

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

104 In brief, $10\mu L$ of the diluted sample was added to $150\mu L$ of Thermo Pierce 660nm protein assay
105 reagent in triplicate and the optical density was read at 660nm.

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

109 The pooled plasma samples were then precipitated by incubation of the sample in 6 times the vol-
110 ume of chilled acetone for 1 hour at $-20^{\circ}C$. The samples were then centrifuged at 6,000G for 10
111 minutes at $4^{\circ}C$, and re-suspended in $200\mu L$ of triethylammonium bicarbonate buffer. Sequencing
112 Grade Modified Trypsin ($10\mu g\text{--}85\mu g$ of protein; Promega, Madison, WI, USA) was then added to the
113 samples for overnight digestion at $37^{\circ}C$. Proteins then underwent reduction and alkylation (ac-
114 cording to the manufacturer's instructions; Applied Biosystems, Bleiswijk, The Netherlands). Tryp-
115 tic digests were labelled with iTRAQ tags (again according to the manufacturer's instructions for the
116 iTRAQ kit), before being pooled into test groups and dried in a vacuum centrifuge. The following
117 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** Each fraction ($10\mu L$) was then analysed by nanoflow LC-ESI-MSMS. The
127 peptides were separated using a nanoLC Ultra 2D plus loading pump and nanoLC AS-2 autosampler
128 chromatography system (Eksigent, Redwood City, CA, USA), using a PepMap RSLC column ($75\mu L \times$
129 $15cm$) and an Acclaim PepMap100 trap ($100\mu m \times 2cm$) (ThermoFisher Scientific, Waltham, MA, USA).
130 After washing the peptides on the trap column for 20 minutes at $5\mu L \text{ min}^{-1}$, the trap was switched
131 in line with the column and the peptides eluted with a gradient of increasing MeCN from 95% buffer
132 A (98% H₂O, 2% MeCN, 0.1% FA), 5% buffer B (2% H₂O, 98% MeCN, 0.1% FA) to 65% buffer A, 35%
133 buffer B over 60 minutes, then to 50% buffer A, 50% buffer B over a further 20 minutes, before
134 increasing the concentration of buffer B to 95% over a further 10 minutes. The column was then
135 washed with 95% buffer B before re-equilibration in 95% buffer A. A flow rate of $300nL \text{ min}^{-1}$ was
136 employed. The eluent was sprayed into a TripleTOF 5600 tandem mass spectrometer (ABSciex,
137 Foster City, CA, USA), using a NANOSpray III source, and analyzed in Information Dependent Ac-
138 quisition (IDA) mode, performing $250ms$ of MS followed by $100ms$ MSMS analyses on the 20 most
139 intense peaks with a charge state of +2 to +5. Parent (MS) ions were accepted with a mass toler-
140 ance of 50 mDa and MSMS was conducted with a rolling collision energy (CE) inclusive of preset
141 iTRAQ CE adjustments. Analyzed parent ions were then excluded from analysis for 13 s after 3
142 occurrences.

143 **3.3.1 Sample preparation and analysis using label-free proteomics**

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

148 To reduce the dynamic range of proteins, ProteoMiner™ beads (BioRad, Hemel Hempstead, UK)
149 were used. Firstly, plasma was treated with $1 \mu\text{g}\cdot\text{mL}^{-1}$ of hyaluronidase. Digestion was confirmed
150 with Coomassie stained 1D-SDS PAGE gel. The supernatant was centrifuged through a $0.22 \mu\text{m}$ cel-
151 lulose acetate membrane (Costar Spin-X, Corning, Tokyo, Japan) tube filter (5000g for 15 minutes)
152 to remove insoluble material. Total protein was quantitated with a Pierce™ 660nm Protein Assay
153 (Thermo Fisher Scientific, Hemel Hempstead, UK), whereupon 5 mg of total protein was applied to
154 ProteoMiner™ beads, and processed as described previously.(Stoscheck 1987)

155 **3.3.1.1 Label free mass spectrometry analysis** Tryptic peptides were subjected to LC-MC/MC
156 via a 2-h gradient on a NanoAcuity™ ultraperformance LC (Waters, Manchester, UK) connected
157 to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Fisher Scientific Hemel Hempstead, UK)
158 as described **previously**.

159 **REWRITE IN BRIEF** The Q-Exactive was operated in a data dependent positive electrospray ion-
160 isation mode, automatically switching between full scan MS and MS/MS acquisition. Survey full
161 scan MS spectra (m/z 300–2000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) fol-
162 lowing accumulation of ions to 1×10^6 target value based on the predictive automatic gain control
163 values from the previous full scan. Dynamic exclusion was set to 20s, the 10 most intense multiply
164 charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by
165 higher energy collisional dissociation (HCD), with a fixed injection time of 100ms and 35,000 res-
166 olution. The following mass spectrometric conditions were used: spray voltage, 1.9kV, no sheath
167 or axillary gas flow; normalised HCD collision energy 30%; heated capillary temperature, 250°C.
168 MS/MS ion selection threshold was set to 1×10^4 count and 2Da isolation width was set.

169 **3.3.2 iTRAQ OpenMS analysis**

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

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

185 To annotate the search results PeptideIndexer and PSMFeatureExtractor were used. For peptide
186 level score estimation and filtering PercolatorAdapter was used with the following arguments:

187 -score_type q-value -enzyme trypsinp. IDFfilter was used to filter to a peptide score of 0.05
188 with -score:pep 0.05

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

197 **3.3.3 Label free OpenMS analysis**

198 For quantification, the raw spectra files were analysed via OpenMS (version 2.6.0) command line
199 tools, with the workflow from the prior section (3.3.2) adapted to suit a label-free analysis. The
200 files were first converted from the proprietary .Raw format to the open .mzML standard with the
201 FileConverter tool via the open-source ThermoRawFileParser.(Röst et al. 2016; Hulstaert et al.
202 2020) Unless otherwise stated, default arguments were used throughout.

203 The decoy database generated in the prior section (iTRAQ OpenMS analysis) was also re-used. The
204 CometAdapter was used to run the search.(Eng, Jahan, and Hoopmann 2013) Fixed modifications
205 were set to "Carbamidomethyl (C)" and "Oxidation (M)" was set as a variable modification. To reflect
206 the instrument the following flags were also set: -precursor_mass_tolerance 20 -isotope_error
207 0/1.

208 To annotate the identified peptides with proteins the PeptideIndexer tool was used. PeptideIndexer
209 and PSMFeatureExtractor were used for annotation. For peptide level score estimation and fil-
210 tering PercolatorAdapter was used with the following flags: -score_type q-value -enzyme
211 trypsin. IDFfilter was used to filter to a peptide score of 0.01 with -score:pep 0.01 followed
212 by IDScoreSwitcher with the following flags: -new_score "MS:1001493" -new_score_orientation
213 lower_better -new_score_type "pep" -old_score "q-value". The ProteomicsLFQ was used for
214 subsequent processing with the flags: -proteinFDR 0.05 -targeted_only true. The -out_msstats
215 flag was also used to produce quantitative data for downstream statistical analysis with the R
216 package MSstats.(Choi et al. 2014)

217 **3.3.4 Enzyme-linked immunosorbent assays**

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

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

232 **3.3.5 Network and pathway analysis**

233 Protein interation networks were created using the Bioconductor package STRINGdb which pro-
234 vides an R interface to STRING version 11.(Szklarczyk et al. 2019) Instantiation of the STRINGdb
235 reference class was done with `species` and `score_threshold` set to 9606, for *Homo sapiens*, and
236 400 respectively. Clustering of networks with STRINGdb used the “fastgreedy” algorithm from the
237 `iGraph` package.

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

241 **4 Results**

242 **4.1 Results**

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

247 In the interest of brevity, only the plots of acute and subactue AIS C improvers VS non-improvers
248 are included here, please see the supplemental data for the other comparisons (section 5.2.2).

249 **4.1.1 Comparing OpenMS and ProteinPilot**

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

254 **4.1.2 iTRAQ analyses**

255 **4.1.3 Differential protein abundances**

256 AIS C improvers had 18 more abundant proteins and 49 less abundant proteins at the acute phase,
257 at the subacute phase, AIS C improvers had 34 more abundant proteins and 34 less abundant pro-
258 teins. The AIS A group had 56 more abundant and 9 less abundant proteins respectively. Acutely,
259 AIS C improvers relative to AIS A and D had 21 and 53 more abundant and 46 and 12 less abundant
260 proteins. Please see the appendix for a full list of protein changes.

261 **4.1.4 Heatmaps**

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

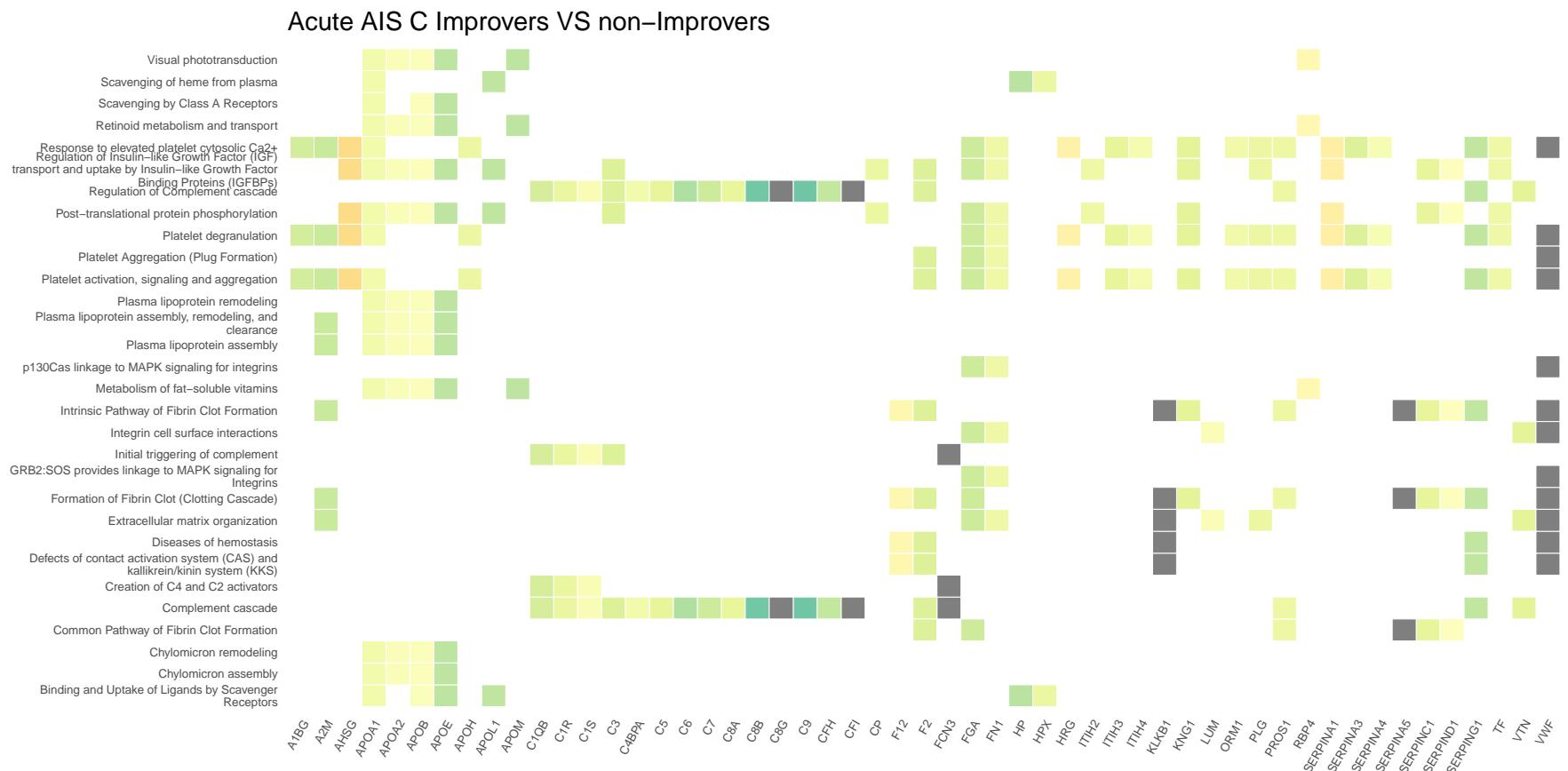


Figure 1. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

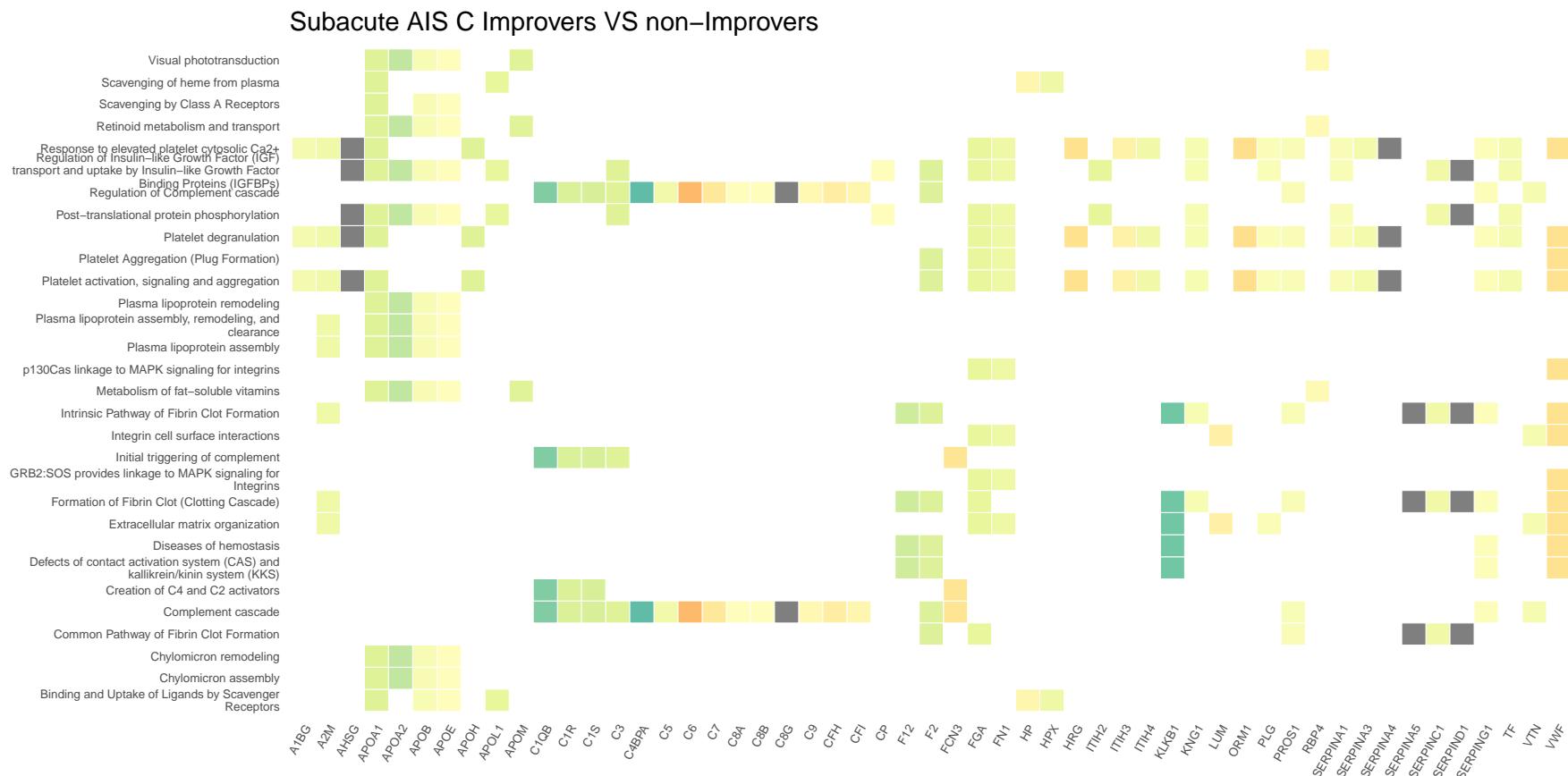


Figure 2. Heatmap denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

²⁶⁶ 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).

²⁶⁸ Please see appendix section 5.5 for additional plots.

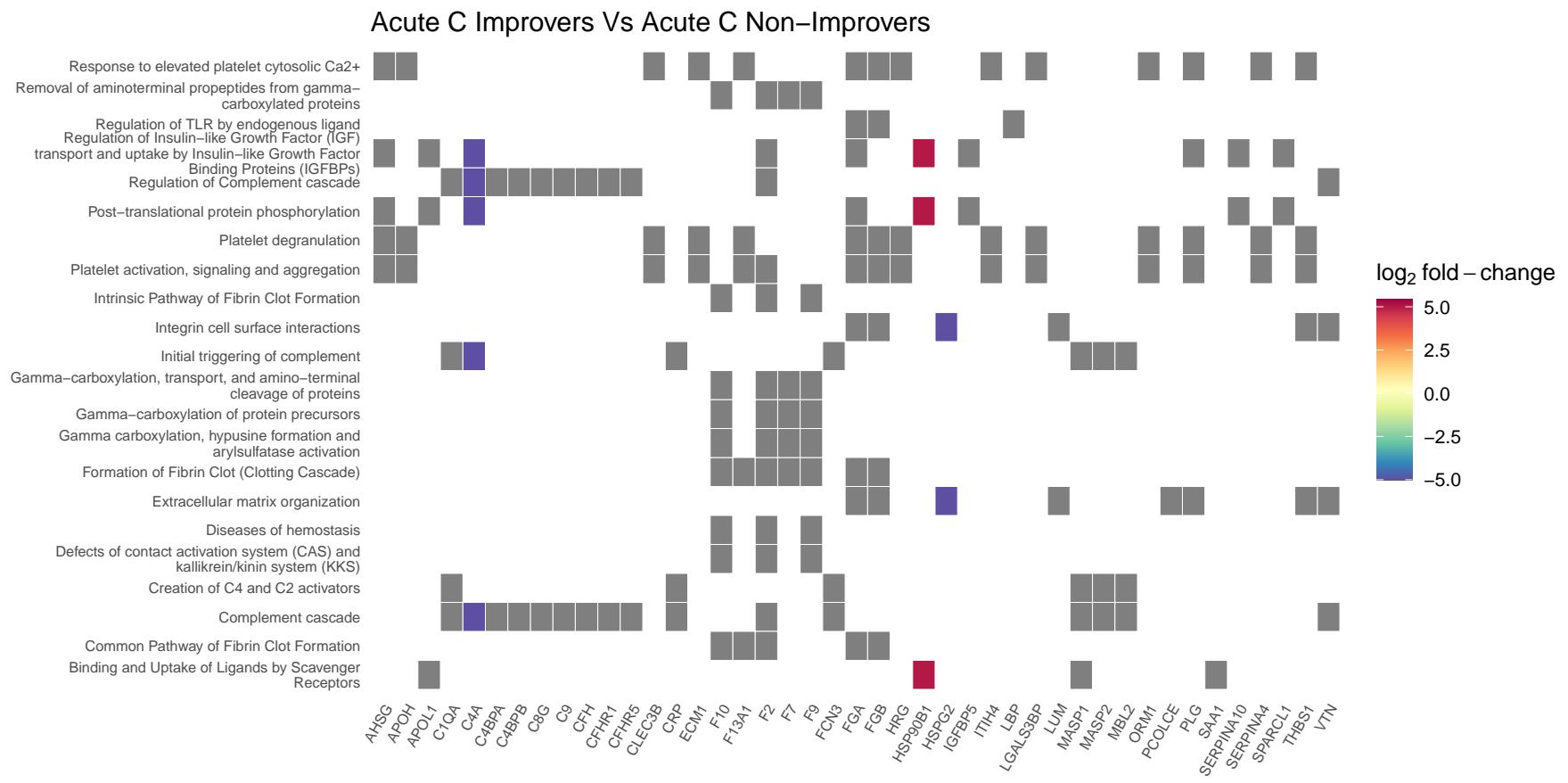


Figure 3. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins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

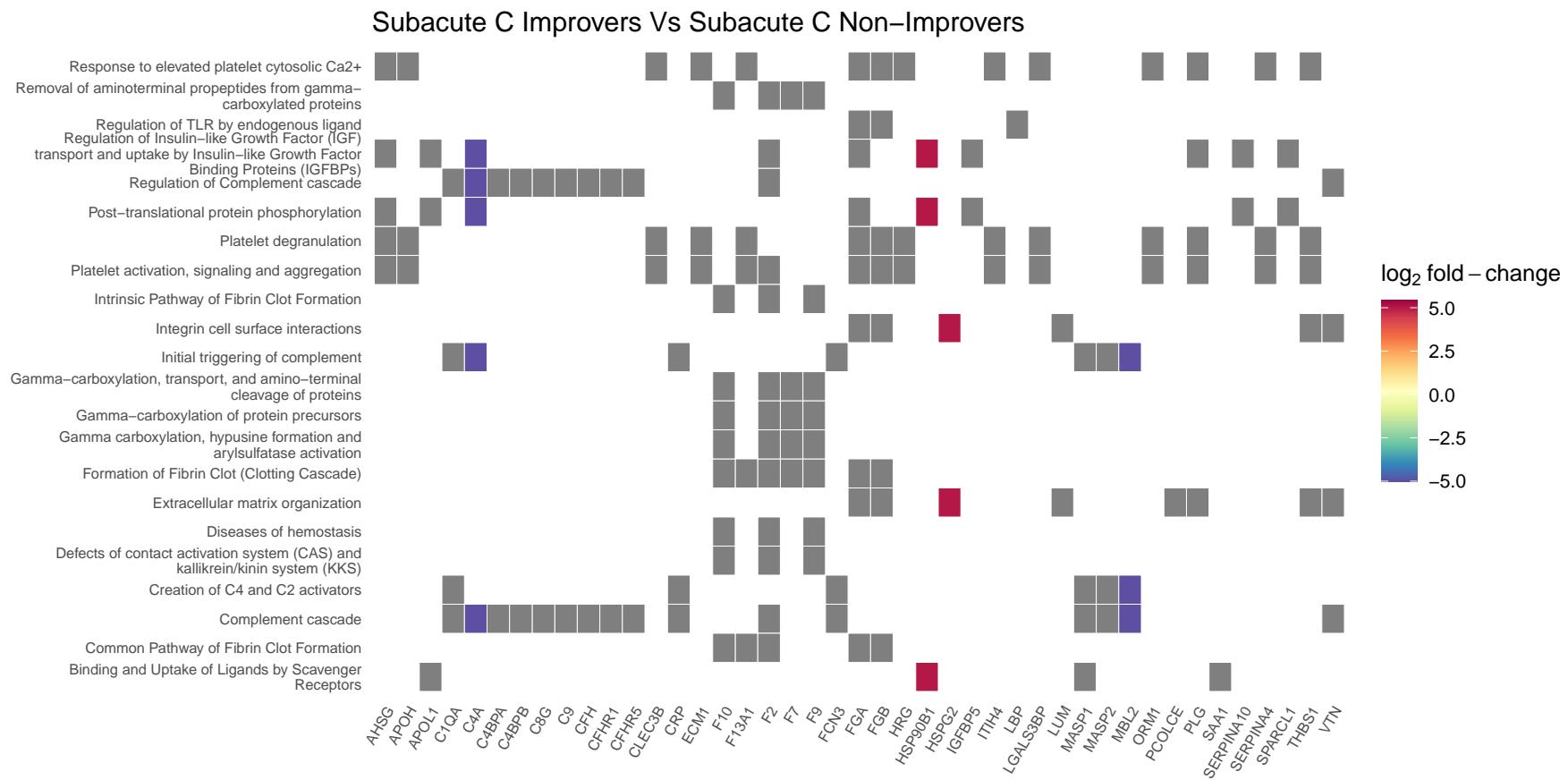


Figure 4. Heatmap denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins not present in the comparison.

²⁶⁹ **4.1.5 Cnetplots**

²⁷⁰ Similar to the heatmaps, network plots highlighted that the majority of proteins changes were
²⁷¹ associated with the complement cascade and pathways linked to platelet activity (Figure 5, 6, S16,
²⁷² S17, S18, S19, S20, S21, S22, S23). Several proteins were also associated with the regulation of
²⁷³ insulin-like growth factor.

