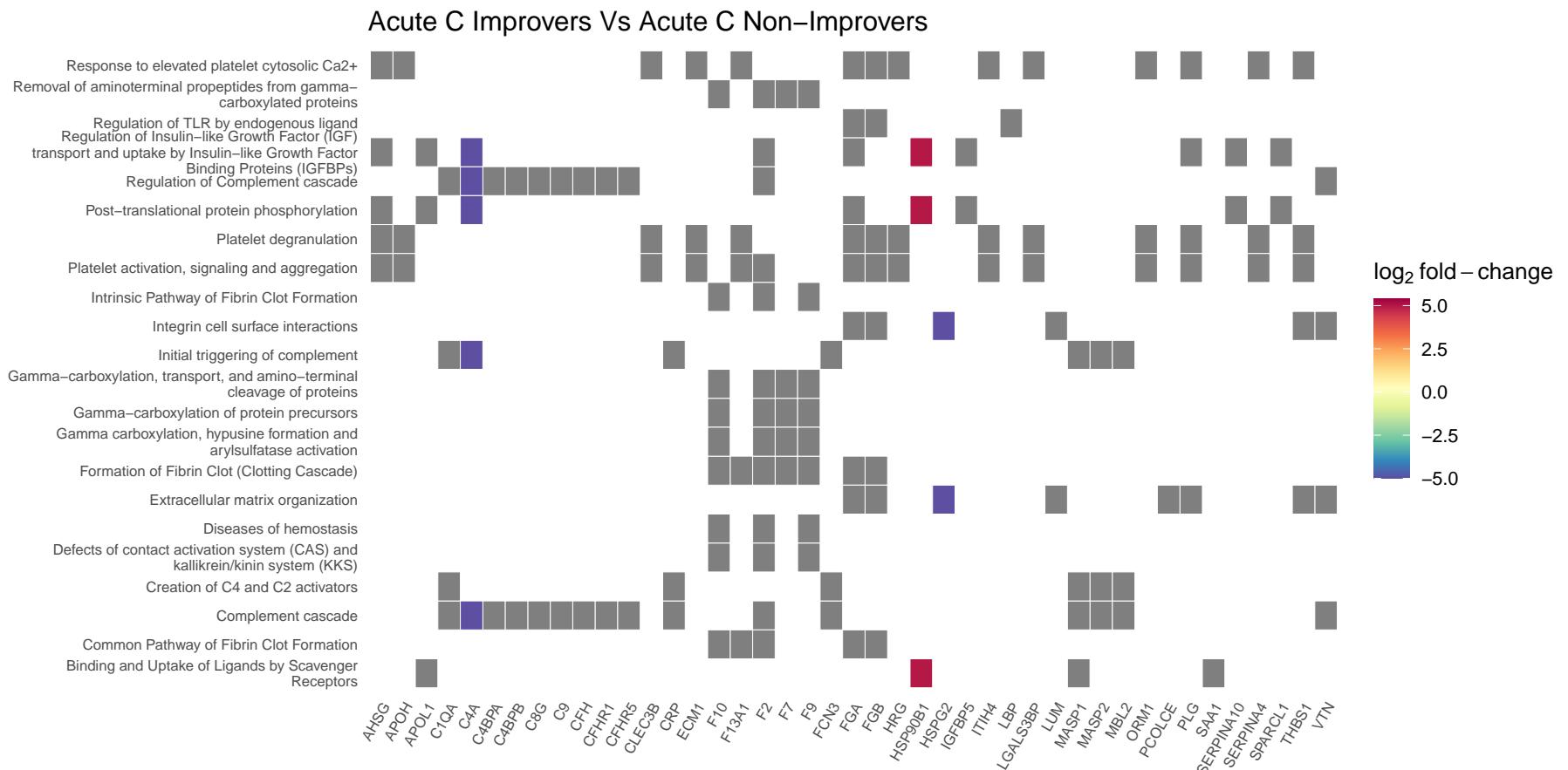


Figure 3. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins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

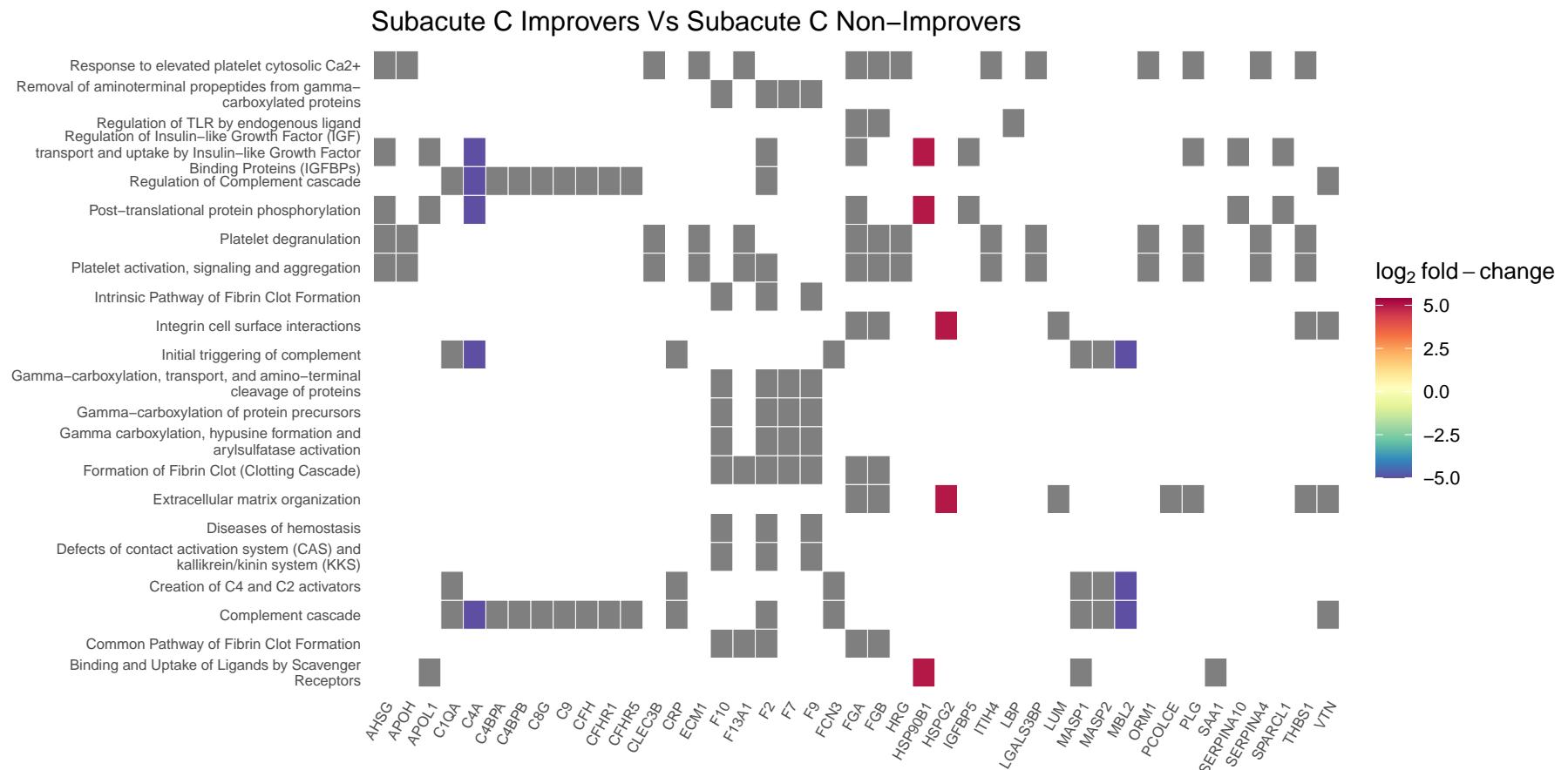


Figure 4. Heatmap denoting the \log_2 fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins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

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

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

Acute AIS C Improvers VS non-Improvers

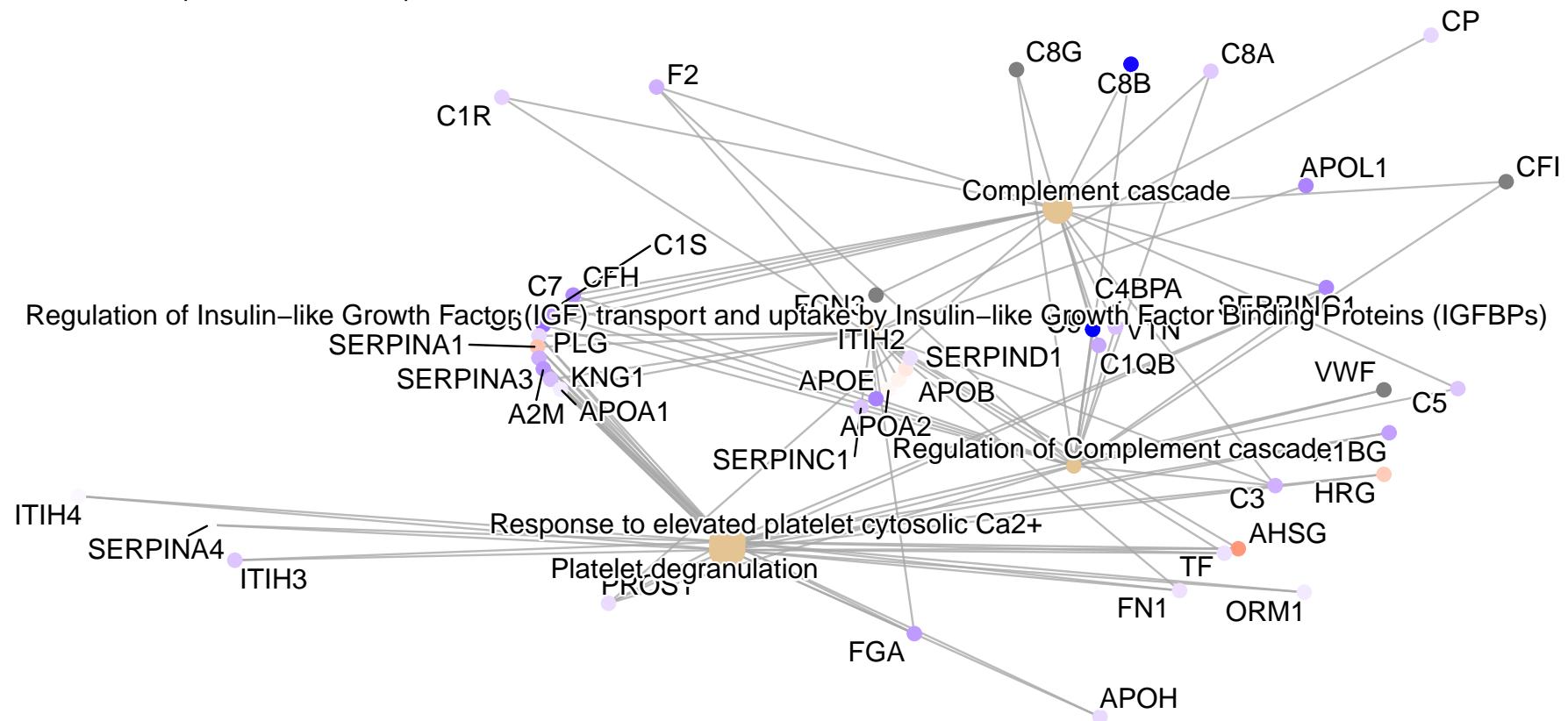


Figure 5. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute AIS C Improvers VS non-Improvers

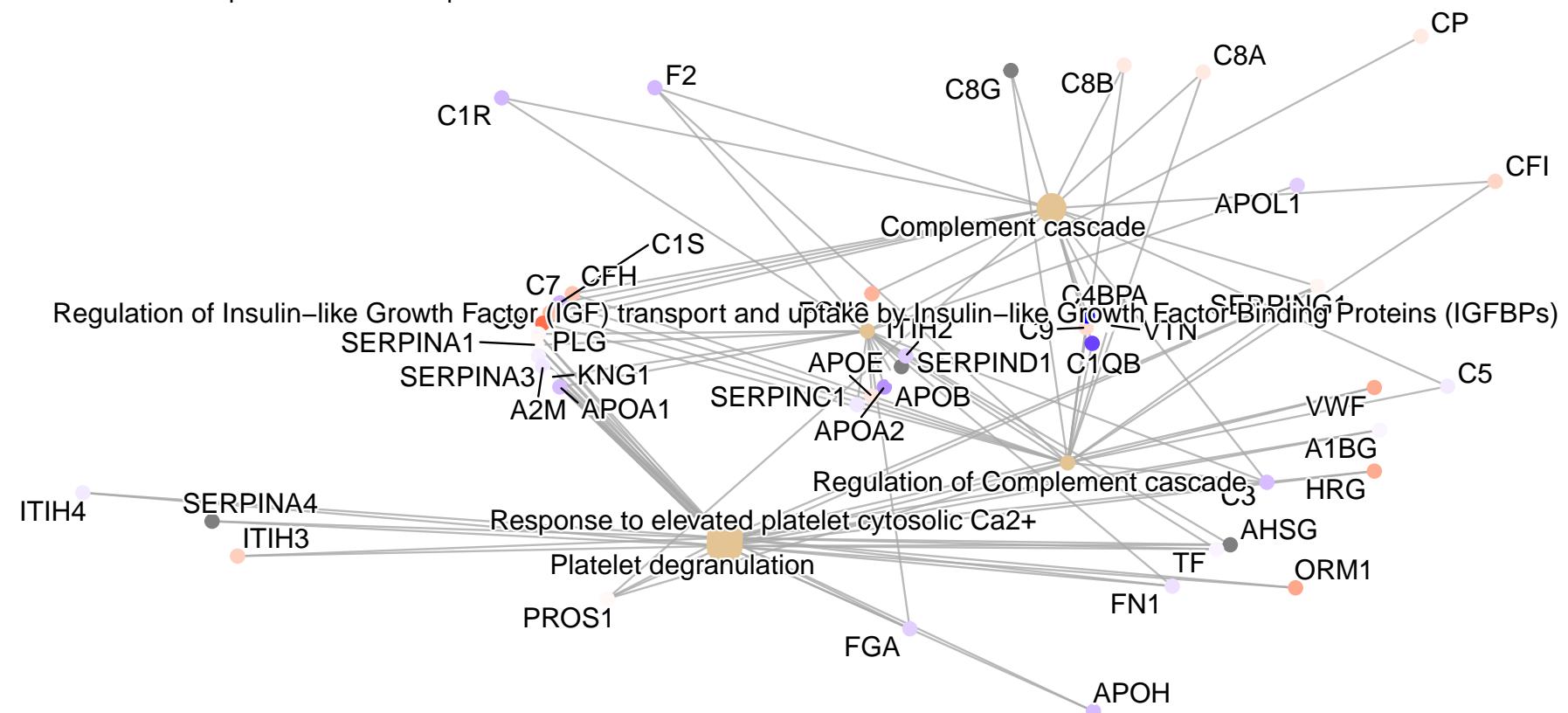


Figure 6. Network plot denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

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

Acute C Improvers Vs Acute C Non-Improvers

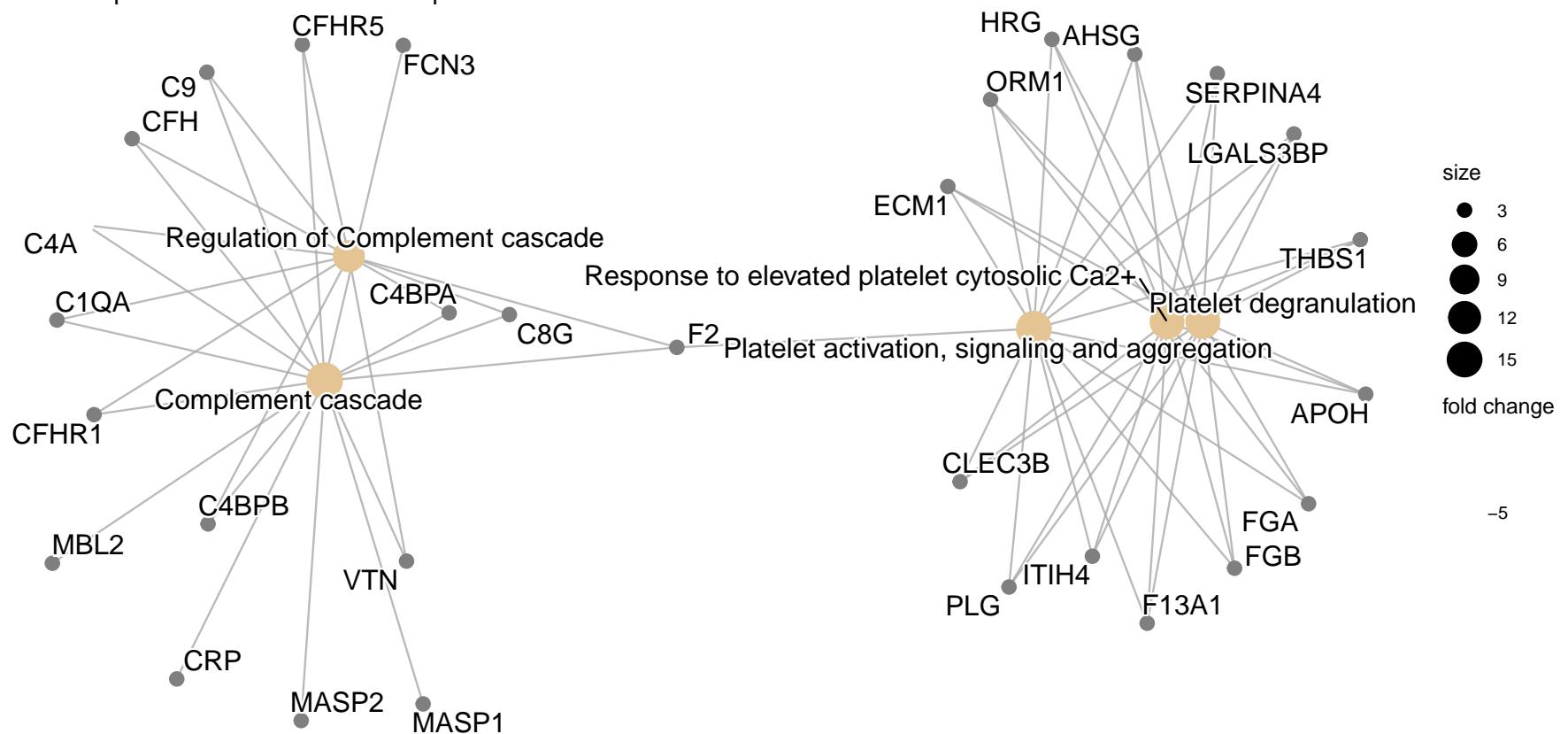


Figure 7. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute C Improvers Vs Subacute C Non-Improvers

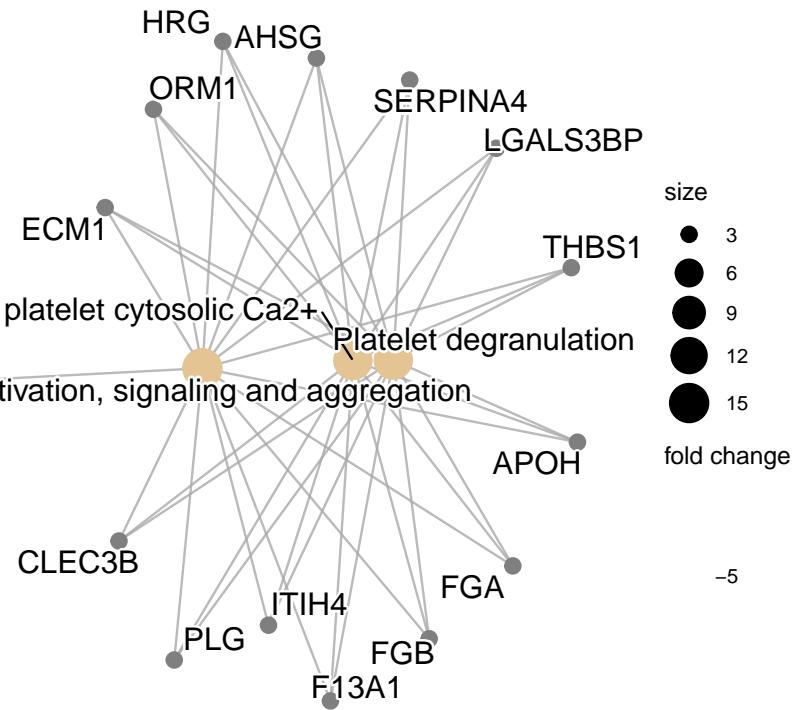
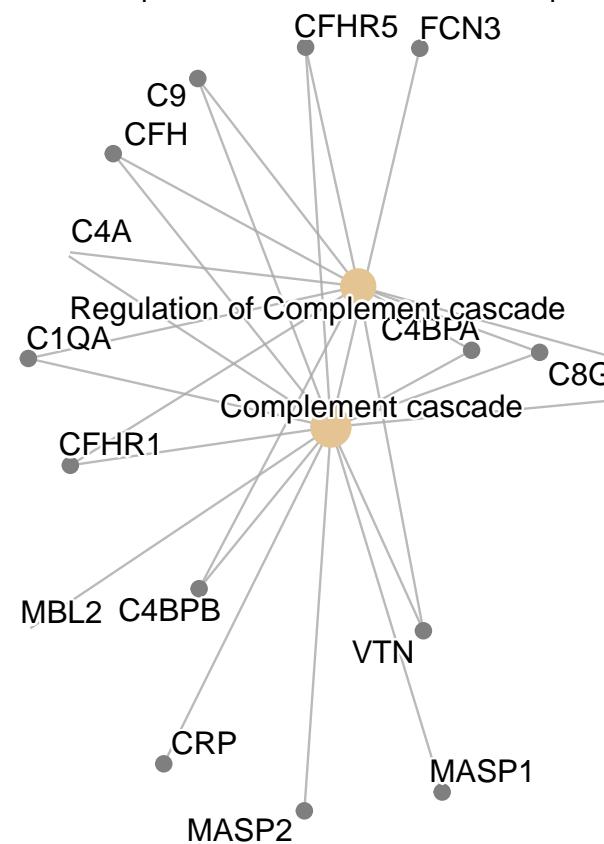


Figure 8. Network plot denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

266 **4.1.6 Pathway analysis of Differentially Abundant Proteins between AIS C improvers and**
 267 **non-improvers**

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

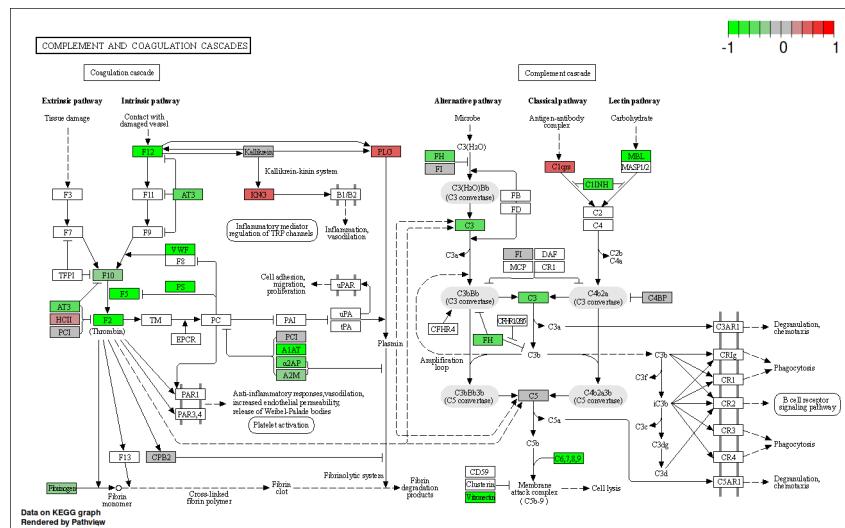


Figure 9. KEGG complement cascade pathway annotated with log₂ fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

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

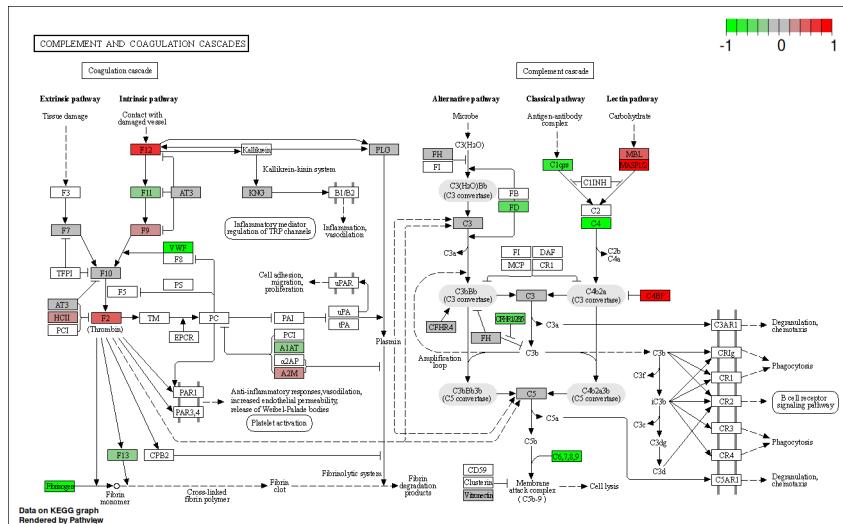


Figure 10. KEGG complement cascade pathway annotated with log₂ fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

4.1.7 Validation of Proteomic Data using ELISA

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

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

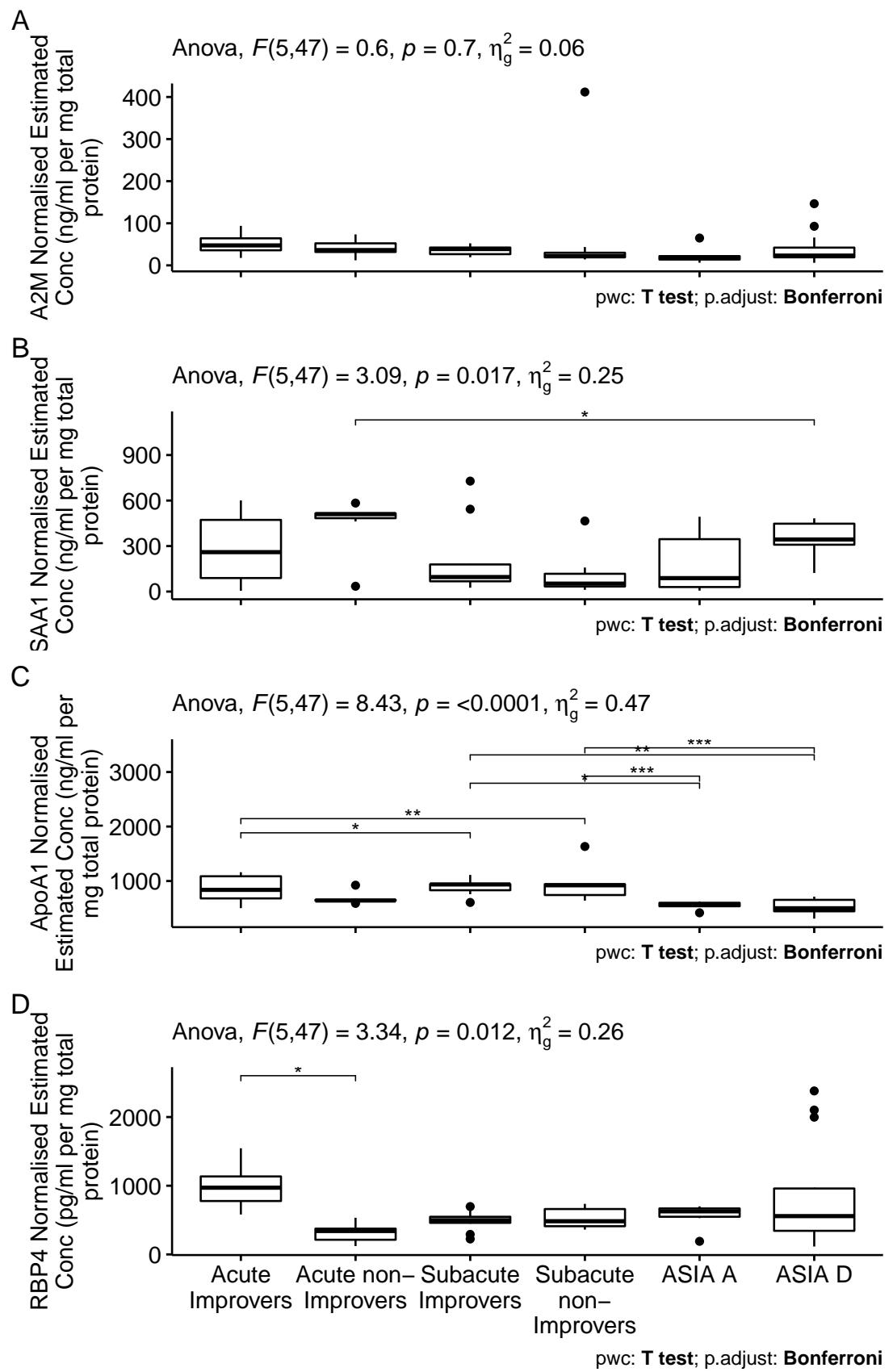


Figure 11. Normalised estimated concentration of α -2-macroglobulin (A), serum amyloid A1 (B), apolipoprotein A1 (C) and retinol binding protein 4 (D). Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled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

288 **4.1.8 STRINGdb plots**

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

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

295 **4.1.9 Volcano plots**

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

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

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

305 **5 Discussion**

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

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

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

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

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

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

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

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

368 5.1 COPIED FROM THESIS

369 5.2 iTRAQ discussion

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

374 **5.2.1 ProteinPilot and OpenMS**

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

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

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

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

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

406 **5.2.2 Proteins identified**

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

409 **5.2.2.1 Alpha-2-macroglobulin** A2M is an inhibitor of an unusually diverse array of proteinases
410 by a unique ‘trapping’ mechanism. The protein achieves this with a peptide stretch, called the
411 “bait region”, which contains specific cleavage sites for different proteinases. When a proteinase
412 cleaves the bait region, a conformational change is induced whereby A2M traps the proteinase.
413 The entrapped enzyme retains active against low molecular weight substrates, whereas activity
414 against high molecular weight substrates is greatly reduced. Following cleavage in the bait region, a
415 thioester bond is hydrolysed and mediates the covalent binding of the protein to the proteinase.(P.
416 K. Hall et al. 1981; Sottrup-Jensen et al. 1984) A2M is unique in it’s ability to inhibit virtually any
417 protease regardless of it’s specificity, origin or catalytic mechanism.(Khan 2004; Lin et al. 2012)

418 Alpha macroglobulins are an integral part of innate immunity and thus are evolutionarily con-
419 served.(Buresova et al. 2009) Alpha macroglobulins have significant primary sequence homology
420 with complement components C3, C4 and C5. The A2M-proteinase complex is cleared from circu-
421 lation primarily by receptors on hepatocytes.(Bond, Cianciolo, and Pizzo 2007; Travis and Salvesen
422 1983) The mammalian receptor for proteinase-reacted A2M is a low-density lipoprotein receptor
423 related protein.(Fujiyoshi et al. 2011; Larios and Marzolo 2012; Wyatt and Wilson 2013)

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

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

437 We observe A2M to be less abundant in AIS C improvers, within 2-weeks post injury and at 3-
438 months, albeit to a lesser extent (Tables S1 and S2). Similarly, A2M was more abundant in AIS As
439 relative to all groups, and whilst A2M was less abundant in AIS C improvers at 2-weeks compared
440 to AIS Ds, AIS C non-improvers had more A2M than AIS Ds. (Table S1). With less A2M there would
441 be more protease activity in these individuals, which may aid in the clearance of damaged tissue,
442 and in particular may lessen the development of an astroglial scar, thus aiding repair. However,
443 glial scaring is not entirely negative, the primary benefit it offerers is minimising the extent of sec-
444 ondary damage to neighbouring areas by functioning as a barrier around the injury site. Animal
445 studies have demonstrated that prevention of astroglial scar formation following CNS injury leads
446 to greater lesion size and poorer function outcomes.(Anderson et al. 2016; Wilhelmsson et al.
447 2006) Interestingly, a rat study using quantitative liquid chromatography-mass spectrometry with
448 CSF, found A2M to be more abundant in moderately injured animals compared to more severe
449 injuries.(Lubieniecka et al. 2011)

450 **5.2.2.2 Apolipoproteins** We found ApoA1, ApoA2, ApoH, ApoL1 and ApoM to be less abundant
451 in AIC improvers at both time points, whereas ApoA4 was more abundant at both time points (Ta-
452 bles S1 and S2). ApoA1 is the main protein component of high-density lipoproteins (HDL). Plasma
453 HDL include two main apolipoproteins, these being ApoA1 and ApoA2 (~70% and ~20% of total
454 HDL protein content respectively), but some HDL particles can also contain small amounts of other
455 apolipoproteins, including ApoA4, ApoA5, ApoC, ApoD, ApoE, ApoJ and ApoL. The primary function
456 of HDL in plasma is the transport of cholesterol, which can have dietary origins, but also be pro-
457 duced endogenously in the liver.