Acute AIS C Improvers VS non-Improvers

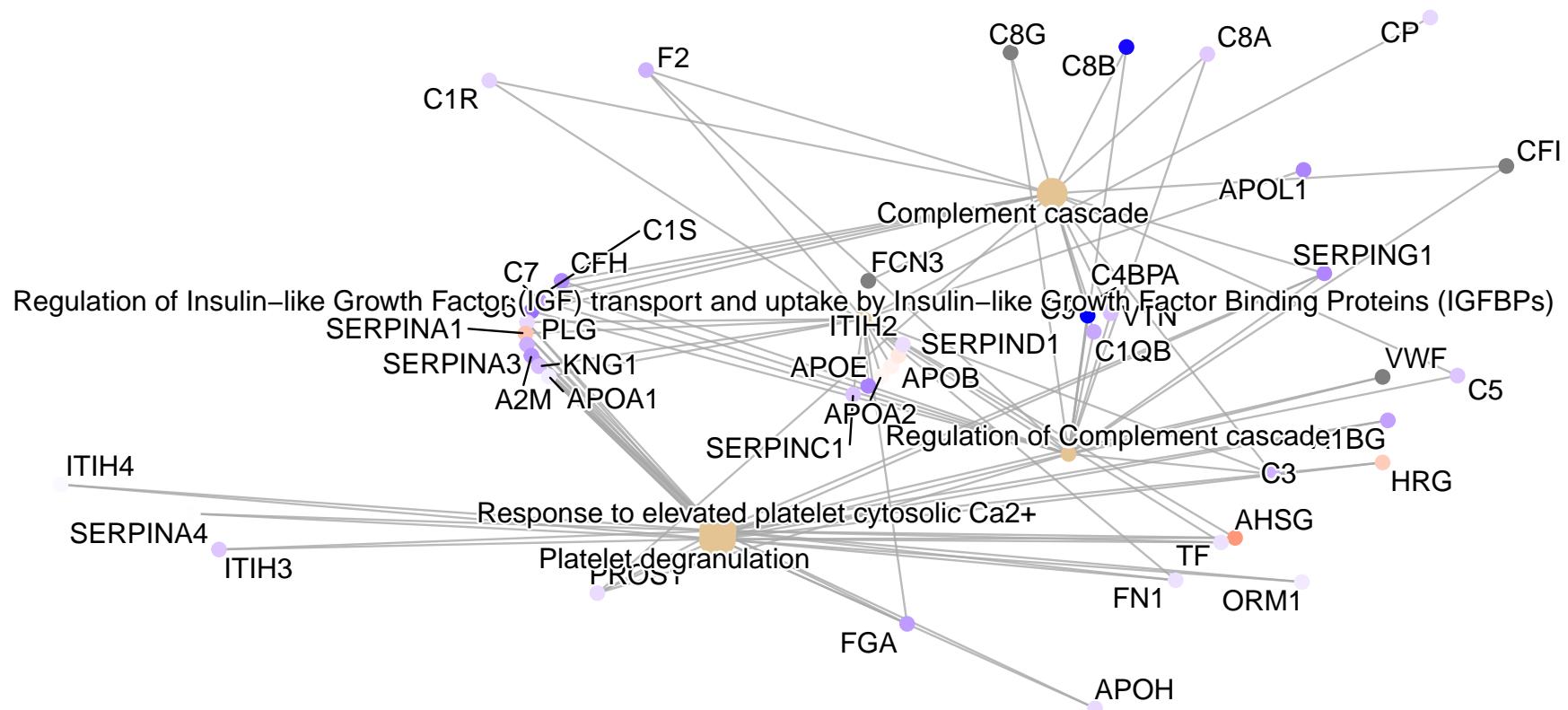


Figure 5. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Subacute AIS C Improvers VS non-Improvers

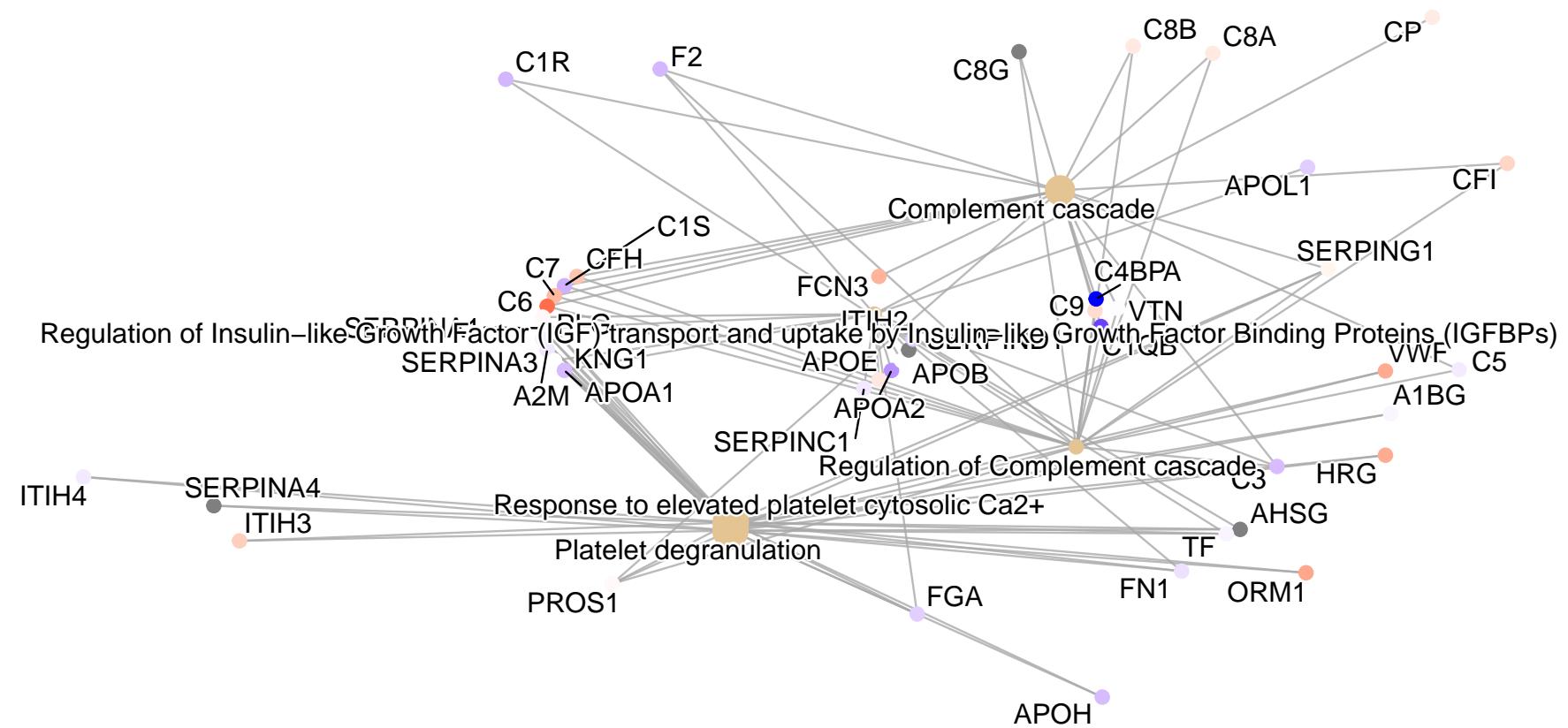
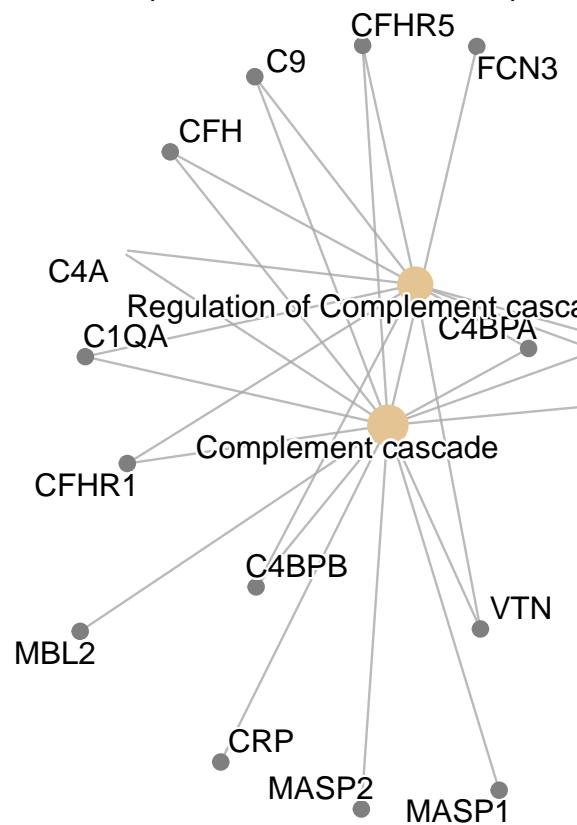


Figure 6. Network plot denoting the log₂ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

²⁷⁴ Similarly to the heatmaps and the iTRAQ data, network plots highlight the majority of differential
²⁷⁵ proteins are associated with the complement cascade and pathways linked to platelets (Figures 7,
²⁷⁶ 8, S24, S25, S26, S27, S28, S29, S30).

²⁷⁷ Please see appendix section 5.6 for additional plots.

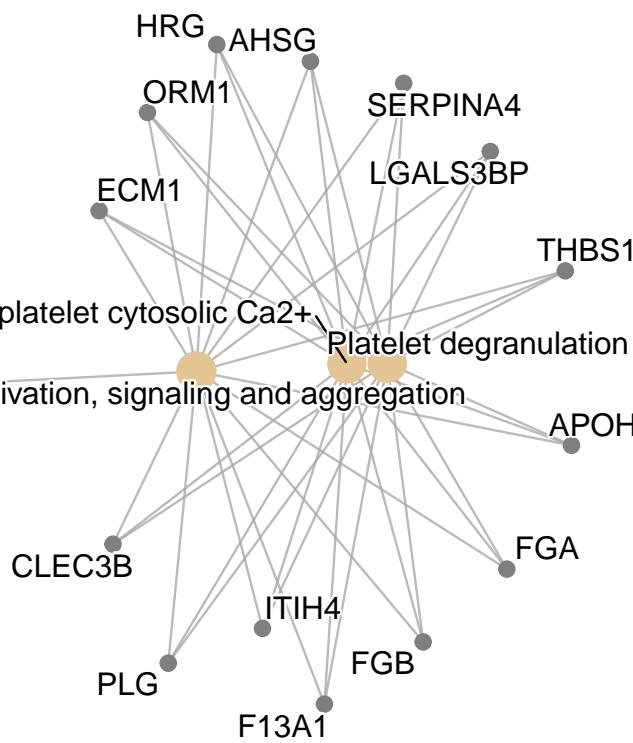
Acute C Improvers Vs Acute C Non-Improvers



Complement cascade
C4BPA

Regulation of Complement cascade
C4A, C1QA, CFHR1, MBL2, CRP, C4BPB, MASP2, MASP1, VTN, C9, CFH, CFHR5, FCN3, F2, C8G

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



size
● 3
● 6
● 9
● 12
● 15
fold change
-5

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

Subacute C Improvers Vs Subacute C Non-Improvers

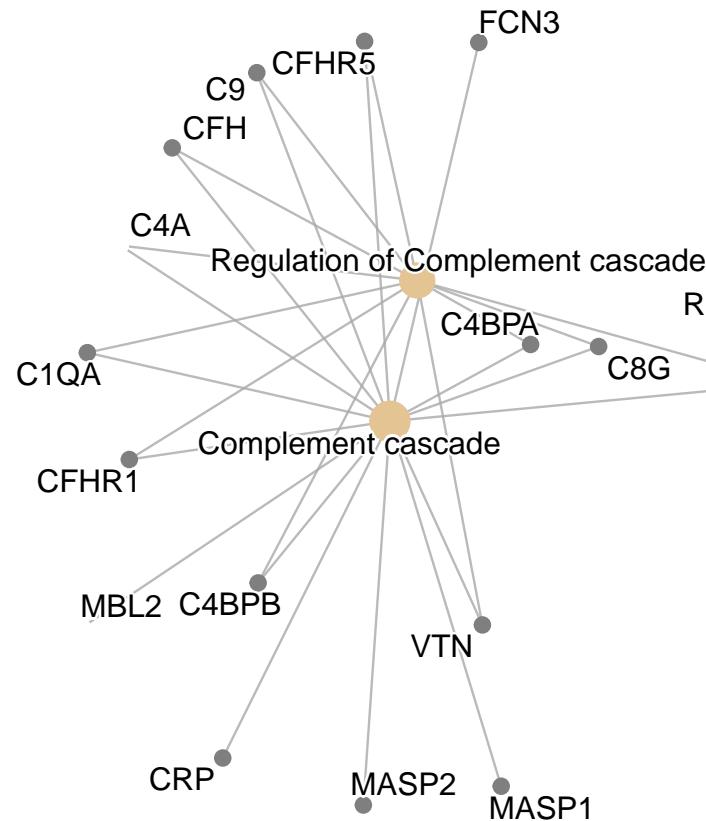
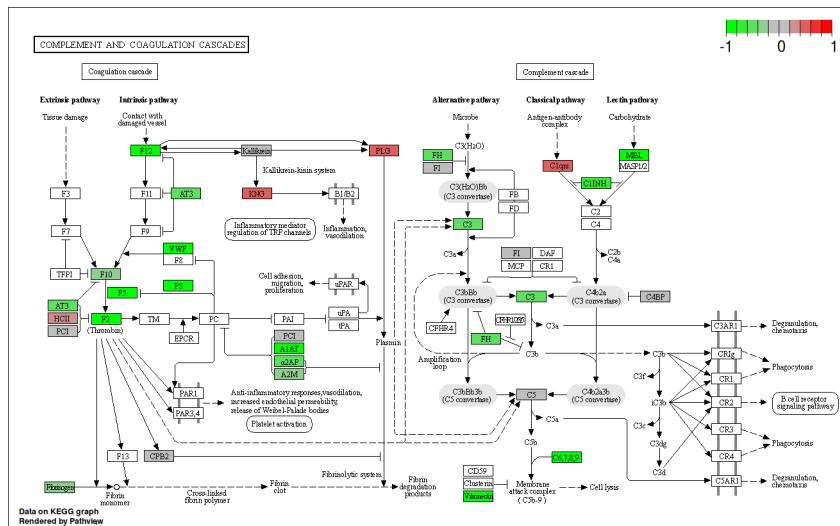


Figure 8. Network plot denoting the \log_2 fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

278 **4.1.6 Pathway analysis**

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



288 **4.1.7 ELISAs**

289 No statistically significant difference between groups for A2M abundance in plasma via Du-
290 oSet® ELISAs, though there were outliers in the AIS A and D groups, and particularly in the AIS
291 C patients at 3-months who did not experience an AIS grade conversion (Figure 11). A significant
292 difference was found between AIS C non-improvers at 2-weeks and AIS D for SAA1, with outliers
293 in AIS C non-improvers at 2-weeks, and both AIS C improvers and non-improvers at 3-months
294 post-injury (Figure 11). For ApoA1 plasma abundance estimated via Quantikine® ELISAs,
295 statistically significant differences were found between AIS C improvers at 2-weeks and both AIS C
296 improvers and non-improvers at 3-months, AIS C 3-month improvers and AIS A and D, and AIS C
297 3-month non-improvers and AIS A and D (Figure 11). A statistically significant difference was also
298 found between AIS C improvers and non-improvers at 2-weeks post-injury for RBP4 (Figure 11).

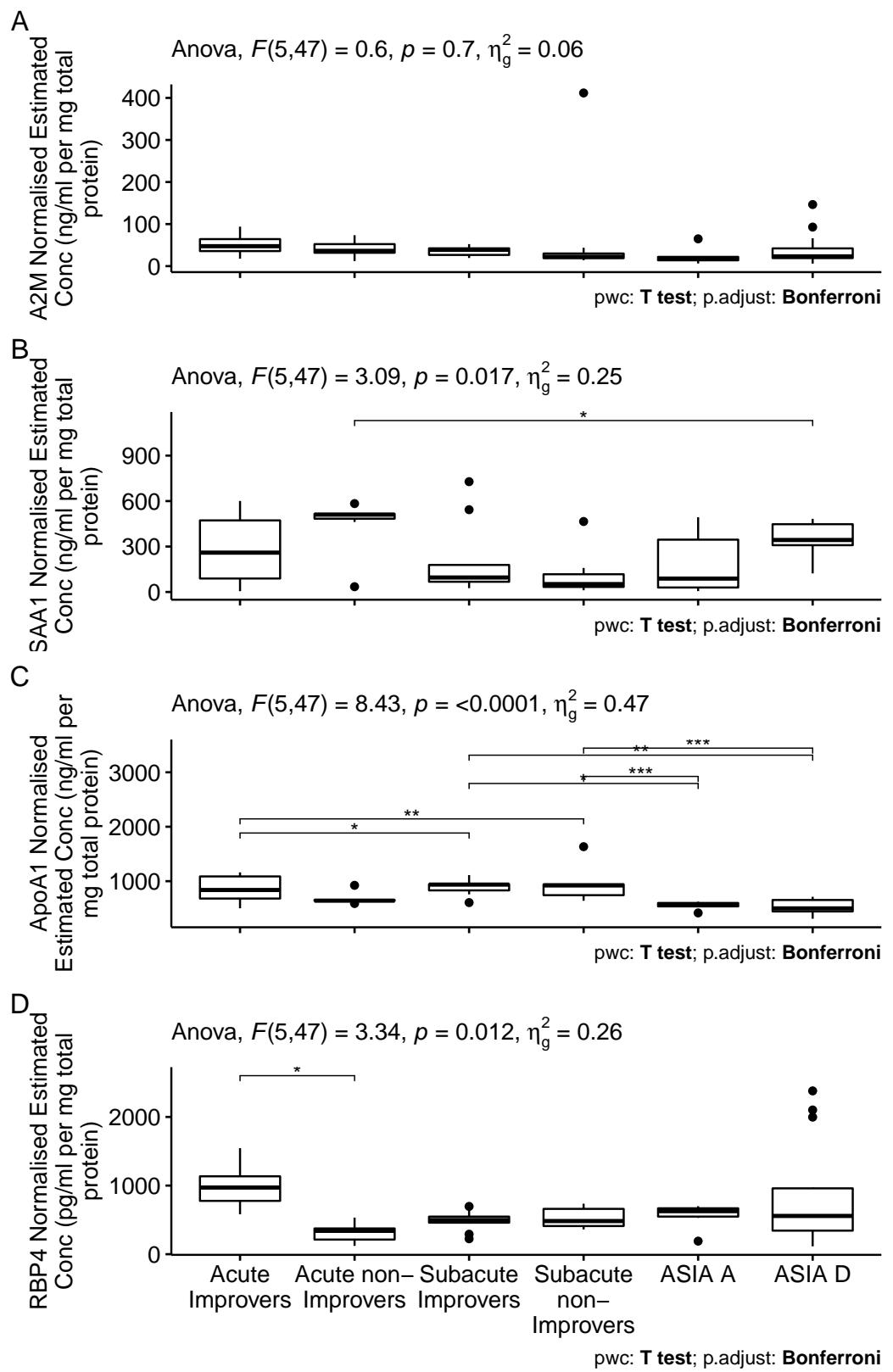


Figure 11. Normalised estimated concentration of α -2-macroglobulin (A), serum amyloid A1 (B), apolipoprotein A1 (C) and retinol binding protein 4 (D). Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled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

299 **4.1.8 STRINGdb plots**

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

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

306 **4.1.9 Volcano plots**

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

313 **4.1.10 Comparing iTRAQ and label-free proteins**

314 A total of 87 and 79 unique proteins were identified across the label-free and iTRAQ experiments
315 respectively, with a modest overlap of 26 proteins found using both techniques (Figure 12).

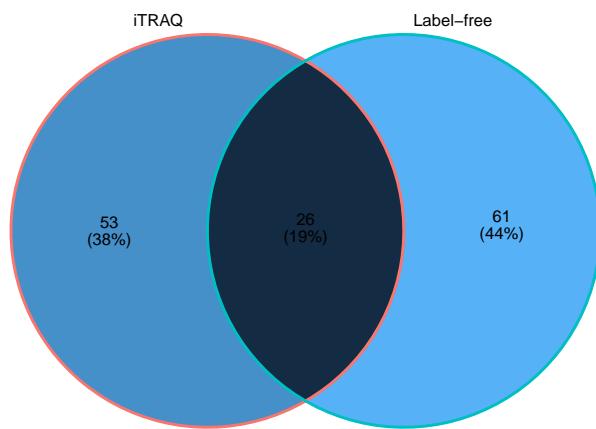


Figure 12. Venn diagram of the overlap in unique proteins identified from iTRAQ and label-free proteomic experiments analysed via OpenMS.

316 **5 Discussion**

317 **5.1 thesis iTRAQ discussion**

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

322 **5.1.1 ProteinPilot and OpenMS**

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

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

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

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

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

354 **5.1.2 Proteins identified**

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

357 **5.1.2.1 Alpha-2-macroglobulin** A2M is an inhibitor of an unusually diverse array of proteinases
358 by a unique 'trapping' mechanism. The protein achieves this with a peptide stretch, called the
359 "bait region", which contains specific cleavage sites for different proteinases. When a proteinase
360 cleaves the bait region, a conformational change is induced whereby A2M traps the proteinase.
361 The entrapped enzyme retains activity against low molecular weight substrates, whereas activity
362 against high molecular weight substrates is greatly reduced. Following cleavage in the bait region, a
363 thioester bond is hydrolysed and mediates the covalent binding of the protein to the proteinase. (P.
364 K. Hall et al. 1981; Sottrup-Jensen et al. 1984) A2M is unique in its ability to inhibit virtually any
365 protease regardless of its specificity, origin or catalytic mechanism. (Khan 2004; Lin et al. 2012)

366 Alpha macroglobulins are an integral part of innate immunity and thus are evolutionarily con-
367 served.(Buresova et al. 2009) Alpha macroglobulins have significant primary sequence homology
368 with complement components C3, C4 and C5. The A2M-proteinase complex is cleared from circu-
369 lation primarily by receptors on hepatocytes.(Bond, Cianciolo, and Pizzo 2007; Travis and Salvesen
370 1983) The mammalian receptor for proteinase-reacted A2M is a low-density lipoprotein receptor
371 related protein.(Fujiyoshi et al. 2011; Larios and Marzolo 2012; Wyatt and Wilson 2013)

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

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

385 We observe A2M to be less abundant in AIS C improvers, within 2-weeks post injury and at 3-
386 months, albeit to a lesser extent (Tables S2 and S3). Similarly, A2M was more abundant in AIS As
387 relative to all groups, and whilst A2M was less abundant in AIS C improvers at 2-weeks compared
388 to AIS Ds, AIS C non-improvers had more A2M than AIS Ds. (Table S2). With less A2M there would
389 be more protease activity in these individuals, which may aid in the clearance of damaged tissue,
390 and in particular may lessen the development of an astroglial scar, thus aiding repair. However,
391 glial scaring is not entirely negative, the primary benefit it offerers is minimising the extent of sec-
392 ondary damage to neighbouring areas by functioning as a barrier around the injury site. Animal
393 studies have demonstrated that prevention of astroglial scar formation following CNS injury leads
394 to greater lesion size and poorer function outcomes.(Anderson et al. 2016; Wilhelmsson et al.
395 2006) Interestingly, a rat study using quantitative liquid chromatography-mass spectrometry with
396 CSF, found A2M to be more abundant in moderately injured animals compared to more severe
397 injuries.(Lubieniecka et al. 2011)

398 **5.1.2.2 Apolipoproteins** We found ApoA1, ApoA2, ApoH, ApoL1 and ApoM to be less abundant
399 in AIC improvers at both time points, whereas ApoA4 was more abundant at both time points (Ta-
400 bles S2 and S3). ApoA1 is the main protein component of high-density lipoproteins (HDL). Plasma
401 HDL include two main apolipoproteins, these being ApoA1 and ApoA2 (~70% and ~20% of total
402 HDL protein content respectively), but some HDL particles can also contain small amounts of other
403 apolipoproteins, including ApoA4, ApoA5, ApoC, ApoD, ApoE, ApoJ and ApoL. The primary function
404 of HDL in plasma is the transport of cholesterol, which can have dietary origins, but also be pro-
405 duced endogenously in the liver.

406 **5.1.2.2.1 HDL Activity** HDLs have serve a wide range of functions, including contributing to
407 anti-inflammatory activity. They can limit chemokine secretion from multiple cells types including
408 endothelial cells and monocytes.(Cockerill Gillian W. et al. 1995; Vorst et al. 2013; Bursill Christina
409 A. et al. 2010) Rats injected with ApoA1 showed significant reduction in expression of CCR2 and
410 CX₃CR1, the receptors for chemokines of the same name, which play a role in leukocyte migration.
411 (Bursill Christina A. et al. 2010)

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

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

423 ApoE was less abundant in AIS C improvers within 2-weeks and more abundant at 3-months, and
424 more abundant in more severe injury, such as AIS A relative to D or C and in AIS C relative to D
425 (Table S2). ApoE is primarily produced by hepatocytes in the liver, but second-most in the brain,
426 synthesised in and secreted by astrocytes, and has been found to an important determinant in
427 response to types of CNS injuries in both animal and human studies.(Teasdale et al. 1997; Poirier
428 1994) A key function of ApoE is as a ligand for the LDL receptor family of proteins, which mediate
429 trafficking of cholesterol to neurons, which is vital for axonal growth, and for synapse formation
430 and remodelling.(Xu, Finkelstein, and Adlard 2014) Additionally, ApoE is implicated in the clearance
431 of neuronal apoptotic bodies.(Elliott et al. 2007) In humans there are three variants/alleles of ApoE:
432 ApoE2, ApoE3 and ApoE4, which have a frequency of 8.4%, 77.9% and 13.7% globally.(C.-C. Liu et
433 al. 2013) The variant proteins differ by one or two amino acids and have been found to result in
434 substantial physiological alterations.(Mahley and Rall 2000; Jha et al. 2008) The presence of the
435 ApoE4 variant has been linked to worse outcomes in SCI and TBI.(Jha et al. 2008; C. Sun et al. 2011;
436 Smith et al. 2006; Friedman et al. 1999) More specifically, the SCI study reported significantly lower
437 change in the median AIS motor score compared the individuals without the ApoE4 allele during
438 rehabilitation.(Jha et al. 2008)

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

456 **5.1.2.3 Serum Amyloid A1** SAA1 was less abundant in AIS C improvers at 2-weeks relative to
457 non-improvers, but more abundance in plasma at 3-months (Table S2. SAA1 was also more abun-
458 dant in AIS A relative to less severe injuries, and in AIS Cs relative to Ds (Table S2. SAA1 is a major
459 acute-phase protein mainly produced in the liver by hepatocytes in response to infection, tissue

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

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

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

484 Macrophages are a major source of SAA in inflammatory tissues, and elevated SAA production has
485 been observed in rheumatoid arthritis, Crohn's disease, Type 2 diabetes and atherosclerosis.(Marzi
486 et al. 2013; Dong et al. 2011; Vallon et al. 2001; C, F, and B 1997; Meek, Urieli-Shoval, and Benditt
487 1994) SAA binding to HDL was reported to increase affinity for macrophages whilst decreasing
488 affinity for hepatocytes.(R. Kisilevsky and Subrahmanyam 1992) This change is thought to favour
489 the removal of cholesterol from site of inflammation.(R. Kisilevsky 1991) SAA inhibits the binding of
490 the scavenger receptor SR-BI and cholesterol efflux is enhanced in a SR-BI-dependent manner.(Cai
491 et al. 2005; van der Westhuyzen et al. 2005) It has been suggested that the SR-BI-mediated re-
492 uptake of cholesterol underpins the role of SAA in cholesterol recycling during tissue repair, where
493 a great deal of cholesterol is required.(Robert Kisilevsky and Manley 2012)

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

504 **5.1.2.4 Retinol-binding protein 4 (RBP4)** In plasma within 2-weeks post-injury, RBP4 was less
505 abundant in AIS C improvers relative to AIS D and A, and more abundant in AIS C non-improvers
506 again, relative to AIS D and A (Table S2. Similarly, AIS A plasma had more RBP4 compared to AIS

507 D, and AIS C improvers were also more abundant in RBP4 compared to non-improvers at both
508 2-weeks and 3-months post-injury (Table S2).

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

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

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

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

538 5.1.3 Metabolism and SCI

539 **5.1.3.1 Acute phase response** The bodies first response to injury or infections, including SCI,
540 is often referred to as the “acute phase response” (APR), which is non-specific, innate reaction
541 that precedes more specific and situational immune reactions.(Gordon and Koj 1985; Gruys et
542 al. 2005) This systemic response is largely coordinated by factors released from the liver, but the
543 APRs effects extend to multiple peripheral organs including the kidneys, lungs and spleen.(Bao et
544 al. 2012; S. J. Campbell, Zahid, et al. 2008; Fleming et al. 2012; Gris, Hamilton, and Weaver 2008)
545 This hepatic response is typically transient and quickly fades, but prolonged liver inflammation and
546 pathology has been observed in rodent SCI models.(Goodus et al. 2018; Sauerbeck et al. 2014)

547 Basic liver functions are chronically impaired by SCI, including metabolising carbohydrates, fats
548 and proteins, storage of minerals vitamins and glycogen and filtering blood from the digestive
549 tract.(García-López et al. 2007; DeLeve 2007; Farkas and Gater 2018; Chow et al. 2012; Sauerbeck
550 et al. 2014) This is likely related to the elevated incidence of metabolic disease in the SCI cohort,
551 including insulin resistance, impaired glucose tolerance and cardiovascular disease.(Bauman and
552 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)

5.1.4 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)

5.1.5 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

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

603 *De novo* lipogenesis occurring within the liver can also drive hepatic steatosis.(Lavoie and Gau-
604 thier 2006) Ceramides are lipid signalling molecules and regulators of apoptosis and inflamma-
605 tion; they can contribute to insulin resistance, oxidative stress and inflammation-induce liver adi-
606 posity through sustained Toll-like-receptor(TRL)-4 activation.(Schilling et al. 2013; Bhargava and
607 Lee 2012; Pagadala et al. 2012) If released into the circulatory system, ceramides can cause CNS
608 toxicity, including oxidative damage and changes to the aggregation of proteins associated with
609 diseases such as Parkinson's, Huntington's and Alzheimer's.(Pagadala et al. 2012; Vidaurre et al.
610 2014; Czubowicz et al. 2019) Mature and precursors of hepatic ceramides and enzymes which
611 contribute to ceramide synthesis are elevated by 1 day post-injury.(Sauerbeck et al. 2014) Endo-
612 toxins can also stimulate the synthesis of ceramides and so the aforementioned "leaky gut" may
613 also contribute to this elevation.(Chang et al. 2011) Ceramide synthesis and lipogenesis genes are
614 also stimulated by TNF- α , which, as touched on in the general introduction (??), has been found
615 to be elevated post-SCI, and associated with differential neurological recovery.(Davies, Hayes, and
616 Dekaban 2007; Hasturk et al. 2009; Biglari et al. 2015; Sauerbeck et al. 2014; Bikman 2012)

617 5.1.6 Chronic liver inflammation in SCI

618 The hepatic APR and associated inflammation that typically follows bodily trauma, subsequently
619 rapidly subsides, whereas post-SCI this hepatic inflammation persists chronically. This chronic
620 phase may be due in part to long-term changes in intestinal permeability via fewer tight junc-
621 tions in intestinal epithelial cells, resulting in gut dysbiosis.(Milosevic et al. 2019; O'Connor et al.
622 2018; Kigerl, Mostacada, and Popovich 2018; Kigerl et al. 2016) Bacterial translocation and gut
623 dysbiosis can be the result of non-mechanical intestinal obstruction, impaired intestinal motility
624 and systemic immune suppression, all of which are potential complications of SCI.(Balzan et al.
625 2007) Specifically, butyrate-producing bacteria have been found to be reduced in SCI relative to a
626 healthy cohort.(Gungor et al. 2016) Butyrate is known to modulate epithelial differentiation and
627 cell growth, and suppress macrophages, including CNS inflammation, thus the reduction in bu-
628 tyrate from bacteria may contribute to recovery post-SCI, though links to the liver specifically have
629 not yet been studied.(H.J. Kim et al. 2007; Arpaia et al. 2013; Park et al. 2005; P. S. Chen et al. 2007)

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

634 5.1.7 Longitudinal metabolic health

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

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

647 **5.1.8 Validation of results**

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

659 **5.1.9 Conclusion**

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

673 **5.2 thesis label-free discussion**

674 As outlined previously (5.1.9), two key limitations of the iTRAQ experiments were the pooling of
675 samples, which prevents statistically robust group-wise comparisons, and the high dynamic range
676 of protein abundances in plasma potentially obscuring less abundant proteins. This work seeks
677 to address these factors by a combination of Proteominer™ beads to shrink the dynamic range of
678 protein abundances, and by not pooling samples.

679 **5.2.1 Proteins identified**

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

684 **5.2.1.0.1 Peroxiredoxins** Peroxiredoxins are a large and highly conserved family of enzymes
685 that reduce peroxides. Peroxiredoxin 2 (PRX-2) is highly abundant in RBCs and intracellularly serves
686 as an important anti-oxidant role in various cell types, including neurons.(Low, Hampton, and

687 Winterbourn 2008) By contrast, extracellular PRX-2 has been suggested to act as an inflamma-
688 tory DAMP, leading microglia and macrophages to release a plethora of pro-inflammatory fac-
689 tors.(Salzano et al. 2014; Garcia-Bonilla and Iadecola 2012; Shichita et al. 2012) An *in vitro* primary
690 neurons and microglia co-culture study reported PRX-2 activating microglia via TLR-4, potentially
691 leading to neuronal apoptosis.(Y. Lu et al. 2018) A mouse study found over-expression of PRX-2 at-
692 tenuated oxidative stress and neuronal apoptosis following subarachnoid haemorrhage.(Y. Lu et al.
693 2019) Over-expression of PRX-2 is speculated to protect again ischaemic neuronal injury by mod-
694 ulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase (ASK) 1 signalling com-
695 plex.(Gan et al. 2012) Several molecular chaperones can interact with ASK1, including thioredoxin
696 and TNF receptor-associated factor 6.(Matsuzawa et al. 2005) The dissociation of the thioredoxin-
697 ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free radicals, whereupon its an-
698 tioxidantive activity is reduced. This inactivation can be reversed by the thioredoxin-thioredoxin
699 reductase system, whereby oxidised PRX-2 can regain its activity by reducing thioredoxin, leading
700 to the dissociation of the thioredoxin-ASK1 complex.(Rhee and Woo 2011) Additionally, oxidised
701 PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding domain on ASK1.(S. Y. Kim,
702 Kim, and Lee 2008)

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

717 **5.2.1.1 Neuroinflammation post-SCI** The neuro-inflammatory response begins immediately
718 post-trauma, and involves a complex series of events that can persist well into the chronic phase.
719 The sudden emergence of necrotic cell debris and associated DAMPs lead surviving CNS-resident
720 cells to produce cytokines, complement factors and ROS. Within minutes CNS cells at the lesion site
721 have been found to secrete several pro-inflammatory mediators, including TNF- α and interleukins,
722 in both rodent models and human patients with SCI.(Pineau and Lacroix 2006; Chandrasekar et al.
723 2017; Dalgard et al. 2012; Bastien et al. 2015) The resulting inflammatory response occurs in
724 parallel to the mechanical destruction of the blood-spinal cord barrier, and the development of
725 tissue oedema and ischaemia combine to propagate damage to parts of the cord spared by the
726 initial trauma.(Maikos and Shreiber 2007; Ahuja et al. 2017)

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

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

735 tem activation and sequential leukocyte migration from the periphery into the injured neural
736 parenchyma.(Brennan et al. 2015; S. L. Peterson and Anderson 2014; Qiao et al. 2006) Curiously,
737 though the breakdown of the BSCB would presumably allow unrestricted access of circulating
738 leukocytes into the injured cord segment, recruitment of these cells remains a highly controlled
739 process.(Beck et al. 2010; Brennan et al. 2019) A mouse study reported lymphocytes, which
740 account for approximately 80% of circulating leukocytes, only enter the cord in substantial
741 numbers at least several weeks to months post-injury.(Beck et al. 2010) Early infiltrate is instead
742 largely comprised of myeloid cells, predominantly neutrophils, which are a minority of circulating
743 cells but are the swiftest peripheral responders to SCI, with studies detecting them at the lesion
744 site within 4 hours of injury.(Wright et al. 2010) Neutrophil numbers have been reported to peak
745 at 1 day post-trauma, but also to remain at the site for a minimum of 42 days post-injury.(Okada
746 2016; Kigerl, McGaughy, and Popovich 2006)

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

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

772 Circulating inflammatory monocytes are also recruited during the first days post-trauma. Adop-
773 tive transfer experiments have shown recruitment to pick up at approximately 3 days post-injury,
774 and peak at 7 days.(Blomster et al. 2013) Whilst monocyte turnover at the lesion appears to be
775 high, infiltrating monocyte-derived macrophages remain at the site of weeks to months post-
776 trauma.(Blomster et al. 2013; Shechter et al. 2009) Interestingly, the timing of monocyte recruit-
777 ment appears to be delayed relative to non-neurological tissue injury. For instance, monocytes
778 are reported to be rapidly recruited to the heart following a myocardial infarction, as early as 1 day
779 post-injury, and their numbers return to baseline by roughly 16 days post-injury.(Nahrendorf et al.
780 2007)

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

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

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

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

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

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

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

838 **5.2.1.1.1 Intravenous immunoglobulin** Intravenous immunoglobulin (IVIG) is increasingly
839 used as an immunomodulatory strategy for managing acute neurological conditions, including
840 neurotrauma. Originally developed as an antibody replacement therapy for immunodeficiency
841 disorders, IVIG is a product comprised primarily of immunoglobulin G (IgG) taken from the blood
842 plasma of healthy donors.(Bayry, Negi, and Kaveri 2011; Schwab and Nimmerjahn 2013) IVIG
843 therapy was found to increase platelet number in idiopathic thrombocytopenic purpura (ITP)
844 patients, which lead to an interest in using it as an immunomodulatory therapy.(Imbach et al.
845 1981) Its potent effects and limited side effects have lead high-dose IVIG therapy to be commonly
846 used in a plethora of inflammatory and autoimmune disorders, including ITP, arthritis, Kawasaki's
847 syndrome and Guillain-Barré syndrome.(Lünemann, Nimmerjahn, and Dalakas 2015; Stangel et
848 al. 1998)

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

862 In TBI mouse models, animals treated with IVIG were shown to have improved neurobehavioural
863 outcomes, and a reduction in neuronal degeneration both acutely and chronically, relative to
864 vehicle-treated controls in rotarod and Morris water maze experiments.(Jeong et al. 2014) Further
865 mouse studies using cerebral artery occlusion, a model of stroke, reported high-dose IVIG signif-
866 icantly reduced infarct volumes, neurological impairment and mortality rates.(Arumugam et al.
867 2007; Widiapradja et al. 2012) Under condition of BBB/BSCB compromise, IVIG has been found to
868 enter the neural parenchyma within hours of injury.(Brennan et al. 2016; Arumugam et al. 2007)
869 SCI studies have found IVIG to localise to oligodendrocytes, astrocytes, neurons, macrophages,
870 microglia, pericytes and blood vessels.(Brennan et al. 2016; Chio et al. 2019) Additionally, reduc-
871 tions in immune cells, as indicated by F4/80⁺ microglia/macrophages and polymorphonuclear
872 cells in brain and spinal injury models respectively, have also been reported.(Jeong et al. 2014;
873 Nguyen et al. 2012; Chio et al. 2019) Relatedly, the aforementioned SCI IVIG mouse study found
874 reduced CD68⁺ macrophages at and surrounding the lesion 35 days post-injury.(Brennan et al.
875 2016) Importantly, these studies do not differentiate between resident microglial and infiltrating
876 monocytes/macrophages. Thus, further research is needed to understand the influence of IVIG
877 on both recruitment and activation states of these cell subsets.

878 **5.2.1.1.2 Speculative mechanisms of action for IVIG in SCI** As IVIG is made from pooled anti-
879 bodies taken from thousands of donors, it includes a vast repertoire of antibodies specific against

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

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

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

903 A separate potential F(ab)'₂-dependent mechanism involves the neutralisation of the cell death
904 mediator Fas (AKA CD95). Studies of Lyell's syndrome, a disorder whereby active Fas ligand binds
905 Fas present on keratinocytes, inducing apoptosis, reported IVIG therapy completely inhibited Fas
906 ligand-induced cell death both *in vitro* and in human patients.(Viard et al. 1998; Altnauer et al.
907 2003) Importantly, IVIG blocked Fas, as opposed to Fas ligand, in these studies, as this result was
908 only observed with cells pre-treated with IVIG. Incubation of IVIG with soluble Fas ligand did not
909 attenuate cell death, implying IVIG contains antibodies specific to Fas.(Viard et al. 1998; Altnauer
910 et al. 2003) This modulatory effect of the Fas-Fas ligand pathway may have relevance in SCI, as a
911 study using knock-out mice lacking Fas showed a reduction in both apoptosis at the lesion site and
912 glial scarring, and improved motor function post-SCI.(Sobrido-Cameán and Barreiro-Iglesias 2018;
913 W. R. Yu and Fehlings 2011) Neurons and glial cells from post-mortem human patients were found
914 to be more Fas- and Fas ligand-positive, but this was limited to the acute phase of SCI, and not
915 observed chronically, suggesting this pathway is more significant immediately post-injury.(W. R. Yu
916 and Fehlings 2011) Therefore, acute IVIG treatment could act by attenuating secondary cell death
917 by blocking Fas, thus disrupting this pathway.

918 Conversely, agonistic anti-Fas antibodies have also been reported with IVIG prepara-
919 tions.(Altnauer et al. 2003) Whilst it remains unknown how these agents may act in SCI,
920 one could postulate a benefit if they induce apoptosis in circulating leukocytes, which could
921 otherwise do harm.(Schneider et al. 2017) Supporting this, papers have found reductions in poly-
922 morphonuclear cell populations within the lesion at 1 day post-injury in rodent models.(Nguyen
923 et al. 2012; Chio et al. 2019; Gok et al. 2009) However, IVIG-induced apoptosis has only been
924 observed in human leukocytes, not in rodents, casting doubt on this idea.(Altnauer et al. 2003;
925 Schneider et al. 2017) Alternatively, the reduced recruitment could be a result of IVIG regulating
926 the expression of adhesion molecules or molecules involved in leukocytes trafficking. A feline
927 ischaemia-reperfusion injury model study found IVIG to down-regulate expression of integrins
928 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 γ receptors (Fc γ Rs), which are present on most leukocytes and resident CNS cells. Many Fc γ 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 γ Rs are expressed by said cell. Myeloid cell all express come combination of these activating Fc γ 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 γ 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 γ Rs in neurons, *in vitro* work with neuronal cultures has detected mRNA for all Fc γ Rs.(Thom et al. 2017) Astrocytes, microglia and oligodendrocyte precursors have also be found to express Fc γ 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 γ 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 γ Rs comes from a mouse study with animals lacking the common γ -chain, and thus no functional Fc γ 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)

5.2.1.1.3 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

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

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

987 With respect to the immunoglobulin heavy variable precursors, 3-15 was present in all groups
988 except acute As and acute C non-improvers, though there was insufficient power to confidently
989 compare the fold change of groups expressing 3-15. Another heavy variable precursor, 1-69, was
990 expressed in subacute As, both acute and subacute C improvers, and both acute and subacute
991 Ds. The final heavy variable precursor, 1-24, was found in all groups except acute C improvers and
992 non-improvers.

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

998 **5.2.2 Conclusion**

999 Much like the iTRAQ experiments (5.1.9), the majority of proteins identified are functionally asso-
1000 ciated with the complement cascade. Unlike the iTRAQ however, many of the proteins where only
1001 detected in one group of the pairwise comparisons, suggesting greater suitability as biomarkers.
1002 PRX-2, a protein associated with oxidative stress, is of particular interest, both as a biomarker for
1003 improvement in acute AIS C patients, but also mechanistically in relation to functional recovery.
1004 Furthermore, several immunoglobulins were identified as differentially abundant, though further
1005 *in vitro/vivo* work is needed to elucidate the pathophysiological relevance of each precursor. The
1006 λ 2-18 and 3-10 precursors are of particular relevance to acute and subacute AIS C improvement
1007 respectively, and both are of interest longitudinally in AIS As, with 2-18 potentially being linked to
1008 severity of injury.

1009 The small number of statistically significant proteins speaks to the variability of human samples,
1010 and is likely exacerbated by the inconstant timing of sample collection relative to injury. Post-hoc
1011 power analysis of the data reveals that to identify a 2.5 fold change with an FDR of 0.5 and a power
1012 of 0.9, 14 biological replicates would be needed, in contrast to the 7-11 replicates used across
1013 groups here. Thus, a repeat of this experiment with a larger sample size will likely reveal many
1014 more proteins of potential interest. Furthermore, a metabolomic analysis with a similar sample
1015 size would greatly compliment this work, particularly with regards to investigating further links to
1016 the liver.

1017 **Supplementary material**

1018 **5.3 Session Information**

```
1019 ## -  
1020 ## platform      aarch64-apple-darwin20  
1021 ## arch          aarch64  
1022 ## os            darwin20  
1023 ## system        aarch64, darwin20  
1024 ## status  
1025 ## major         4  
1026 ## minor         1.3  
1027 ## year          2022  
1028 ## month         03  
1029 ## day           10  
1030 ## svn rev       81868  
1031 ## language      R  
1032 ## version.string R version 4.1.3 (2022-03-10)  
1033 ## nickname      One Push-Up
```

Table S1. Packages Used

package	version	date
base	4.1.3	2022-03-18
MSstats	4.2.0	2021-05-31
STRINGdb	2.6.5	2020-01-10
ReactomePA	1.38.0	2021-10-26
rlang	1.0.2	2022-03-04
bookdown	0.25	2022-03-16
lime	0.5.2	2021-02-24
RColorBrewer	1.1.3	2022-04-03
ggVennDiagram	1.2.0	2021-10-19
DiagrammeR	1.0.9	2022-03-04
lubridate	1.8.0	2021-10-03
patchwork	1.1.1	2020-12-15
cowplot	1.1.1	2020-12-15
readxl	1.4.0	2022-03-28
BiocManager	1.30.16	2021-06-15
knitr	1.38	2022-03-25
rmarkdown	2.13	2022-03-09
data.table	1.14.2	2021-09-23
naniar	0.6.1	2021-05-14
psych	2.2.3	2022-03-17
Hmisc	4.6.0	2021-10-05
Formula	1.2.4	2020-10-16
survival	3.2.13	2021-08-23
lattice	0.20.45	2021-09-18
bibtex	0.4.2.3	2020-09-19
captioner	2.2.3	2015-07-15

forcats	0.5.1	2021-01-27
stringr	1.4.0	2019-02-09
dplyr	1.0.8	2022-02-07
purrr	0.3.4	2020-04-16
readr	2.1.2	2022-01-30
tidyr	1.2.0	2022-01-27
tibble	3.1.6	2021-10-25
ggplot2	3.3.5	2021-06-24
tidyverse	1.3.1	2021-04-15
kableExtra	1.3.4	2021-02-19

1034 **5.4 Fold changes**

Table S2. 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 S2. 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 S2. 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 S3. 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 S3. 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 S3. 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