458 **5.2.2.2.1 HDL Activity** HDLs have serve a wide range of functions, including contributing to
459 anti-inflammatory activity. They can limit chemokine secretion from multiple cells types including
460 endothelial cells and monocytes.(Cockerill Gillian W. et al. 1995; Vorst et al. 2013; Bursill Christina
461 A. et al. 2010) Rats injected with ApoA1 showed significant reduction in expression of CCR2 and
462 CX₃CR1, the receptors for chemokines of the same name, which play a role in leukocyte migration.
463 (Bursill Christina A. et al. 2010)

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

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

475 ApoE was less abundant in AIS C improvers within 2-weeks and more abundant at 3-months, and
476 more abundant in more severe injury, such as AIS A relative to D or C and in AIS C relative to D
477 (Table S1). ApoE is primarily produced by hepatocytes in the liver, but second-most in the brain,
478 synthesised in and secreted by astrocytes, and has been found to an important determinant in
479 response to types of CNS injuries in both animal and human studies.(Teasdale et al. 1997; Poirier
480 1994) A key function of ApoE is as a ligand for the LDL receptor family of proteins, which mediate
481 trafficking of cholesterol to neurons, which is vital for axonal growth, and for synapse formation
482 and remodelling.(Xu, Finkelstein, and Adlard 2014) Additionally, ApoE is implicated in the clearance
483 of neuronal apoptotic bodies.(Elliott et al. 2007) In humans there are three variants/alleles of ApoE:
484 ApoE2, ApoE3 and ApoE4, which have a frequency of 8.4%, 77.9% and 13.7% globally.(C.-C. Liu et
485 al. 2013) The variant proteins differ by one or two amino acids and have been found to result in
486 substantial physiological alterations.(Mahley and Rall 2000; Jha et al. 2008) The presence of the
487 ApoE4 variant has been linked to worse outcomes in SCI and TBI.(Jha et al. 2008; C. Sun et al. 2011;
488 Smith et al. 2006; Friedman et al. 1999) More specifically, the SCI study reported significantly lower
489 change in the median AIS motor score compared the individuals without the ApoE4 allele during
490 rehabilitation.(Jha et al. 2008)

491 Prior *in vivo* rodent studies have demonstrated up-regulation of ApoE following SCI and TBI, though
492 ApoE is not observed in neurons of rodents under normal neuropathology, and they only posses
493 a single ApoE allele.(Iwata et al. 2005; Seitz et al. 2003; Mahley, Weisgraber, and Huang 2006) A
494 separate rodent study reported ApoE levels decreased for the first 3 days post-injury, and then in-
495 creased peak expression at 7 days post-injury, a similar pattern to our results.(X. Yang et al. 2018)
496 Furthermore, mouse studies have demonstrated replacement of ApoE in neurons with human
497 ApoE4 have impaired neurite outgrowth compared to replacement with ApoE2 or ApoE3, suggest-
498 ing ApoE4 interferes with neuroplasticity.(Seitz et al. 2003; White et al. 2001) The underlying mech-
499 anism/s by which ApoE and its alleles effect neuroplasticity is not currently known, but proposals
500 have been made. One possibility is reduced lipid transport from astrocytes to neurons, poten-
501 tially impeding the membrane generation required to support axon growth or dendrite sprouting.
502 ApoE has anti-oxidant properties, so others have suggested impaired anti-oxidant activity may con-
503 tribute. ApoE4 has been found to be both secreted less than ApoE2 or ApoE3, and to have inferior
504 anti-oxidant abilities, lending some credence to this idea.(Mishra and Brinton 2018; Miyata and
505 Smith 1996) Knowing this, whilst ApoE may make for a useful biomarker for SCI, it will be impor-
506 tant that particular variants of ApoE a given patient has could be just as important, if not more so,
507 than simple abundance.

508 **5.2.2.3 Serum Amyloid A1** SAA1 was less abundant in AIS C improvers at 2-weeks relative to
509 non-improvers, but more abundance in plasma at 3-months (Table S1. SAA1 was also more abun-
510 dant in AIS A relative to less severe injuries, and in AIS Cs relative to Ds (Table S1. SAA1 is a major
511 acute-phase protein mainly produced in the liver by hepatocytes in response to infection, tissue

512 injury and malignancy.(L. Sun and Ye 2016) SAA1 is a precursor of amyloid A (AA), the aberrant
513 deposition of which leads to inflammatory amyloidosis.(Tape et al. 1988) There are 5 known SAA1
514 variants, though currently, no indication of substantial functional differences have been identi-
515 fied.(J. Lu et al. 2014) However, some alleles have been linked to disease, including increased amy-
516 loidogenesis and tumour suppression.[van der Hilst et al. (2008); lung_saa1_2015]

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

529 SAA1 can both induce anti-inflammatory interleukin 10 (IL-10)-secreting neutrophils, but also pro-
530 motes the interaction of invariant natural killer T cells with those neutrophils, which limits their
531 suppressive activity by diminishing the production of IL-10 and enhancing the production of IL-12,
532 indicating that SAA1 can have both pro- and anti-inflammatory effects.(Santo et al. 2010) There has
533 however been conflicting results reported of SAAs cytokine induction abilities, and some studies
534 have suggested that recombinant human SAA1 provided by some vendors may have additional
535 cytokine-inducing actiity due the altered amino acid sequence.(M.-H. Kim et al. 2013)

536 Macrophages are a major source of SAA in inflammatory tissues, and elevated SAA production has
537 been observed in rheumatoid arthritis, Crohn's disease, Type 2 diabetes and atherosclerosis.(Marzi
538 et al. 2013; Dong et al. 2011; Vallon et al. 2001; C, F, and B 1997; Meek, Urieli-Shoval, and Benditt
539 1994) SAA binding to HDL was reported to increase affinity for macrophages whilst decreasing
540 affinity for hepatocytes.(R. Kisilevsky and Subrahmanyam 1992) This change is thought to favour
541 the removal of cholesterol from site of inflammation.(R. Kisilevsky 1991) SAA inhibits the binding of
542 the scavenger receptor SR-BI and cholesterol efflux is enhanced in a SR-BI-dependent manner.(Cai
543 et al. 2005; van der Westhuyzen et al. 2005) It has been suggested that the SR-BI-mediated re-
544 uptake of cholesterol underpins the role of SAA in cholesterol recycling during tissue repair, where
545 a great deal of cholesterol is required.(Robert Kisilevsky and Manley 2012)

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

556 **5.2.2.4 Retinol-binding protein 4 (RBP4)** In plasma within 2-weeks post-injury, RBP4 was less
557 abundant in AIS C improvers relative to AIS D and A, and more abundant in AIS C non-improvers
558 again, relative to AIS D and A (Table S1. Similarly, AIS A plasma had more RBP4 compared to AIS

559 D, and AIS C improvers were also more abundant in RBP4 compared to non-improvers at both
560 2-weeks and 3-months post-injury (Table S1).

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

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

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

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

590 5.2.3 Metabolism and SCI

591 **5.2.3.1 Acute phase response** The bodies first response to injury or infections, including SCI,
592 is often referred to as the “acute phase response” (APR), which is non-specific, innate reaction
593 that precedes more specific and situational immune reactions.(Gordon and Koj 1985; Gruys et
594 al. 2005) This systemic response is largely coordinated by factors released from the liver, but the
595 APRs effects extend to multiple peripheral organs including the kidneys, lungs and spleen.(Bao et
596 al. 2012; S. J. Campbell, Zahid, et al. 2008; Fleming et al. 2012; Gris, Hamilton, and Weaver 2008)
597 This hepatic response is typically transient and quickly fades, but prolonged liver inflammation and
598 pathology has been observed in rodent SCI models.(Goodus et al. 2018; Sauerbeck et al. 2014)

599 Basic liver functions are chronically impaired by SCI, including metabolising carbohydrates, fats
600 and proteins, storage of minerals vitamins and glycogen and filtering blood from the digestive
601 tract.(García-López et al. 2007; DeLeve 2007; Farkas and Gater 2018; Chow et al. 2012; Sauerbeck
602 et al. 2014) This is likely related to the elevated incidence of metabolic disease in the SCI cohort,
603 including insulin resistance, impaired glucose tolerance and cardiovascular disease.(Bauman and
604 Spungen 2001; Maruyama et al. 2008; Lee et al. 2004; J. Myers, Lee, and Kiratli 2007) Long-term

survival is noticeably lower relative to the general population and, whilst mortality in the first 2 year following SCI has decreased in recent decades, long-term survival has not.(Strauss et al. 2006; Shavelle et al. 2015) More recently, a longitudinal study found SCI patients had a significantly higher incidence of acute pancreatitis relative to a matched healthy cohort.(Ho, Yeh, and Pan 2021)

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

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

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

5.2.4 Microbiome & SCI

Circulating factors from the injury site are not the only potential driver of hepatic inflammation. Within 24 hours post-SCI in rodents tight junctions between epithelial cells become more permeable, thus allowing gut bacteria and the endotoxins they can produce to enter the bloodstream.(J. Liu et al. 2004) This will reach the liver through the portal vein where Kupffer cells function as a "first line of defence".(Jenne and Kubes 2013; M. L. Balmer et al. 2014) It has been proposed that elevated LPS+ endotoxins caused by the post-SCI "leaky gut" causes acute liver inflammation by overloading hepatic filtrations capacity, allowing microbes to bypass the liver and elicit systemic inflammation.(J. Liu et al. 2004; O'Connor et al. 2018) The binding of LPS to Kupffer cells results in the production of a range of growth factors, including TNF- α , multiple interleukins and reactive oxygen species (ROS), stimulating bone-marrow-derived monocytes and neutrophils to infiltrate the liver.(S. A. Myers et al. 2019; Milosevic et al. 2019; Kazankov et al. 2019) A rodent study found transcription factors for tight junctions down-regulated following SCI, and that application of probiotics improved neurological outcomes.(Kigerl, Mostacada, and Popovich 2018; Kigerl et al. 2016) Human studies of the microbiome post-SCI have also demonstrated dysbiosis, both chronically and more acutely post-injury.(Zhang et al. 2018; Gungor et al. 2016; Bazzocchi et al. 2021)

5.2.5 Drivers of liver steatosis

Steatosis, the abnormal retention of lipids within cells or organs, most commonly associated with the liver, has been observed to increase in rodents during the first week post-injury.(Sauerbeck

651 et al. 2014) The liver takes up circulating fatty acids, and when levels exceed the oxidative and
652 secretory limits of the liver, hepatocytes store the excess as triglycerides.(Diraison and Beylot 1998)
653 Adipose tissue lipolysis during elevated sympathetic activity leading to spikes in circulating fatty
654 acids has been reported in human subjects following SCI.(Karlsson 1999)

655 *De novo* lipogenesis occurring within the liver can also drive hepatic steatosis.(Lavoie and Gau-
656 thier 2006) Ceramides are lipid signalling molecules and regulators of apoptosis and inflamma-
657 tion; they can contribute to insulin resistance, oxidative stress and inflammation-induce liver adi-
658 posity through sustained Toll-like-receptor(TRL)-4 activation.(Schilling et al. 2013; Bhargava and
659 Lee 2012; Pagadala et al. 2012) If released into the circulatory system, ceramides can cause CNS
660 toxicity, including oxidative damage and changes to the aggregation of proteins associated with
661 diseases such as Parkinson's, Huntington's and Alzheimer's.(Pagadala et al. 2012; Vidaurre et al.
662 2014; Czubowicz et al. 2019) Mature and precursors of hepatic ceramides and enzymes which
663 contribute to ceramide synthesis are elevated by 1 day post-injury.(Sauerbeck et al. 2014) Endo-
664 toxins can also stimulate the synthesis of ceramides and so the aforementioned "leaky gut" may
665 also contribute to this elevation.(Chang et al. 2011) Ceramide synthesis and lipogenesis genes are
666 also stimulated by TNF- α , which, as touched on in the general introduction (??), has been found
667 to be elevated post-SCI, and associated with differential neurological recovery.(Davies, Hayes, and
668 Dekaban 2007; Hasturk et al. 2009; Biglari et al. 2015; Sauerbeck et al. 2014; Bikman 2012)

669 5.2.6 Chronic liver inflammation in SCI

670 The hepatic APR and associated inflammation that typically follows bodily trauma, subsequently
671 rapidly subsides, whereas post-SCI this hepatic inflammation persists chronically. This chronic
672 phase may be due in part to long-term changes in intestinal permeability via fewer tight junc-
673 tions in intestinal epithelial cells, resulting in gut dysbiosis.(Milosevic et al. 2019; O'Connor et al.
674 2018; Kigerl, Mostacada, and Popovich 2018; Kigerl et al. 2016) Bacterial translocation and gut
675 dysbiosis can be the result of non-mechanical intestinal obstruction, impaired intestinal motility
676 and systemic immune suppression, all of which are potential complications of SCI.(Balzan et al.
677 2007) Specifically, butyrate-producing bacteria have been found to be reduced in SCI relative to a
678 healthy cohort.(Gungor et al. 2016) Butyrate is known to modulate epithelial differentiation and
679 cell growth, and suppress macrophages, including CNS inflammation, thus the reduction in bu-
680 tyrate from bacteria may contribute to recovery post-SCI, though links to the liver specifically have
681 not yet been studied.(H.J. Kim et al. 2007; Arpaia et al. 2013; Park et al. 2005; P. S. Chen et al. 2007)

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

686 5.2.7 Longitudinal metabolic health

687 Prior work has found at least 25% of acute SCI patients to be obese, which is well known to induce
688 low-level systemic inflammation, and that this cohort has significantly worse outcomes compared
689 to non-obese SCI patients (Stenson et al. 2011). Alcohol abuse has also been associated with
690 poorer SCI neurological outcomes (Elliot et al. 2002). Furthermore, advancing age is associated with
691 increased liver inflammation and the SCI population has followed the general populations ageing
692 trend (Bertolotti et al. 2014; Y. Chen, He, and DeVivo 2016). Taken together, it is not unreasonable
693 to assume that a large number of SCI patients may have pre-existing liver inflammation at injury.
694 This may be an important differentiator that contributes to the degree of neurological recovery
695 a given patient may experience. Future experiments investigating neurological outcomes of SCI
696 may benefit from establishing parameters of metabolic health, including the composition of the

697 microbiome, as close to injury as possible, and potentially monitoring changes in these parameters
698 longitudinally.

699 **5.2.8 Validation of results**

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

711 **5.2.9 Conclusion**

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

725 **5.3 thesis label-free discussion**

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

731 **5.3.1 Proteins identified**

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

736 **5.3.1.0.1 Peroxiredoxins** Peroxiredoxins are a large and highly conserved family of enzymes
737 that reduce peroxides. Peroxiredoxin 2 (PRX-2) is highly abundant in RBCs and intracellularly serves
738 as an important anti-oxidant role in various cell types, including neurons.(Low, Hampton, and

739 Winterbourn 2008) By contrast, extracellular PRX-2 has been suggested to act as an inflamma-
740 tory DAMP, leading microglia and macrophages to release a plethora of pro-inflammatory fac-
741 tors.(Salzano et al. 2014; Garcia-Bonilla and Iadecola 2012; Shichita et al. 2012) An *in vitro* primary
742 neurons and microglia co-culture study reported PRX-2 activating microglia via TLR-4, potentially
743 leading to neuronal apoptosis.(Y. Lu et al. 2018) A mouse study found over-expression of PRX-2 at-
744 tenuated oxidative stress and neuronal apoptosis following subarachnoid haemorrhage.(Y. Lu et al.
745 2019) Over-expression of PRX-2 is speculated to protect again ischaemic neuronal injury by mod-
746 ulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase (ASK) 1 signalling com-
747 plex.(Gan et al. 2012) Several molecular chaperones can interact with ASK1, including thioredoxin
748 and TNF receptor-associated factor 6.(Matsuzawa et al. 2005) The dissociation of the thioredoxin-
749 ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free radicals, whereupon its an-
750 tioxidantive activity is reduced. This inactivation can be reversed by the thioredoxin-thioredoxin
751 reductase system, whereby oxidised PRX-2 can regain its activity by reducing thioredoxin, leading
752 to the dissociation of the thioredoxin-ASK1 complex.(Rhee and Woo 2011) Additionally, oxidised
753 PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding domain on ASK1.(S. Y. Kim,
754 Kim, and Lee 2008)

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

769 **5.3.1.1 Neuroinflammation post-SCI** The neuro-inflammatory response begins immediately
770 post-trauma, and involves a complex series of events that can persist well into the chronic phase.
771 The sudden emergence of necrotic cell debris and associated DAMPs lead surviving CNS-resident
772 cells to produce cytokines, complement factors and ROS. Within minutes CNS cells at the lesion site
773 have been found to secrete several pro-inflammatory mediators, including TNF- α and interleukins,
774 in both rodent models and human patients with SCI.(Pineau and Lacroix 2006; Chandrasekar et al.
775 2017; Dalgard et al. 2012; Bastien et al. 2015) The resulting inflammatory response occurs in
776 parallel to the mechanical destruction of the blood-spinal cord barrier, and the development of
777 tissue oedema and ischaemia combine to propagate damage to parts of the cord spared by the
778 initial trauma.(Maikos and Shreiber 2007; Ahuja et al. 2017)

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

786 The following hours and days post-injury are characterised by a substantive complement sys-

787 tem activation and sequential leukocyte migration from the periphery into the injured neural
788 parenchyma.(Brennan et al. 2015; S. L. Peterson and Anderson 2014; Qiao et al. 2006) Curiously,
789 though the breakdown of the BSCB would presumably allow unrestricted access of circulating
790 leukocytes into the injured cord segment, recruitment of these cells remains a highly controlled
791 process.(Beck et al. 2010; Brennan et al. 2019) A mouse study reported lymphocytes, which
792 account for approximately 80% of circulating leukocytes, only enter the cord in substantial
793 numbers at least several weeks to months post-injury.(Beck et al. 2010) Early infiltrate is instead
794 largely comprised of myeloid cells, predominantly neutrophils, which are a minority of circulating
795 cells but are the swiftest peripheral responders to SCI, with studies detecting them at the lesion
796 site within 4 hours of injury.(Wright et al. 2010) Neutrophil numbers have been reported to peak
797 at 1 day post-trauma, but also to remain at the site for a minimum of 42 days post-injury.(Okada
798 2016; Kigerl, McGaughy, and Popovich 2006)

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

816 Moving forward in the SCI pathology, newly proliferated and recruited microglia begin ac-
817 tively phagocytosing necrotic cell debris, and begin accumulating around the lesion epicen-
818 tre.(Greenhalgh and David 2014; Bellver-Landete et al. 2019; Pineau and Lacroix 2006) The
819 presence of microglia appears to be vital, particularly during the first week post-SCI, as depletion
820 via the colony stimulating factor-1 inhibitor PLX5622 has been linked to substantially worsened
821 functional outcomes.(Bellver-Landete et al. 2019; Brennan et al. 2018) Relatedly, another
822 mouse SCI model study found early enhancement of microglial activation can reduce secondary
823 pathology.(Stirling et al. 2014)

824 Circulating inflammatory monocytes are also recruited during the first days post-trauma. Adop-
825 tive transfer experiments have shown recruitment to pick up at approximately 3 days post-injury,
826 and peak at 7 days.(Blomster et al. 2013) Whilst monocyte turnover at the lesion appears to be
827 high, infiltrating monocyte-derived macrophages remain at the site of weeks to months post-
828 trauma.(Blomster et al. 2013; Shechter et al. 2009) Interestingly, the timing of monocyte recruit-
829 ment appears to be delayed relative to non-neurological tissue injury. For instance, monocytes
830 are reported to be rapidly recruited to the heart following a myocardial infarction, as early as 1 day
831 post-injury, and their numbers return to baseline by roughly 16 days post-injury.(Nahrendorf et al.
832 2007)

833 Owing to the diversity of monocyte subsets and macrophage phenotypes, a complete un-
834 derstanding of their role with respect to SCI pathology is still lacking, and requires under-
835 active research.(David and Kroner 2011) Some polarisation states associated with recruited

836 macrophages are thought to be implicated in propagating secondary injury via fibrotic scar
837 formation and demyelination of axons.(Kigerl et al. 2009; Popovich et al. 1999; Zhu et al. 2015)
838 Similarly, several studies have reported a reduction in infiltration of monocytes/macrophages
839 is associated with better SCI outcomes.(Kigerl et al. 2009; Zhu et al. 2015; Horn et al. 2008)
840 Conversely, others have found depletion o circulating monocytes/macrophages significantly
841 increased lesion size and results in worse function outcome, with restoration of blood monocyte
842 numbers attenuating this phenotype.(Shechter et al. 2009) More recent *in vitro* studies suggested
843 blood-derived macrophages can suppress microglial phagocytosis without reducing microglial
844 proliferation and extension of processes.(Greenhalgh and David 2014; Greenhalgh et al. 2018)
845 This literature represents and ongoing controversy over the role of monocytes/macrophages in
846 relation to recovery post-SCI. Importantly, many of these studies are based on somewhat crude
847 depletion of cell types, with little discrimination paid toward any potential subpopulations and/or
848 cell polarisation status. Given the shear complexity of the pathology at play, more nuanced
849 approaches will likely be needed in future studies to paint a more complete picture.

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

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

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

876 There are fewer studies that attempt to elucidate the underlying mechanisms driving this non-
877 resolving inflammatory response in the chronic phase of SCI. One study suggested communica-
878 tion with infiltrating monocytes suppresses chronic microglial activation and inflammation after
879 SCI.(Greenhalgh et al. 2018) Interruption of this communication was linked to worsened func-
880 tion outcomes, implying the initial microglial response to trauma may be beneficial, their pro-
881 tracted activation can eventually become detrimental.(Bellver-Landete et al. 2019; Greenhalgh et
882 al. 2018) Furthermore, a rodent model study of chronic SCI, found use of the anti-inflammatory
883 drug licoferone, applied daily for 1 month at 8 months post-injury, observed some improvement
884 to metabolic functions, but no benefit to locomotor function.(Dulin et al. 2013) To summarise, un-

885 derstanding of persistent inflammation during the chronic phase of SCI is lacking, and particularly
886 complicated by the plateaus in locomotive recovery that typically occurs well before the chronic
887 SCI phase is reached. Thus, there is a need for further studies to uncover the role of the various
888 immune cell populations with respect to ongoing neurological dysfunction and pathology during
889 the chronic phase of SCI.

890 **5.3.1.1.1 Intravenous immunoglobulin** Intravenous immunoglobulin (IVIG) is increasingly
891 used as an immunomodulatory strategy for managing acute neurological conditions, including
892 neurotrauma. Originally developed as an antibody replacement therapy for immunodeficiency
893 disorders, IVIG is a product comprised primarily of immunoglobulin G (IgG) taken from the blood
894 plasma of healthy donors.(Bayry, Negi, and Kaveri 2011; Schwab and Nimmerjahn 2013) IVIG
895 therapy was found to increase platelet number in idiopathic thrombocytopenic purpura (ITP)
896 patients, which lead to an interest in using it as an immunomodulatory therapy.(Imbach et al.
897 1981) Its potent effects and limited side effects have lead high-dose IVIG therapy to be commonly
898 used in a plethora of inflammatory and autoimmune disorders, including ITP, arthritis, Kawasaki's
899 syndrome and Guillain-Barré syndrome.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et
900 al. 1998)

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

914 In TBI mouse models, animals treated with IVIG were shown to have improved neurobehavioural
915 outcomes, and a reduction in neuronal degeneration both acutely and chronically, relative to
916 vehicle-treated controls in rotarod and Morris water maze experiments.(Jeong et al. 2014) Further
917 mouse studies using cerebral artery occlusion, a model of stroke, reported high-dose IVIG signif-
918 icantly reduced infarct volumes, neurological impairment and mortality rates.(Arumugam et al.
919 2007; Widiapradja et al. 2012) Under condition of BBB/BSCB compromise, IVIG has been found to
920 enter the neural parenchyma within hours of injury.(Brennan et al. 2016; Arumugam et al. 2007)
921 SCI studies have found IVIG to localise to oligodendrocytes, astrocytes, neurons, macrophages,
922 microglia, pericytes and blood vessels.(Brennan et al. 2016; Chio et al. 2019) Additionally, reduc-
923 tions in immune cells, as indicated by F4/80⁺ microglia/macrophages and polymorphonuclear
924 cells in brain and spinal injury models respectively, have also been reported.(Jeong et al. 2014;
925 Nguyen et al. 2012; Chio et al. 2019) Relatedly, the aforementioned SCI IVIG mouse study found
926 reduced CD68⁺ macrophages at and surrounding the lesion 35 days post-injury.(Brennan et al.
927 2016) Importantly, these studies do not differentiate between resident microglial and infiltrating
928 monocytes/macrophages. Thus, further research is needed to understand the influence of IVIG
929 on both recruitment and activation states of these cell subsets.

930 **5.3.1.1.2 Speculative mechanisms of action for IVIG in SCI** As IVIG is made from pooled anti-
931 bodies taken from thousands of donors, it includes a vast repertoire of antibodies specific against

932 millions of unique antigens, allowing for a diverse variety of effects in differing disease contexts.
933 Whilst there is extensive research of IVIG and autoimmune disorders, such as Guillain-Barré syn-
934 drome, the immune pathology found in the acute phase of CNS injury is not typically considered
935 to be driven by autoimmune processes.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et
936 al. 1998) There may be some overlap in therapeutic mechanism, but it seems more likely any
937 benefits are conferred through modulation of the innate rather than adaptive immune responses.
938 The potential mechanisms of IVIG can be split between those mediated via the IgG constant (Fc)
939 fragment, which binds the Fc receptors, and the F(ab)'₂ fragment, which governs antigen recogni-
940 tion.(Schwab and Nimmerjahn 2013) In the context of neurological diseases, mechanisms related
941 to F(ab)'₂ are thought to potentially bind and therefore neutralise cell surface receptors, comple-
942 ment, cytokines and autoantibodies. By contrast, Fc-dependent mechanisms are speculated to in-
943 clude regulation of Fc receptor expression, saturation of the neonatal Fc receptor, block activation
944 of Fc receptors, and modulate T cells.(Schwab and Nimmerjahn 2013; Lünemann, Nimmerjahn,
945 and Dalakas 2015; Dalakas 2014) Furthermore, models of neurological injury suggest both F(ab)'₂
946 and Fc-dependent signalling cascades could be involved in the modulation of several chemokines
947 and cytokines.(Dalakas 2014)

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

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

955 A separate potential F(ab)'₂-dependent mechanism involves the neutralisation of the cell death
956 mediator Fas (AKA CD95). Studies of Lyell's syndrome, a disorder whereby active Fas ligand binds
957 Fas present on keratinocytes, inducing apoptosis, reported IVIG therapy completely inhibited Fas
958 ligand-induced cell death both *in vitro* and in human patients.(Viard et al. 1998; Altnauer et al.
959 2003) Importantly, IVIG blocked Fas, as opposed to Fas ligand, in these studies, as this result was
960 only observed with cells pre-treated with IVIG. Incubation of IVIG with soluble Fas ligand did not
961 attenuate cell death, implying IVIG contains antibodies specific to Fas.(Viard et al. 1998; Altnauer
962 et al. 2003) This modulatory effect of the Fas-Fas ligand pathway may have relevance in SCI, as a
963 study using knock-out mice lacking Fas showed a reduction in both apoptosis at the lesion site and
964 glial scarring, and improved motor function post-SCI.(Sobrido-Cameán and Barreiro-Iglesias 2018;
965 W. R. Yu and Fehlings 2011) Neurons and glial cells from post-mortem human patients were found
966 to be more Fas- and Fas ligand-positive, but this was limited to the acute phase of SCI, and not
967 observed chronically, suggesting this pathway is more significant immediately post-injury.(W. R. Yu
968 and Fehlings 2011) Therefore, acute IVIG treatment could act by attenuating secondary cell death
969 by blocking Fas, thus disrupting this pathway.

970 Conversely, agonistic anti-Fas antibodies have also been reported with IVIG prepara-
971 tions.(Altnauer et al. 2003) Whilst it remains unknown how these agents may act in SCI,
972 one could postulate a benefit if they induce apoptosis in circulating leukocytes, which could
973 otherwise do harm.(Schneider et al. 2017) Supporting this, papers have found reductions in poly-
974 morphonuclear cell populations within the lesion at 1 day post-injury in rodent models.(Nguyen
975 et al. 2012; Chio et al. 2019; Gok et al. 2009) However, IVIG-induced apoptosis has only been
976 observed in human leukocytes, not in rodents, casting doubt on this idea.(Altnauer et al. 2003;
977 Schneider et al. 2017) Alternatively, the reduced recruitment could be a result of IVIG regulating
978 the expression of adhesion molecules or molecules involved in leukocytes trafficking. A feline
979 ischaemia-reperfusion injury model study found IVIG to down-regulate expression of integrins
980 on leukocyte cell surfaces, inhibiting adhesion and subsequent extravasation of the cells into the

damaged site.(Gill et al. 2005) Again however, these finding are contradicted by an experimental stroke study where IVIG was found to increase leukocyte and platelet trafficking to the injury, leading to formation of aggregates within cerebral vasculature.(Lapointe et al. 2004)

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

Modulation via the constant Fc region

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

Studies utilising just the Fc fragment have been found to be equally effective as normal IVIG in several non-neurological autoimmune diseases, including nephrotoxic nephritis, ITP and K/BxN arthritis models, suggesting $Fc\gamma$ Rs play a key role in the mechanism of IVIG.(Samuelsson, Towers, and Ravetch 2001; I. K. Campbell et al. 2014; Kaneko et al. 2006) With respect to CNS injury, some evidence suggesting a role of $Fc\gamma$ Rs comes from a mouse study with animals lacking the common γ -chain, and thus no functional $Fc\gamma$ Rs, which were found to be protected from experimental stroke and SCI.(Ankeny, Guan, and Popovich 2009; Komine-Kobayashi et al. 2004)

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

5.3.1.1.3 Immunoglobulins Several immunoglobulin components were identified here, including 3 λ variable precursors (3-19, 3-10 and 2-18), 3 heavy variable precursors (3-15, 1-69 and 1-24) and 2 heavy constant gamma regions (2 and 4). For the λ variable precursors, acute AIS C improvers the precursors 3-19 and 3-10 were detected, whereas 3-10 and 2-18 were detected in acute C non-improvers. That acute C non-improvers expressed the 2-18 precursor whilst the improvers did not, suggests potential as a biomarker of poorer functional outcomes. It is difficult to comment on the biological mechanisms that may be a play here from this data, but one could infer that it is indicative of either a more robust, or a more maladaptive, immune response to the trauma. Given that the injuries are of the same severity by AIS grade, the latter seems more likely, though

1028 again, further research is needed to highlight the precise nature of this difference. Interestingly,
1029 whilst the acute C improvers do not express precursor 2-18, both the subacute C improvers and
1030 non-improvers, and subacute As do, whereas acute or subacute Ds do not, seemingly implying this
1031 precursor is also indicative of more severe injury in the latter phases of SCI.

1032 In addition of acute C improvers, subacute As and acute Ds also express the 3-19 precursor, with
1033 subacute As possessing the greatest abundance. Again, this would seem to suggest this marker
1034 is indicative of positive outcomes or less severe injury in the acute phase, but may be more detri-
1035 mental in the latter phases. The final λ precursor, 3-10, is present in acute As, subacute As and
1036 both subacute C groups as well as the aforementioned acute C improvers. The curious absence
1037 of 3-10 in both AIS D groups and C non-improvers groups suggests the marker is implicated in a
1038 more beneficial response, but perhaps this is limited to more severe injuries.

1039 With respect to the immunoglobulin heavy variable precursors, 3-15 was present in all groups
1040 except acute As and acute C non-improvers, though there was insufficient power to confidently
1041 compare the fold change of groups expressing 3-15. Another heavy variable precursor, 1-69, was
1042 expressed in subacute As, both acute and subacute C improvers, and both acute and subacute
1043 Ds. The final heavy variable precursor, 1-24, was found in all groups except acute C improvers and
1044 non-improvers.

1045 For the two immunoglobulin heavy constant γ s, 4 was significant in acute C improvers and non-
1046 improvers, relative to subacute As, whereas γ 2 was only significant in acute C improvers relative to
1047 subacute Ds. Both acute C improvers and non-improvers had a lower abundance of γ 4 relative to
1048 subacute As (-2.2 and -2.7 respectively), whilst γ 2 had a -1.8 fold change between acute C improvers
1049 and subacute Ds.

1050 **5.3.2 Conclusion**

1051 Much like the iTRAQ experiments (5.2.9), the majority of proteins identified are functionally asso-
1052 ciated with the complement cascade. Unlike the iTRAQ however, many of the proteins where only
1053 detected in one group of the pairwise comparisons, suggesting greater suitability as biomarkers.
1054 PRX-2, a protein associated with oxidative stress, is of particular interest, both as a biomarker for
1055 improvement in acute AIS C patients, but also mechanistically in relation to functional recovery.
1056 Furthermore, several immunoglobulins were identified as differentially abundant, though further
1057 *in vitro/vivo* work is needed to elucidate the pathophysiological relevance of each precursor. The
1058 λ 2-18 and 3-10 precursors are of particular relevance to acute and subacute AIS C improvement
1059 respectively, and both are of interest longitudinally in AIS As, with 2-18 potentially being linked to
1060 severity of injury.

1061 The small number of statistically significant proteins speaks to the variability of human samples,
1062 and is likely exacerbated by the inconstant timing of sample collection relative to injury. Post-hoc
1063 power analysis of the data reveals that to identify a 2.5 fold change with an FDR of 0.5 and a power
1064 of 0.9, 14 biological replicates would be needed, in contrast to the 7-11 replicates used across
1065 groups here. Thus, a repeat of this experiment with a larger sample size will likely reveal many
1066 more proteins of potential interest. Furthermore, a metabolomic analysis with a similar sample
1067 size would greatly compliment this work, particularly with regards to investigating further links to
1068 the liver.

1069 **Supplementary material**

1070 **5.4 Session Information**

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1085 ## nickname     One Push-Up

1086 Packages Used

1087 package

1088 version

1089 date

1090 base

1091 4.1.3

1092 2022-03-18

1093 MSstats

1094 4.2.0

1095 2021-05-31

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1097 2.6.5

1098 2020-01-10

1099 ReactomePA

1100 1.38.0

1101 2021-10-26

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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	NA	-4.365158	-5.248075	NA	NA	NA	
SERPIN F2	NA	NA	NA	NA	NA	-4.207266	NA	-3.467369	NA	NA	
SERPIN G1	NA	-2.535129	NA	2.964831	-1.836538	-4.365158	NA	-2.488857	2.187762	5.248075	

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

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

1205 **5.6 Heatmaps**

1206 **5.6.1 iTRAQ data**

AIS C Improvers acute vs subacute

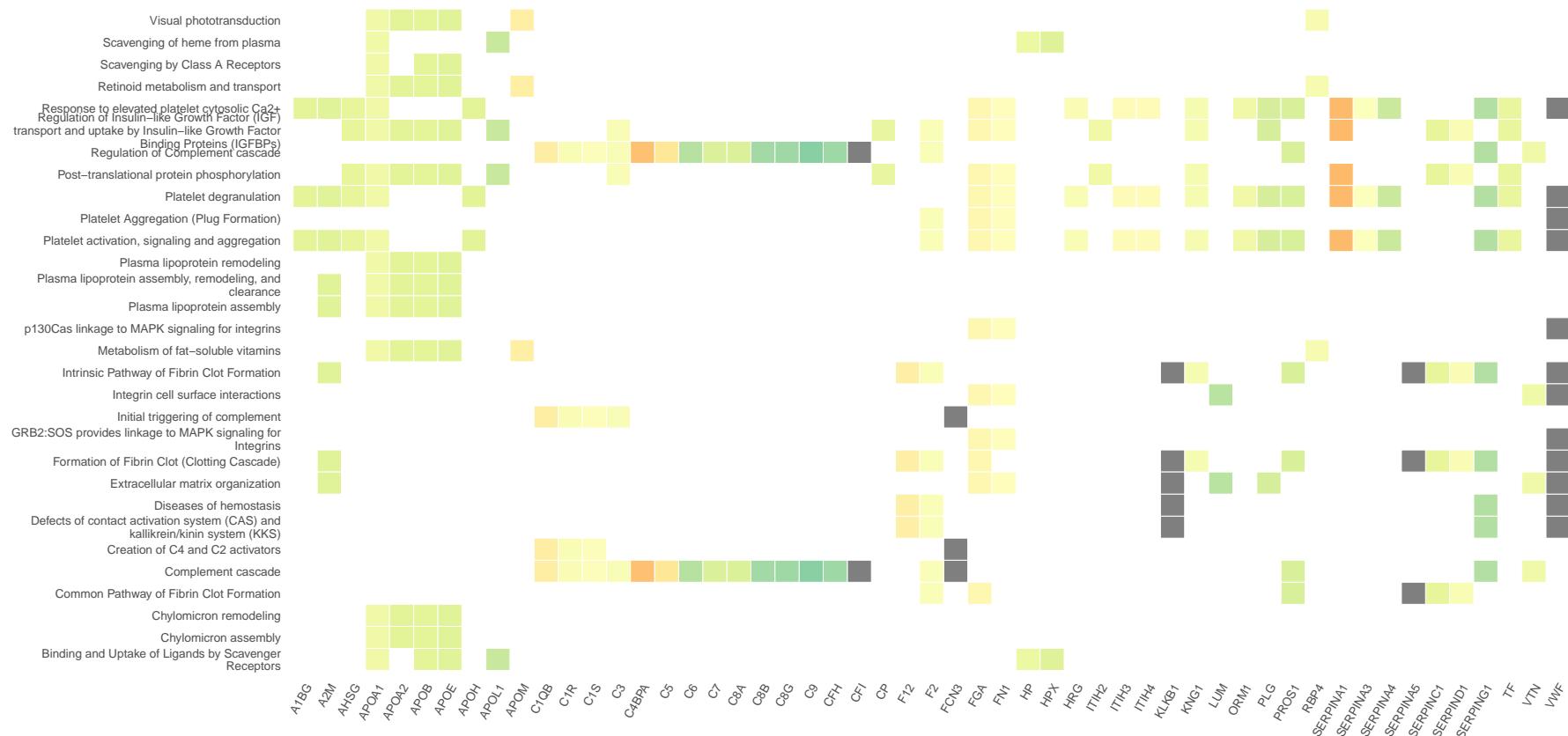


Figure S1. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

AIS C non-Improvers acute vs subacute

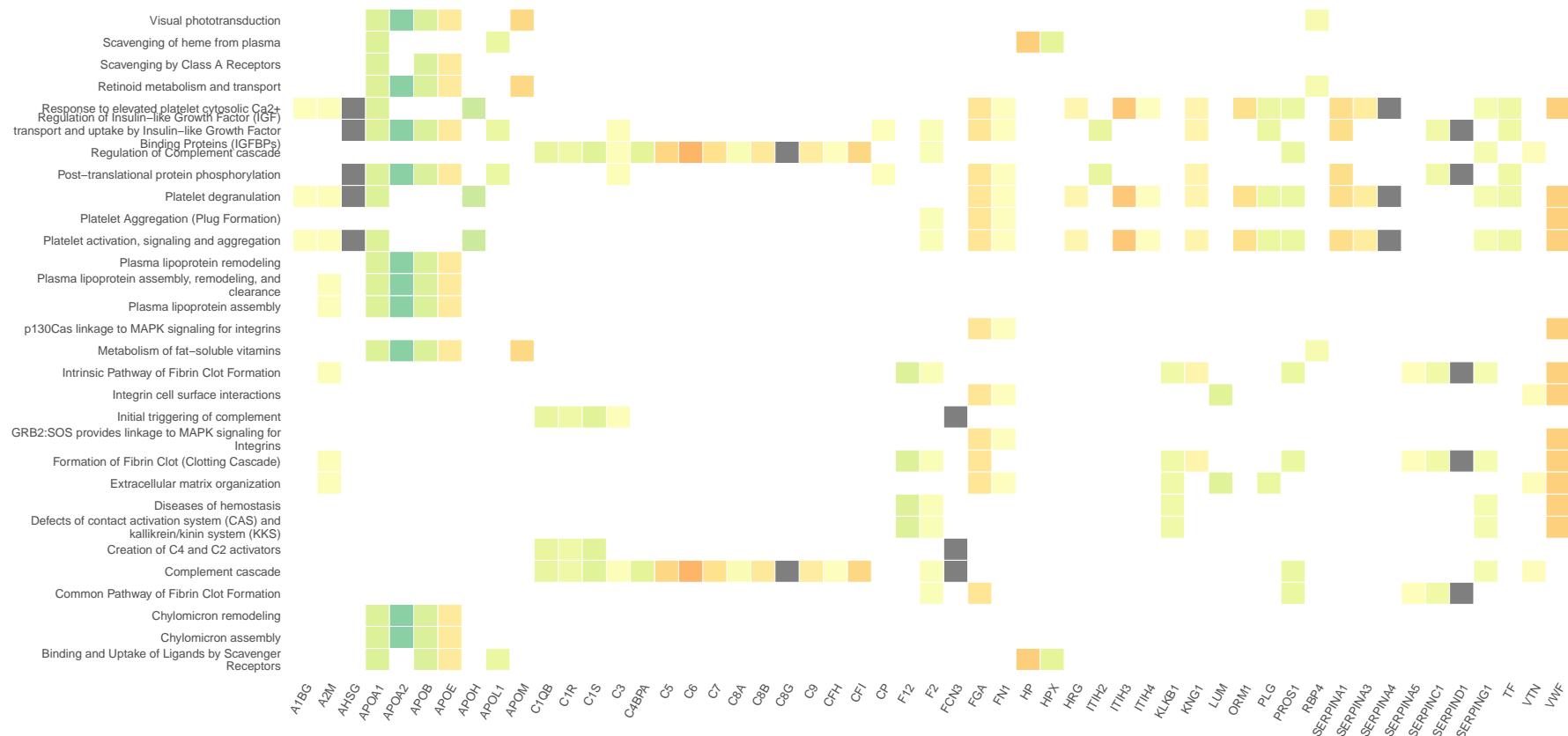


Figure S2. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

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.

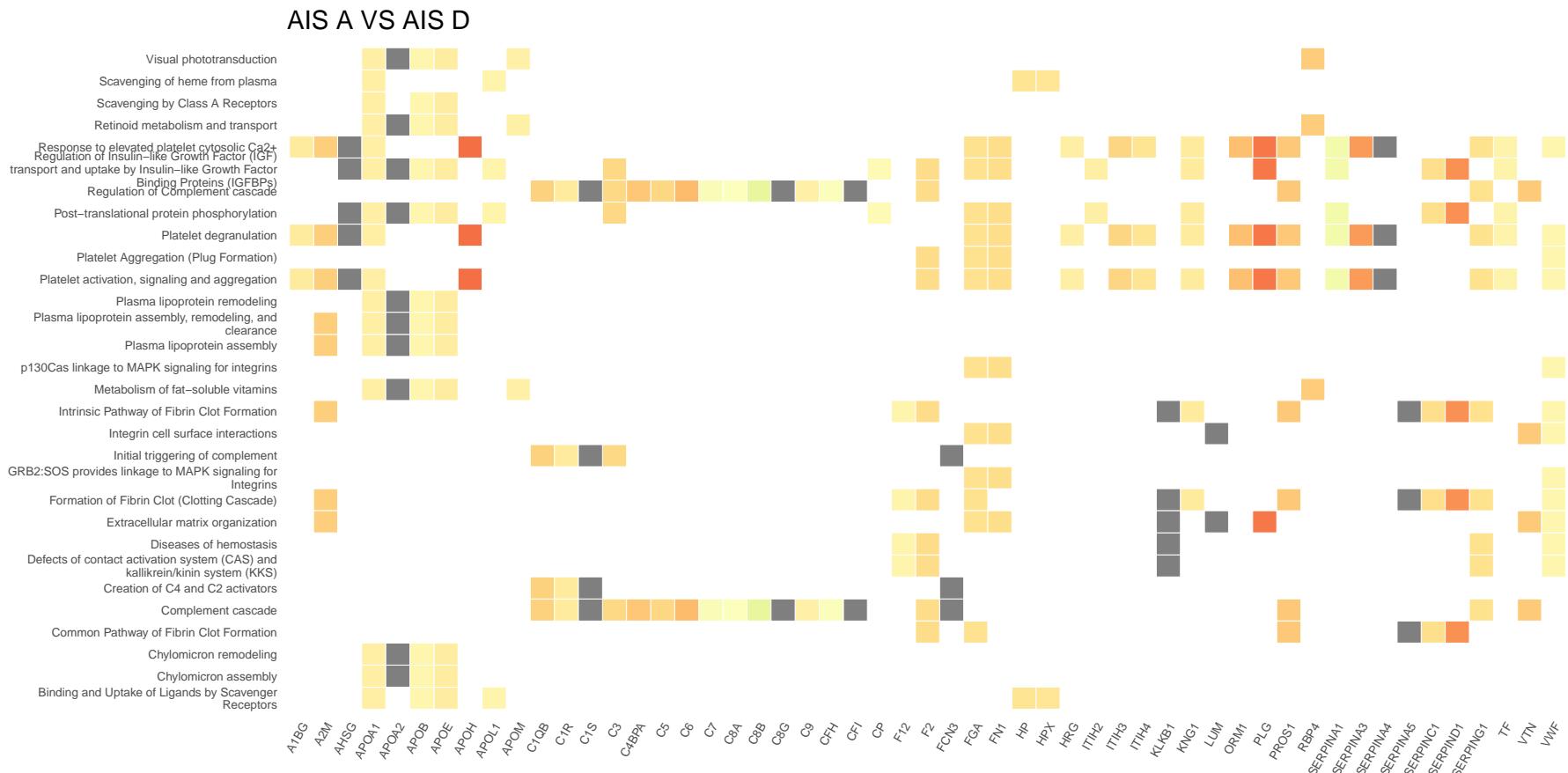


Figure S4. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Acute AIS C Improvers VS AIS D

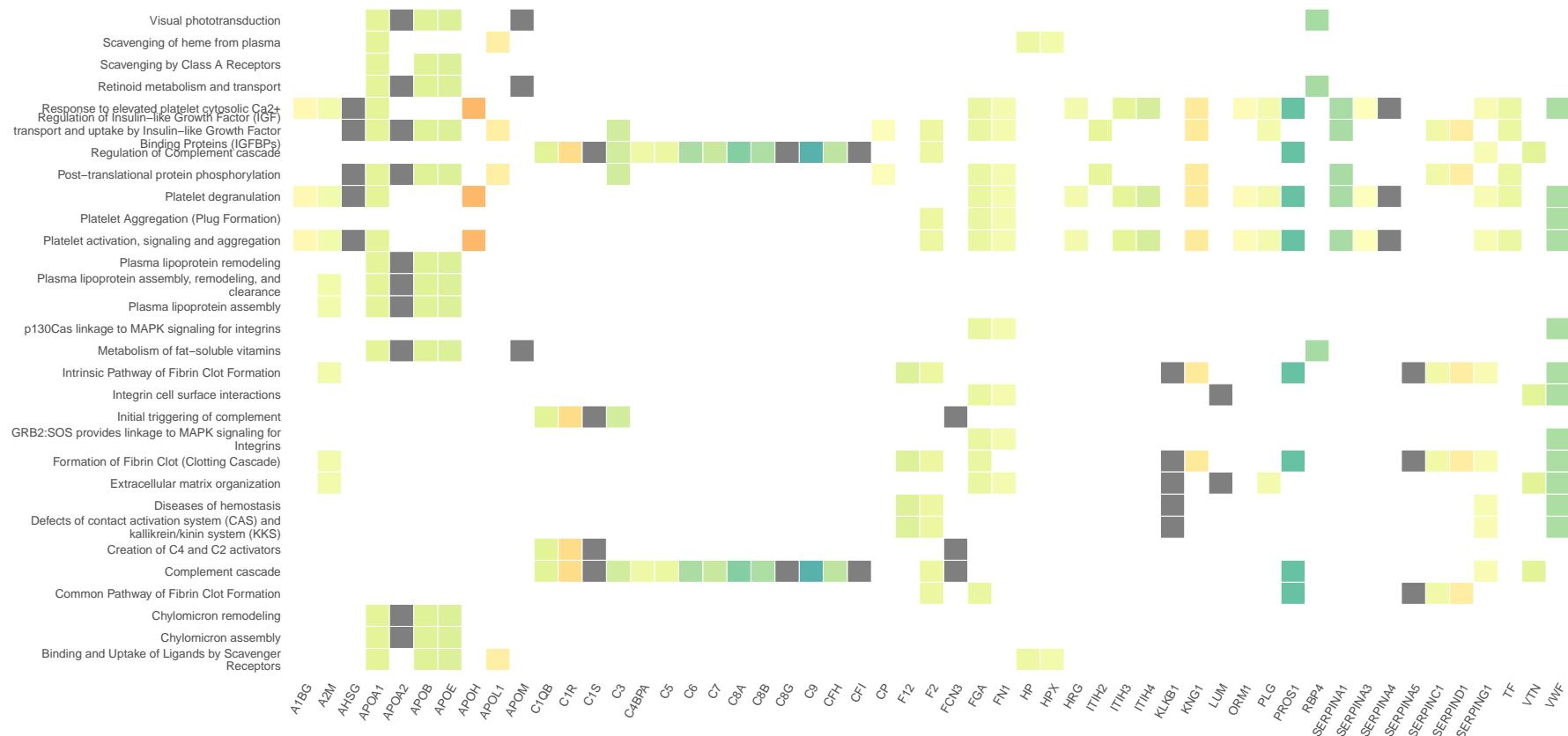


Figure S5. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

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

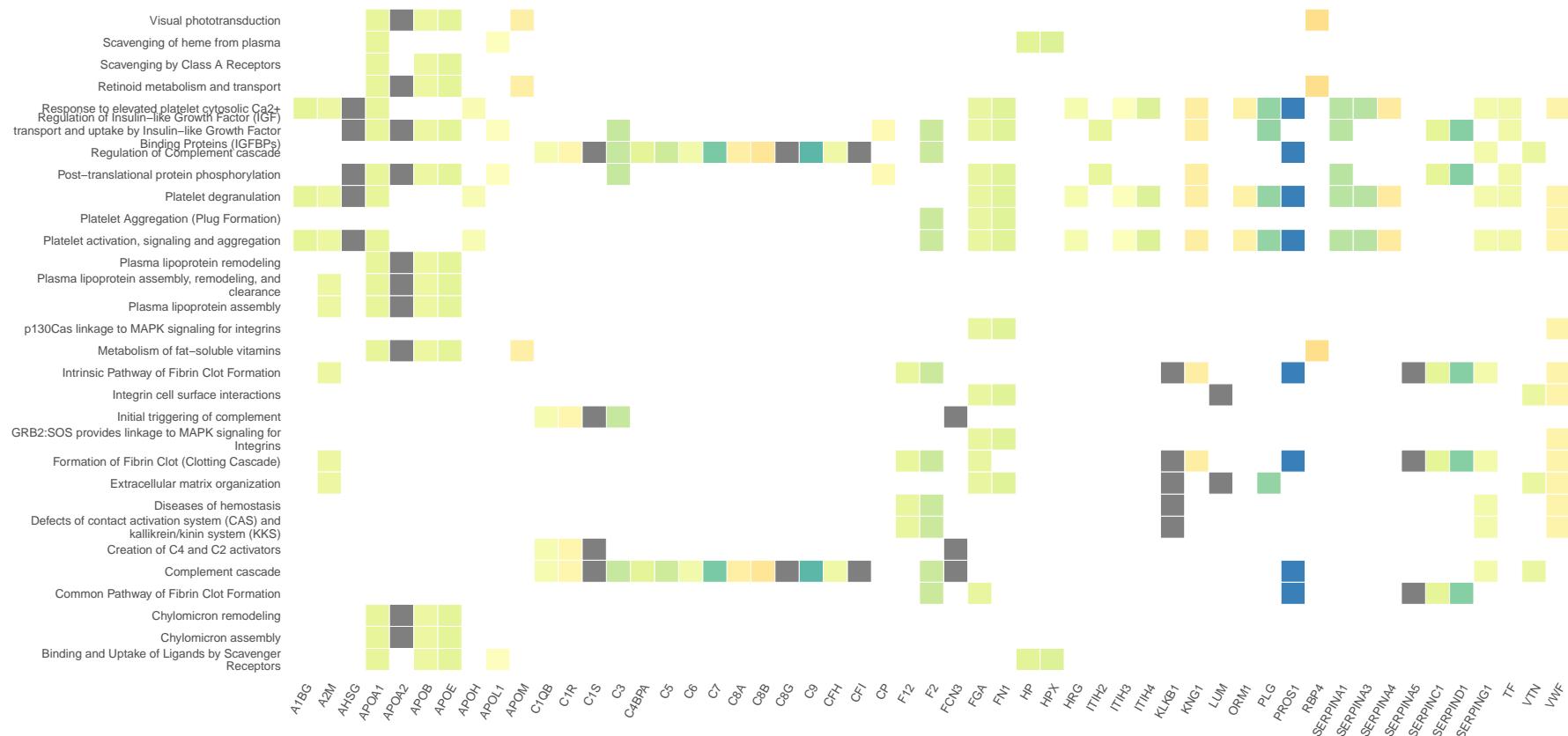


Figure S7. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D

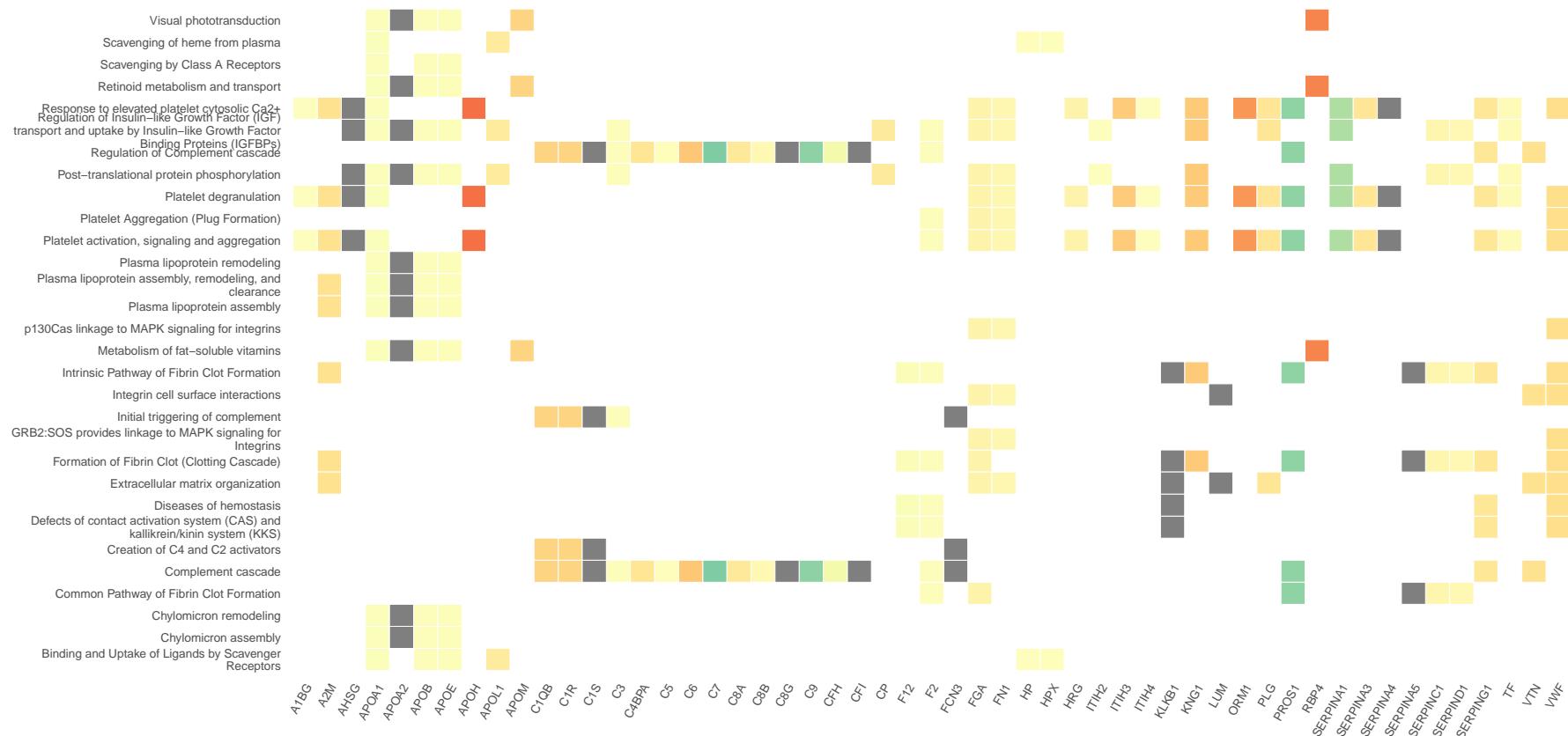


Figure S8. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

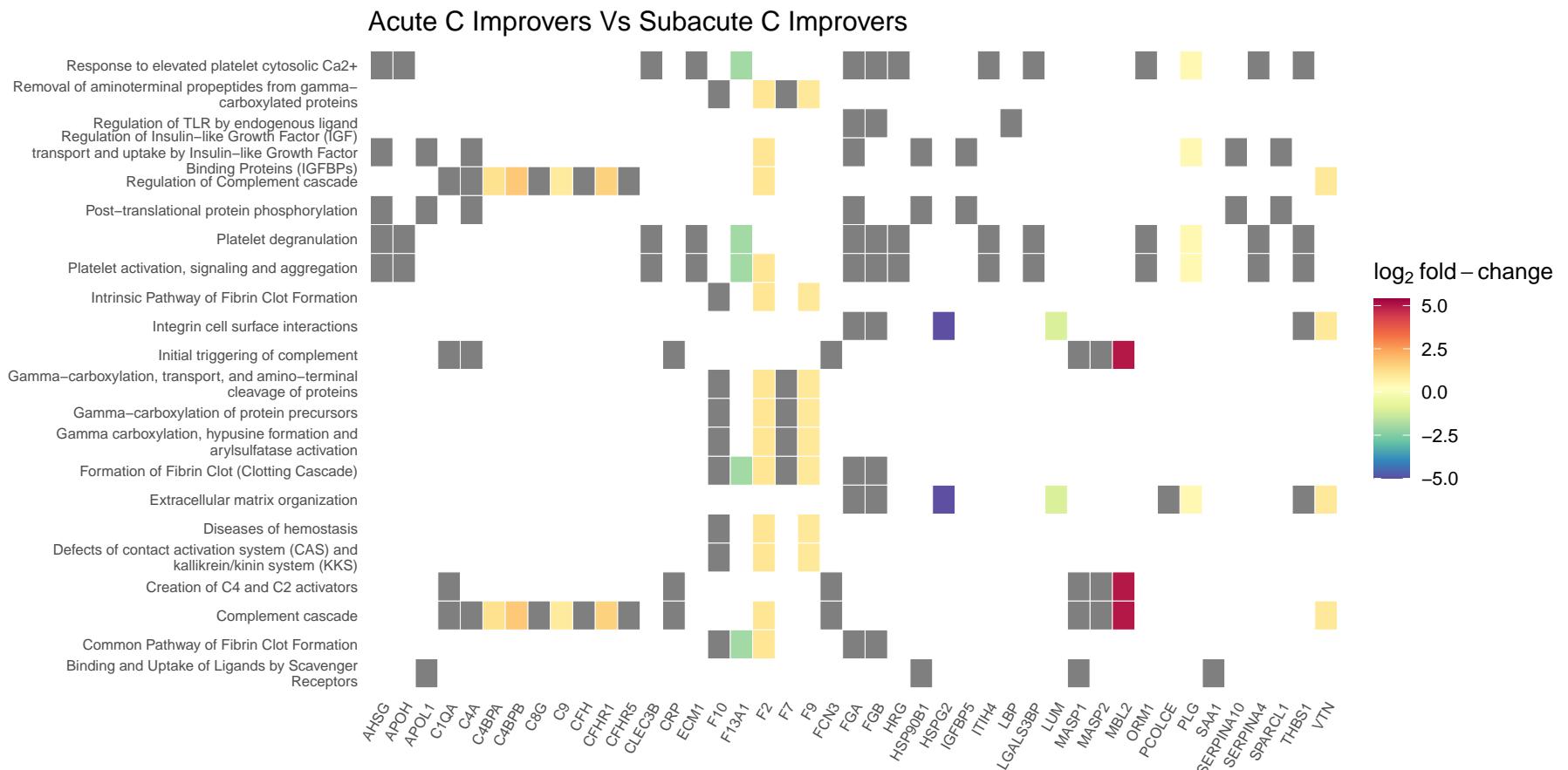


Figure S9. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury. Grey blocks denote proteins not present in the comparison.