1035 **5.5 Heatmaps**

1036 **5.5.1 iTRAQ data**

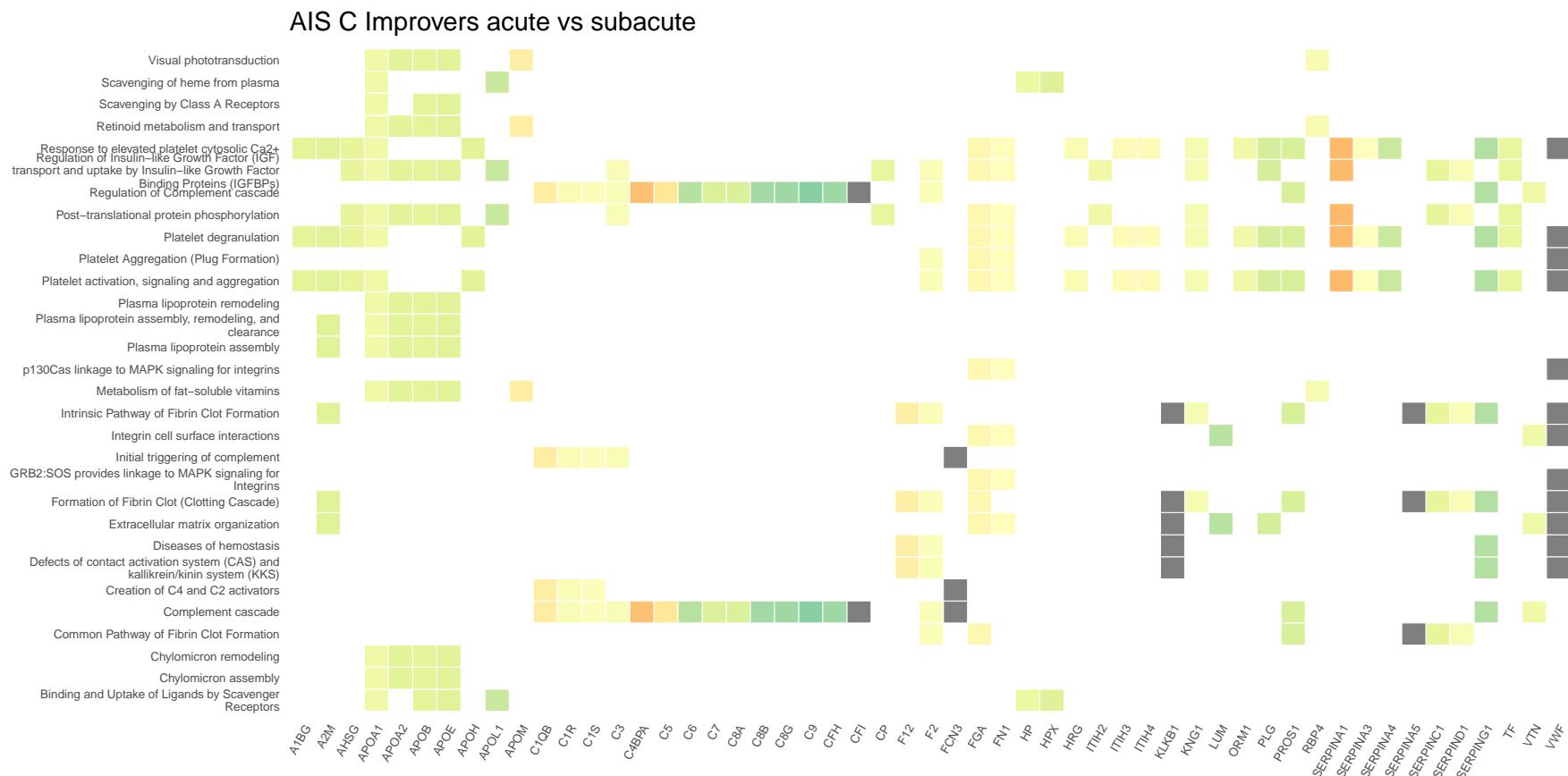


Figure S1. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

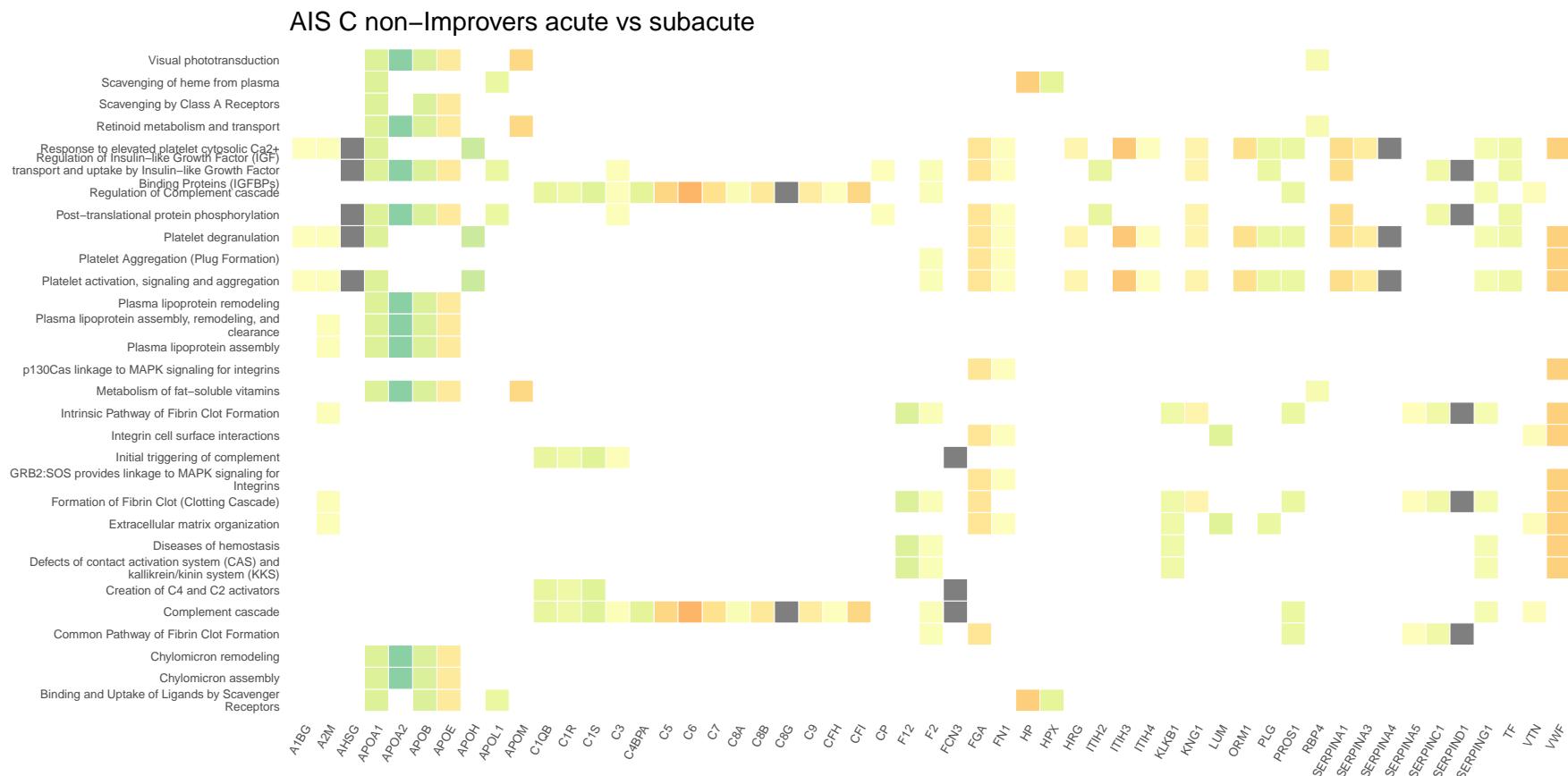


Figure S2. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

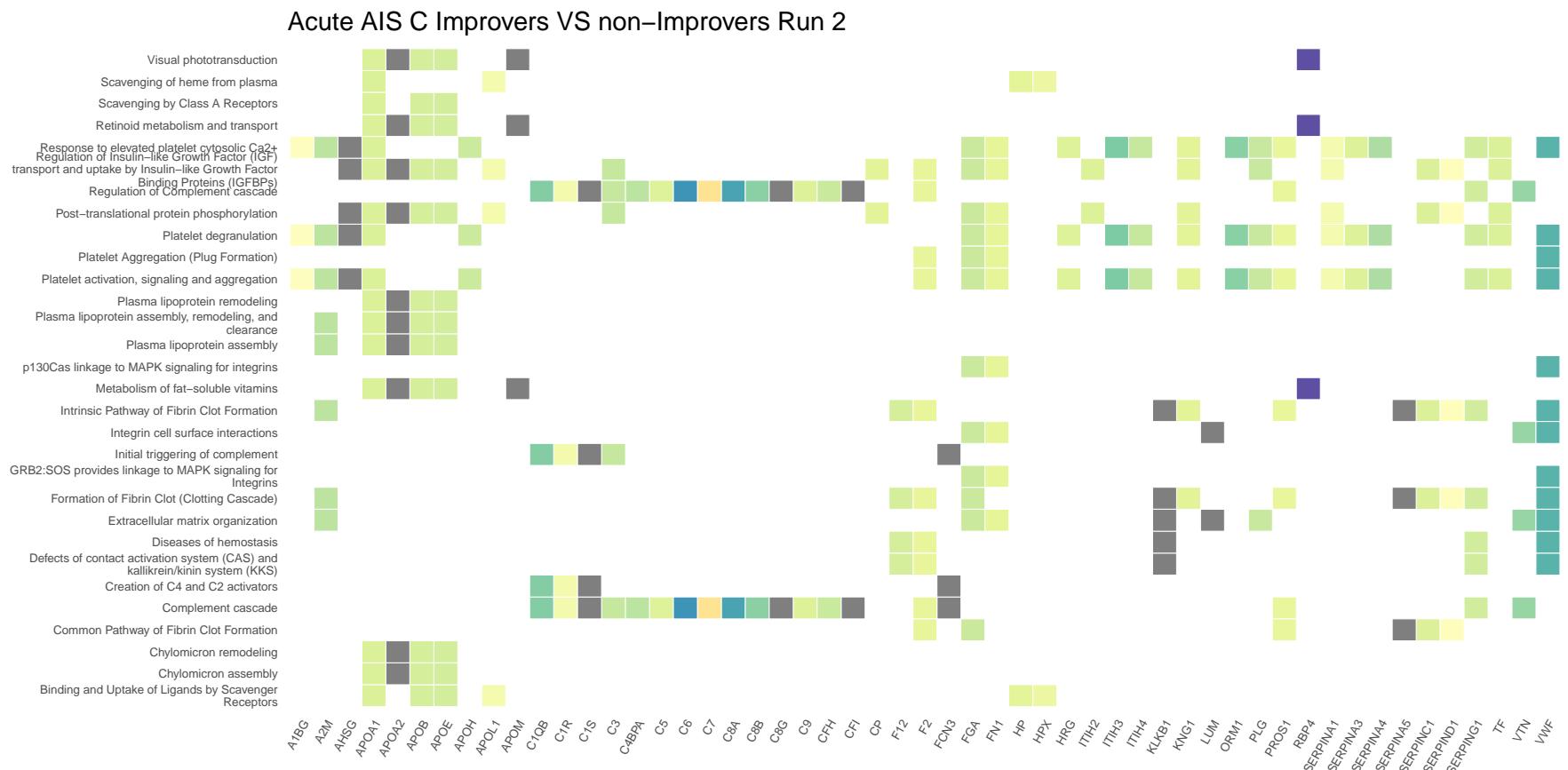


Figure S3. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.

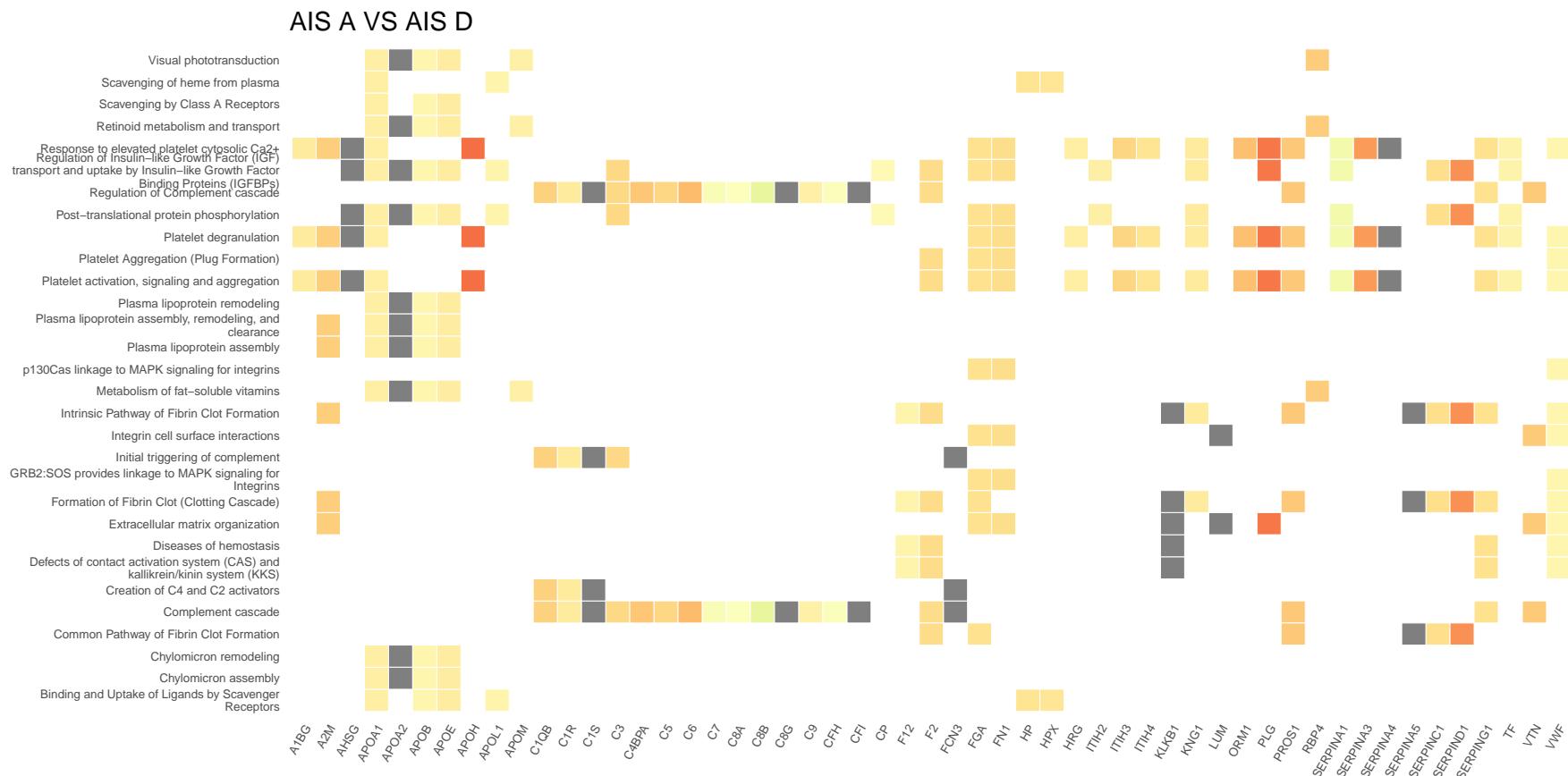


Figure S4. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

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.