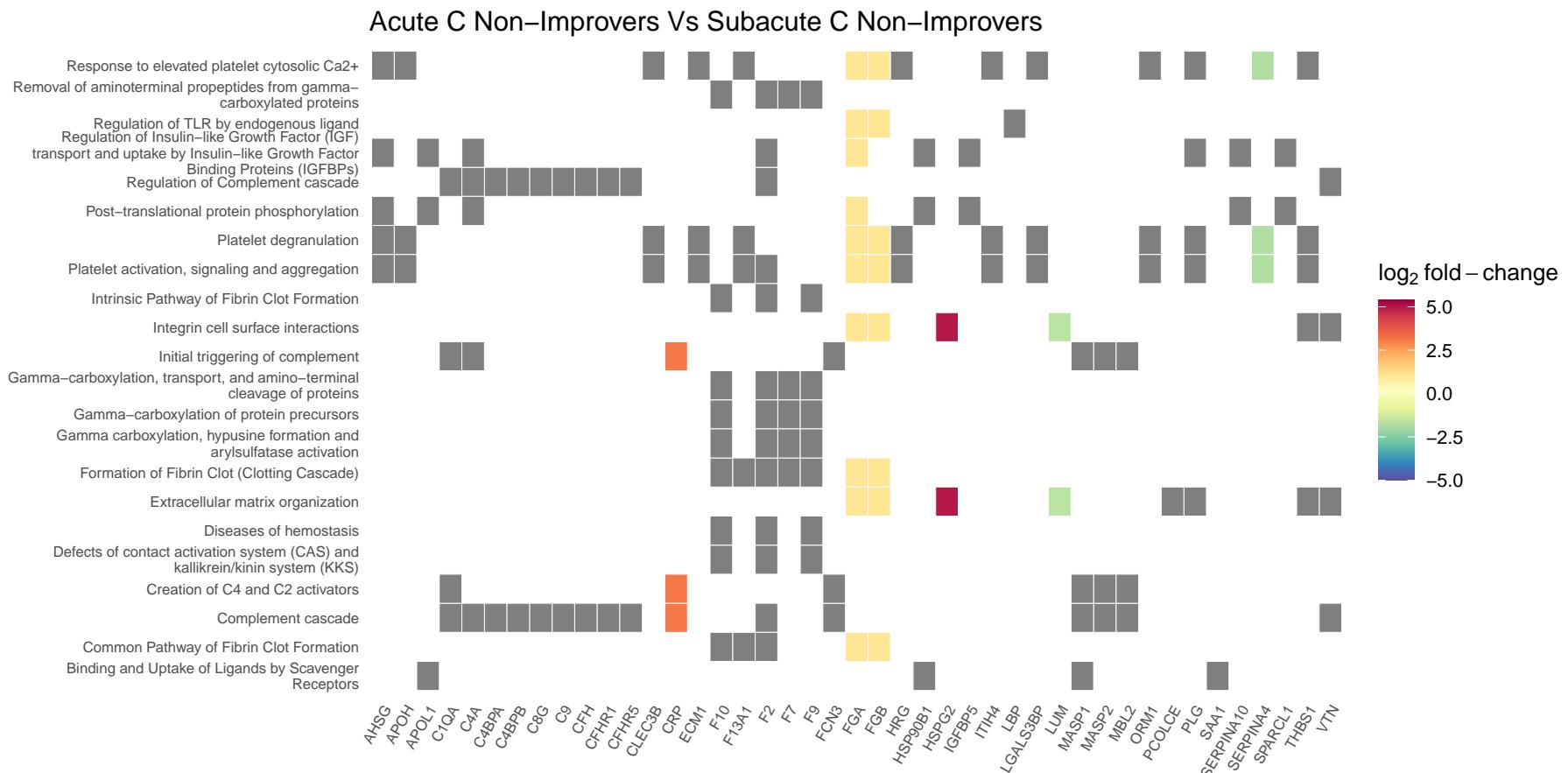


Figure S10. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.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

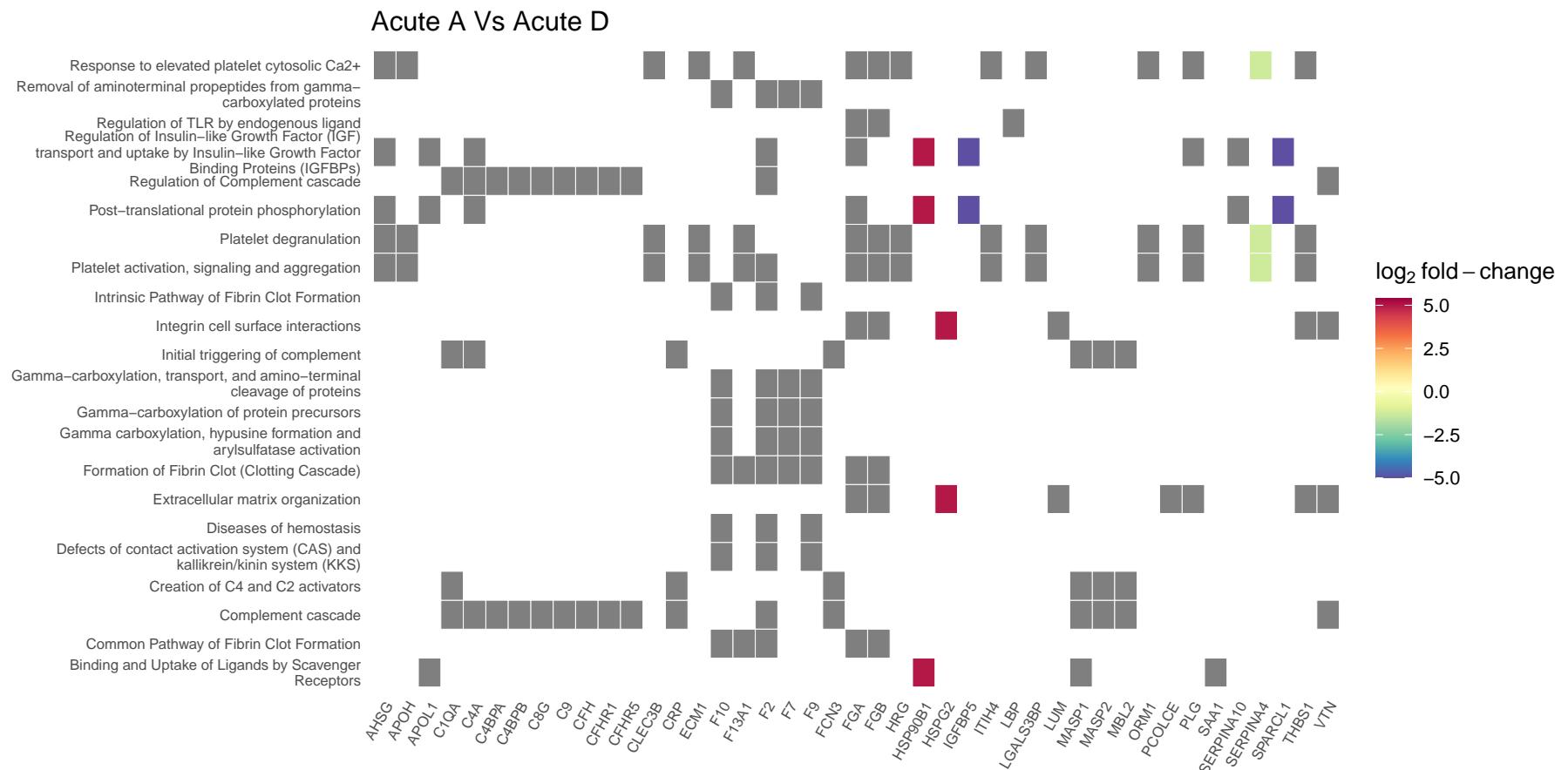


Figure S11. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients. Grey blocks denote proteins not present in the comparison.

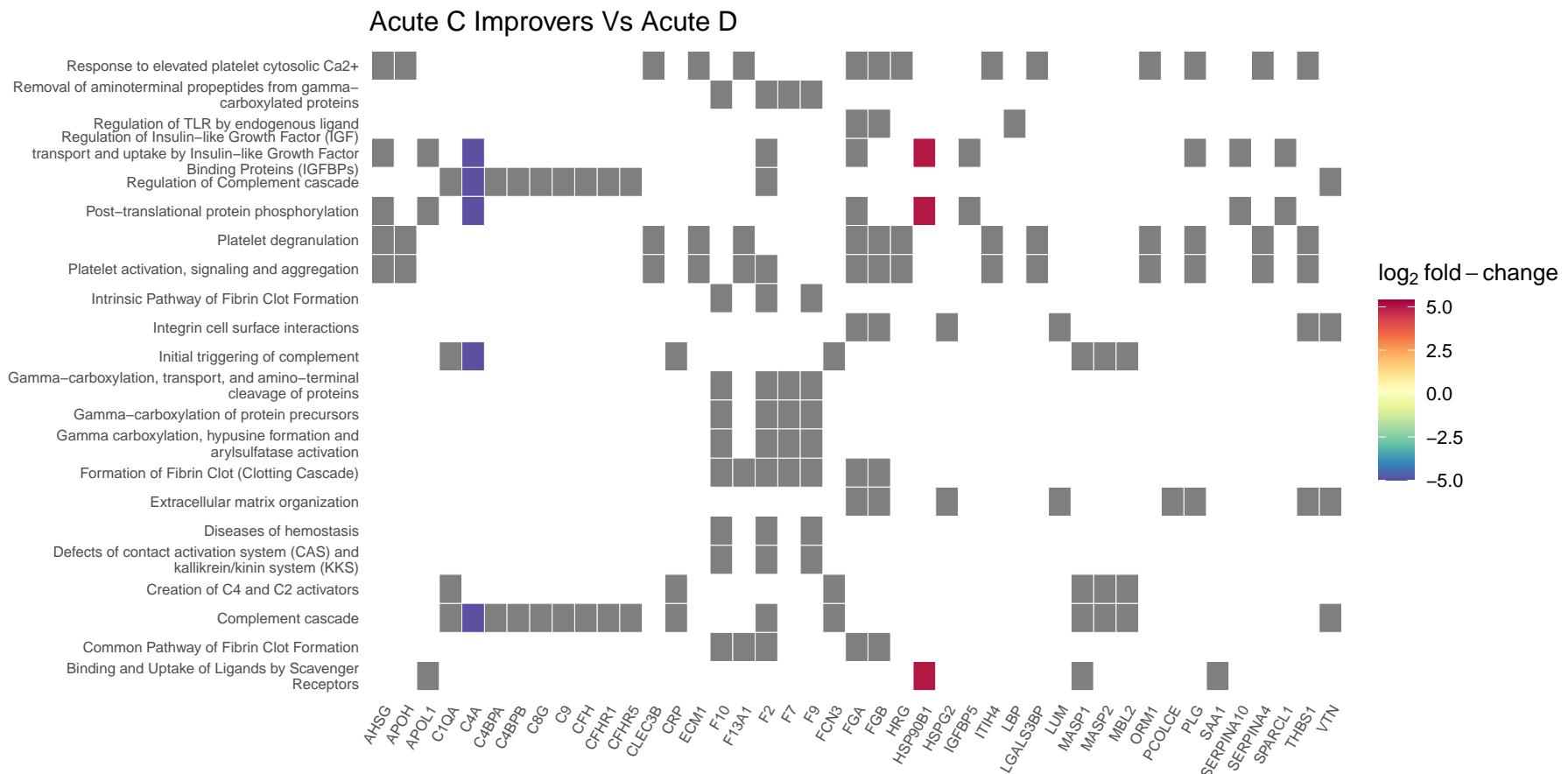


Figure S12. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients. Grey blocks denote proteins not present in the comparison.

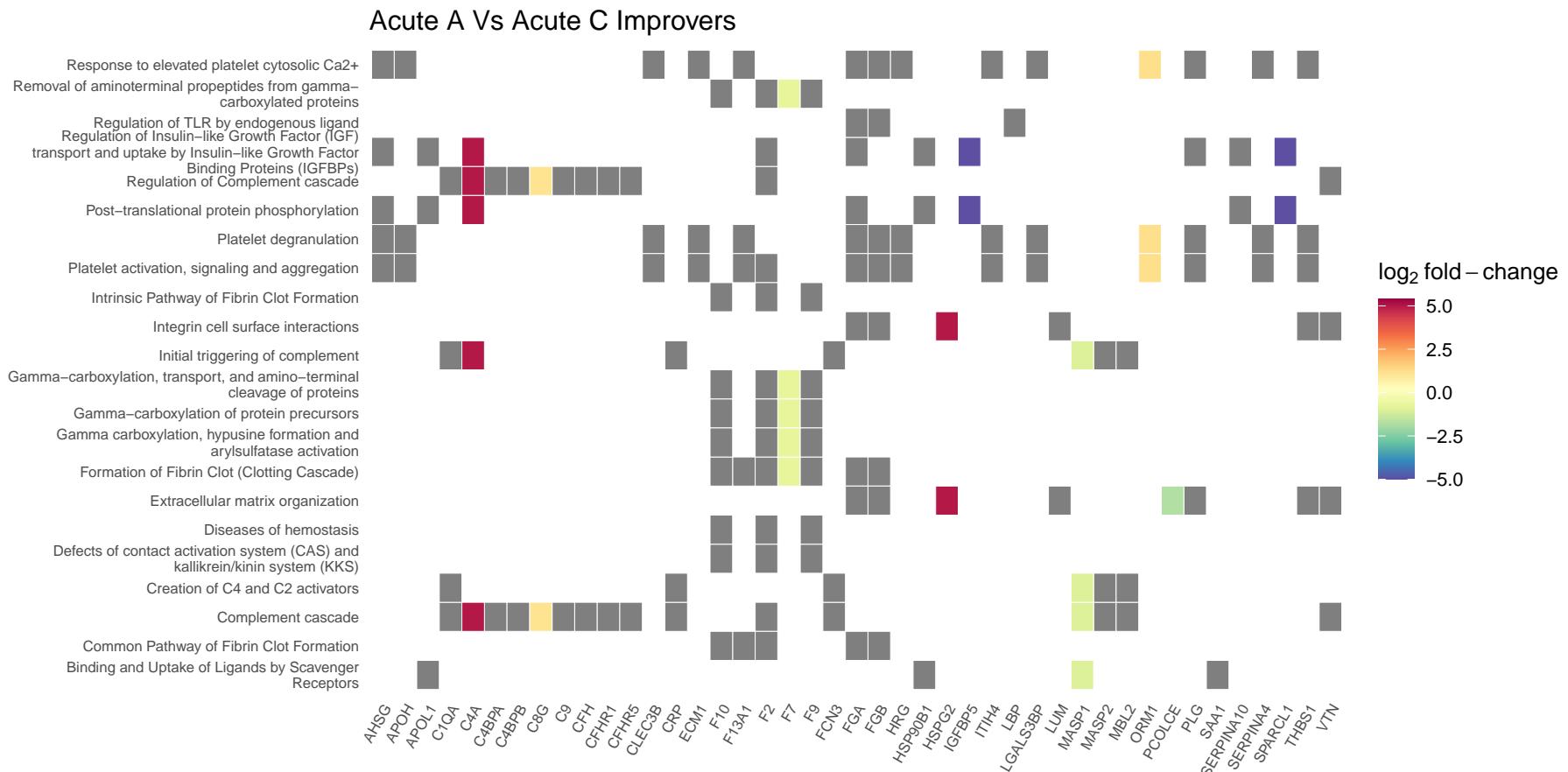


Figure S13. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.

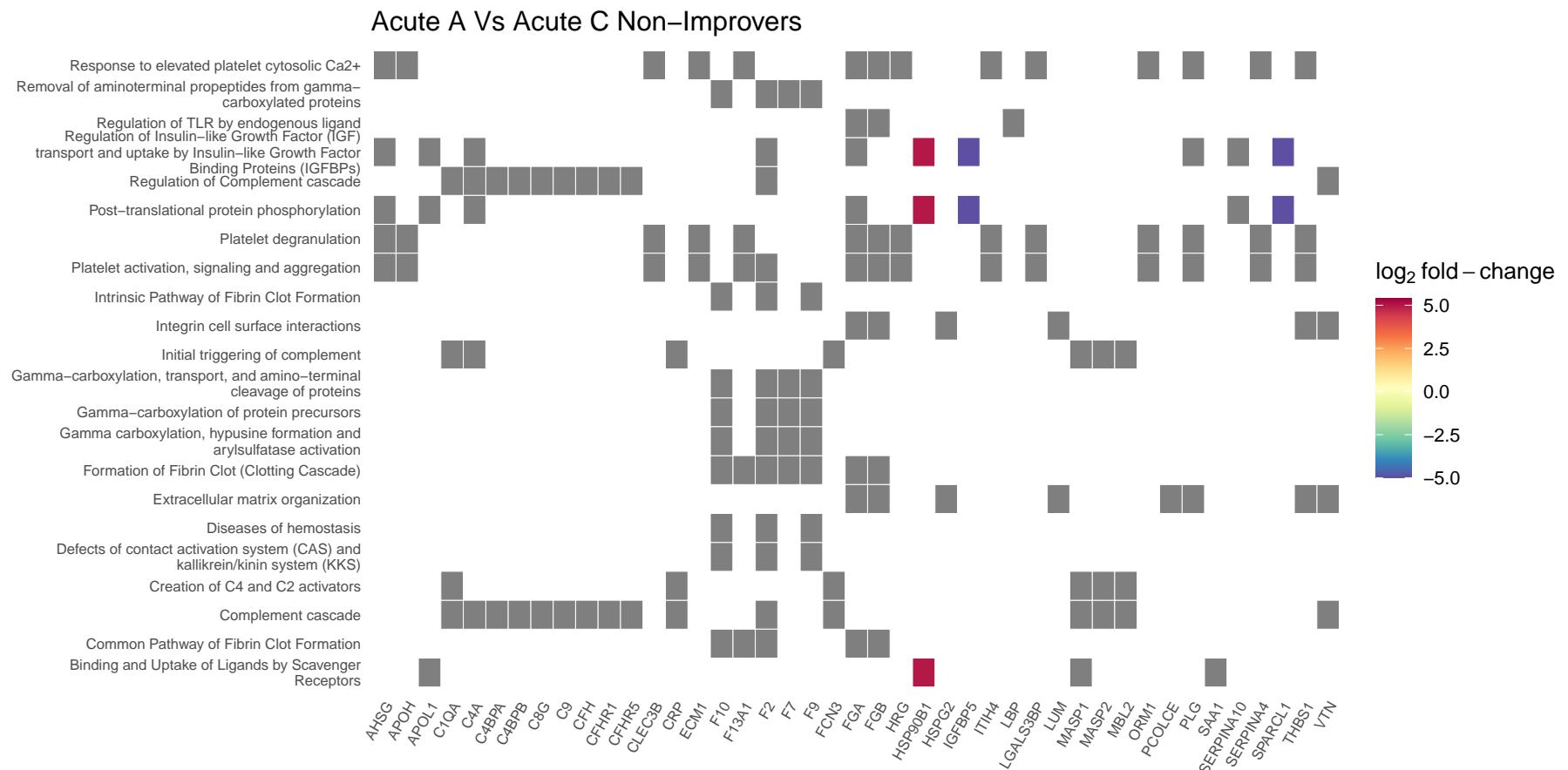


Figure S14. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.

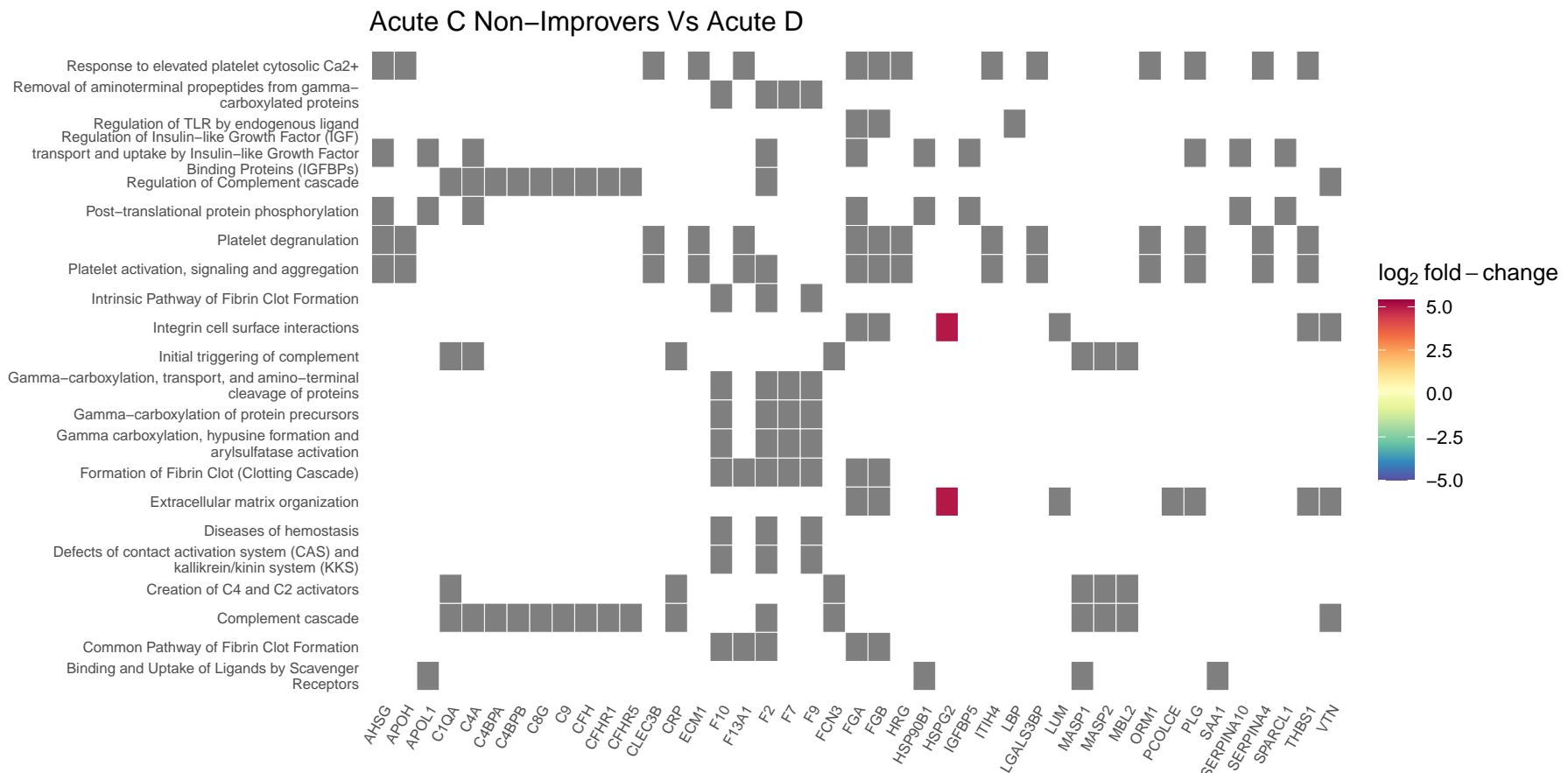


Figure S15. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients. Grey blocks denote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

1208 **5.7 Cnetplots**

1209 **5.7.1 iTRAQ data**

AIS C Improvers acute vs subacute

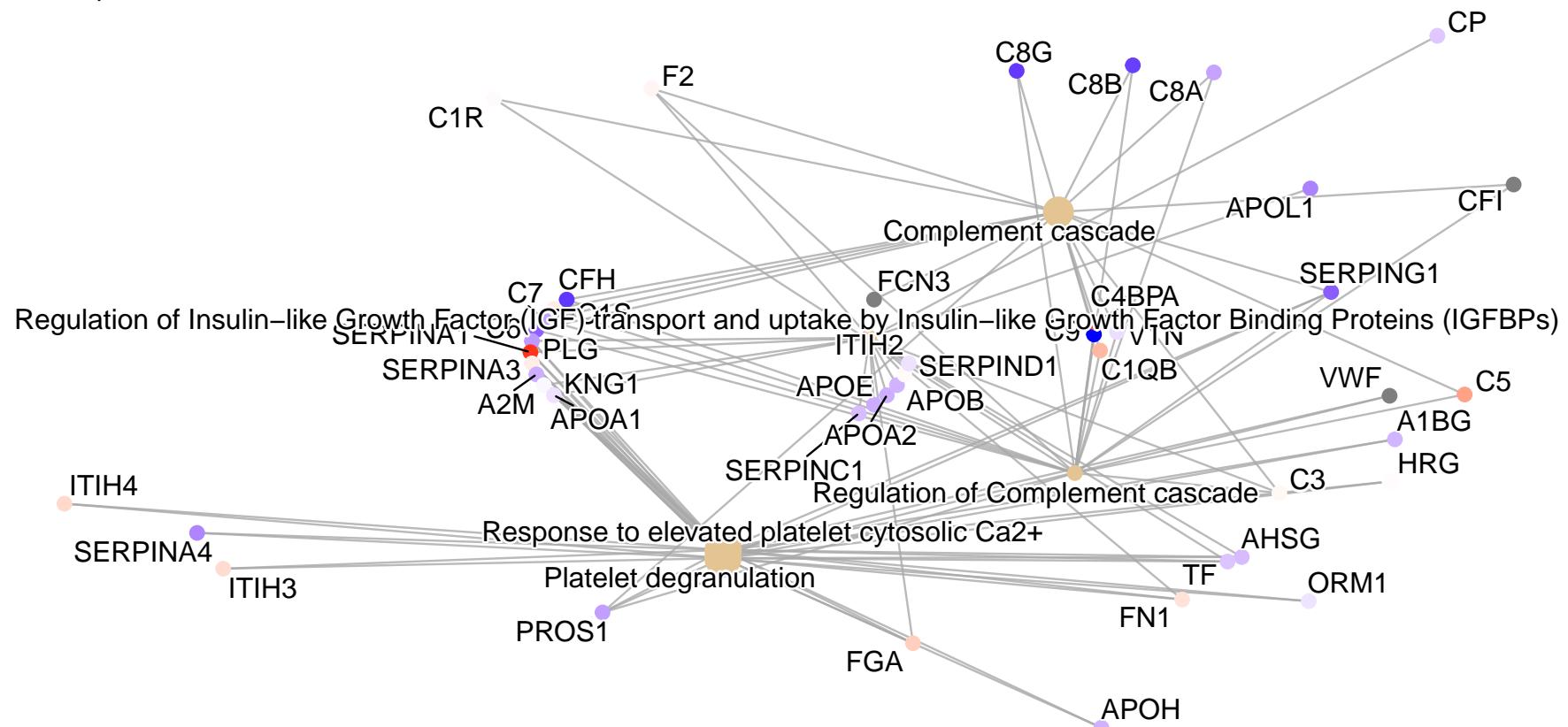


Figure S16. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

AIS C non-Improvers acute vs subacute

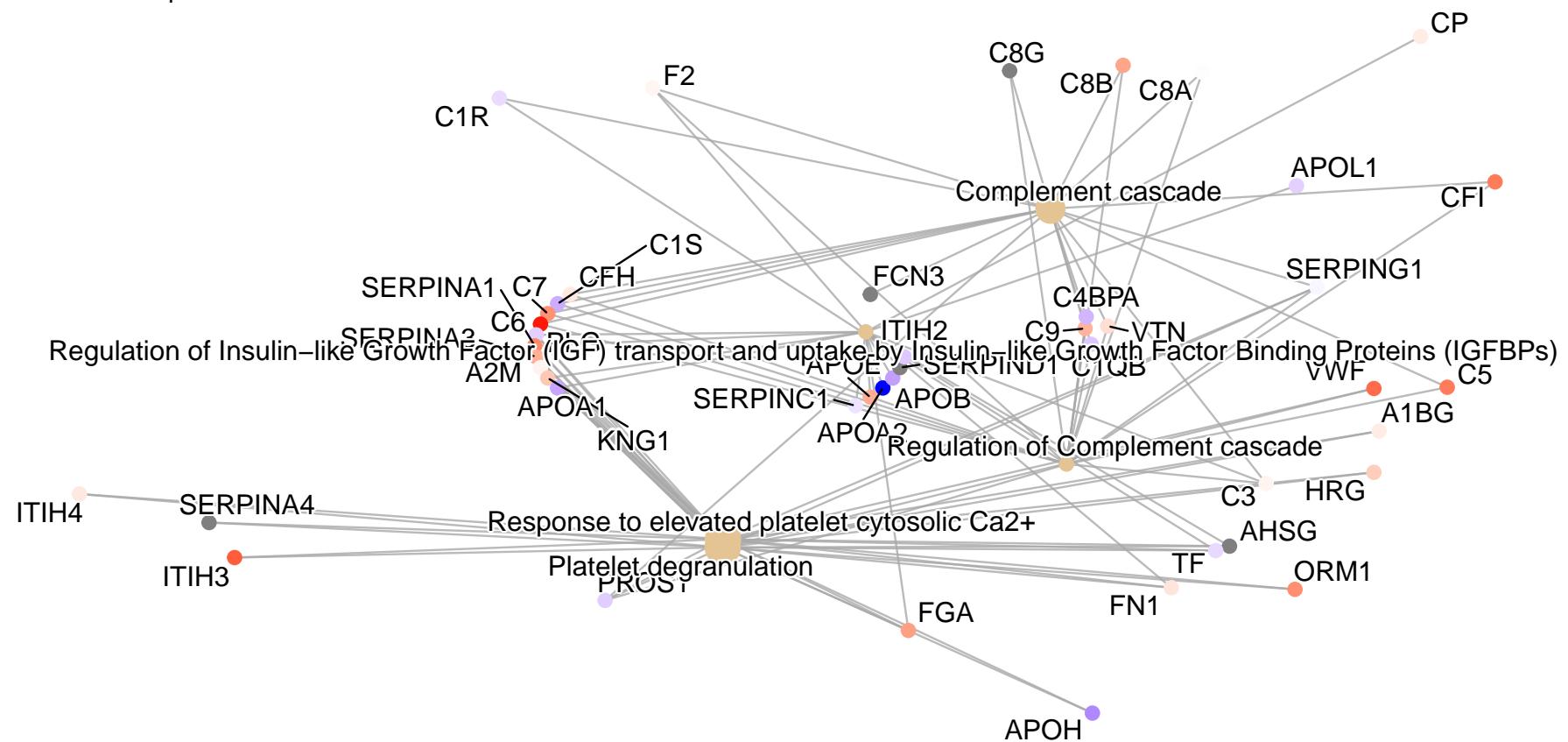


Figure S17. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute AIS C Improvers VS non-Improvers Run 2

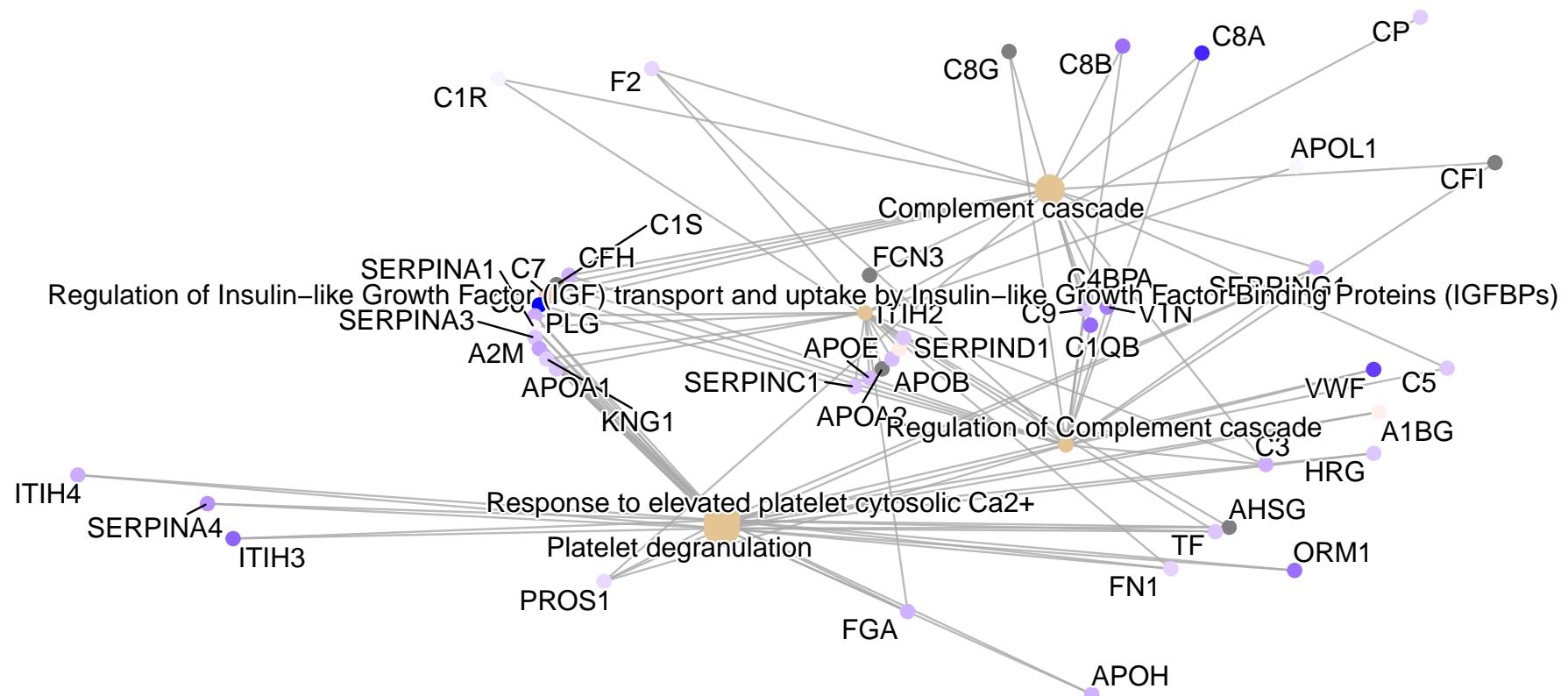


Figure S18. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex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

AIS A VS AIS D

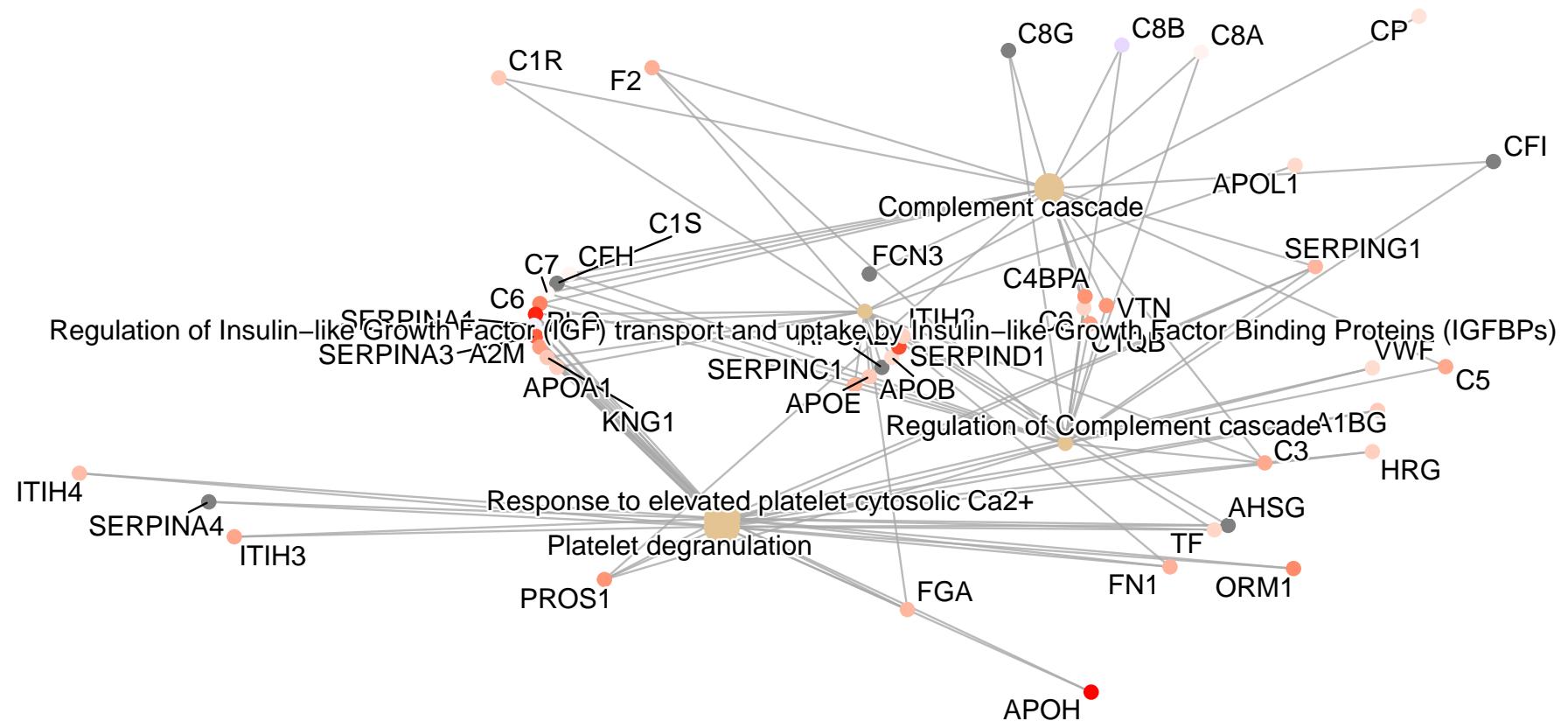


Figure S19. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Acute AIS C Improvers VS AIS D

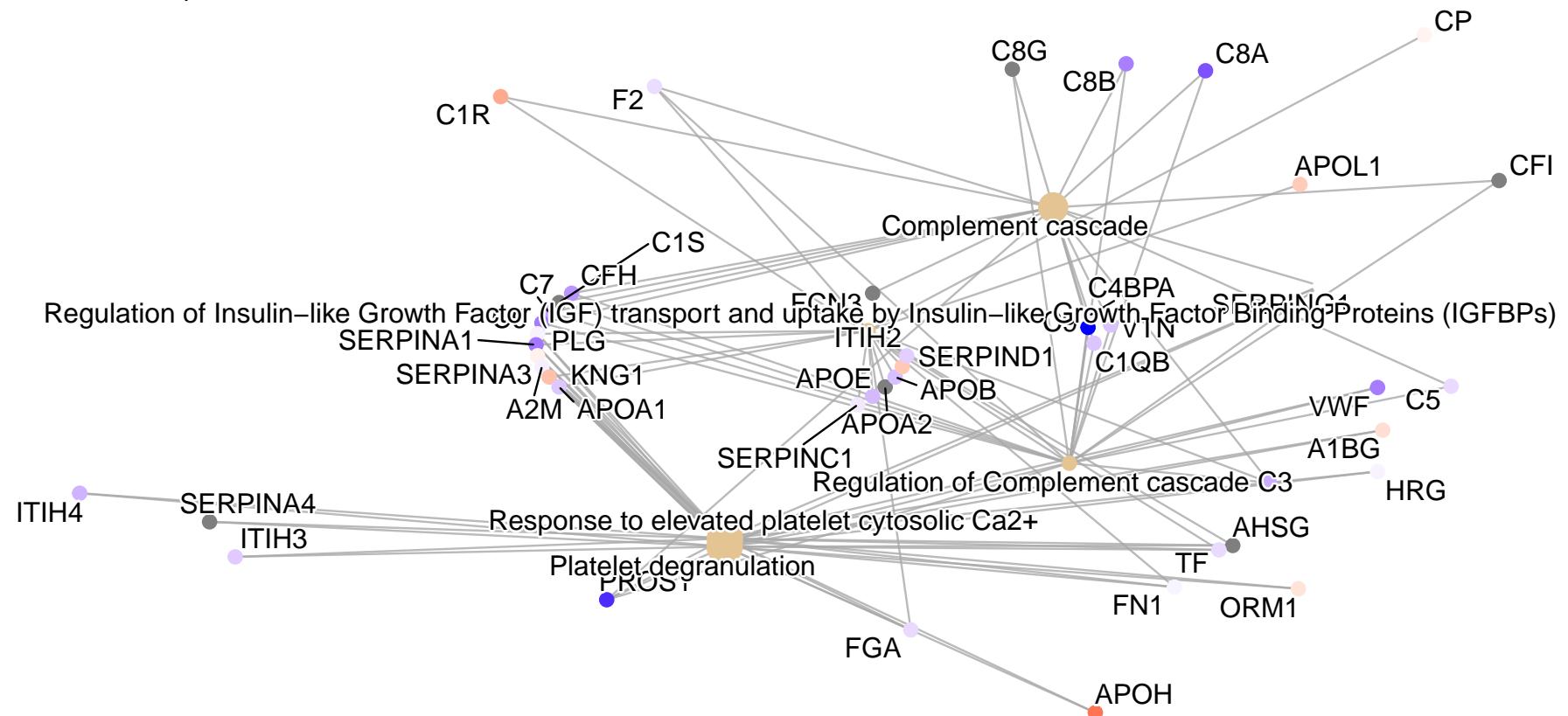


Figure S20. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Acute AIS C Improvers VS AIS A

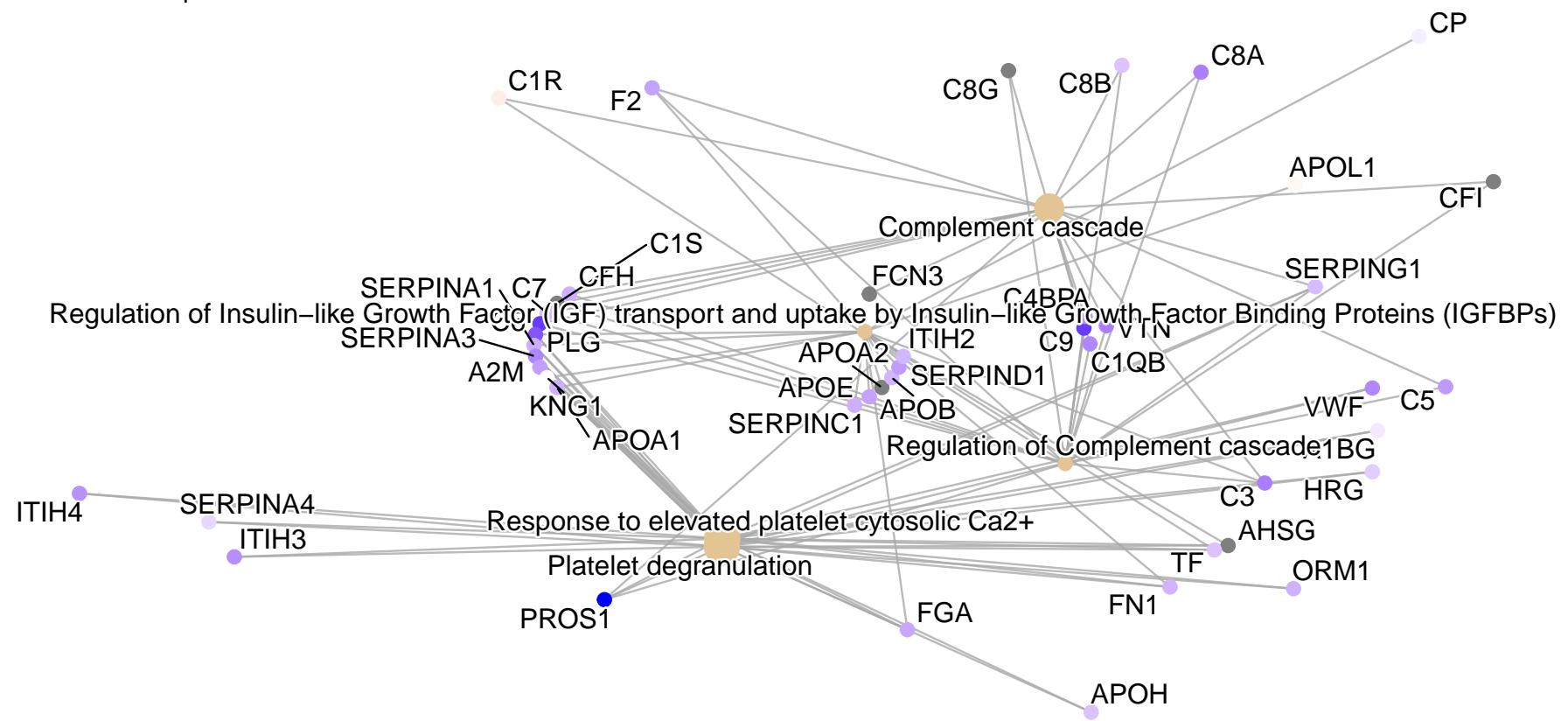


Figure S21. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS A

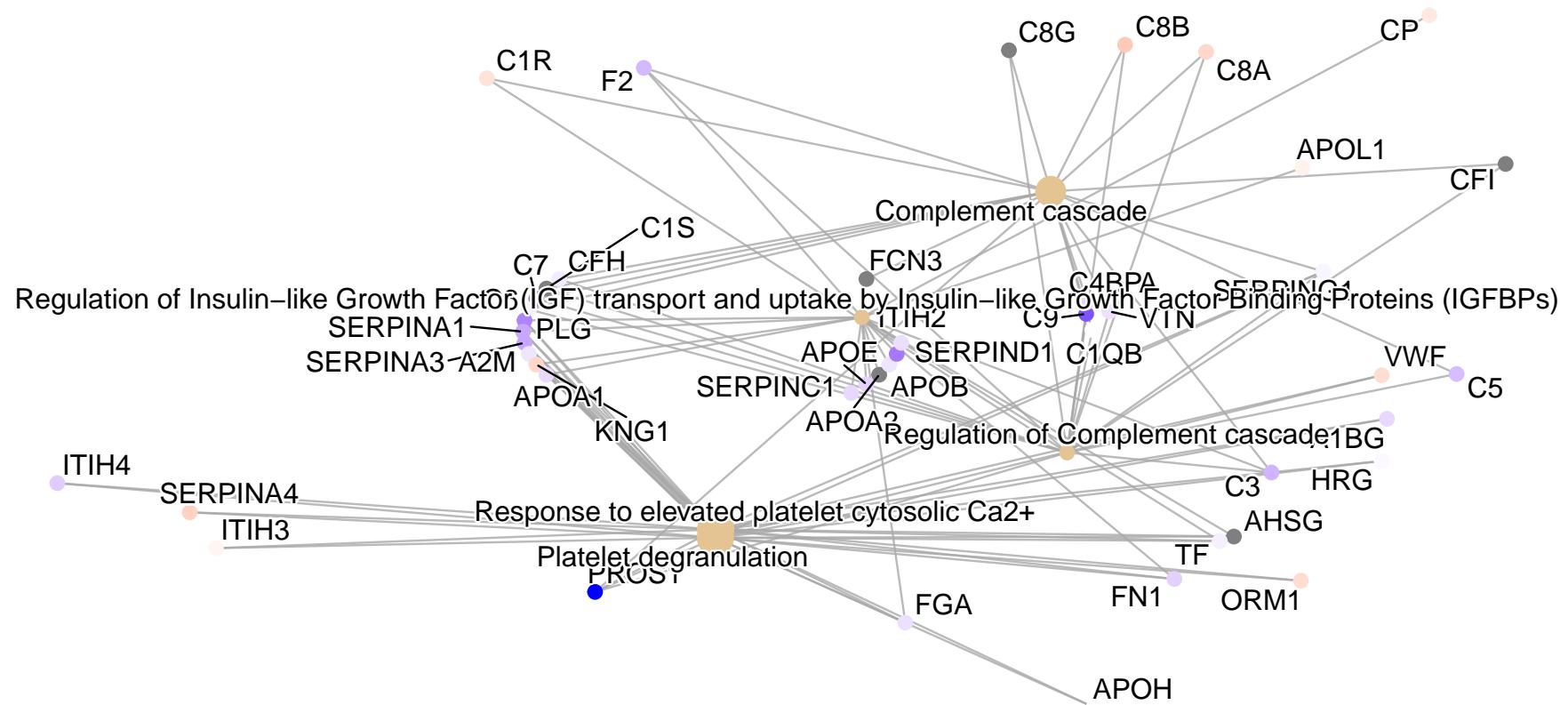


Figure S22. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D

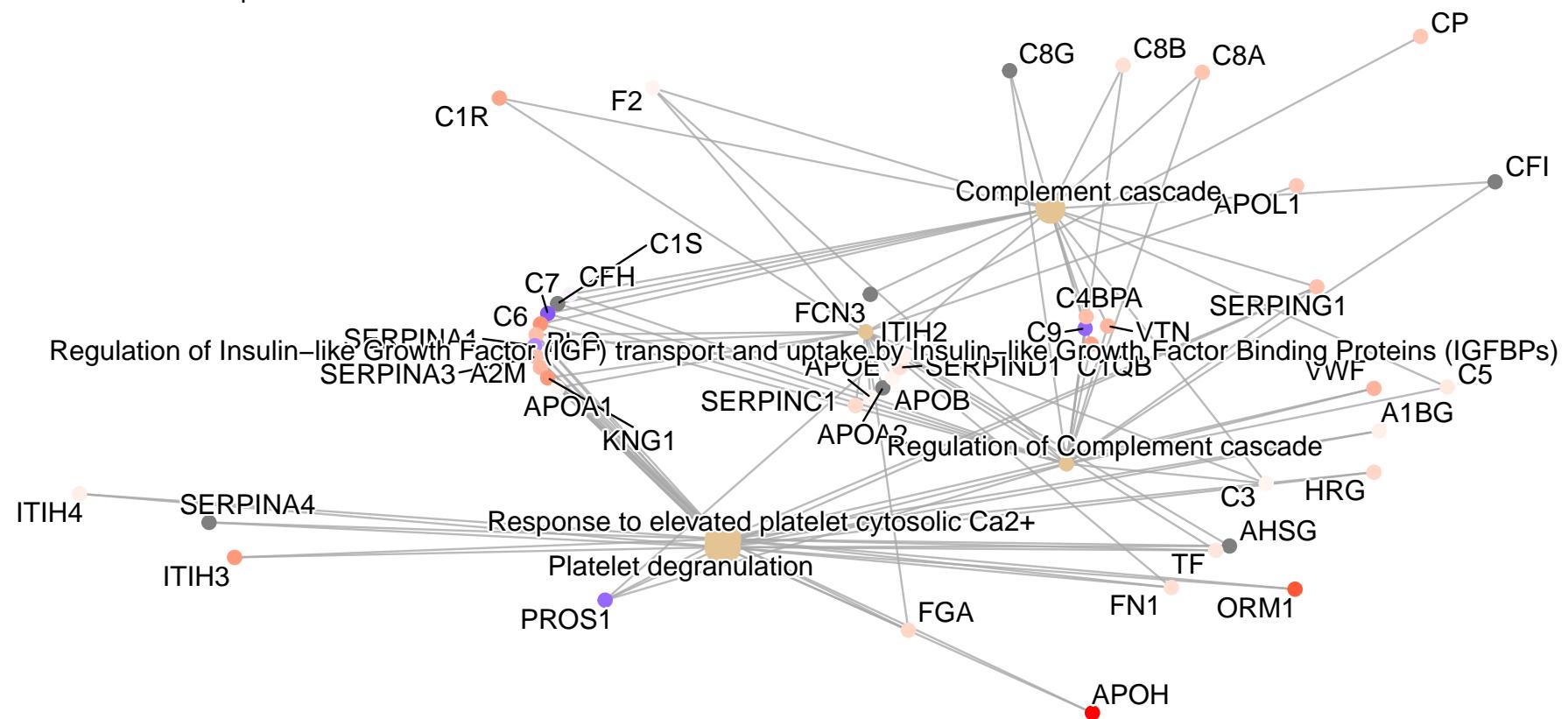


Figure S23. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

Acute C Improvers Vs Subacute C Improvers

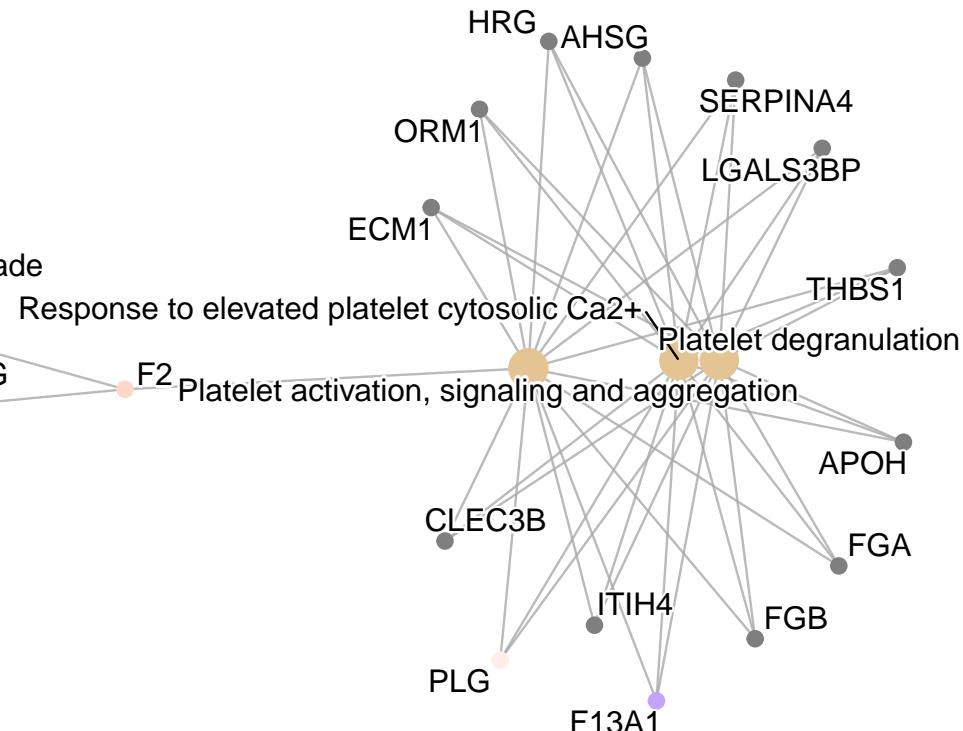
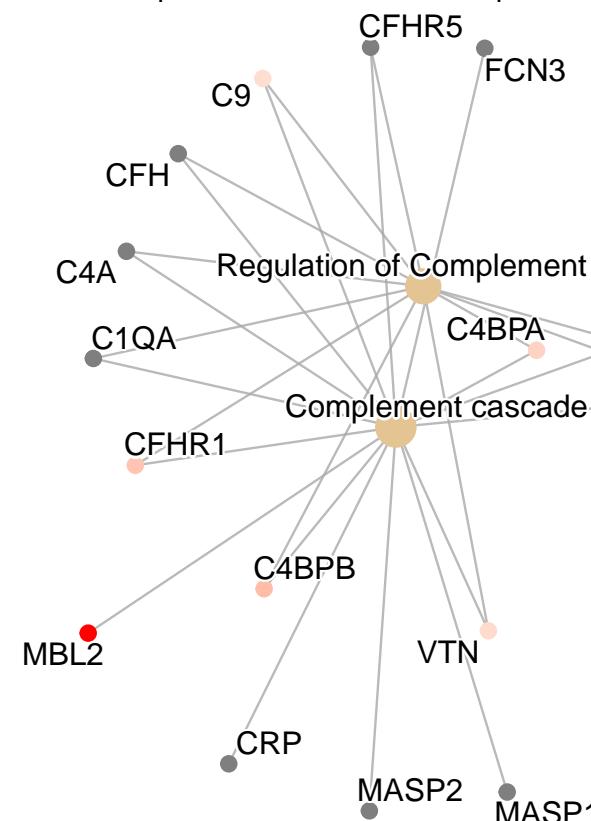


Figure S24. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute C Non-Improvers Vs Subacute C Non-Improvers

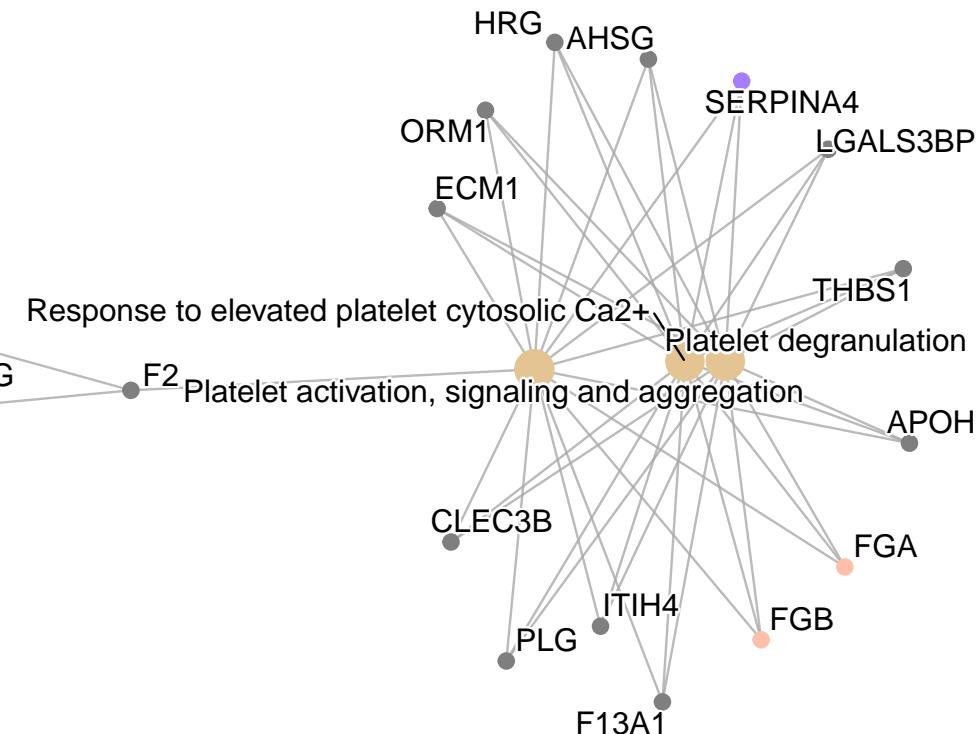
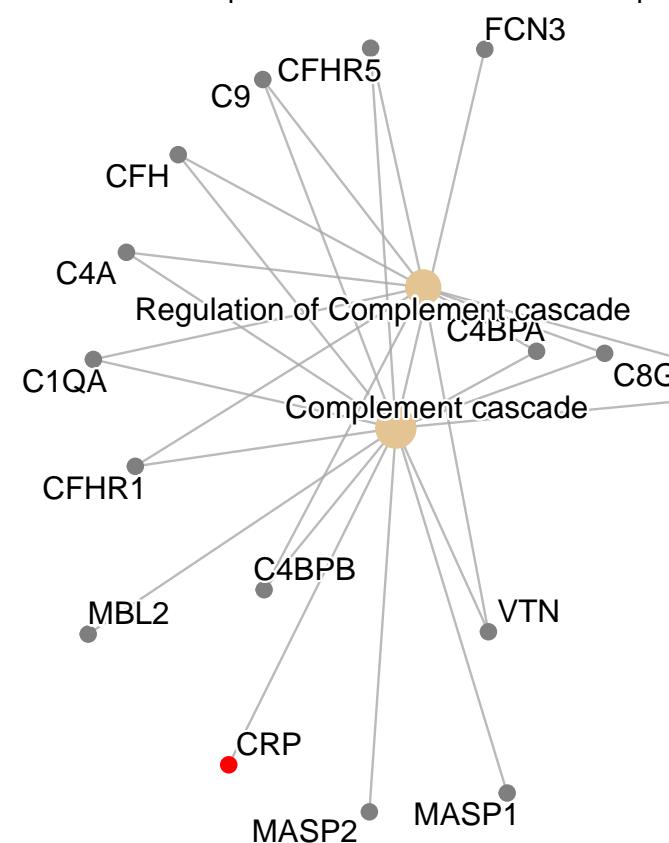
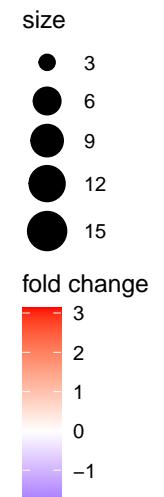


Figure S25. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months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