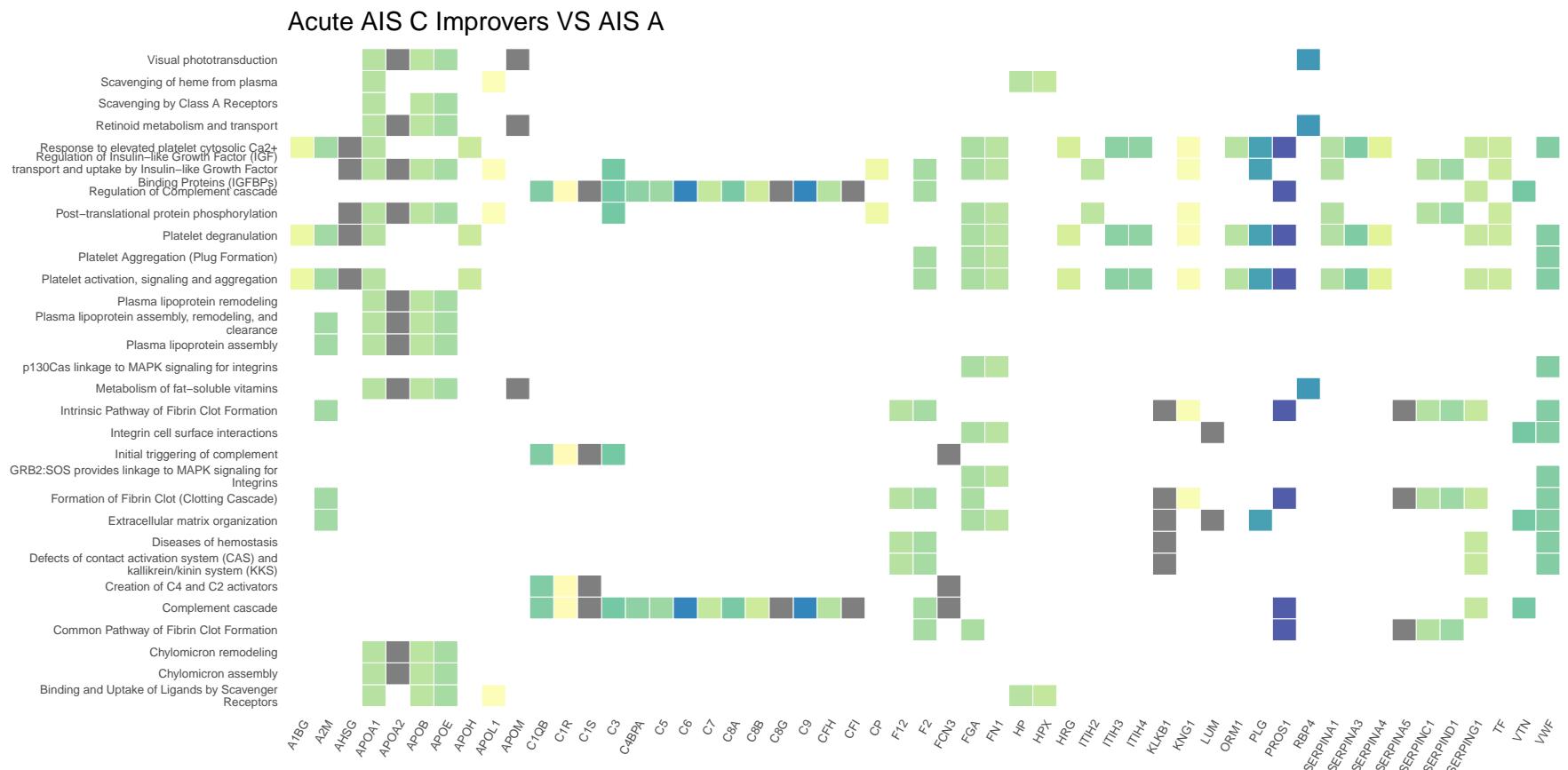


Figure S6. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

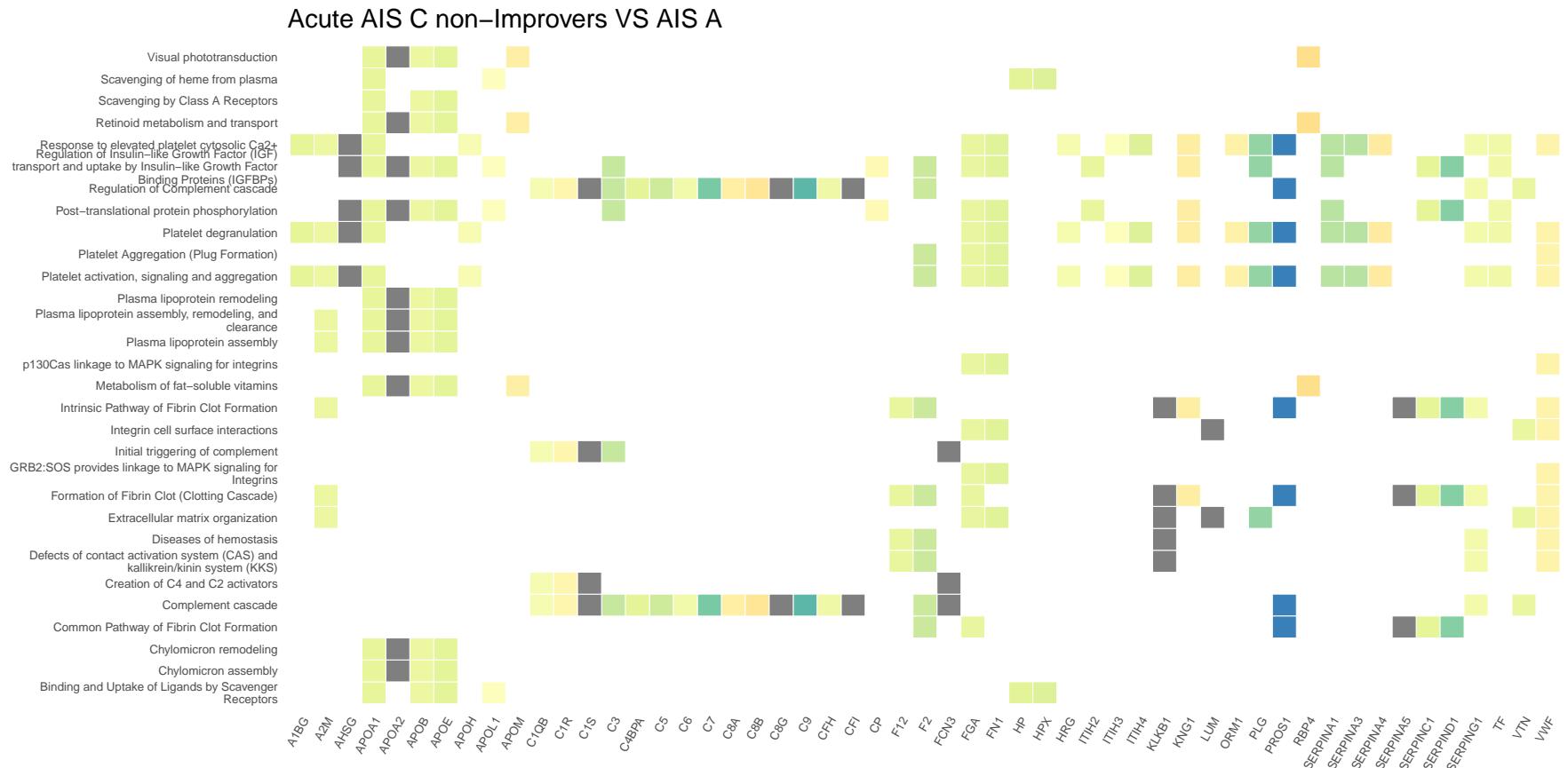


Figure S7. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

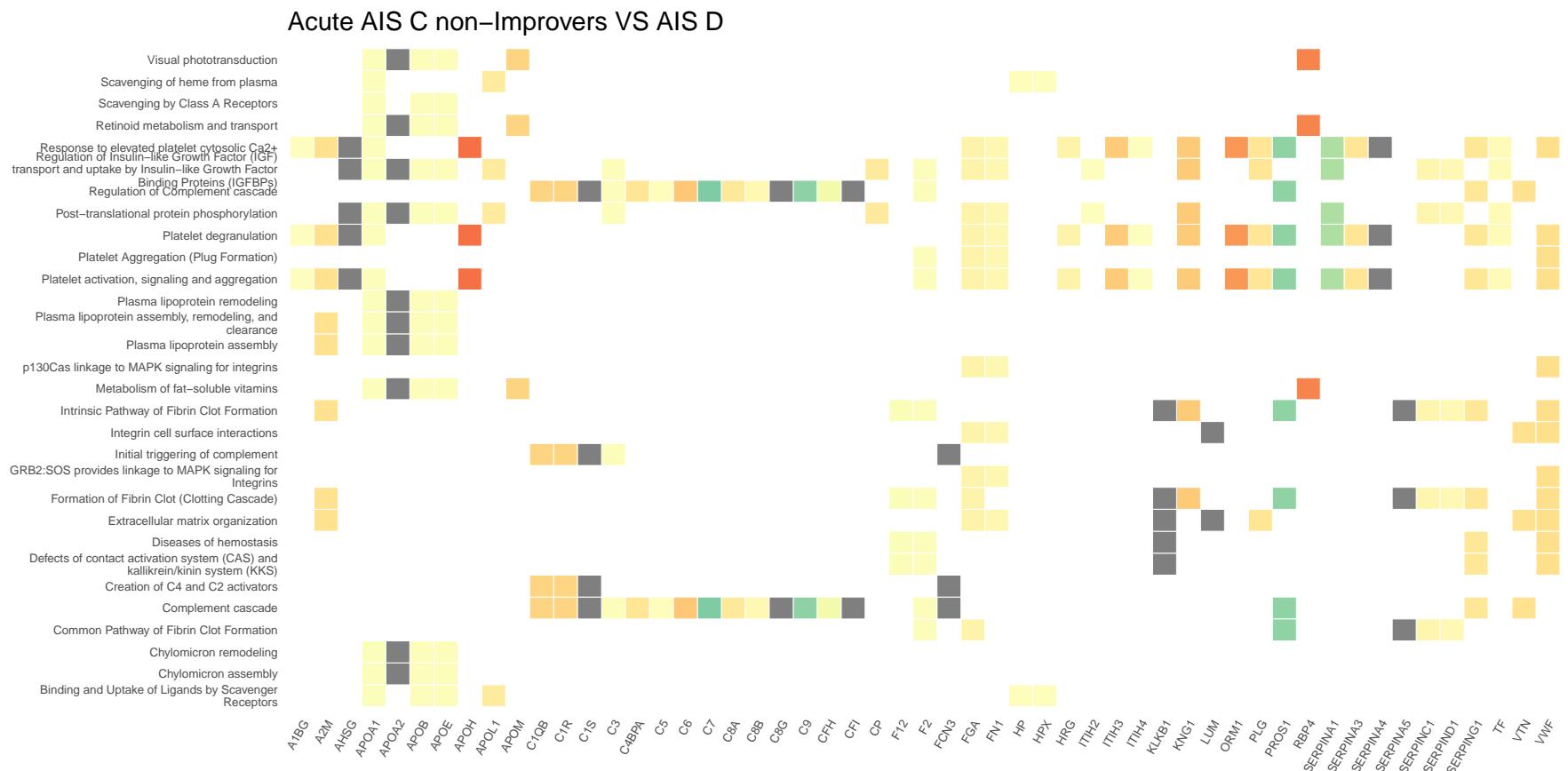


Figure S8. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

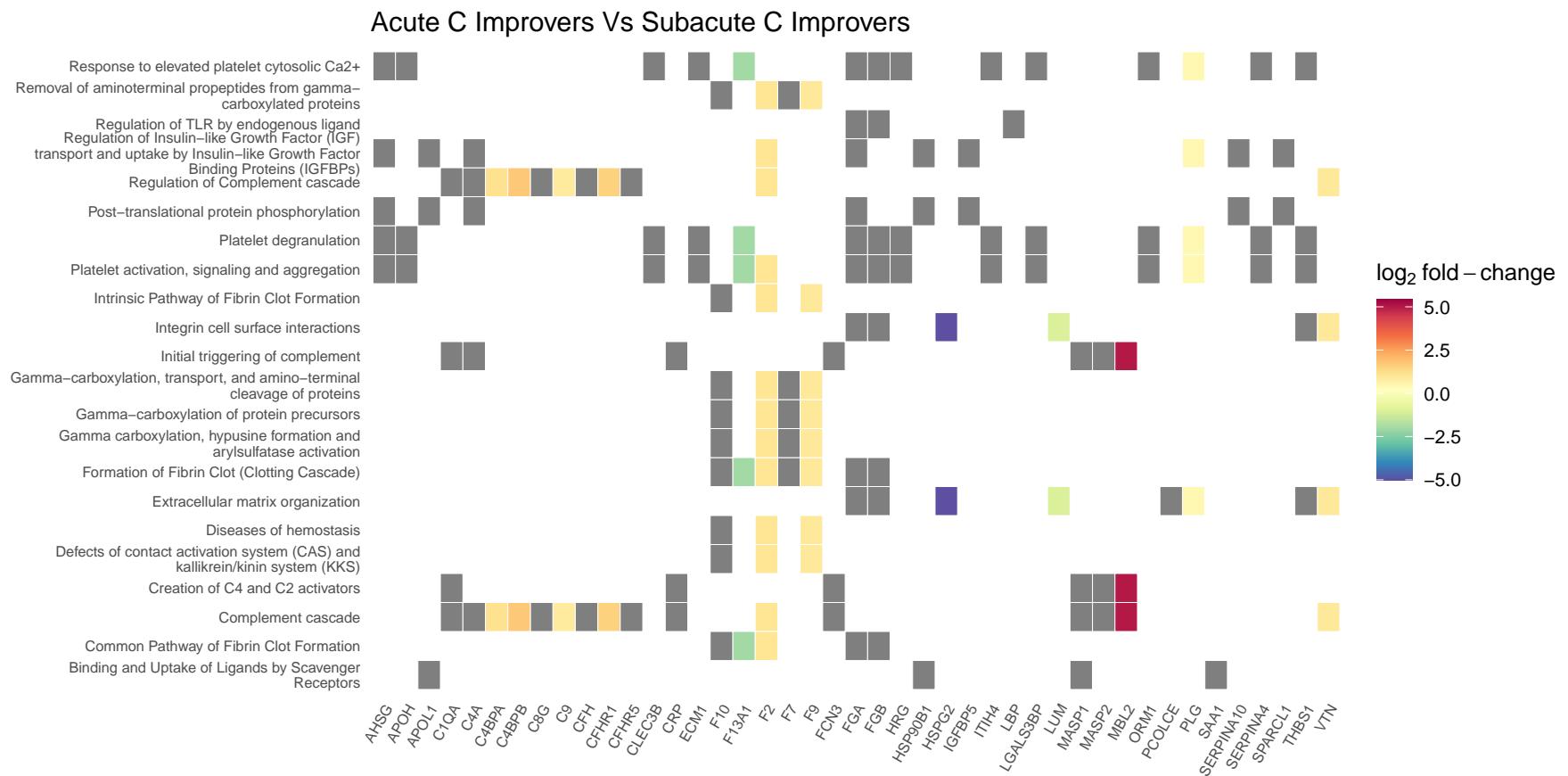


Figure S9. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.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

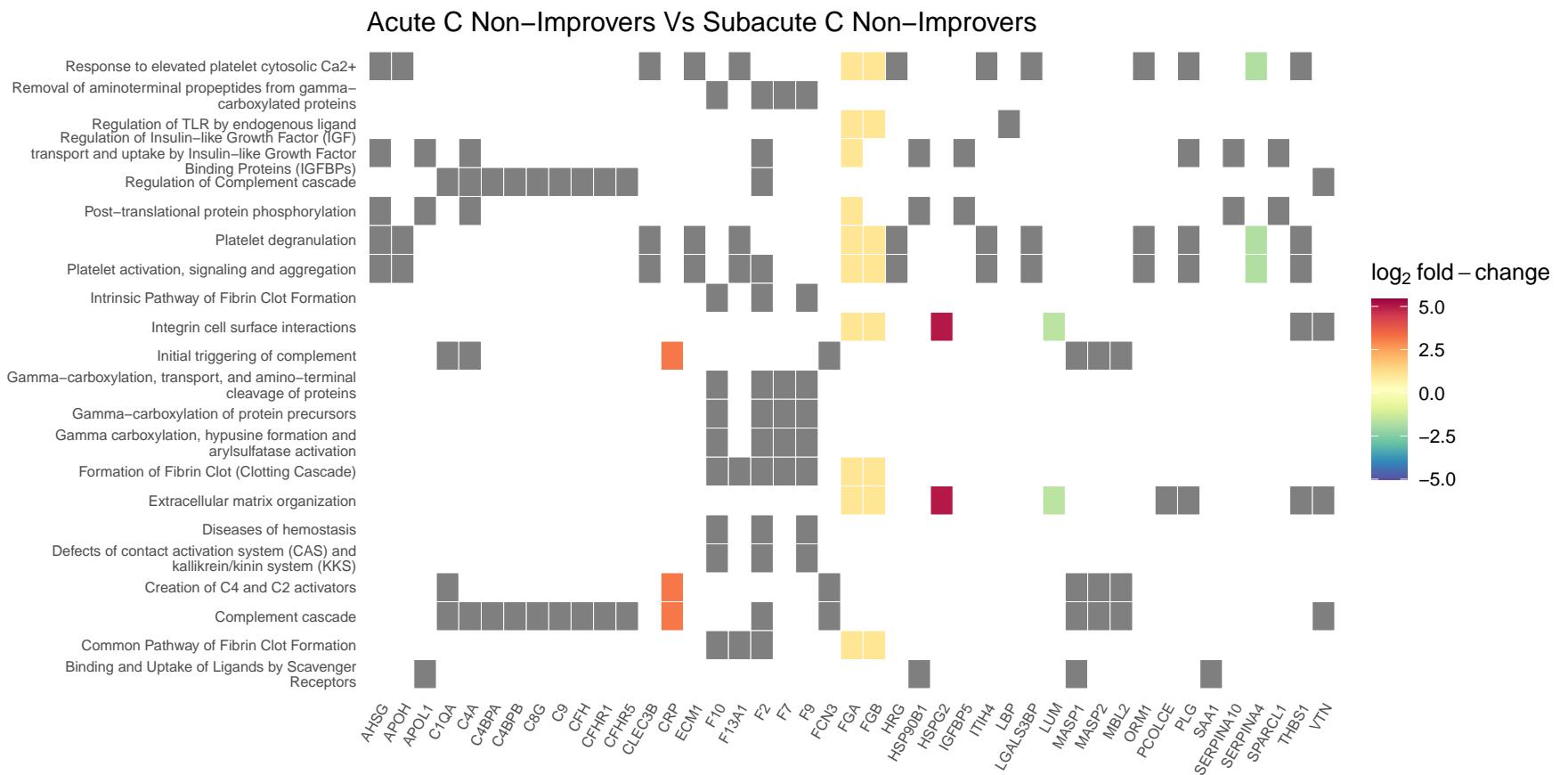


Figure S10. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.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

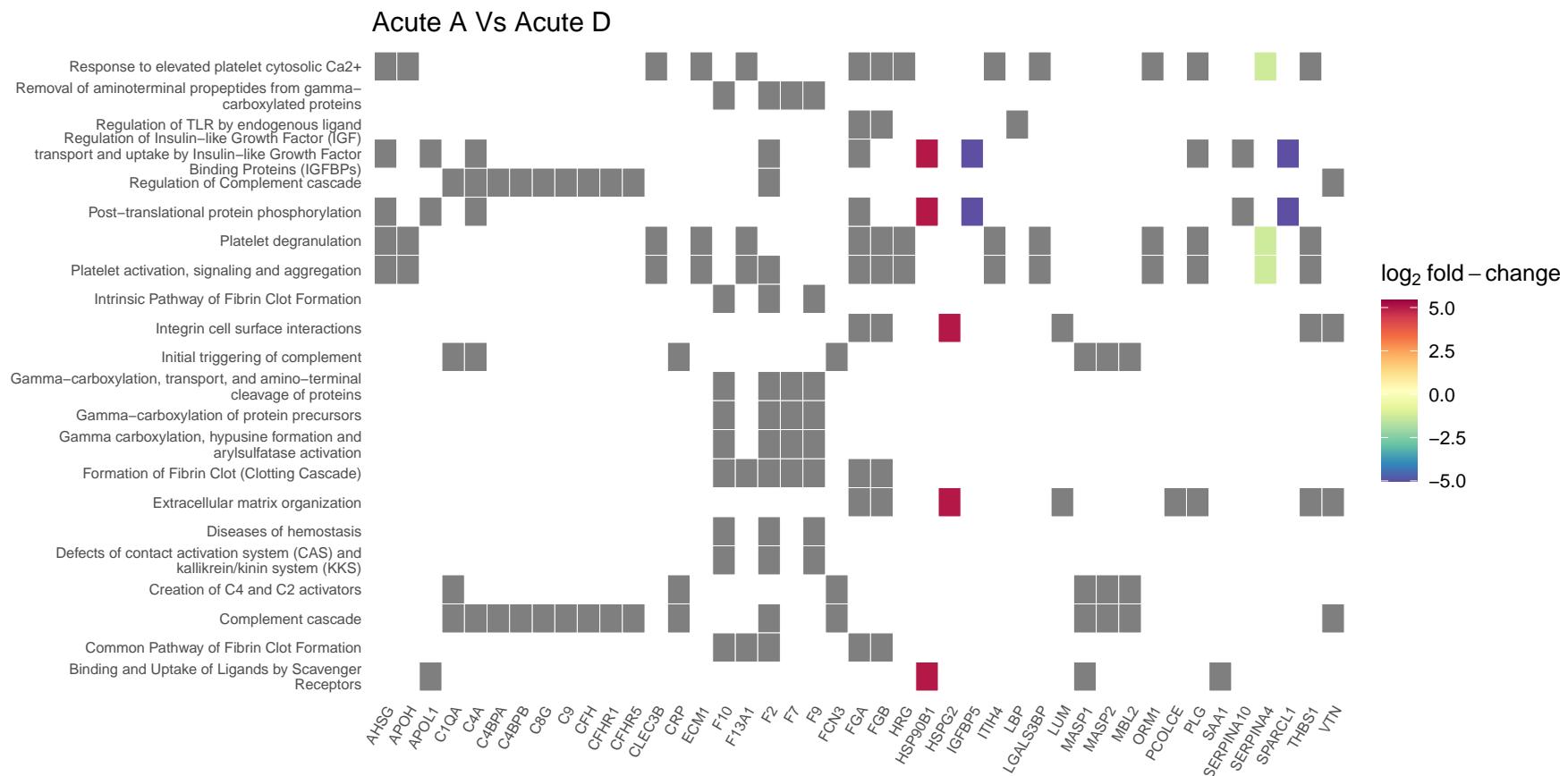


Figure S11. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients. Grey blocks denote proteins not present in the comparison.

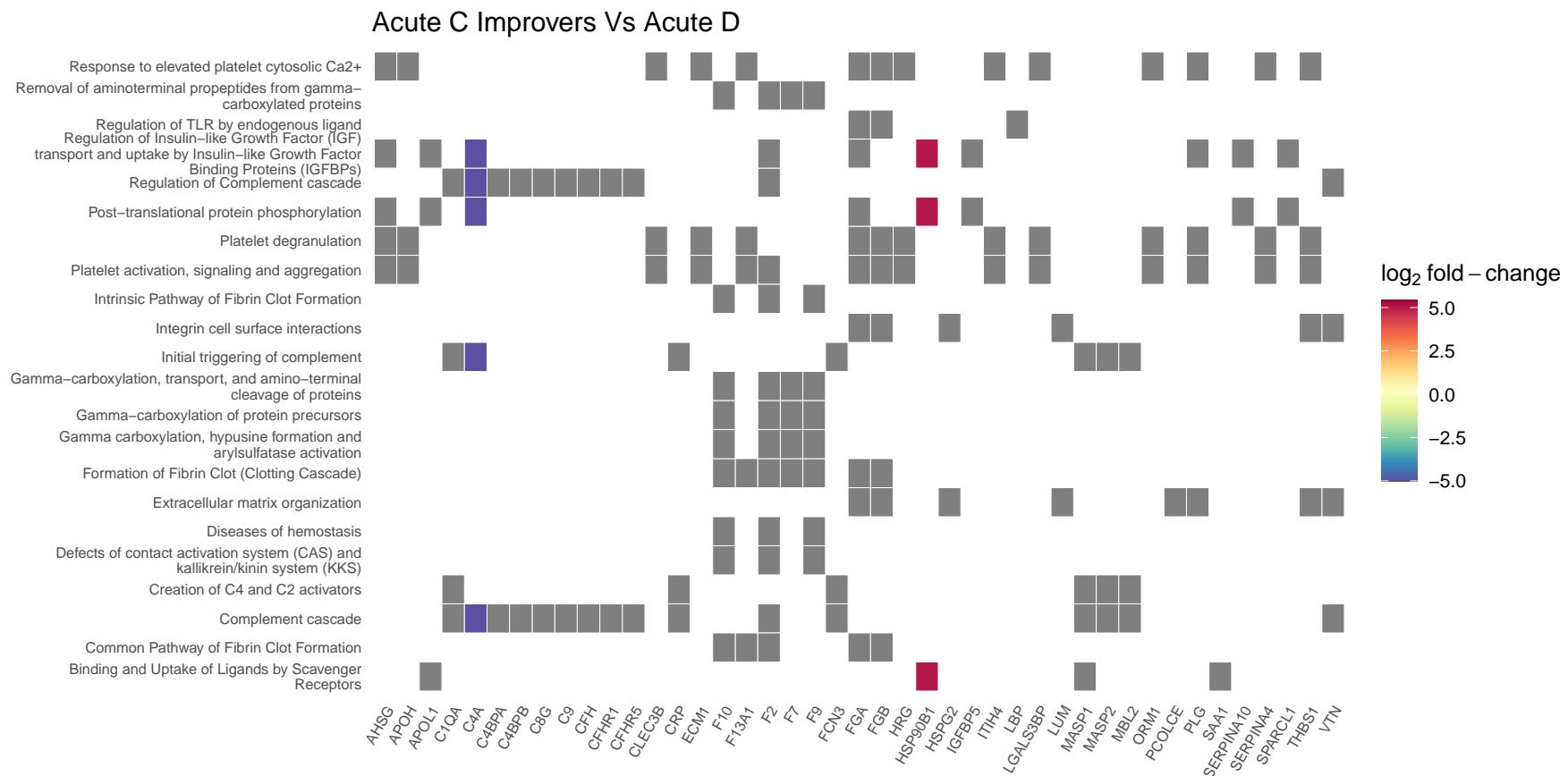


Figure S12. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients. Grey blocks denote proteins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

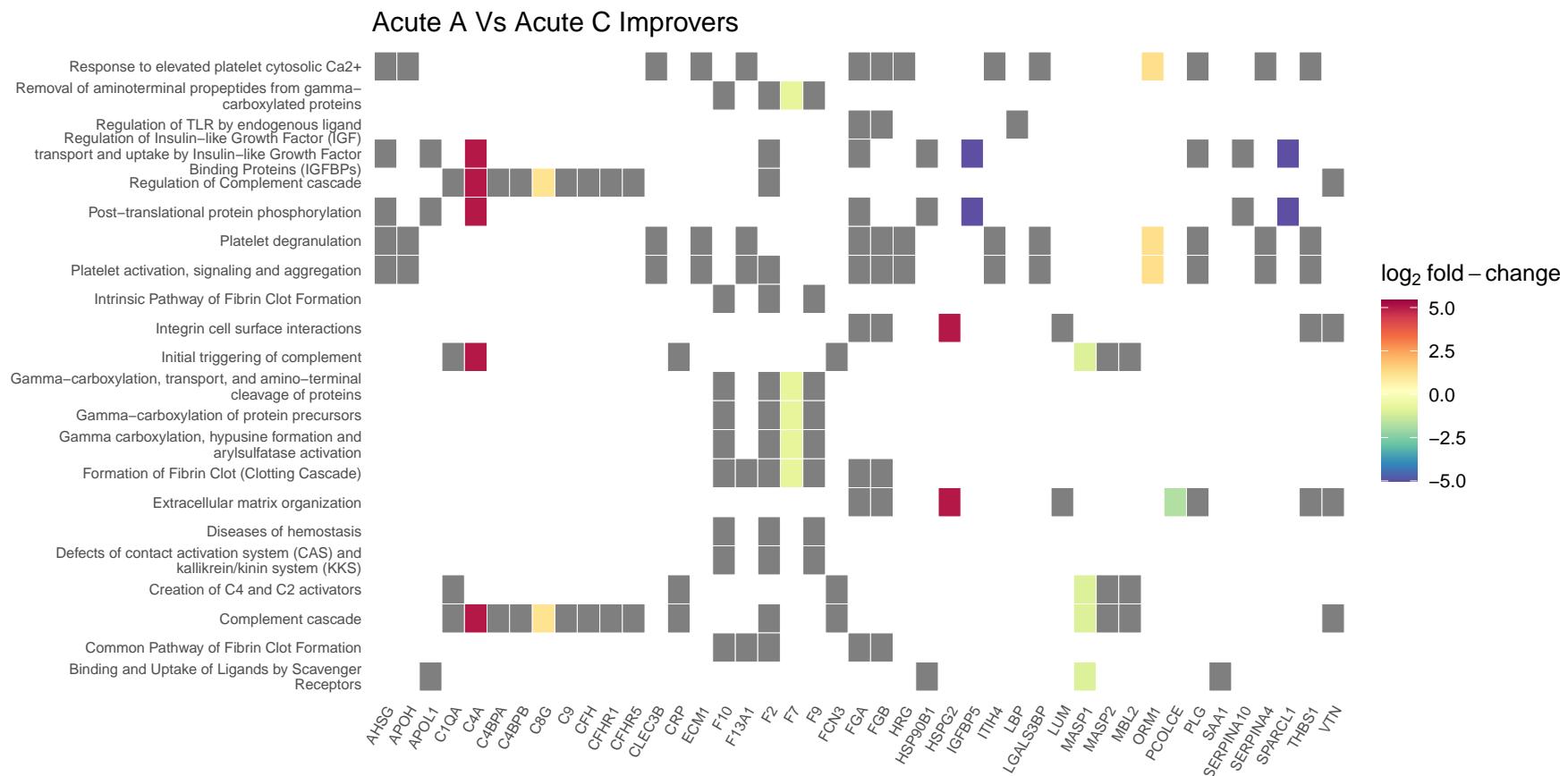


Figure S13. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients. Grey blocks denote proteins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

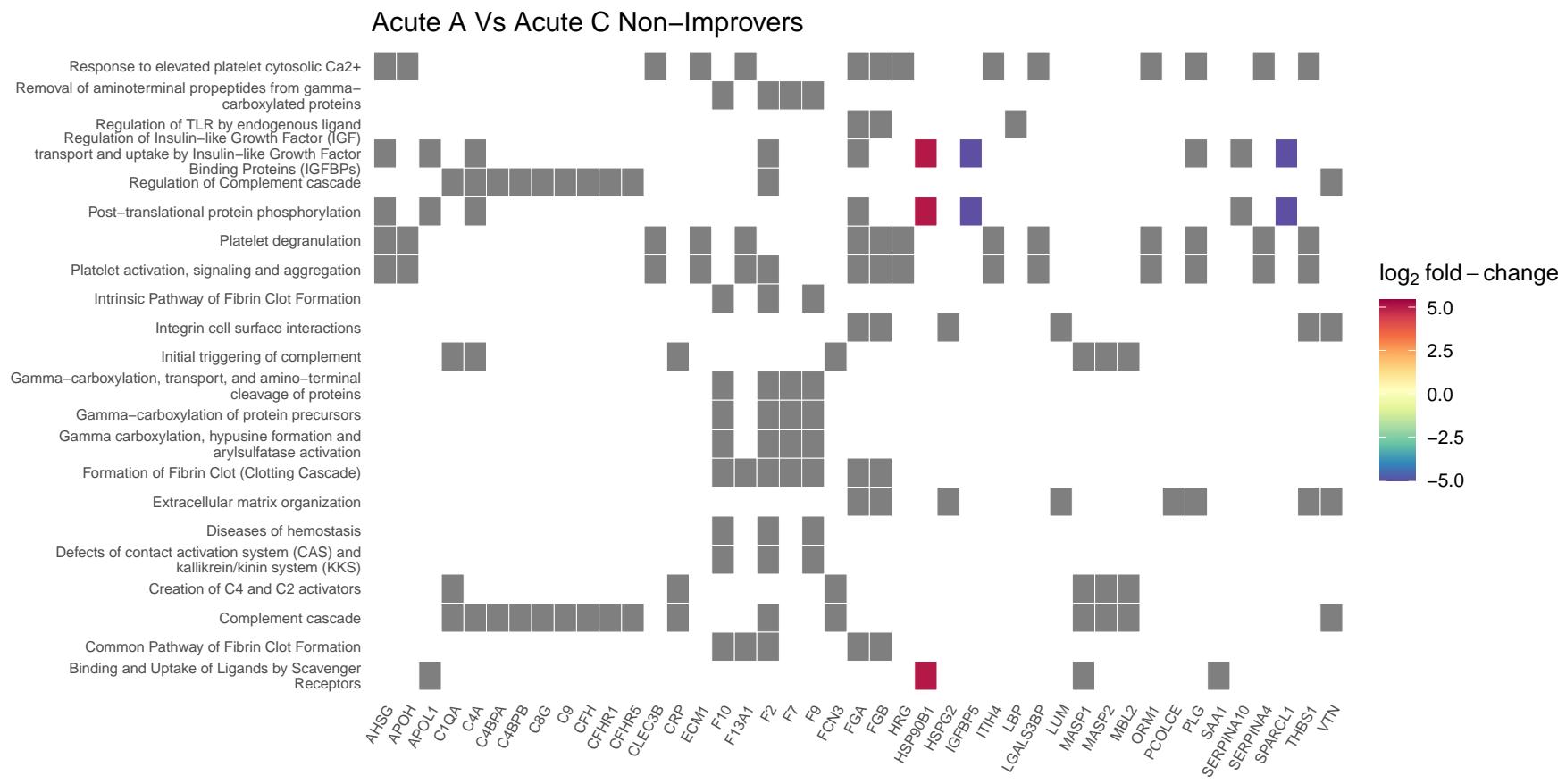


Figure S14. Heatmap denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients. Grey blocks denote proteins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