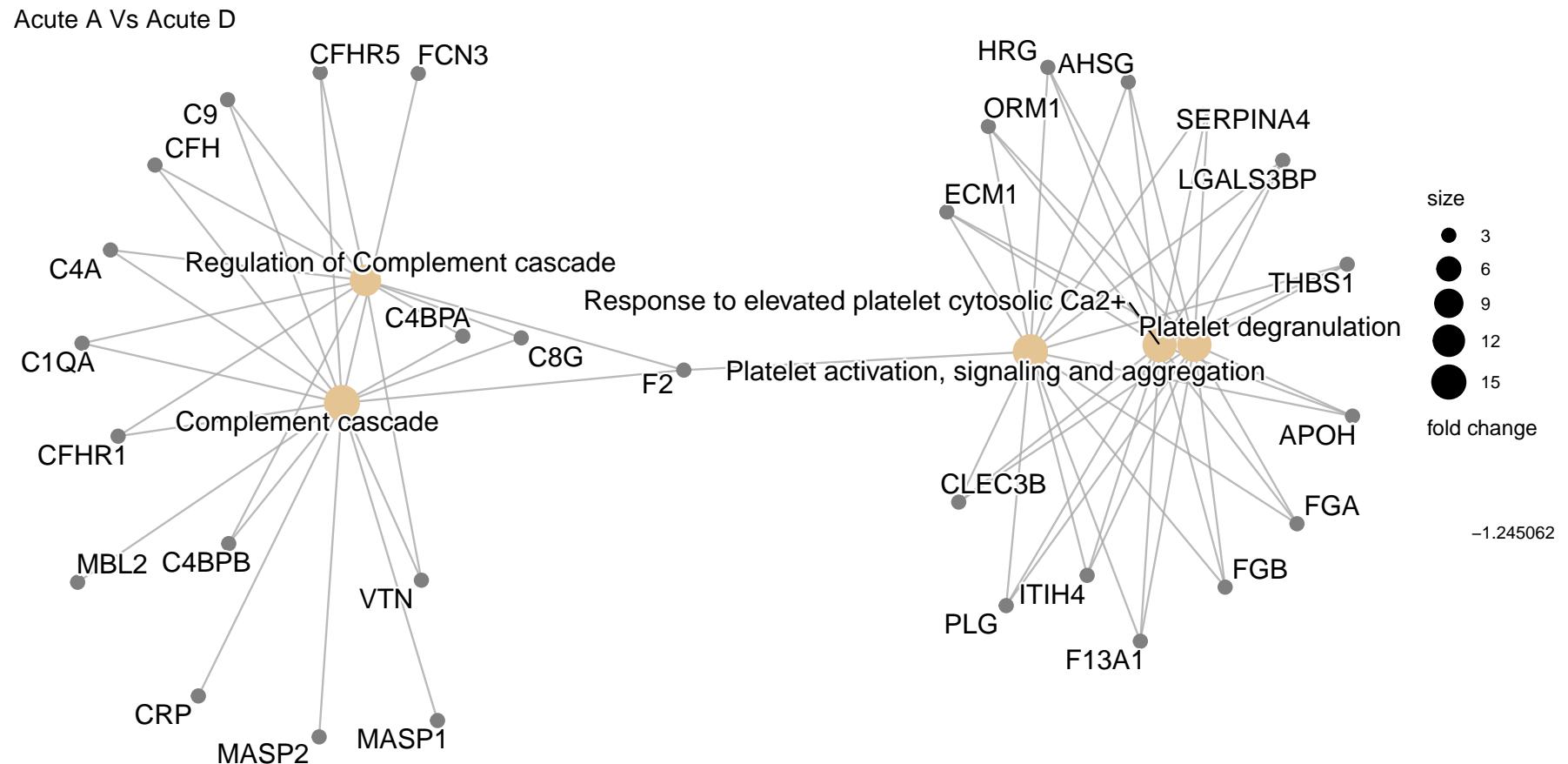
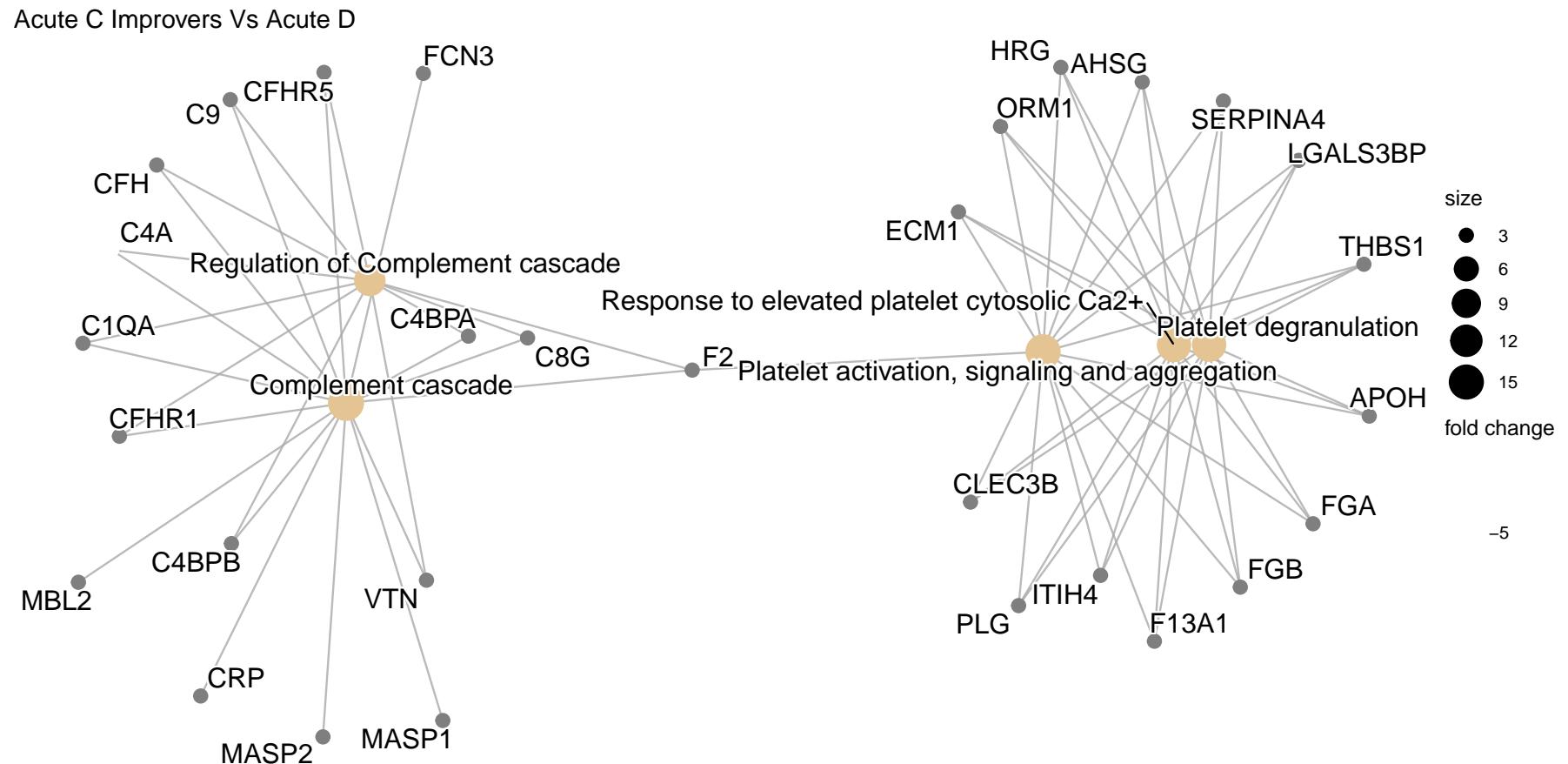


Figure S26. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.



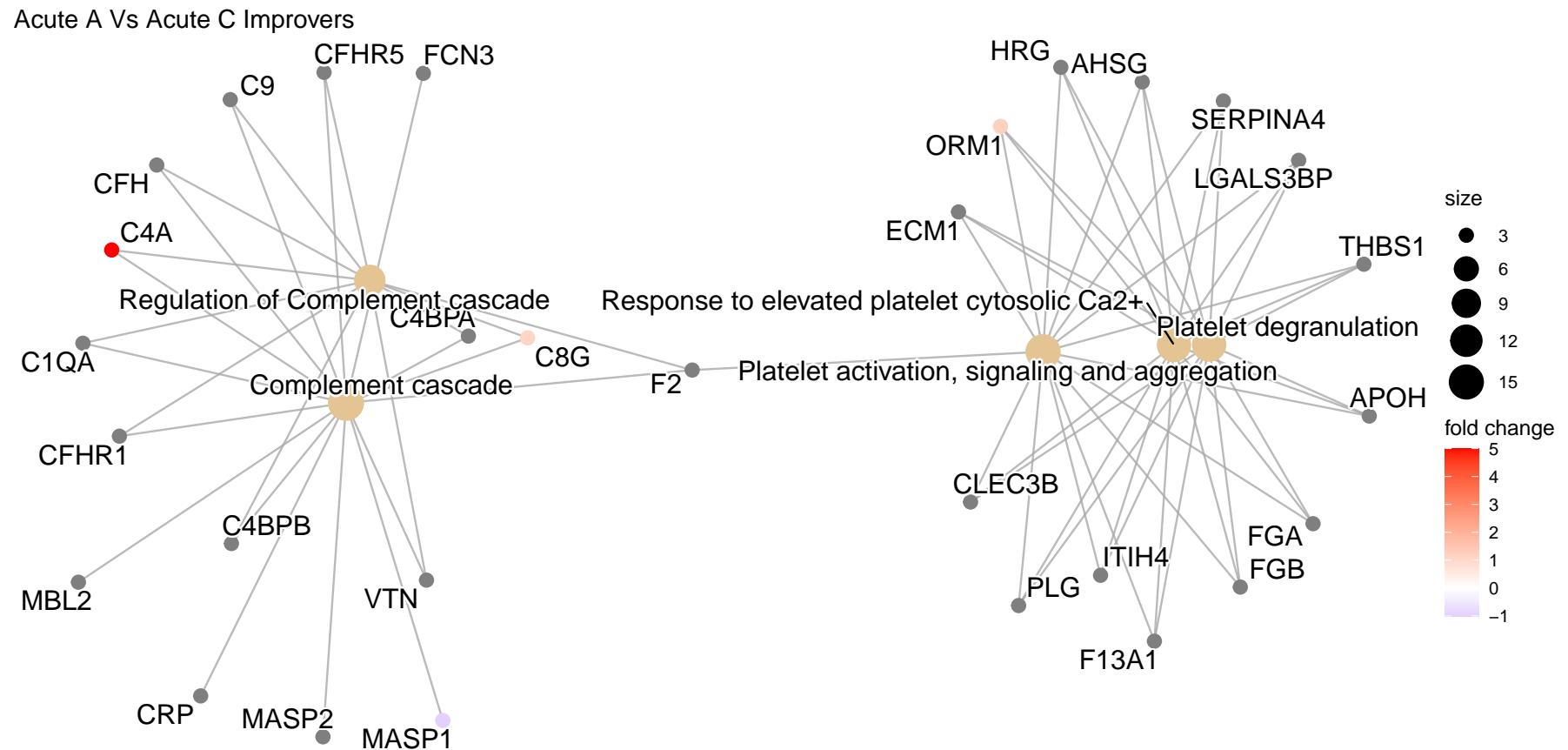
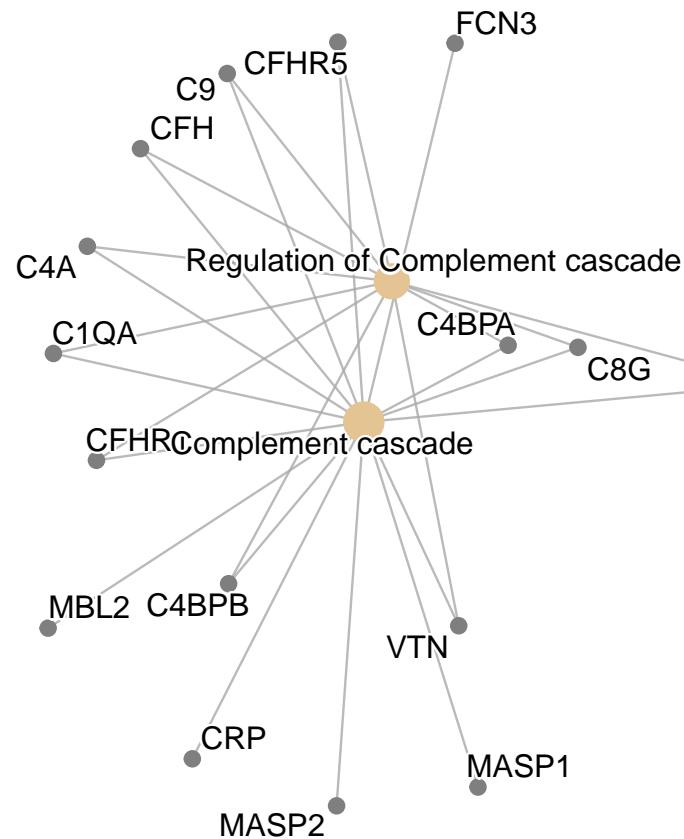


Figure S28. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute A Vs Acute C Non-Improvers



Response to elevated platelet cytosolic Ca²⁺

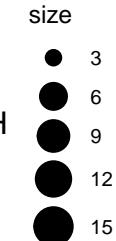
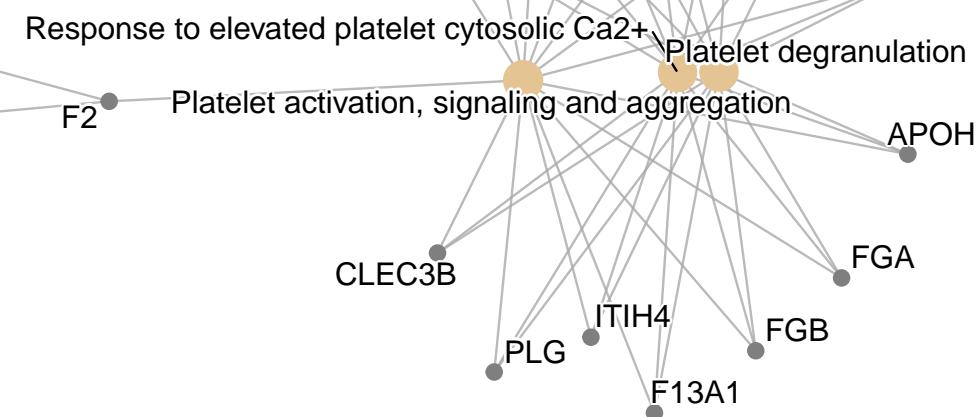


Figure S29. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute C Non-Improvers Vs Acute D

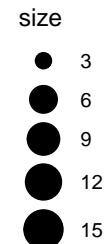
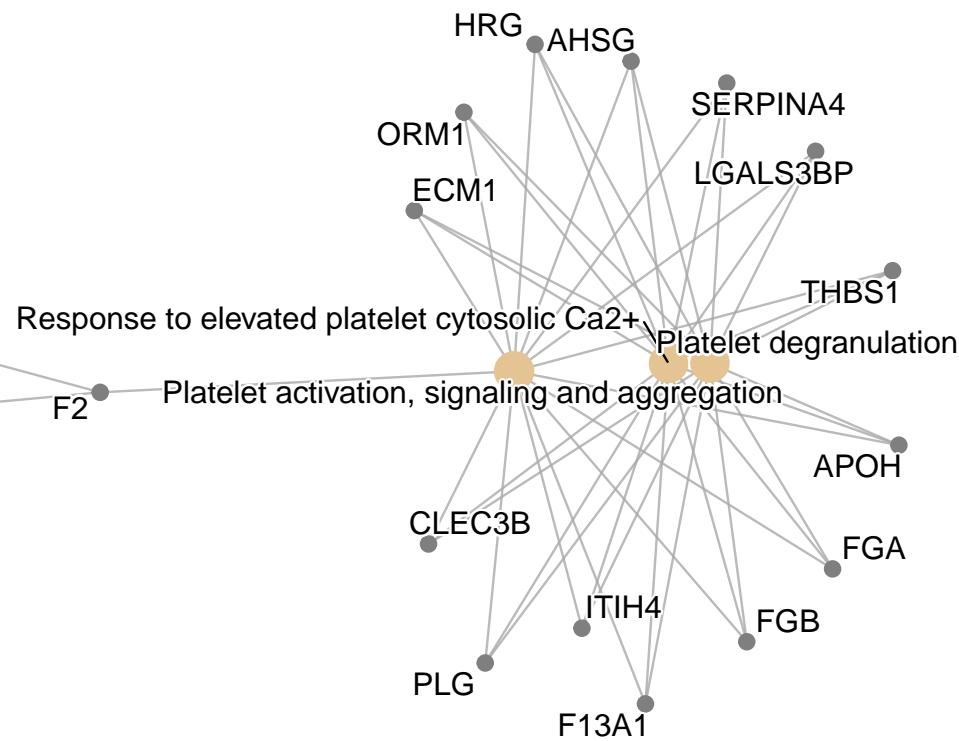
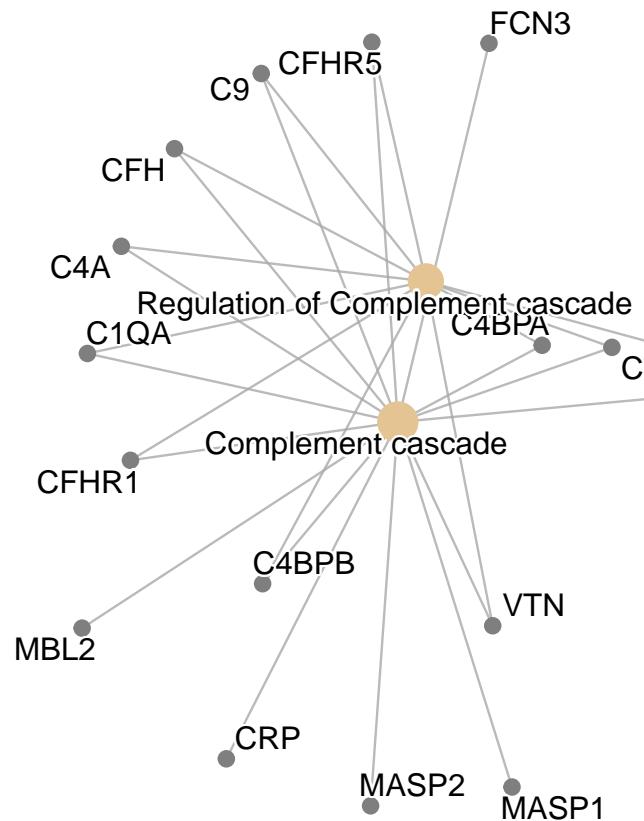


Figure S30. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

1211 5.8 STRINGdb network plots

1212 5.8.1 iTRAQ data

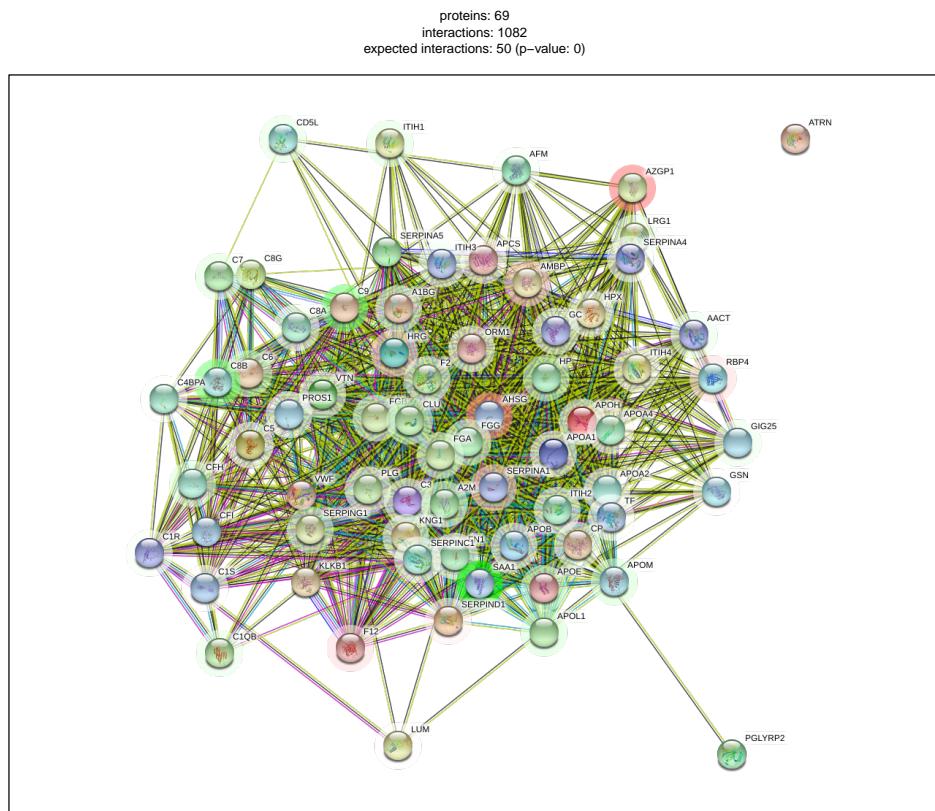


Figure S31. The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates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

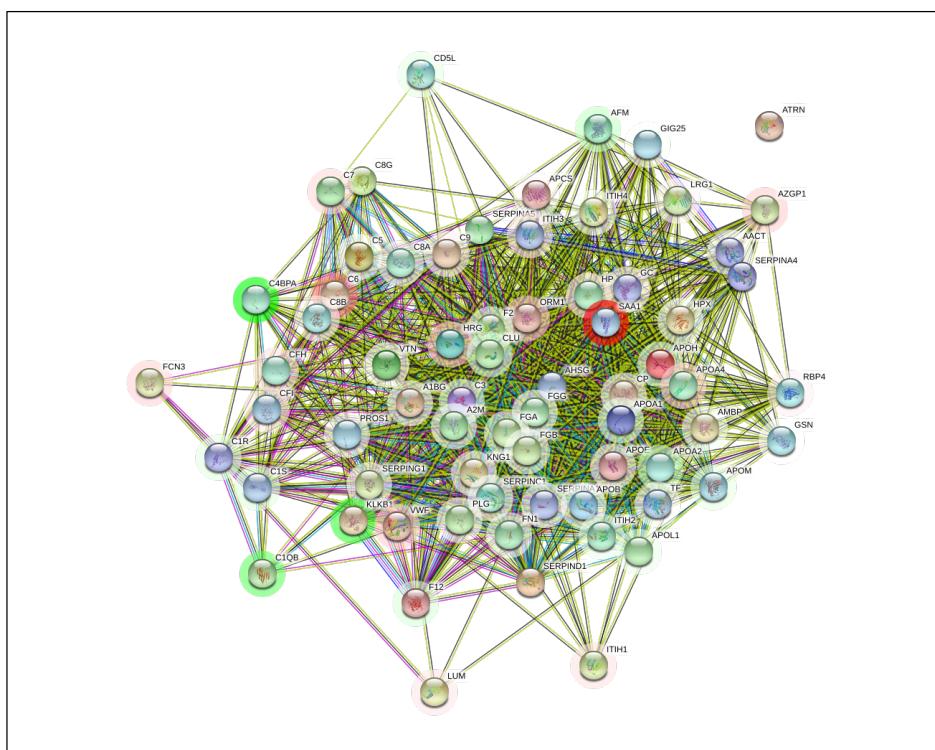


Figure S32. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there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

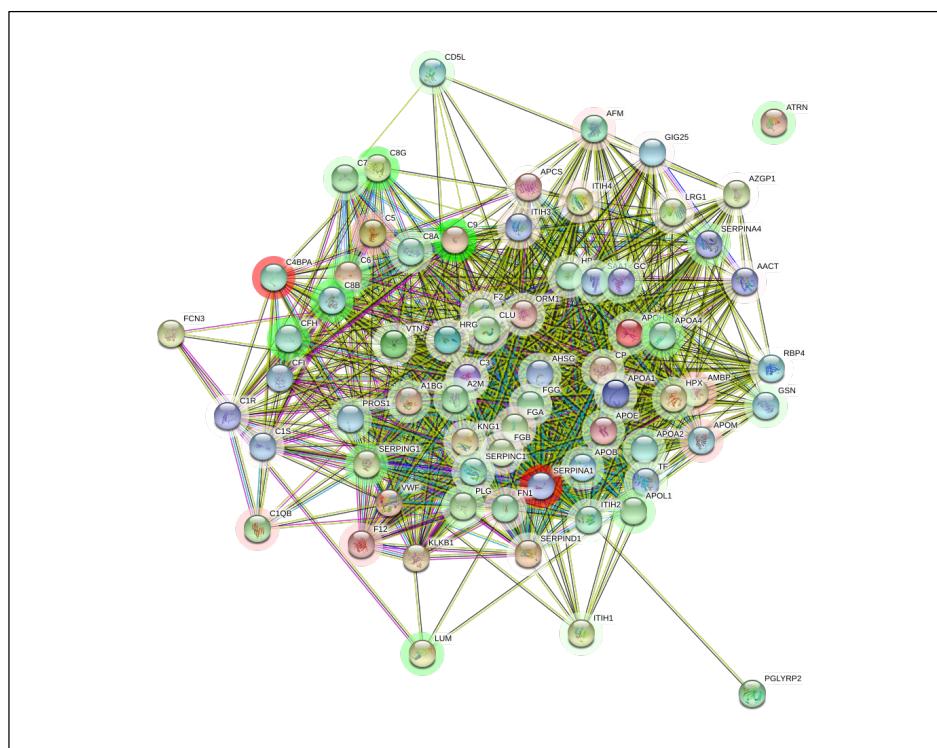


Figure S33. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance.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

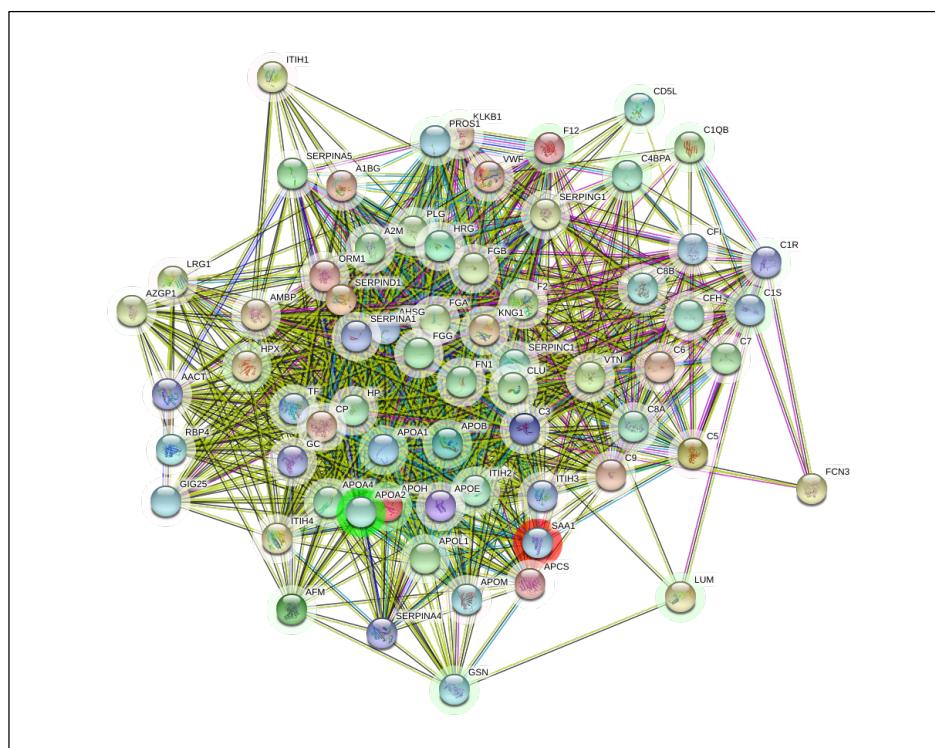


Figure S34. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater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