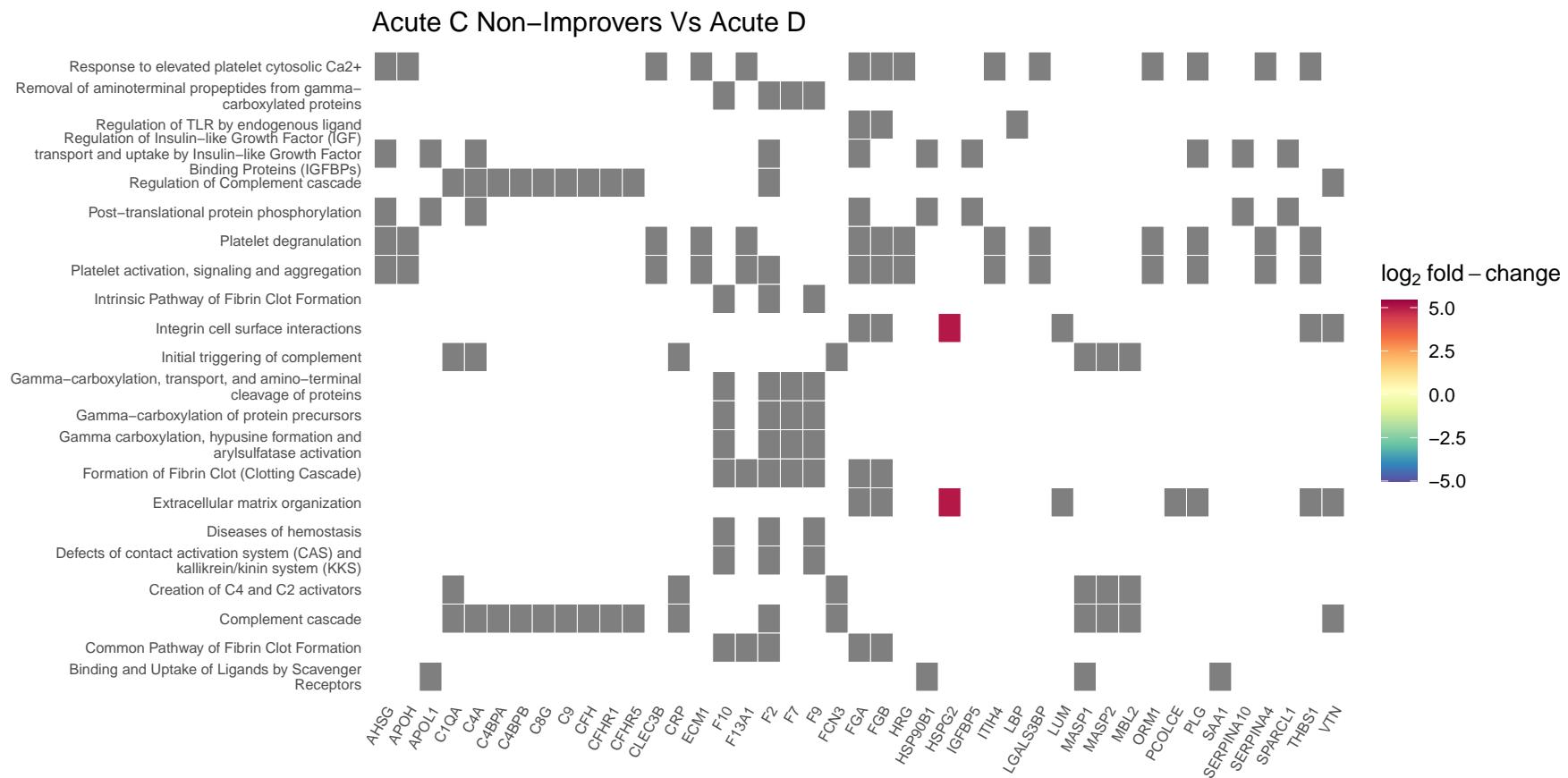


Figure S15. Heatmap denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients. Grey blocks denote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

¹⁰³⁸ **5.6 Cnetplots**

¹⁰³⁹ **5.6.1 iTRAQ data**

AIS C Improvers acute vs subacute

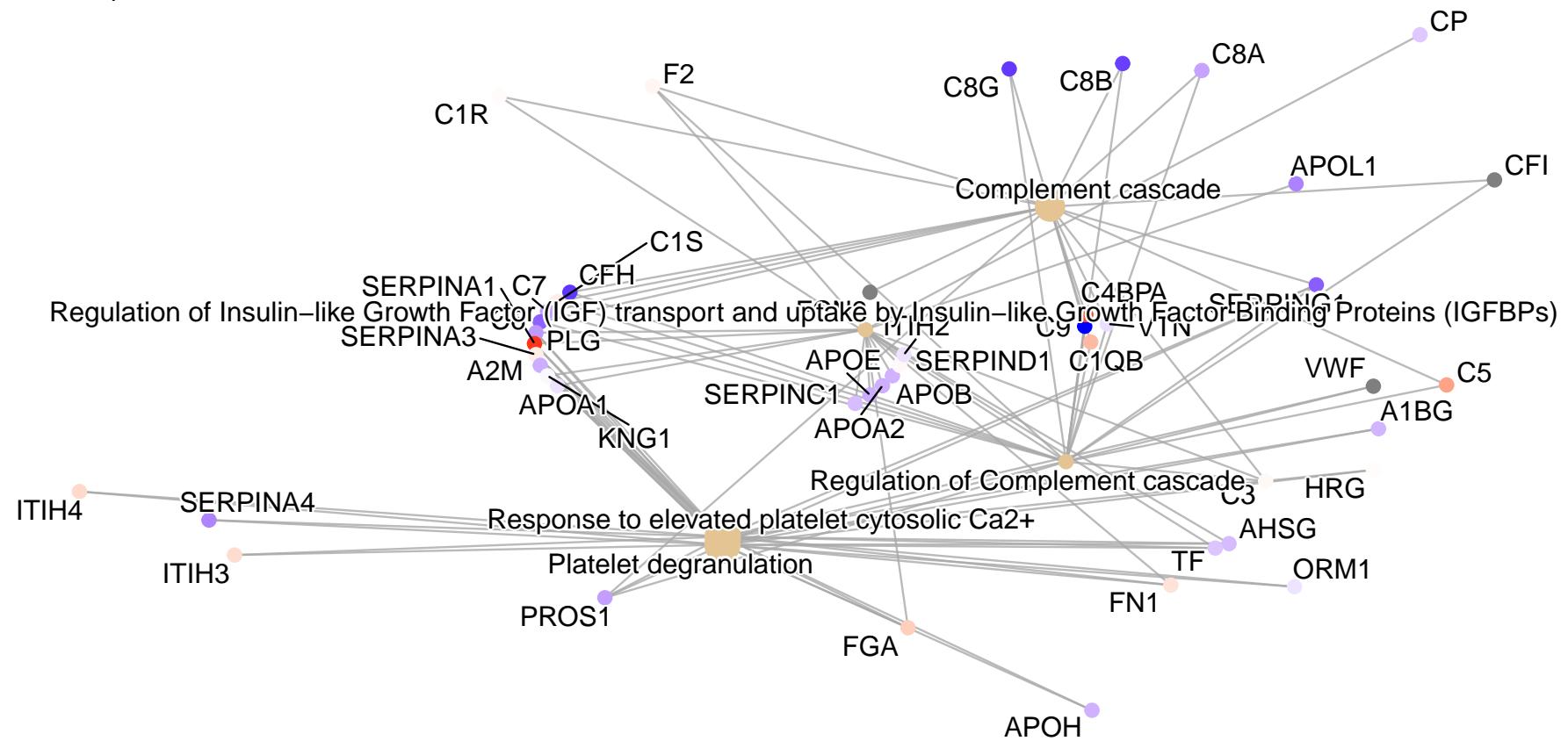


Figure S16. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

AIS C non-Improvers acute vs subacute

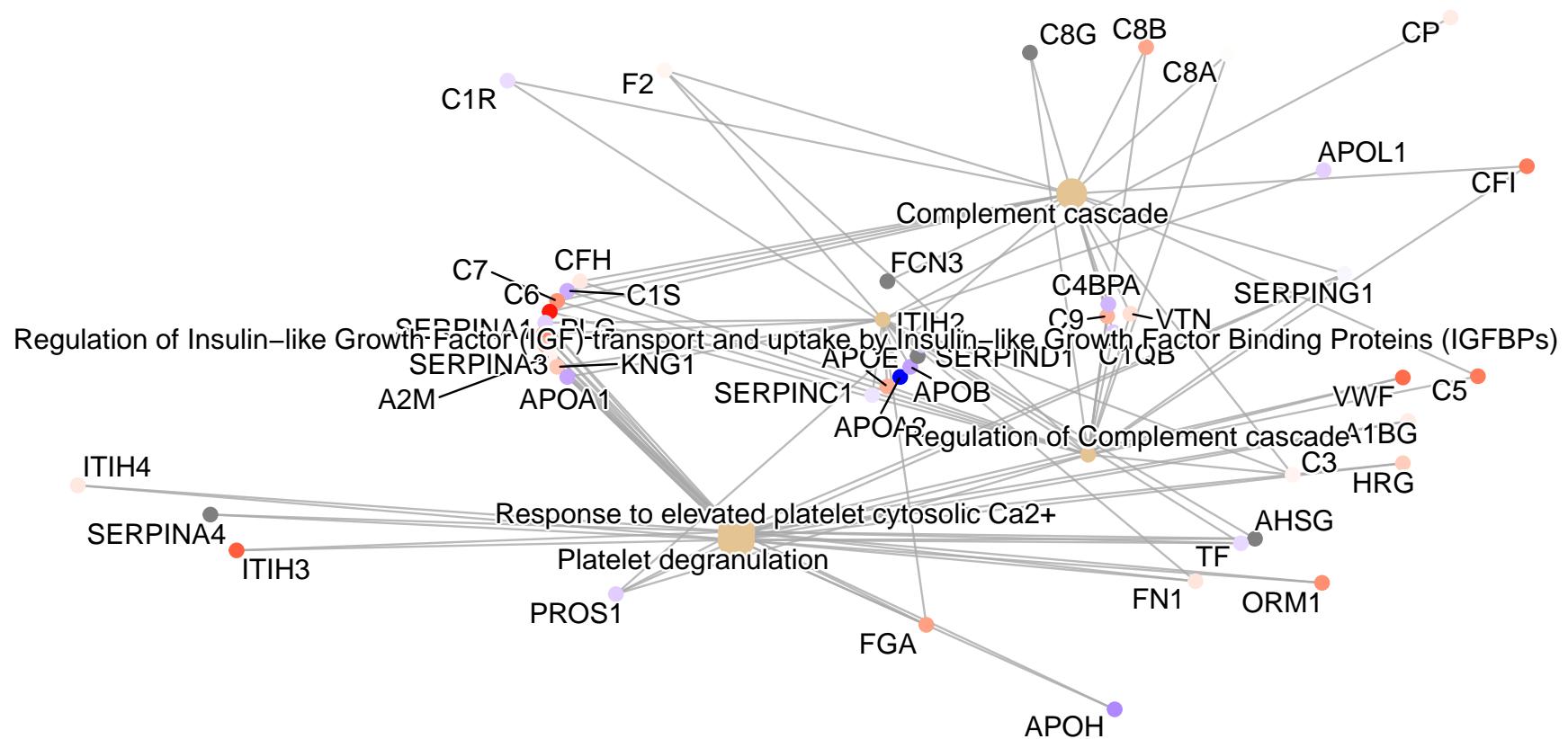


Figure S17. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months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

Acute AIS C Improvers VS non-Improvers Run 2

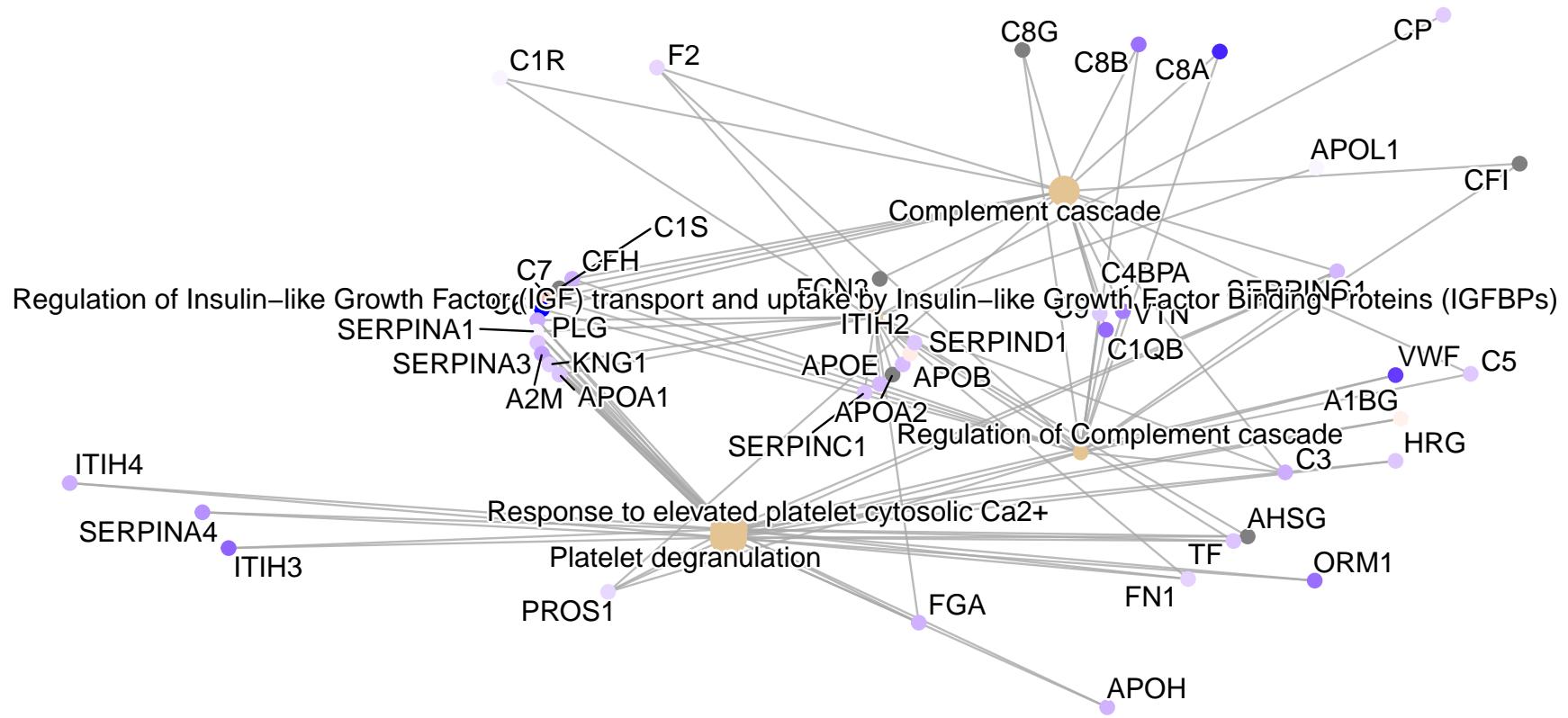


Figure S18. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.

AIS A VS AIS D

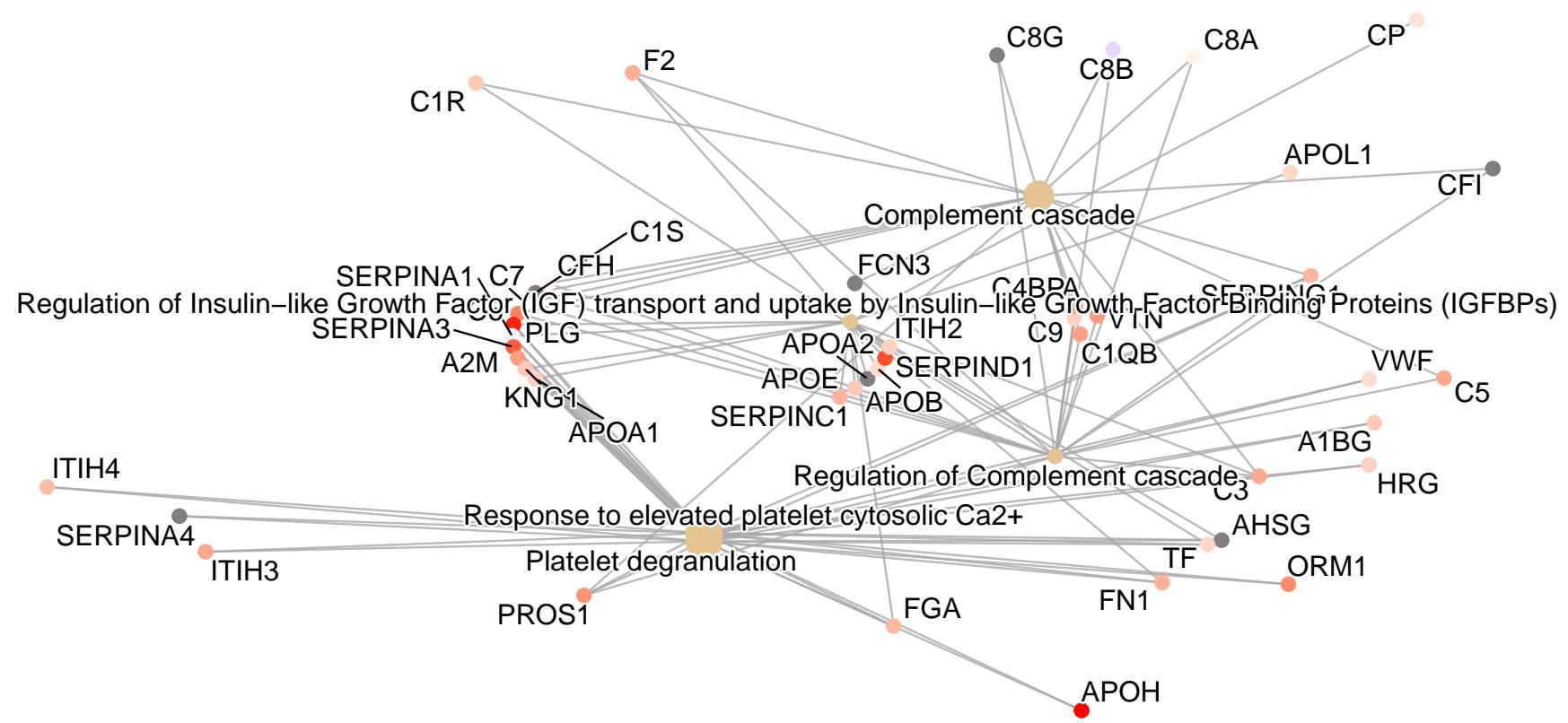


Figure S19. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Acute AIS C Improvers VS AIS D

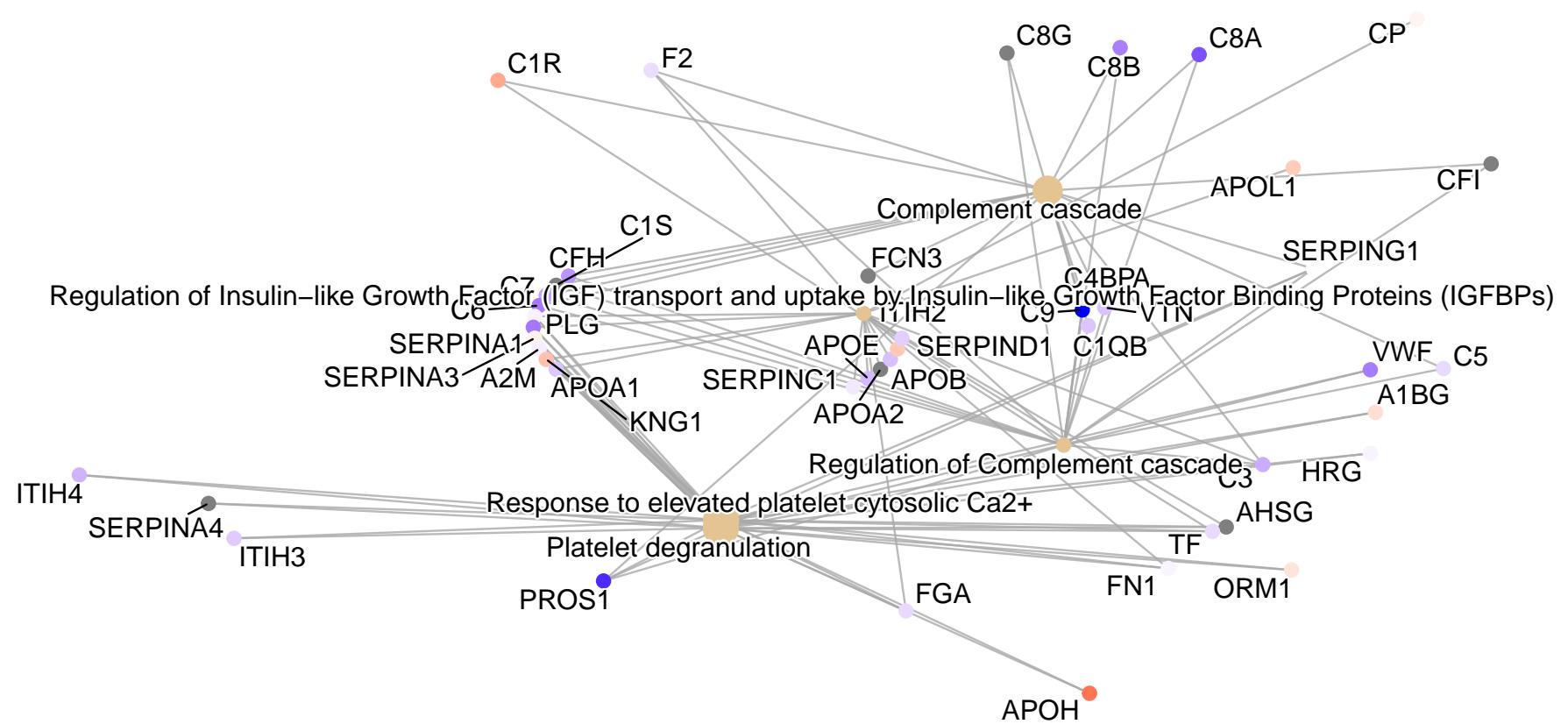


Figure S20. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Acute AIS C Improvers VS AIS A