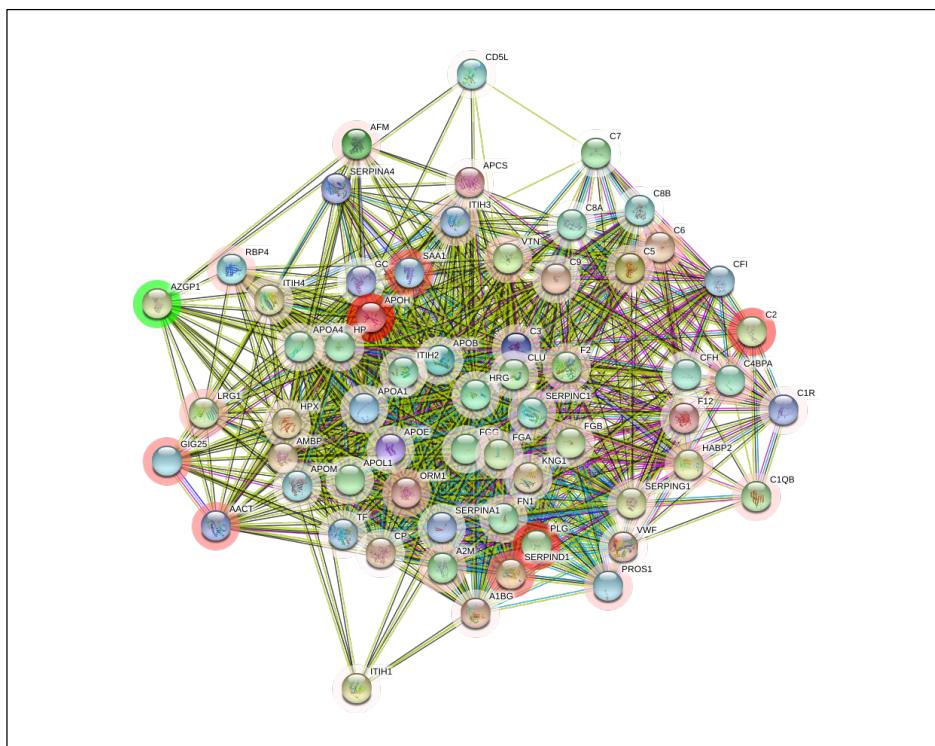


Figure S35. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

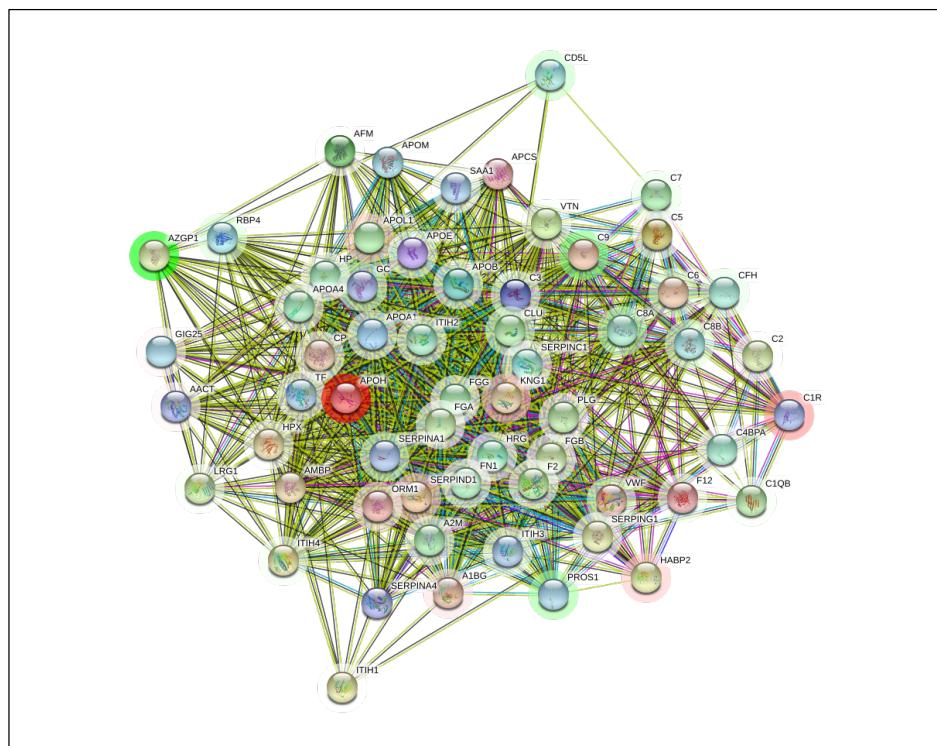


Figure S36. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

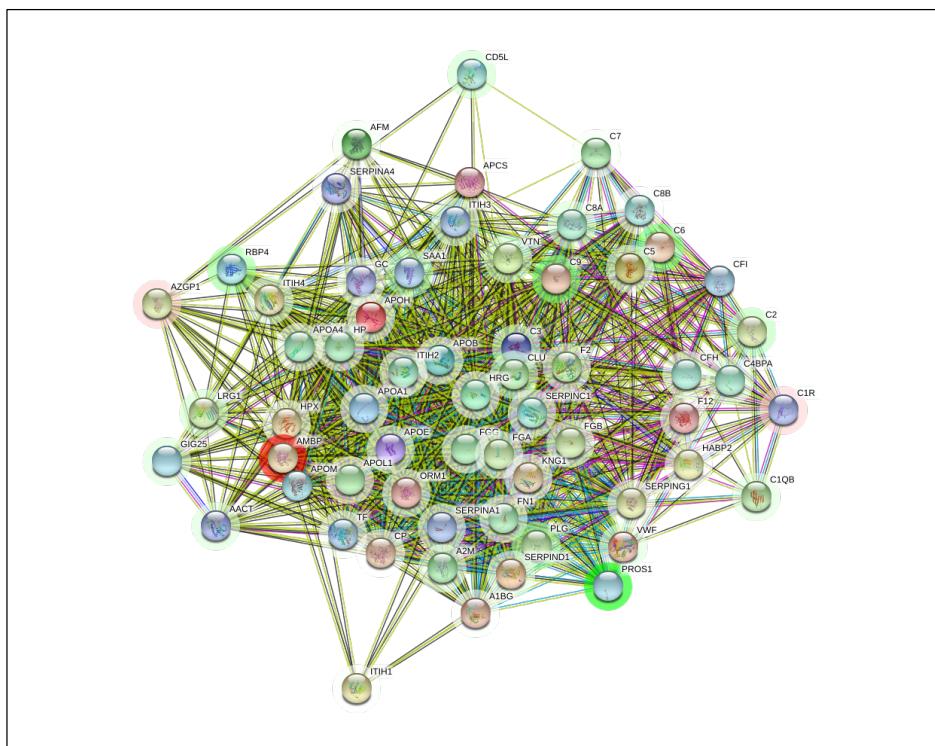


Figure S37. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

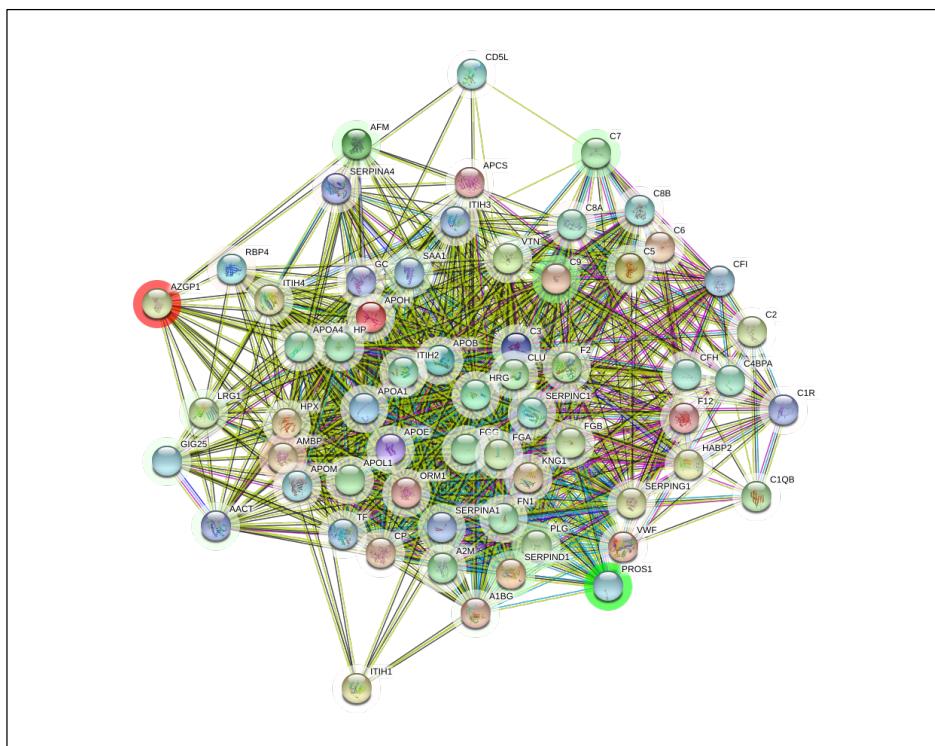


Figure S38. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

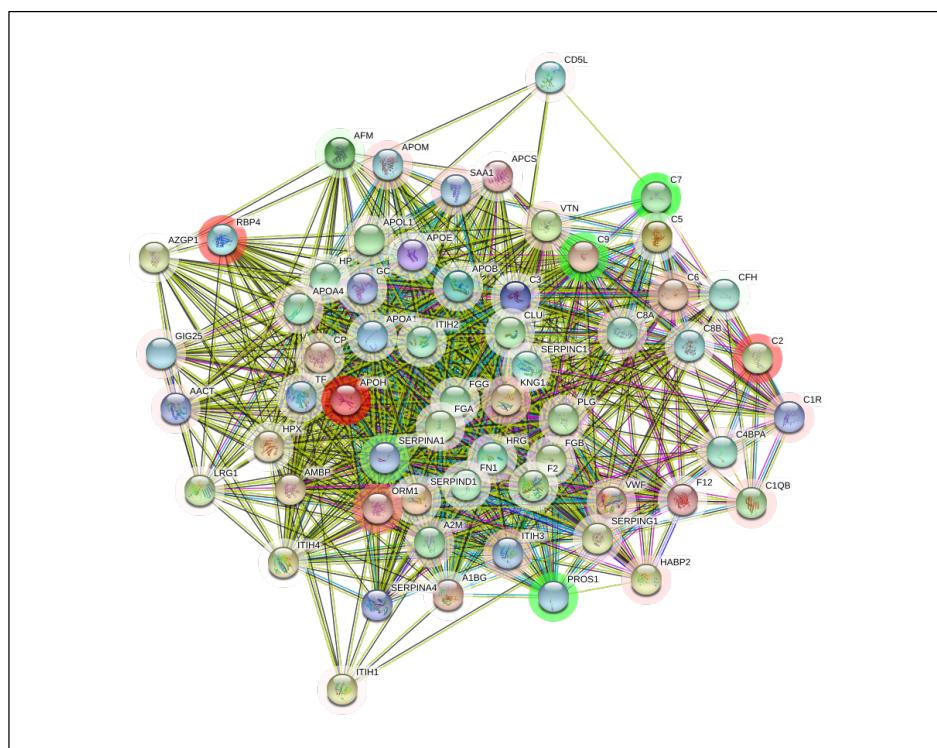


Figure S39. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

1213 5.8.2 Label-free data



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

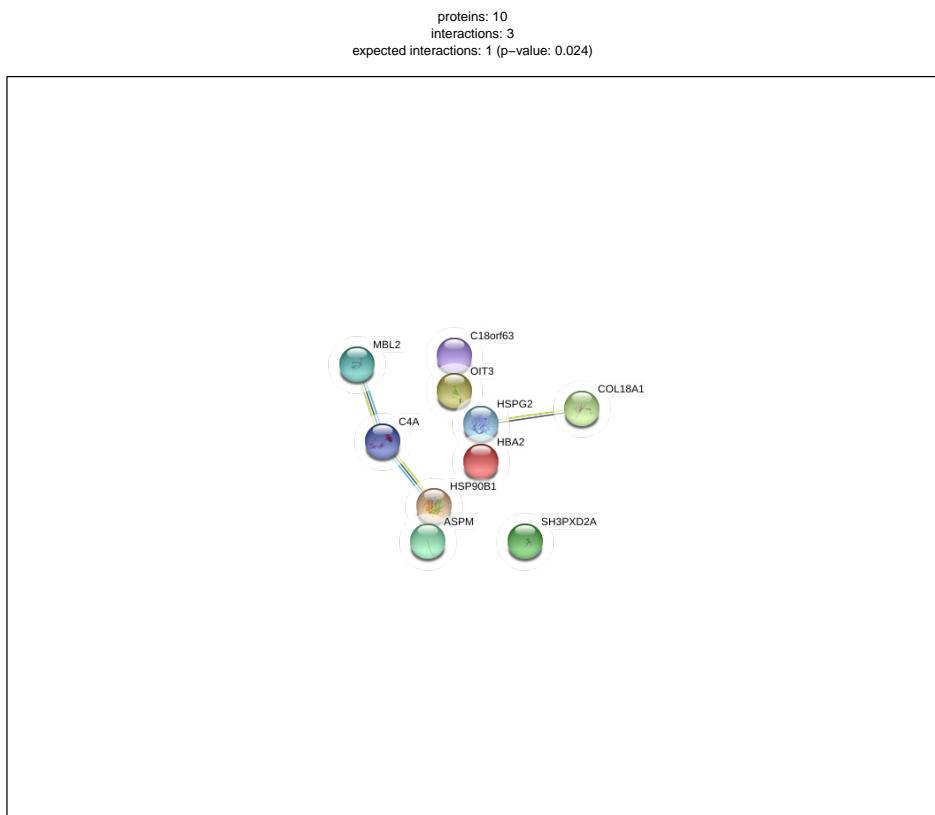


Figure S41. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there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

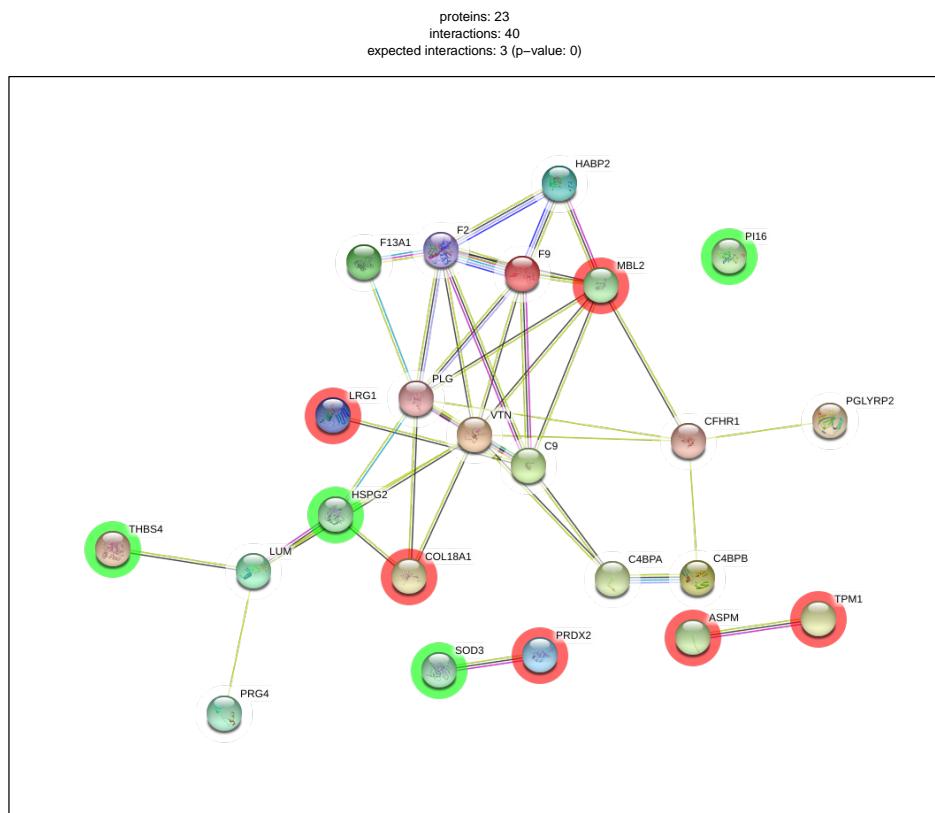


Figure S42. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance.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

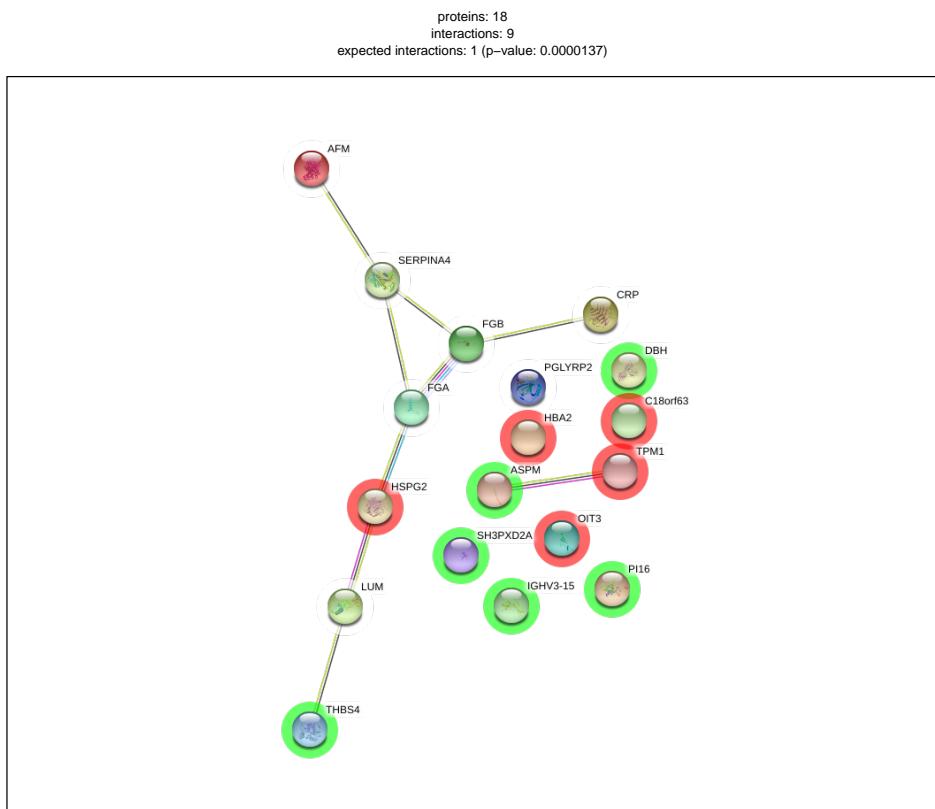


Figure S43. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater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

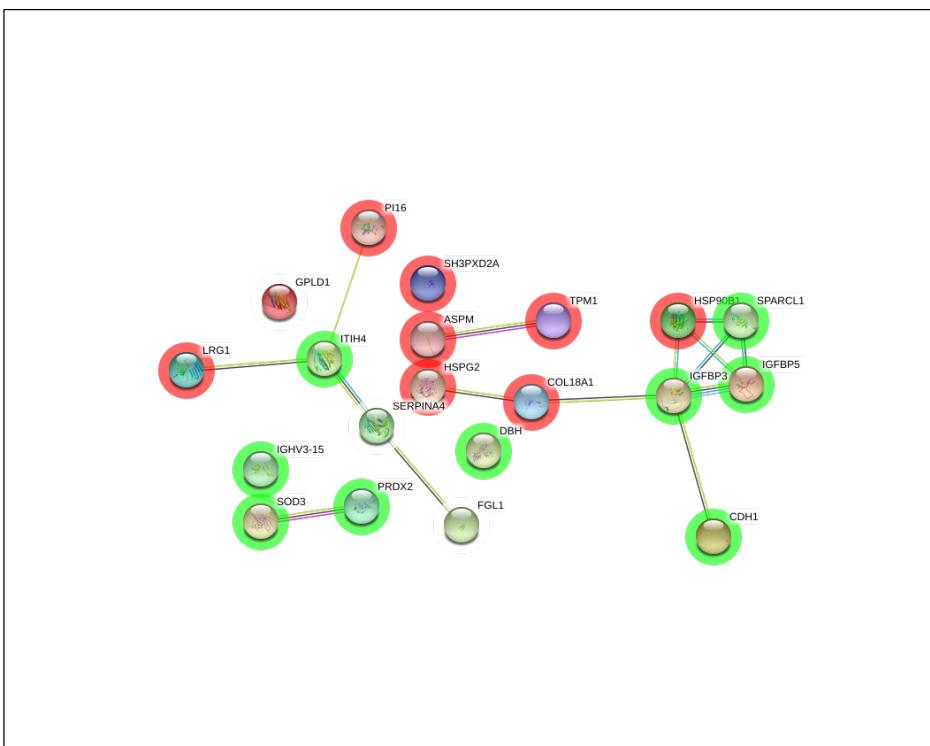


Figure S44. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

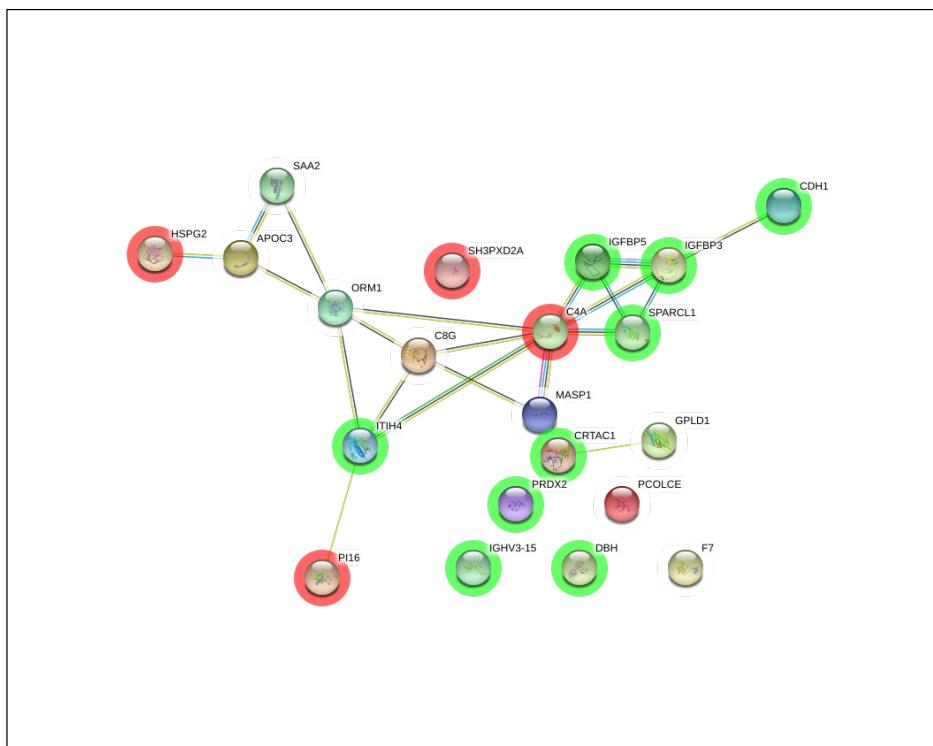


Figure S46. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

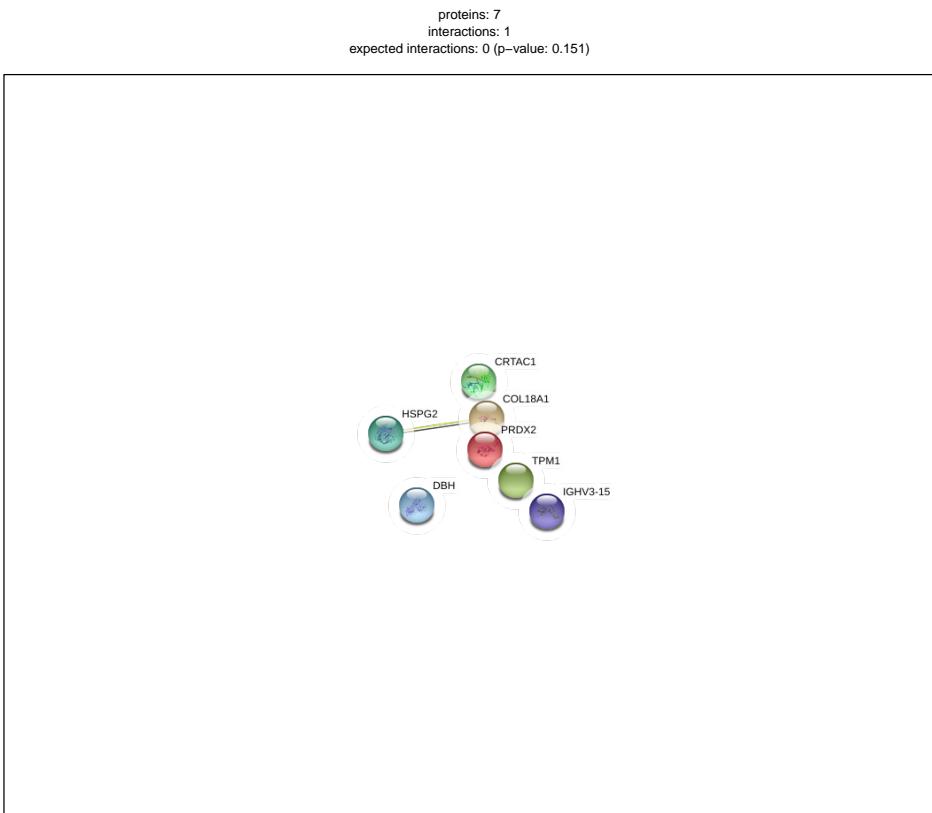


Figure S48. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

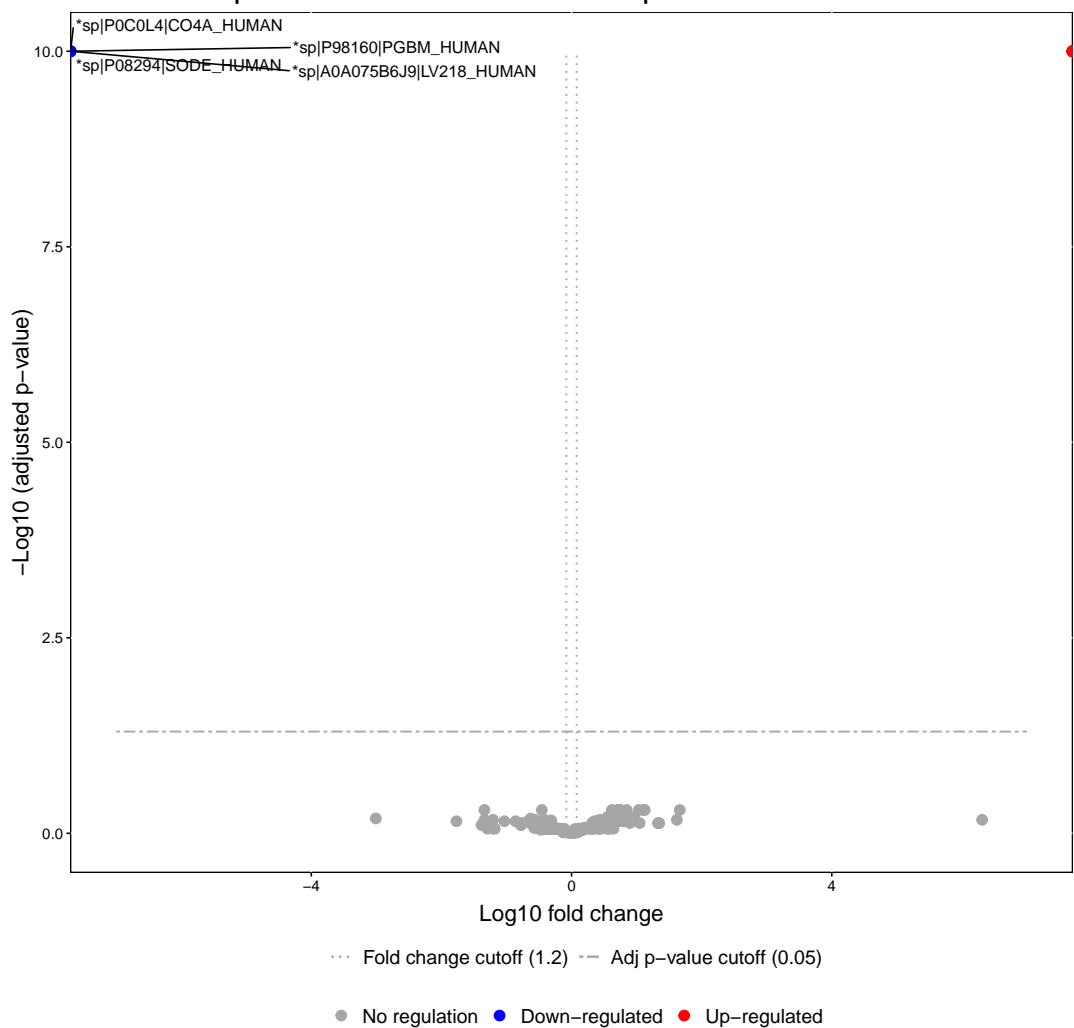


Figure S49. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Subacute C Improvers Vs Subacute C Non-Improvers

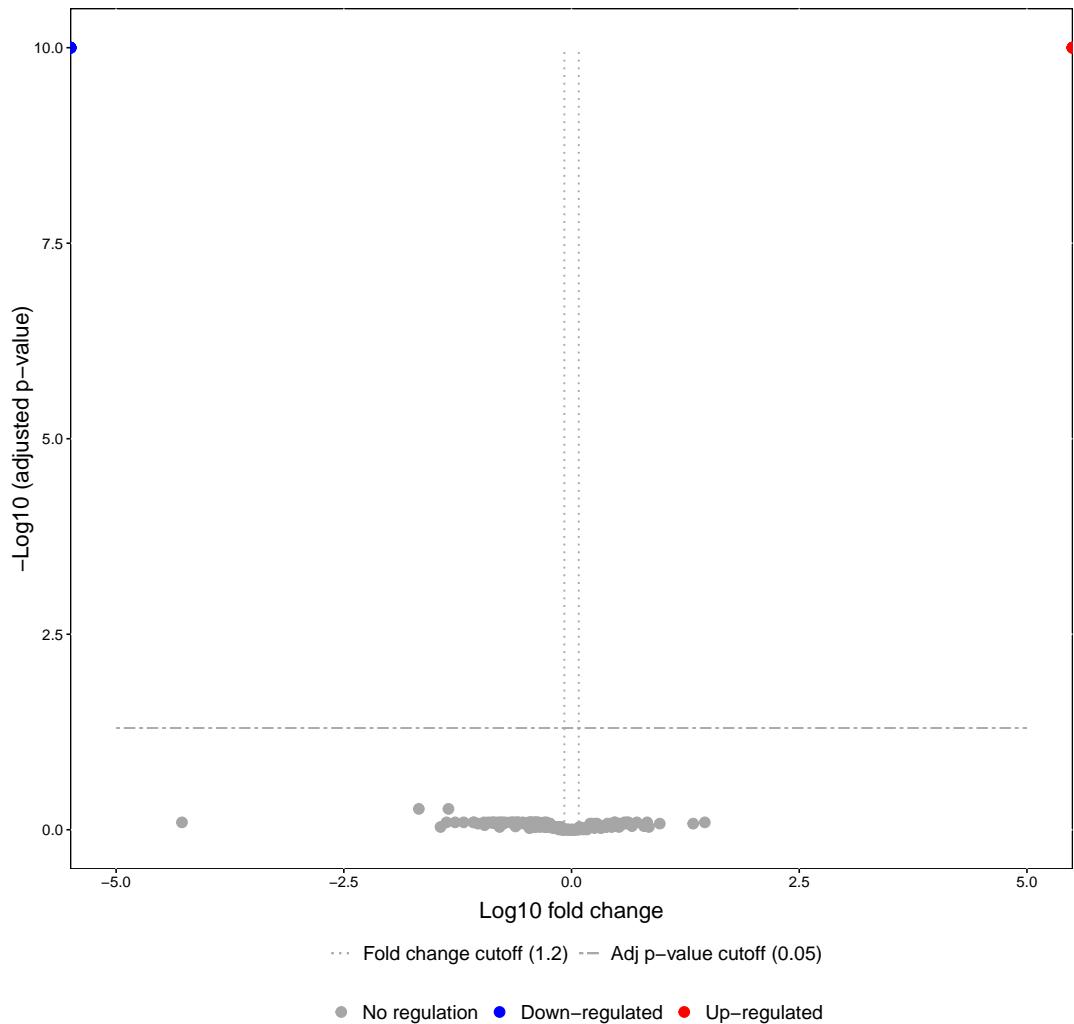


Figure S50. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 3-months post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Subacute C Improvers