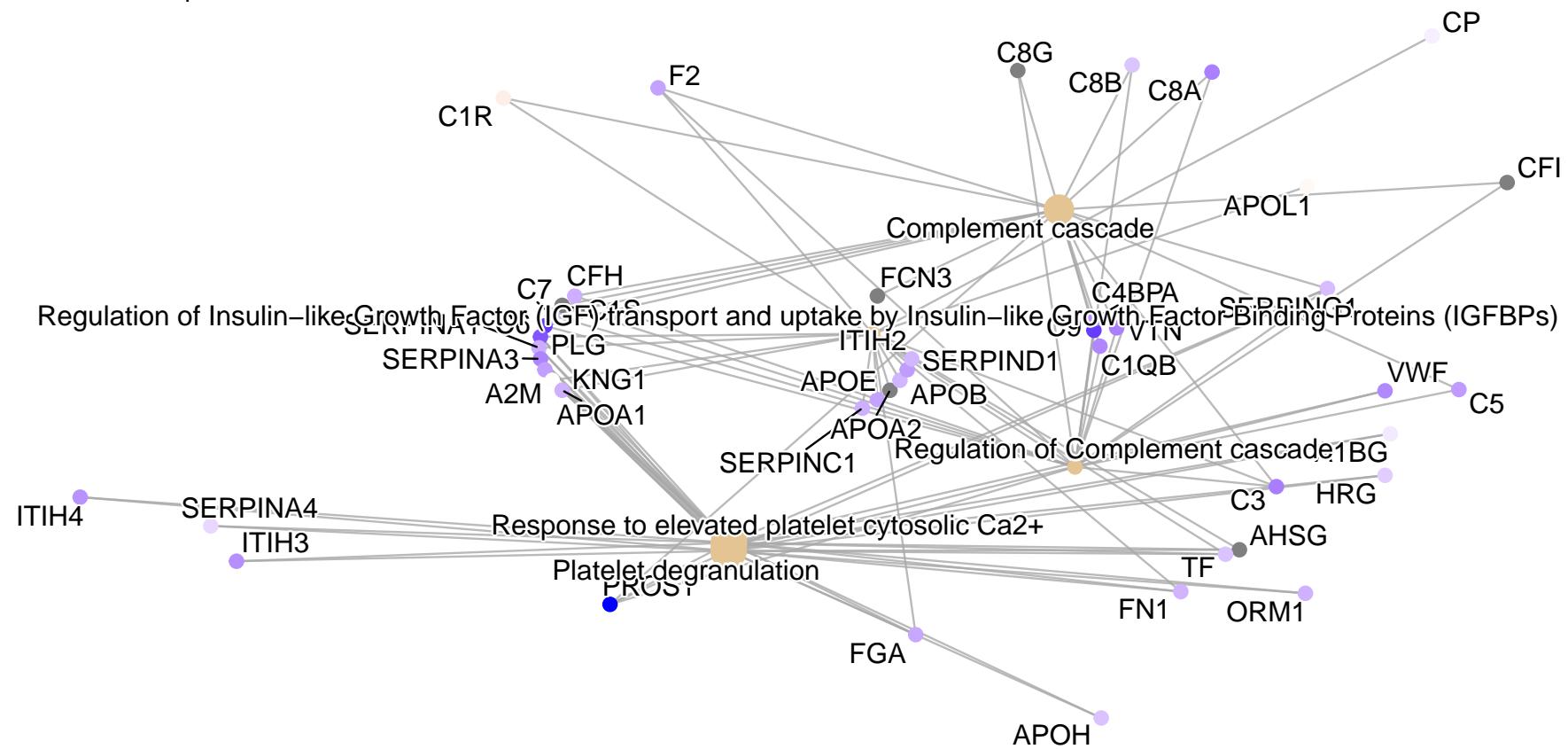


Figure S21. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS A

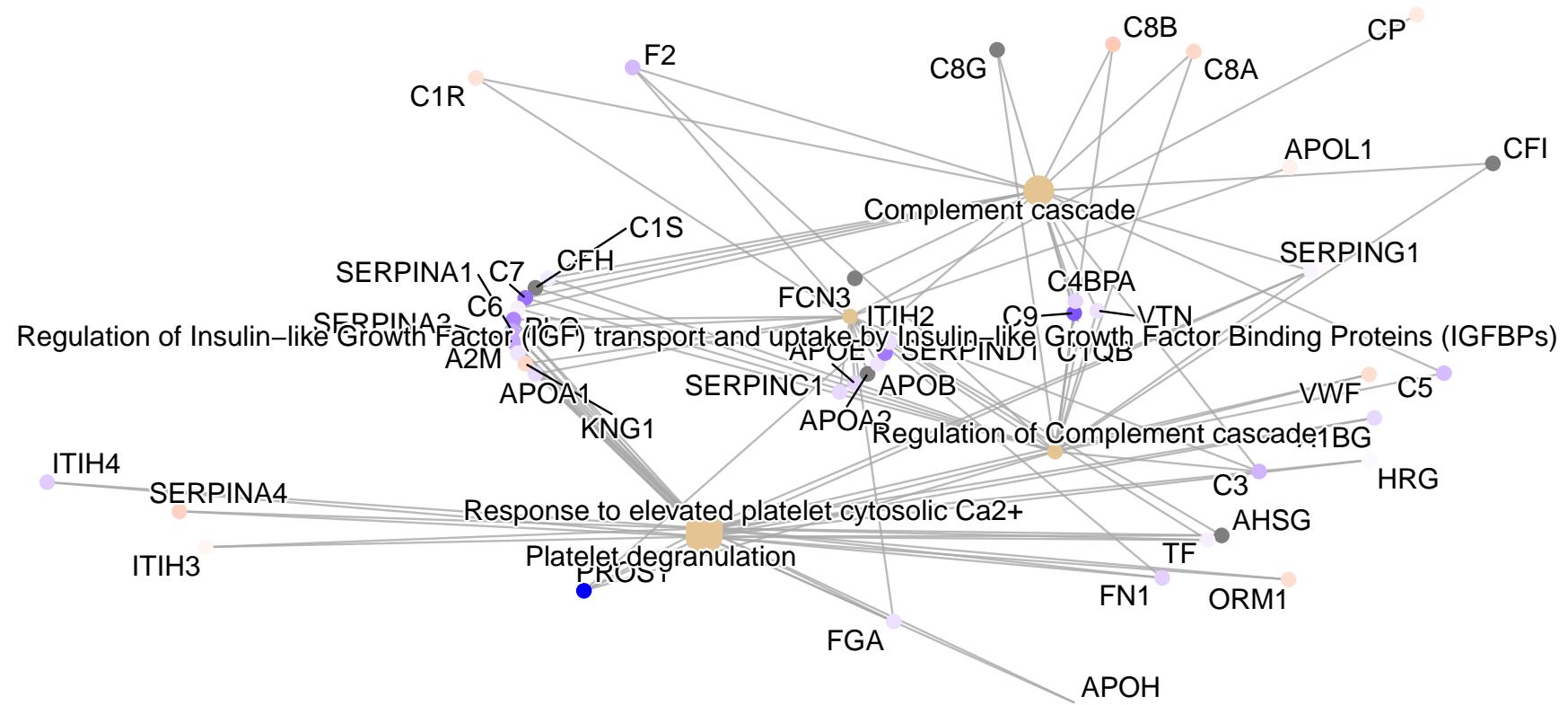


Figure S22. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

Acute AIS C non-Improvers VS AIS D

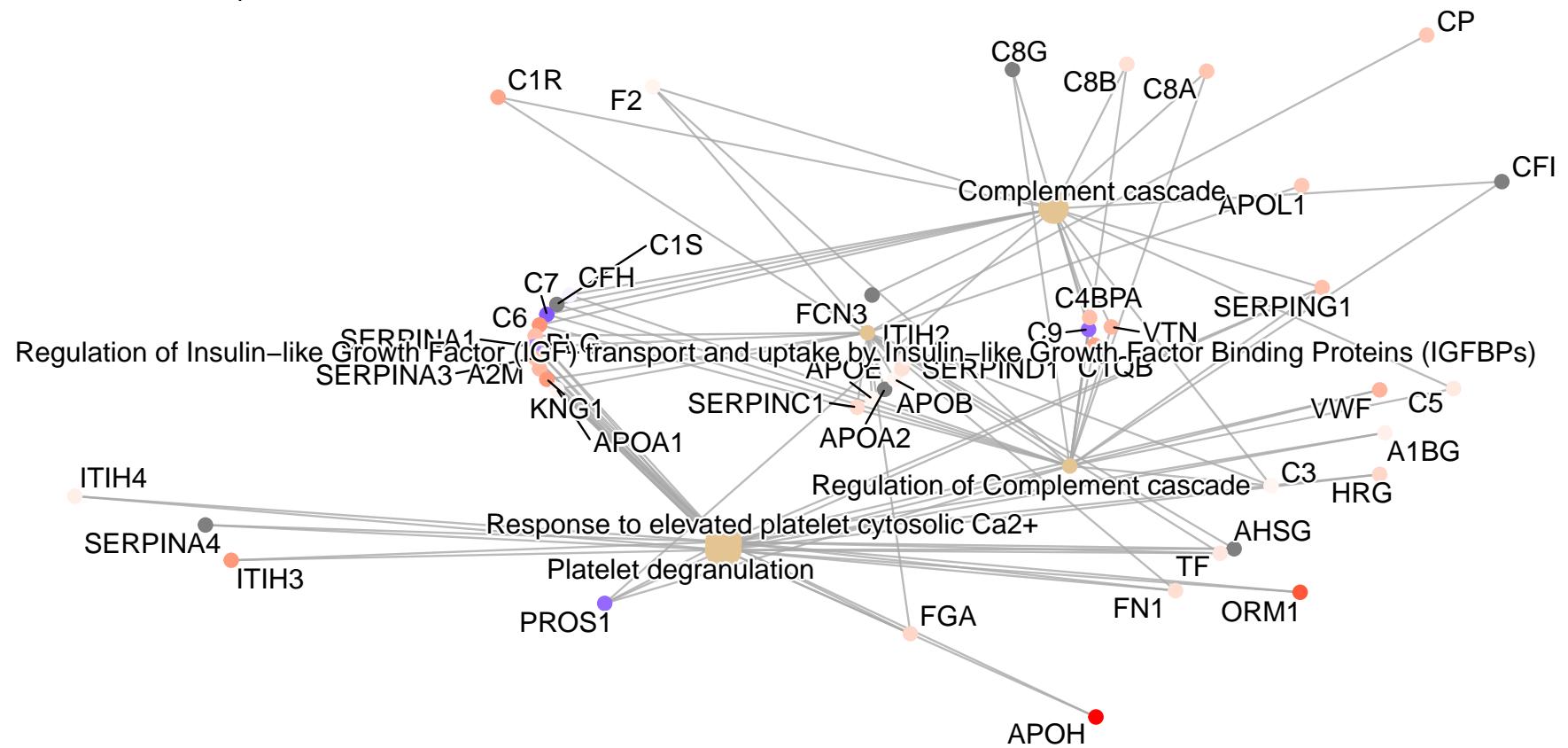


Figure S23. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

Acute C Improvers Vs Subacute C Improvers

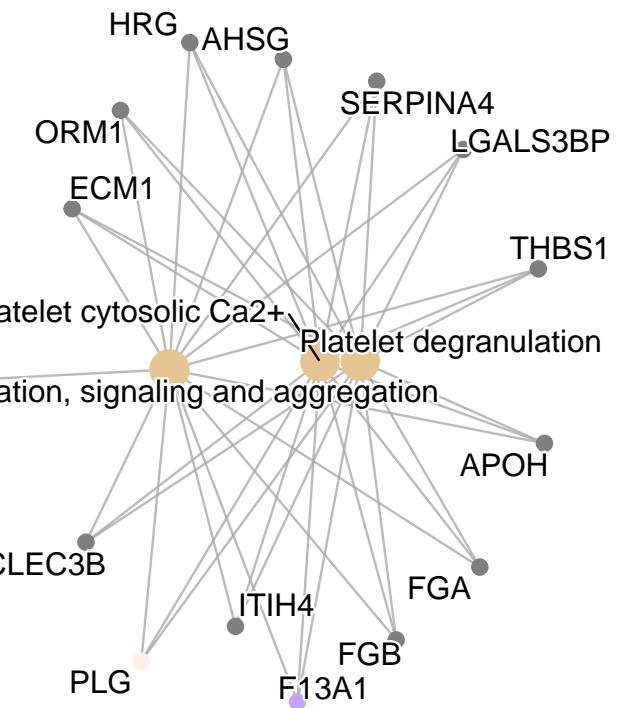
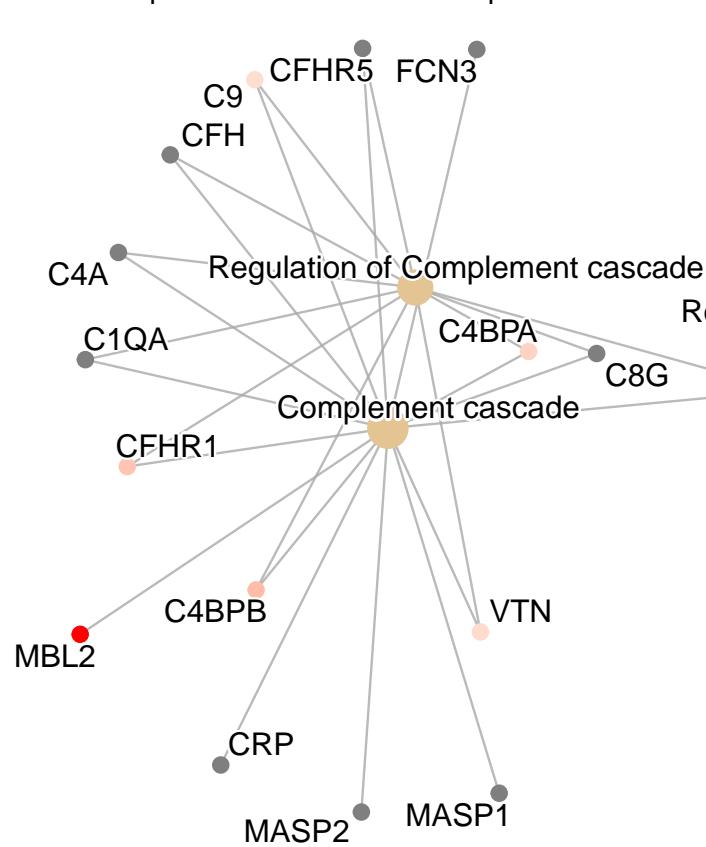


Figure S24. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

Acute C Non-Improvers Vs Subacute C Non-Improvers

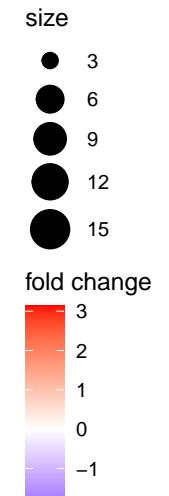
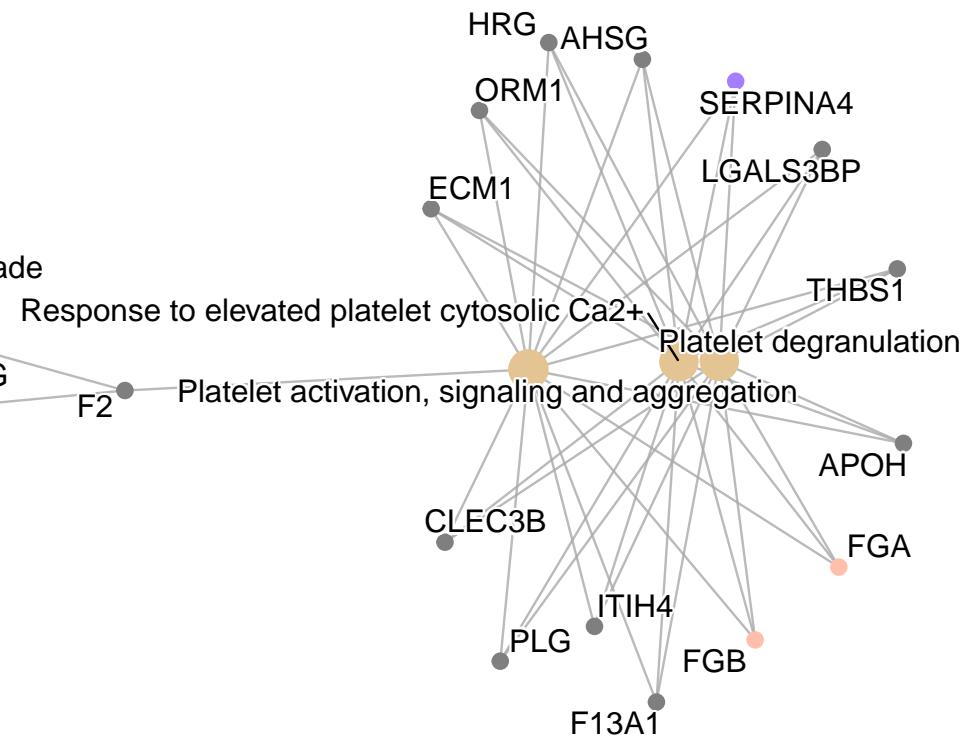
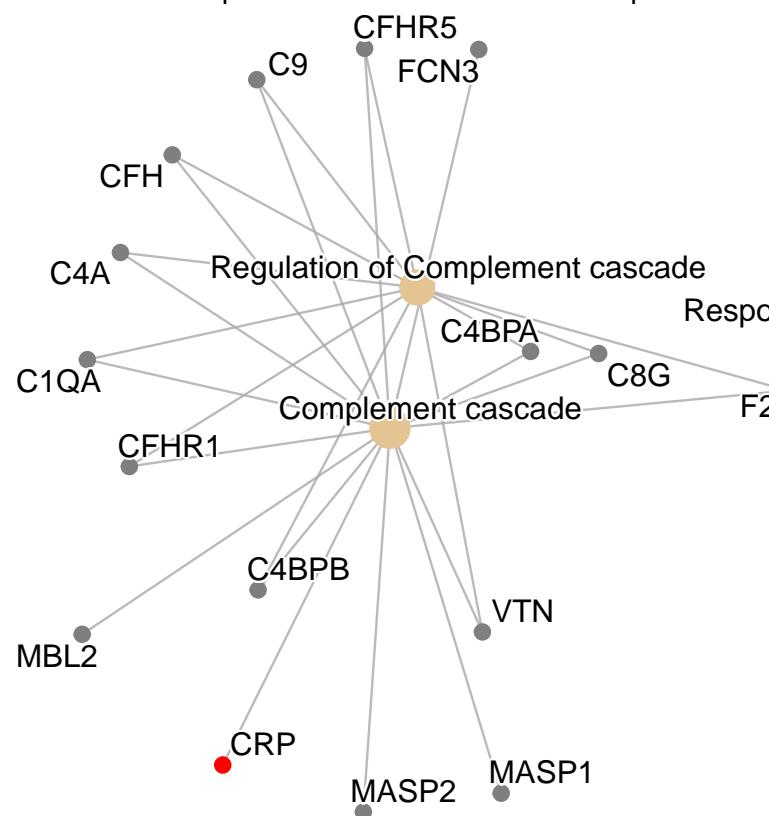
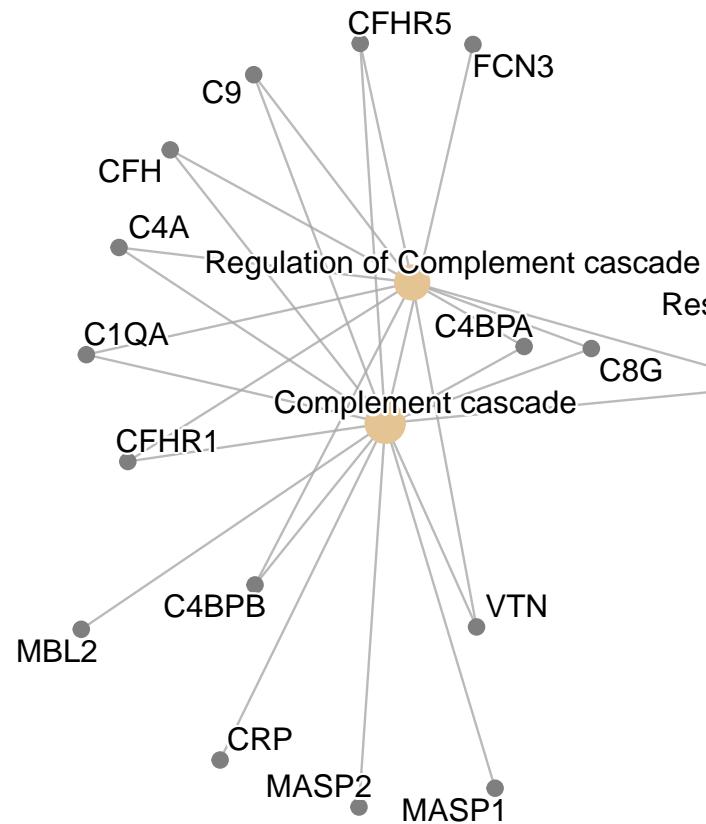
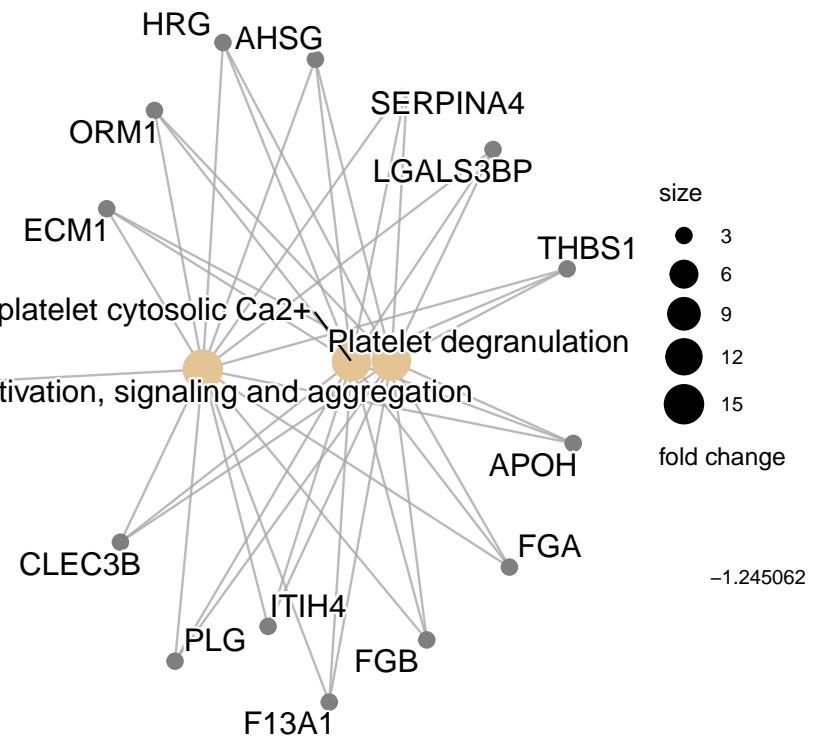


Figure S25. Network plot denoting the log₂ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months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

Acute A Vs Acute D



Complement cascade
Regulation of Complement cascade



size
● 3
● 6
● 9
● 12
● 15
fold change
-1.245062

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.

Acute C Improvers Vs Acute D

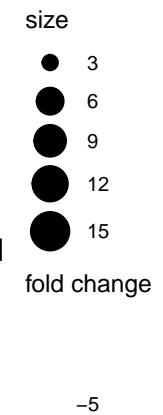
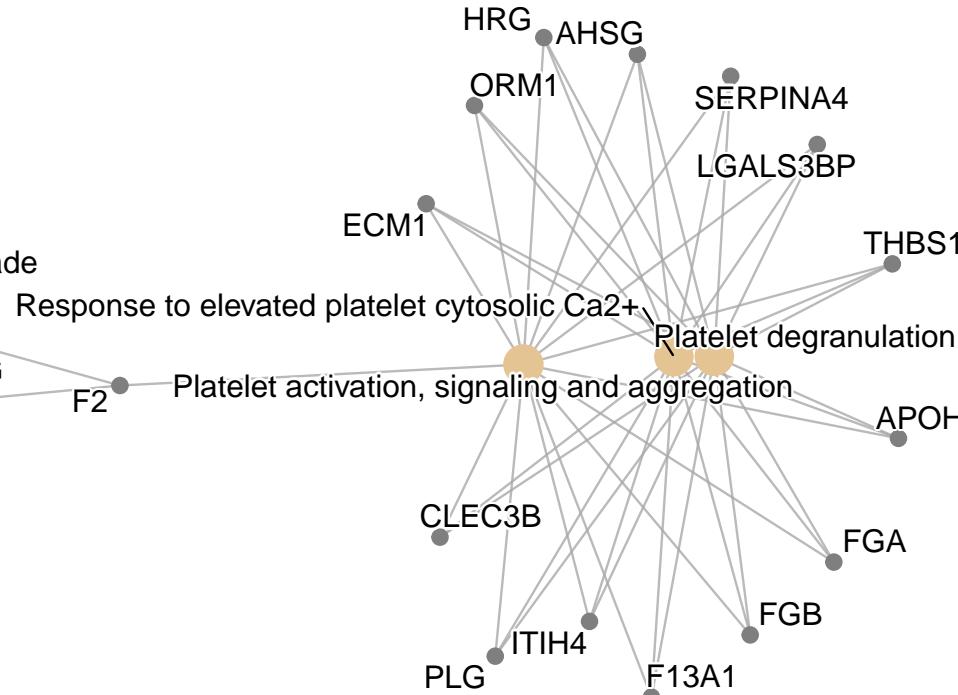
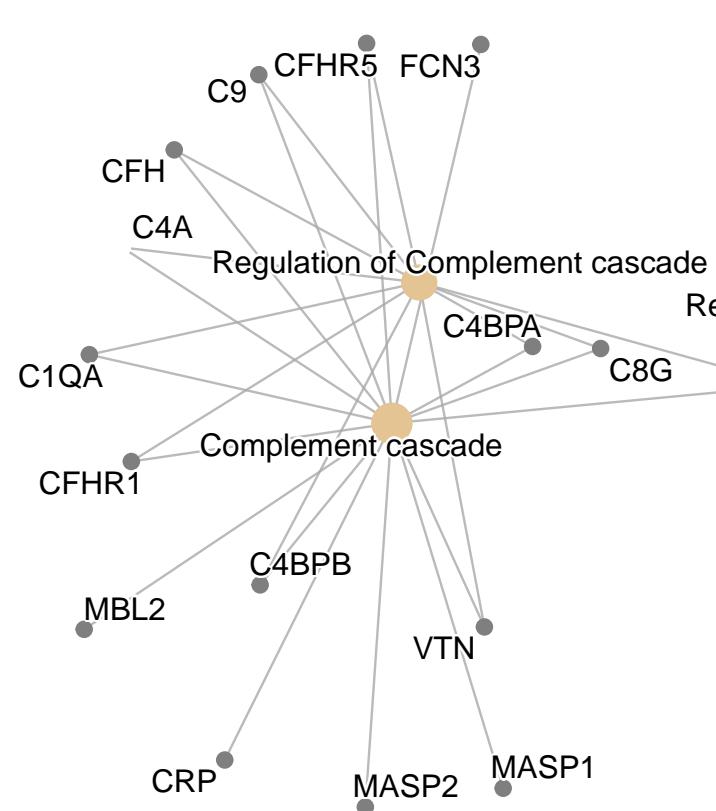
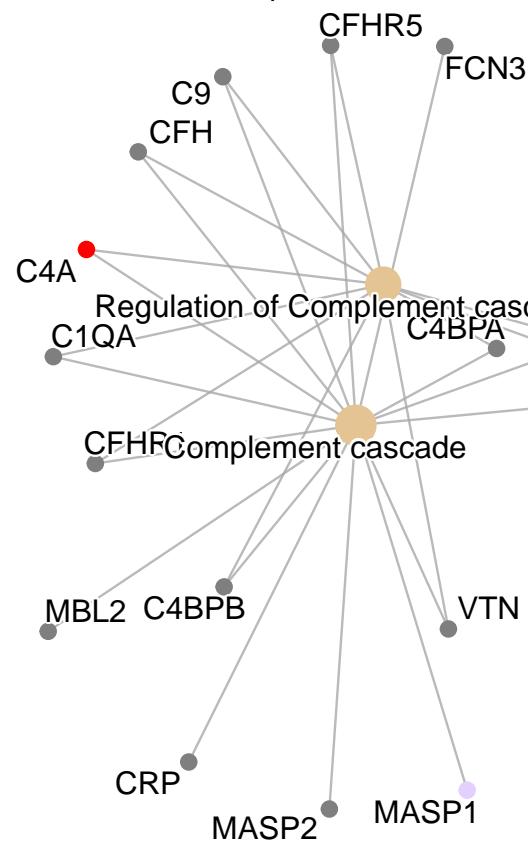