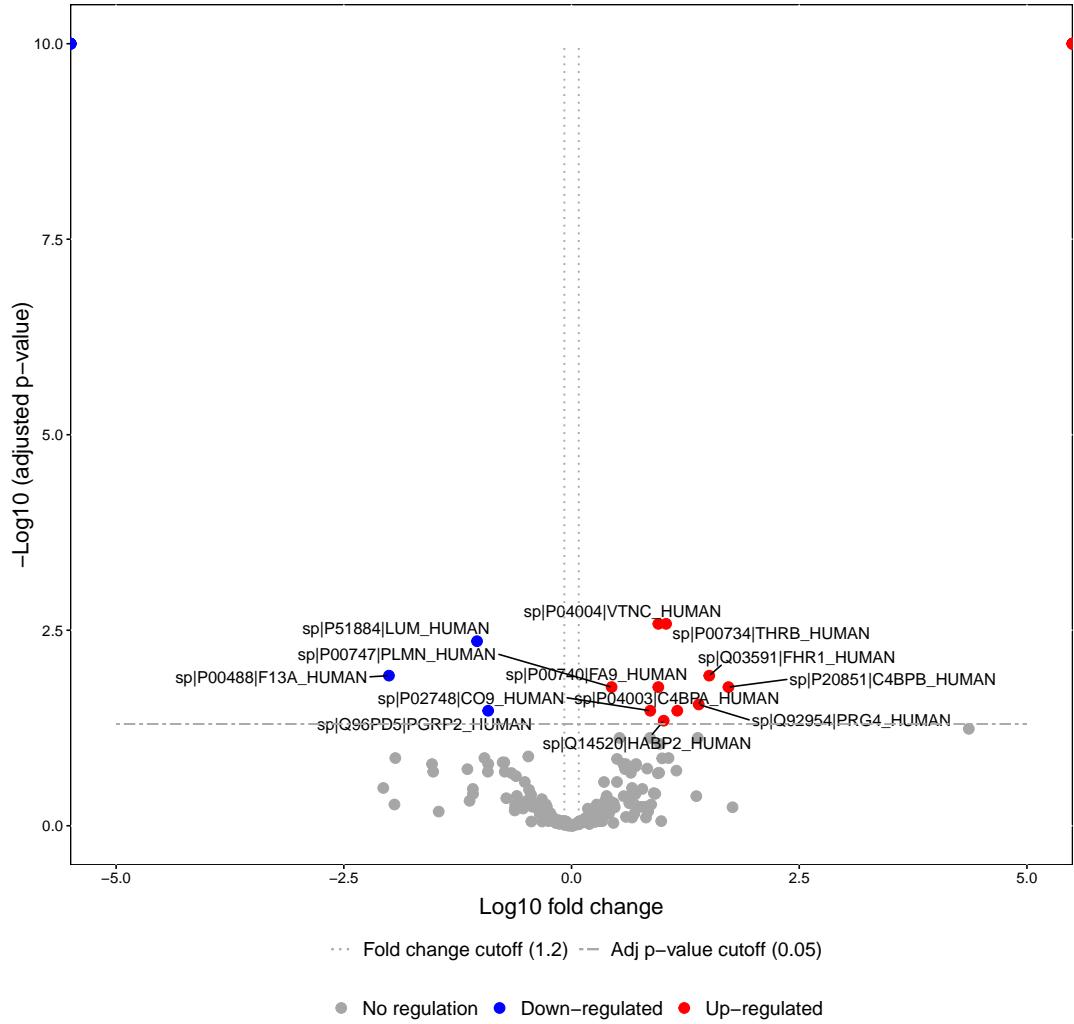


Figure S51. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Non-Improvers Vs Subacute C Non-Improvers

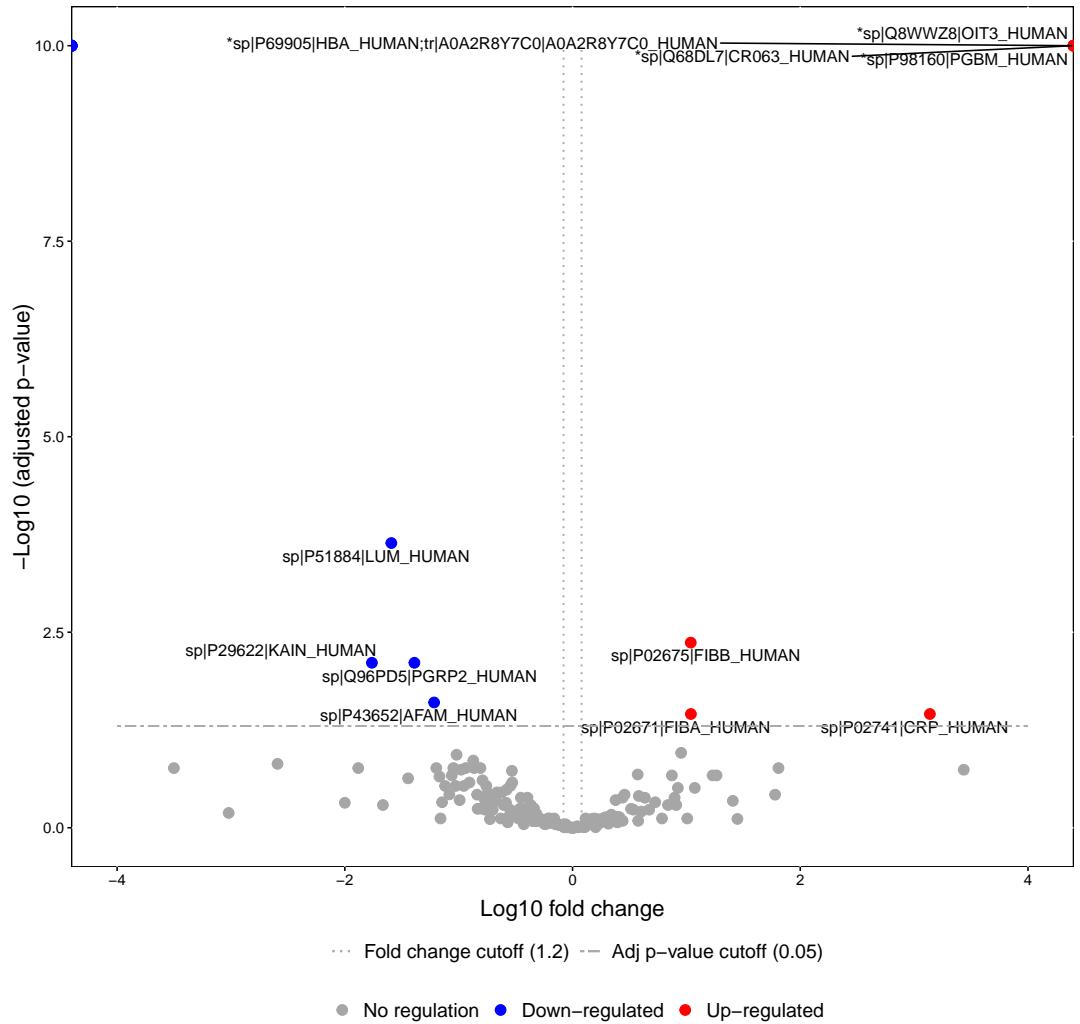


Figure S52. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute D

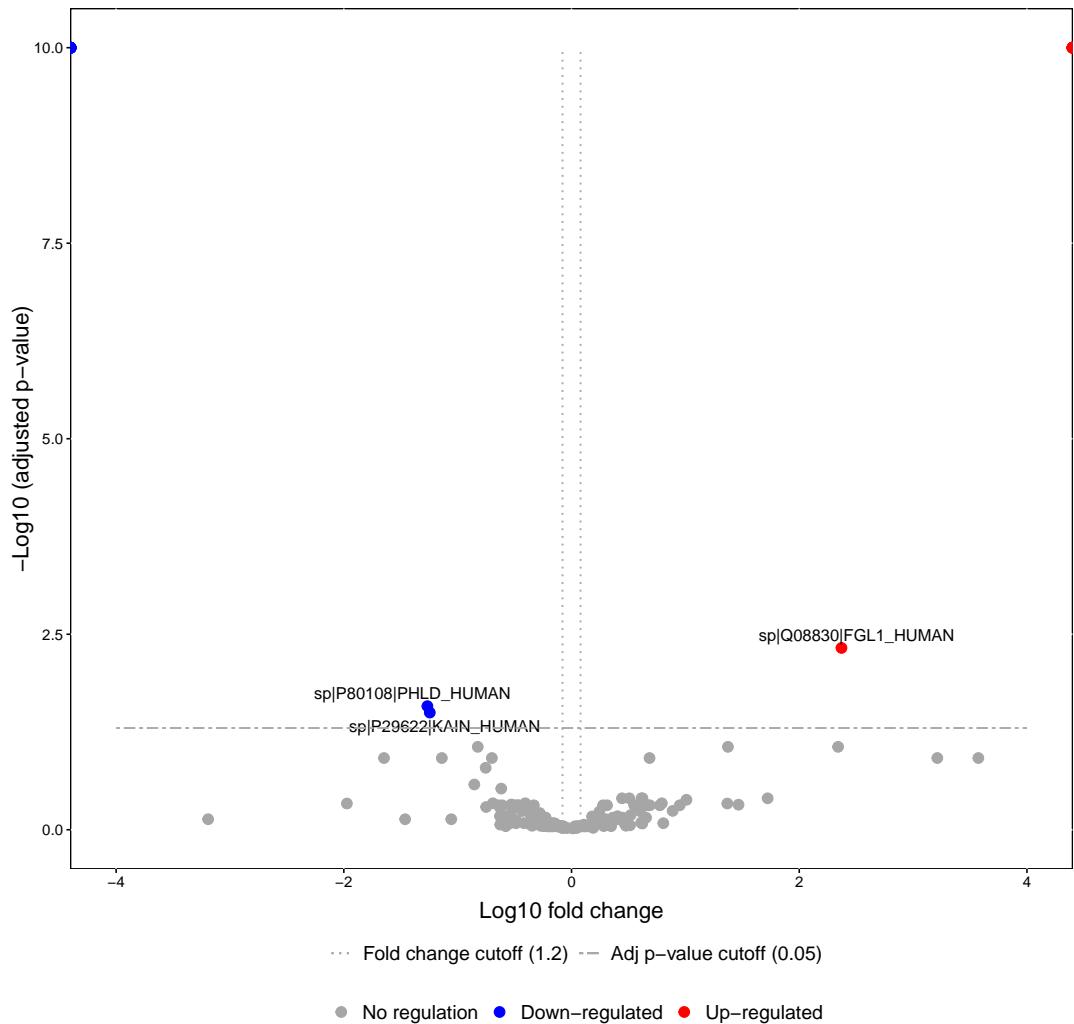


Figure S53. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Subacute A

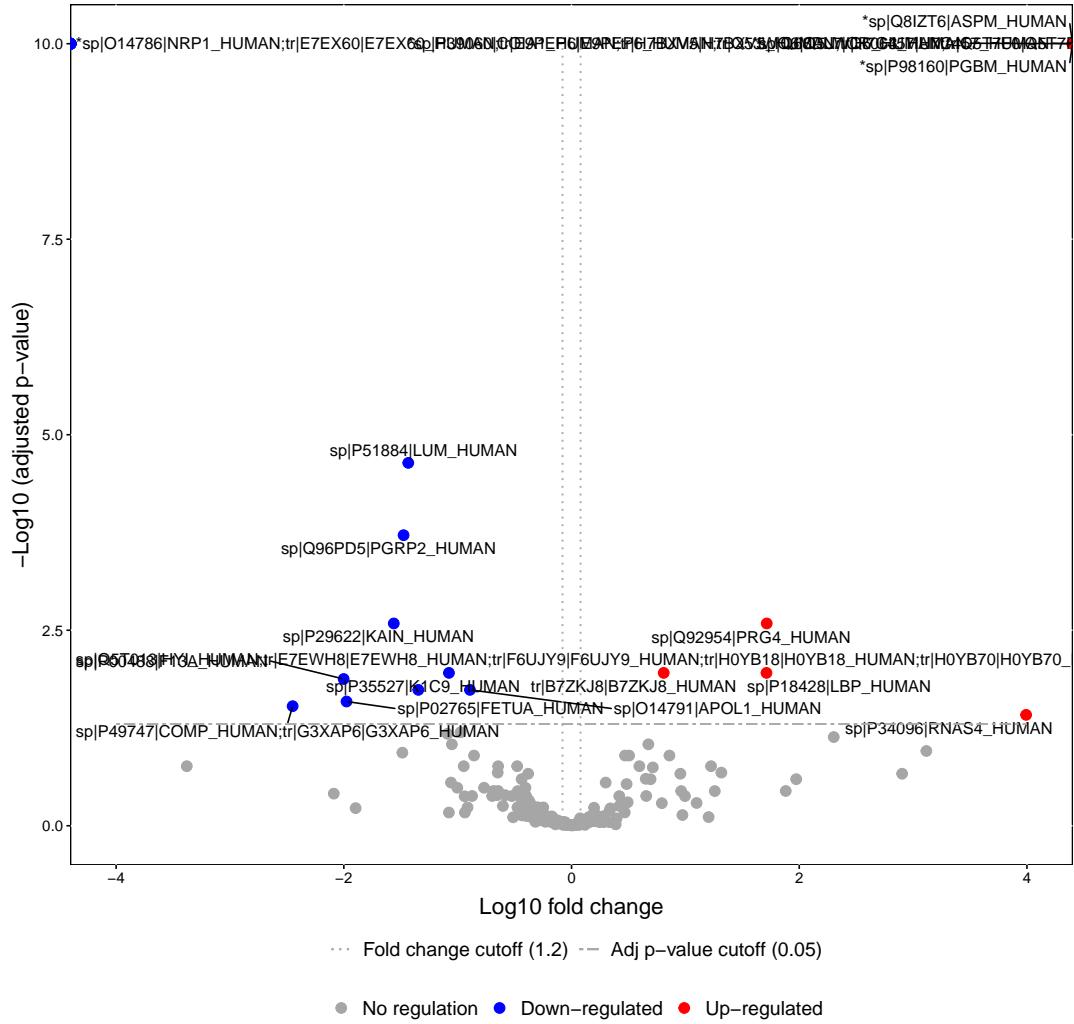


Figure S54. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS A patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute D Vs Subacute D

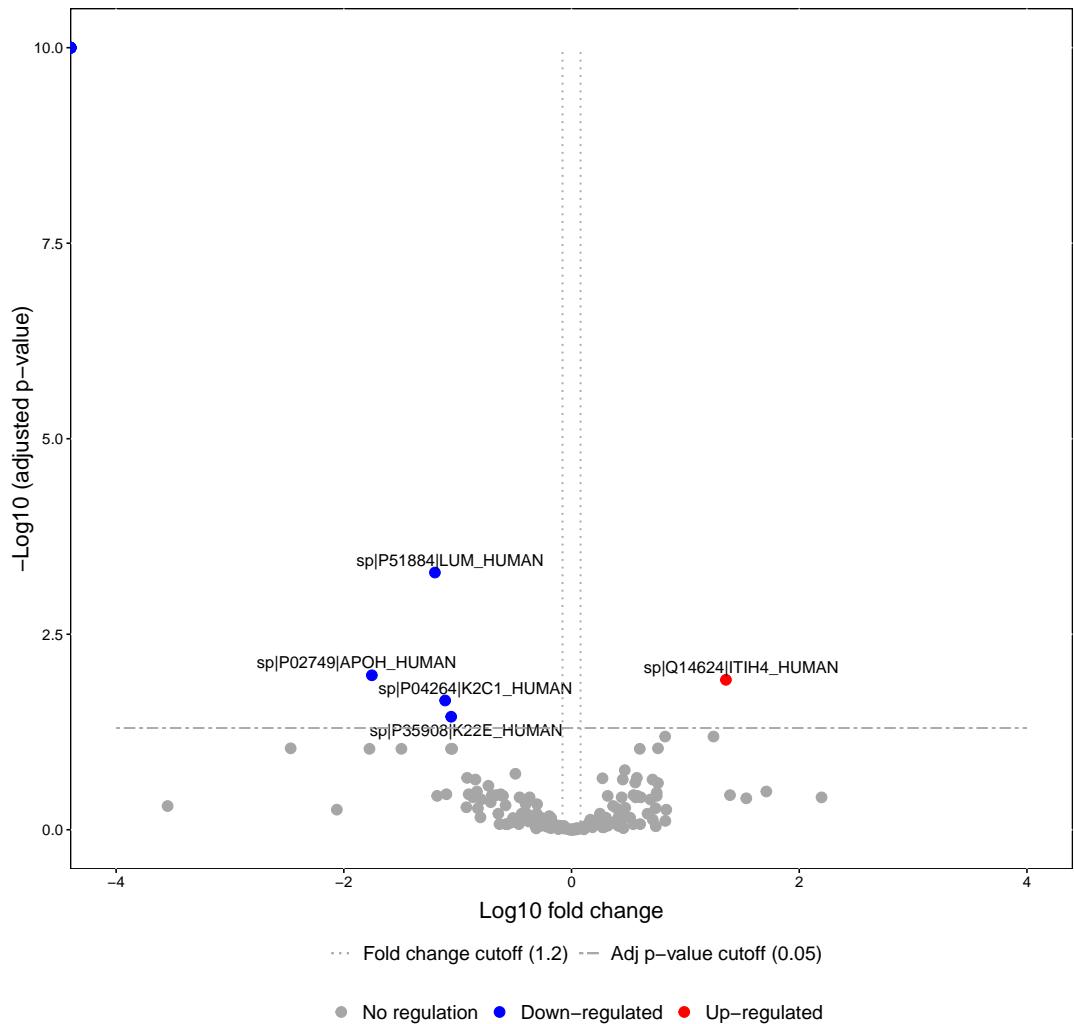


Figure S55. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS D patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Acute D

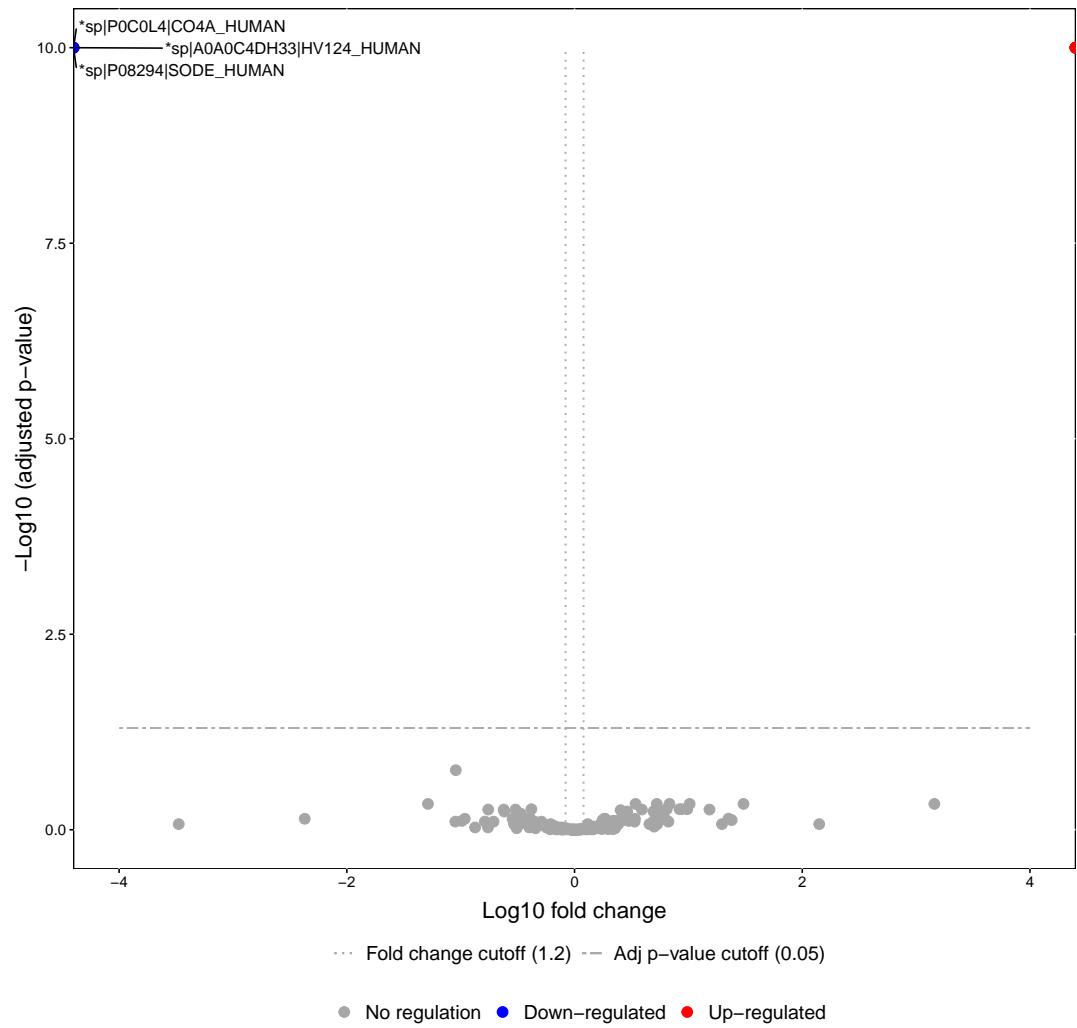


Figure S56. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute C Improvers

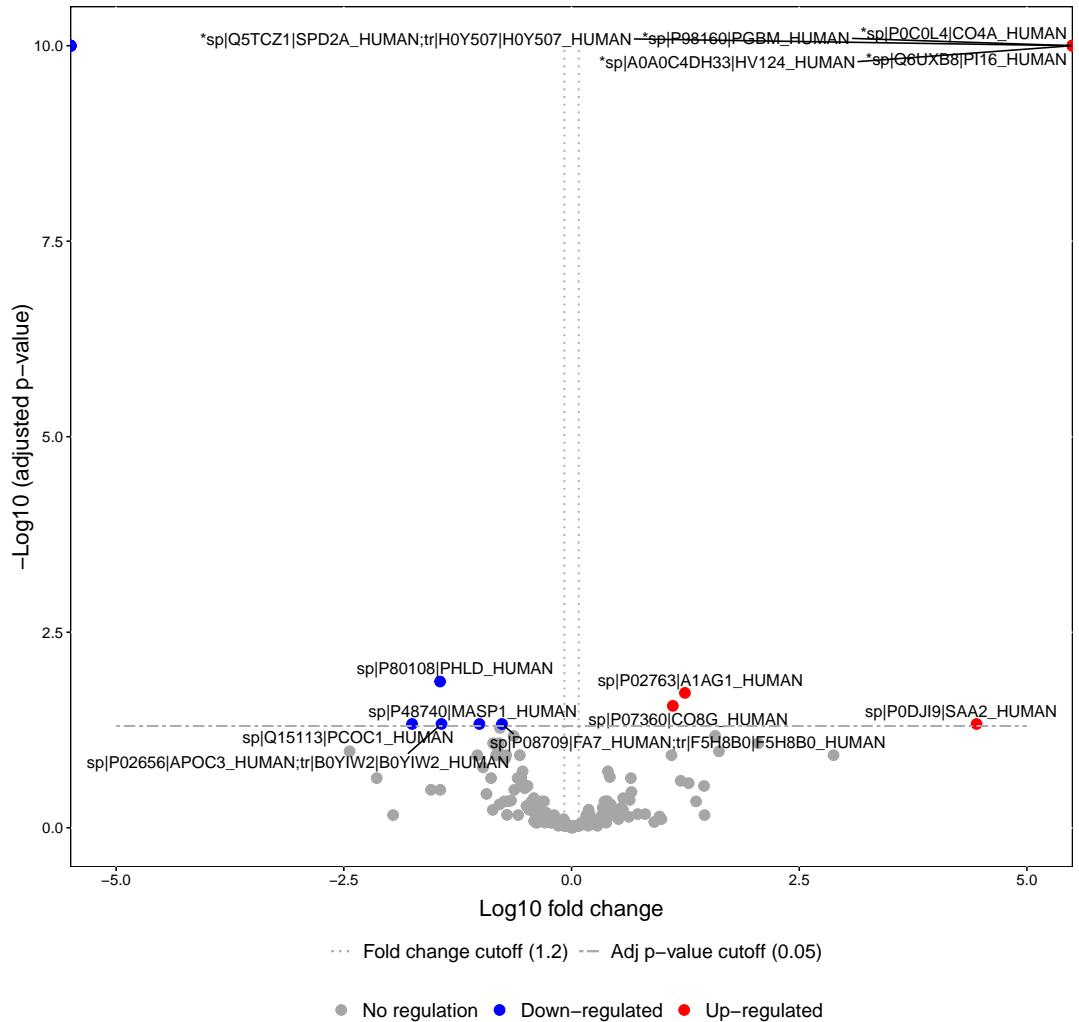


Figure S57. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

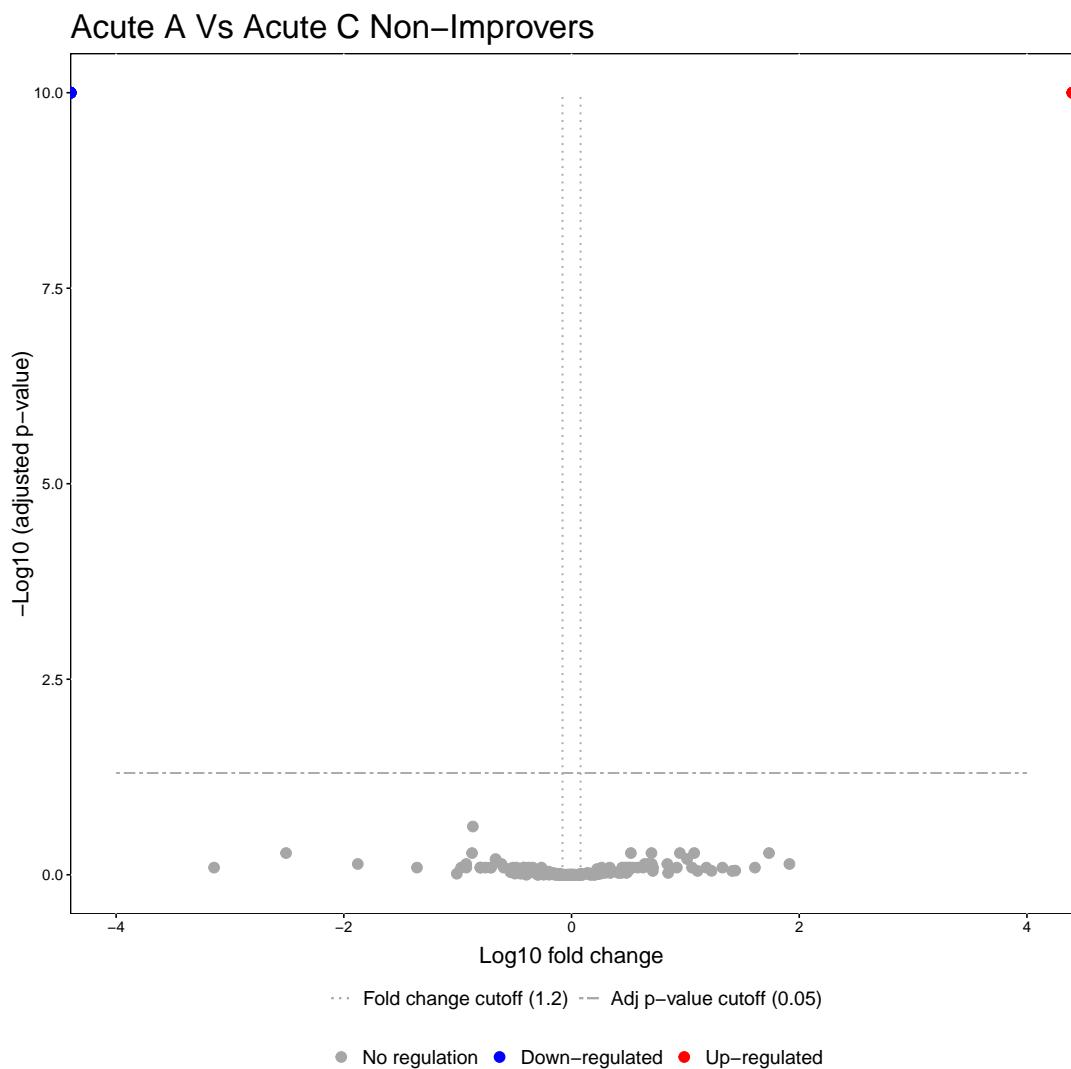


Figure S58. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

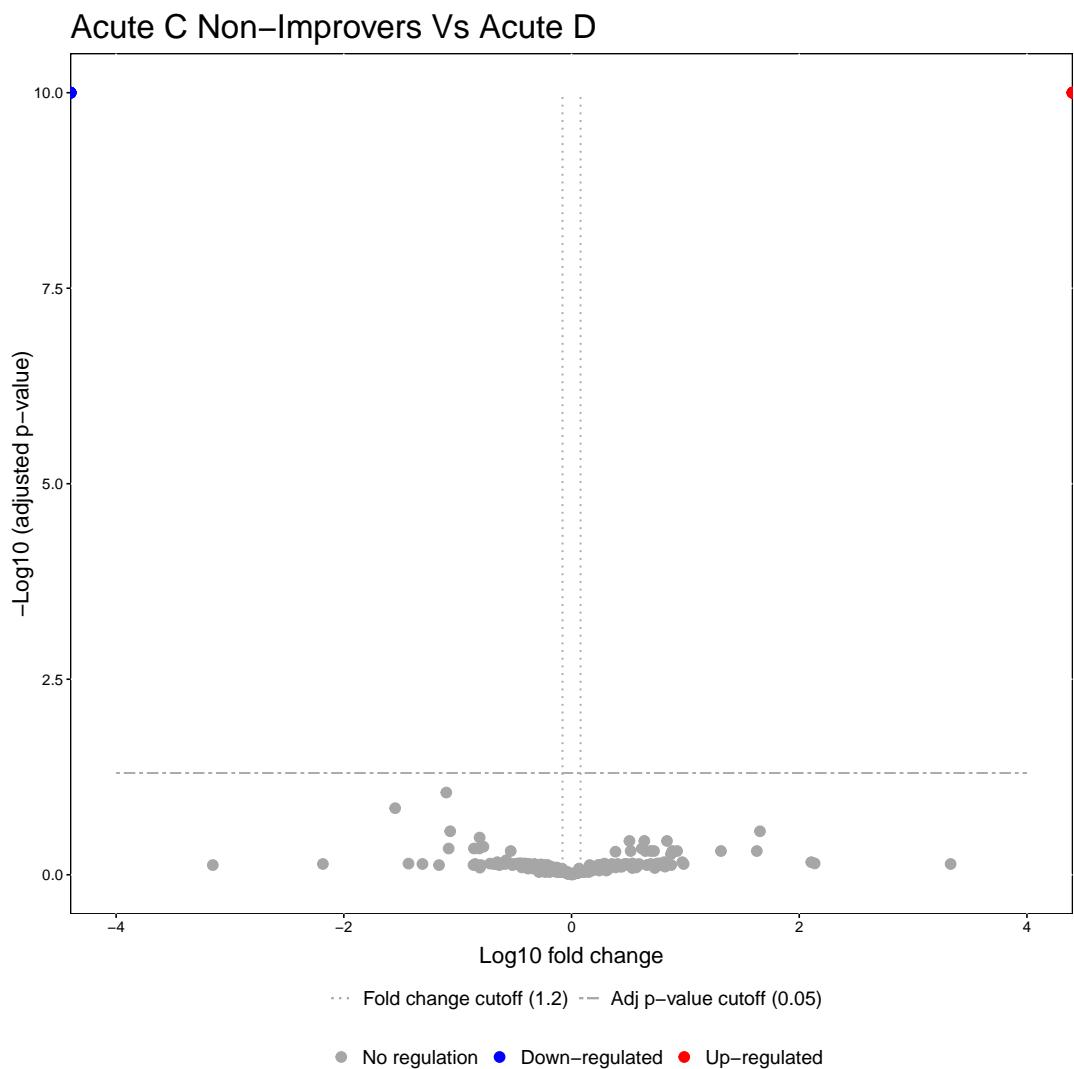


Figure S59. Volcano plot of log₁₀ fold change and log₁₀ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who did not experience an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

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