Figure S27. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Acute A Vs Acute C Improvers



Regulation of Complement cascade

C4BPA

C8G

F2

CFHR5
Complement cascade

FCN3

MASP1

VTN

CRP

MASP2

CFH

C9

C4A

C1QA

MBL2

C4BPB

ECM1

ORM1

HRG

AHSG

SERPINA4

LGALS3BP

THBS1

APOH

FGA

FGB

F13A1

ITIH4

PLG

CLEC3B

Platelet activation, signaling and aggregation

Platelet degranulation

Response to elevated platelet cytosolic Ca²⁺

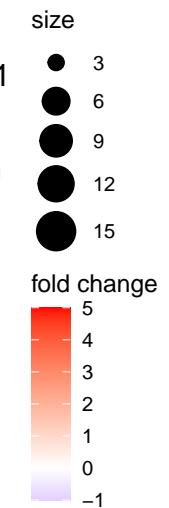


Figure S28. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

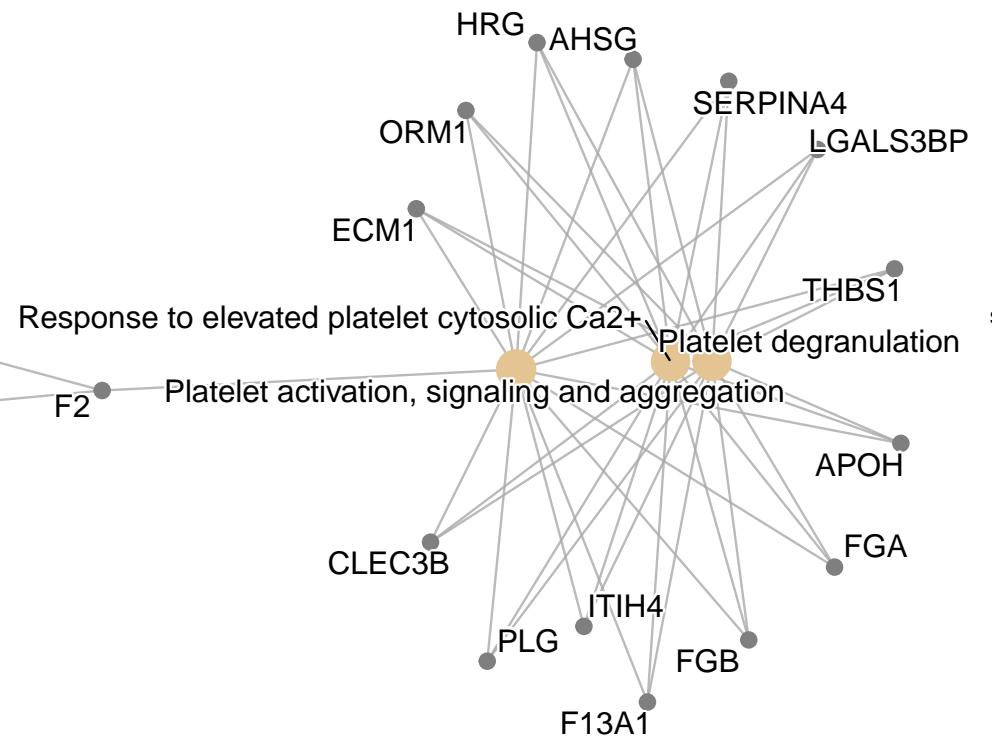
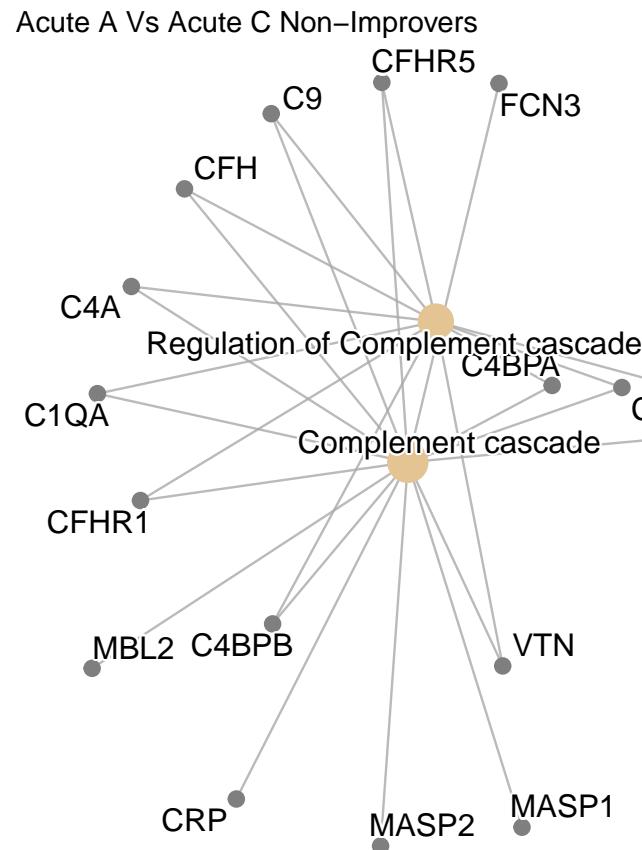
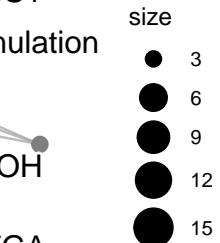


Figure S29. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.



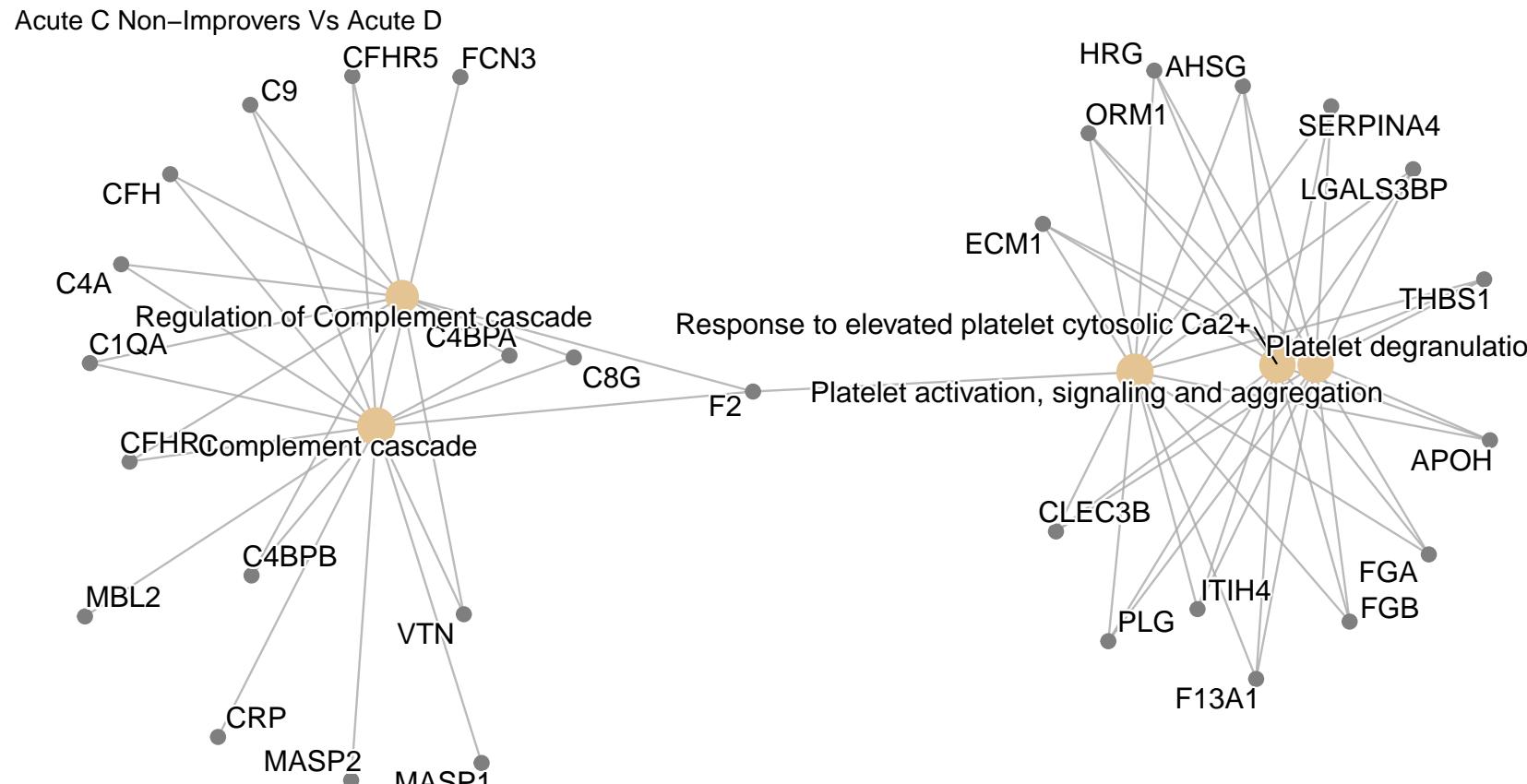


Figure S30. Network plot denoting the \log_2 fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

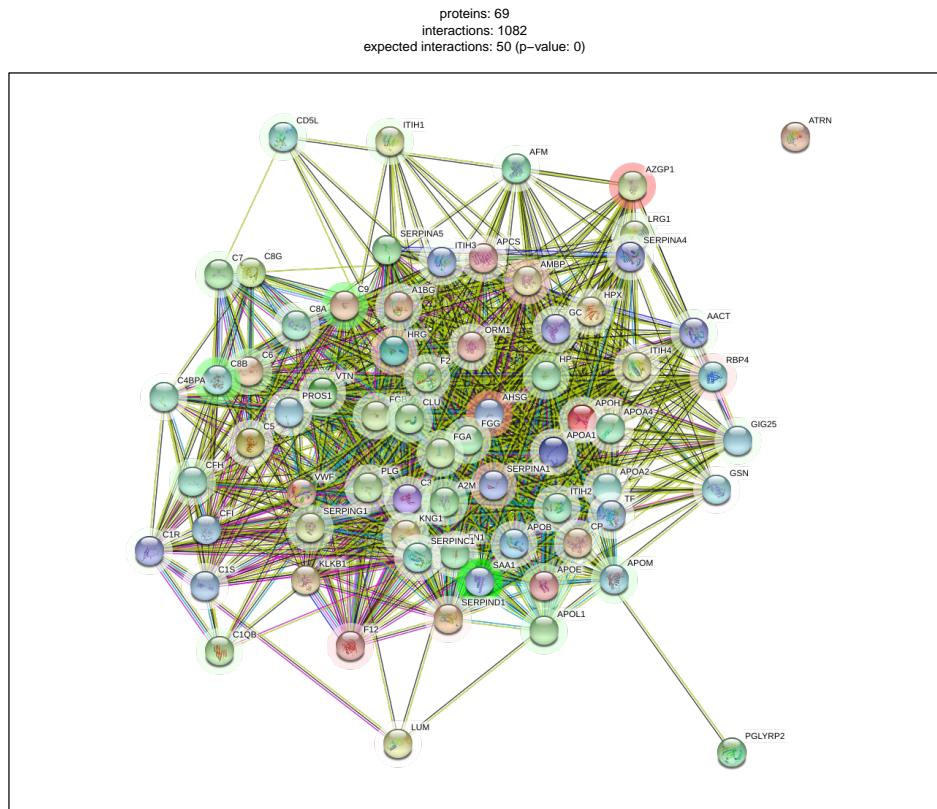


Figure S31. The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates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

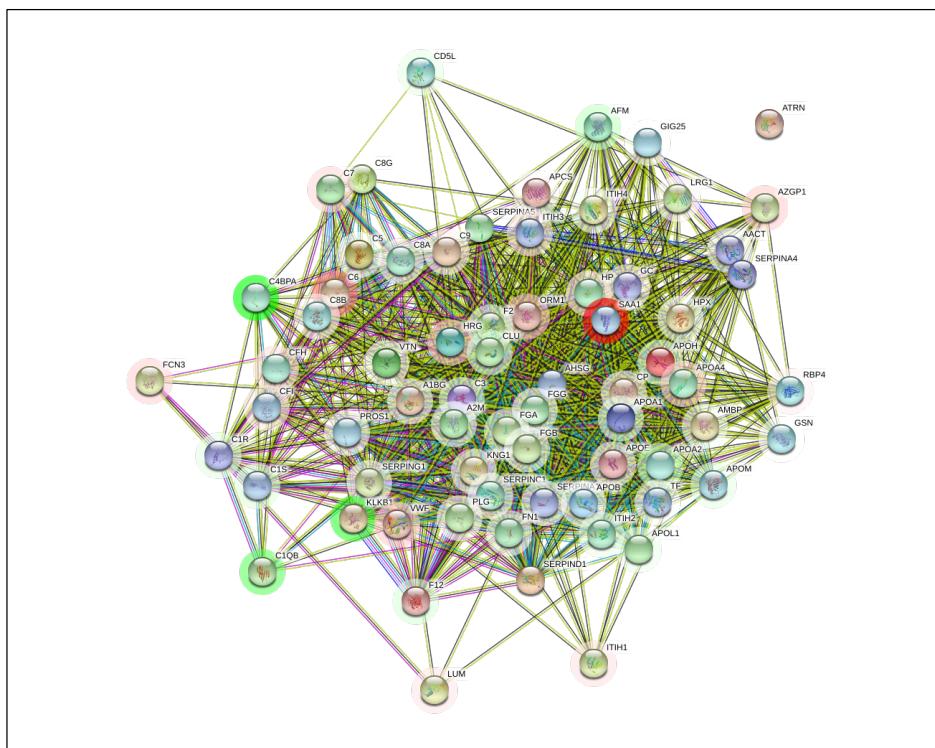


Figure S32. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there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

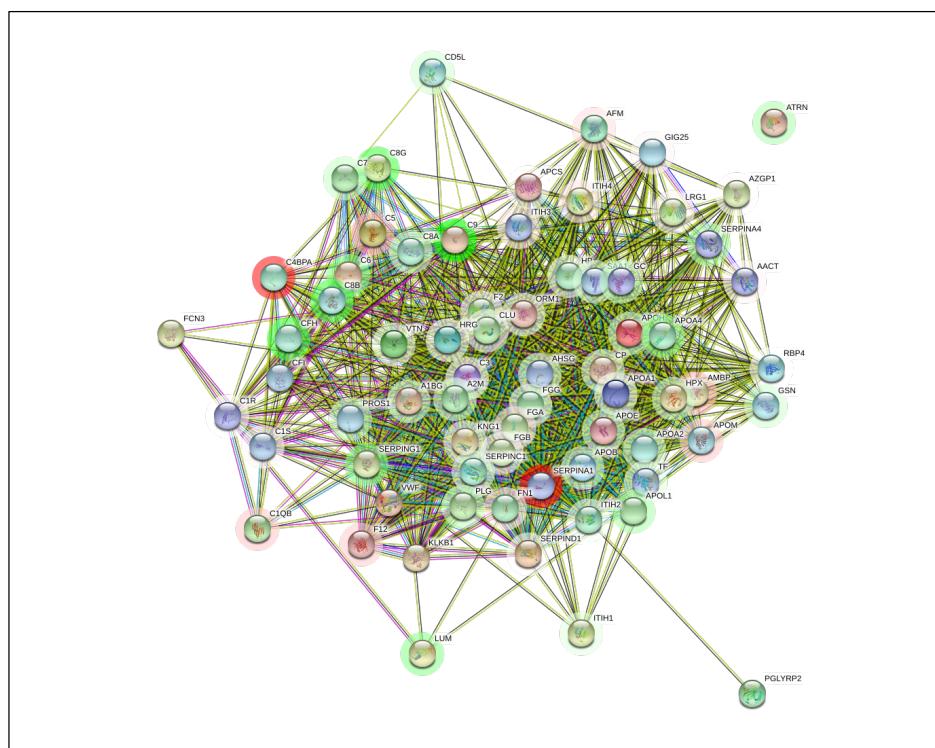
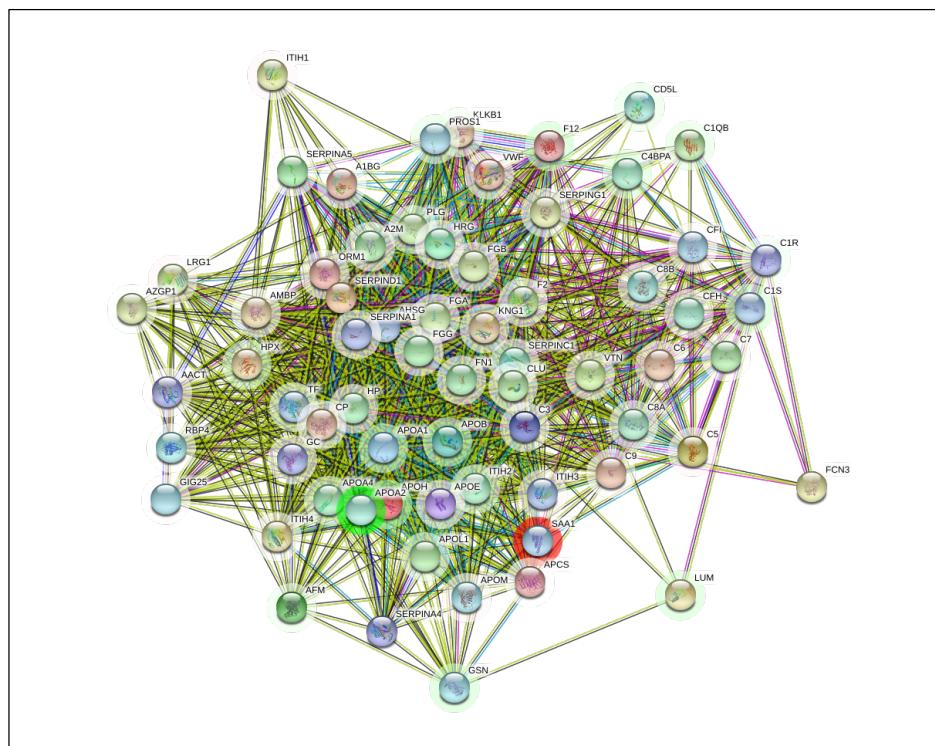


Figure S33. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance.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



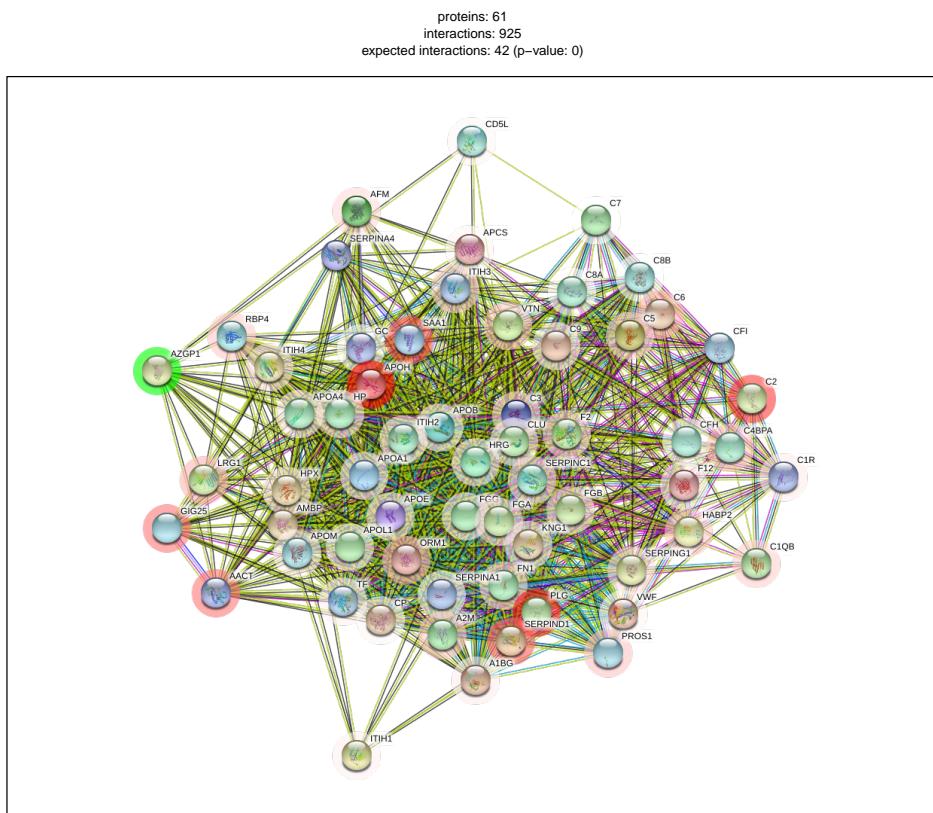


Figure S35. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

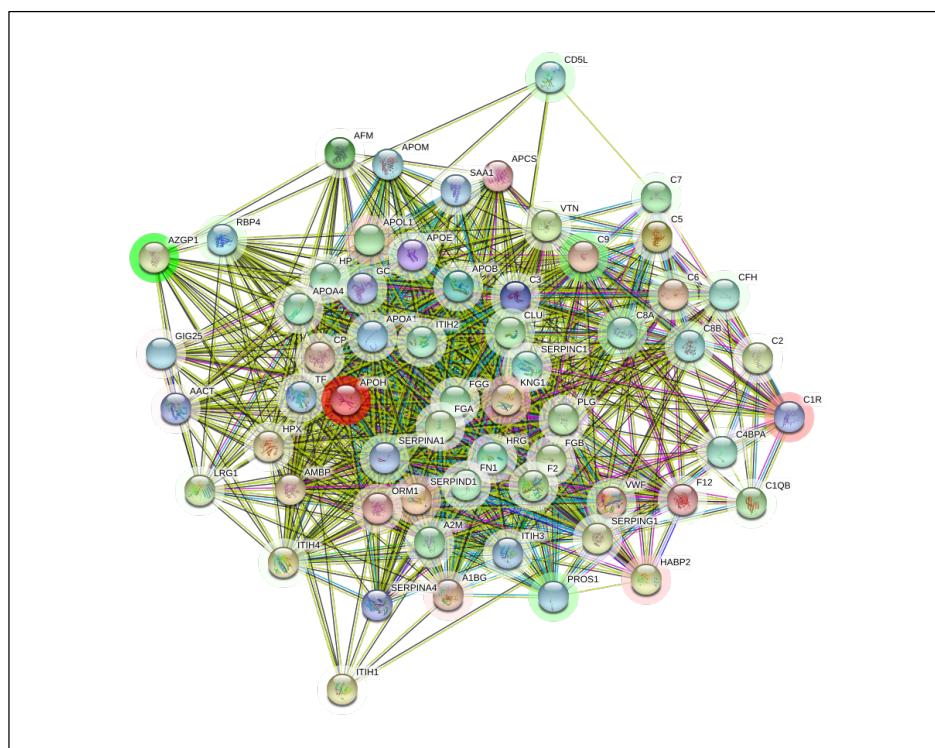


Figure S36. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

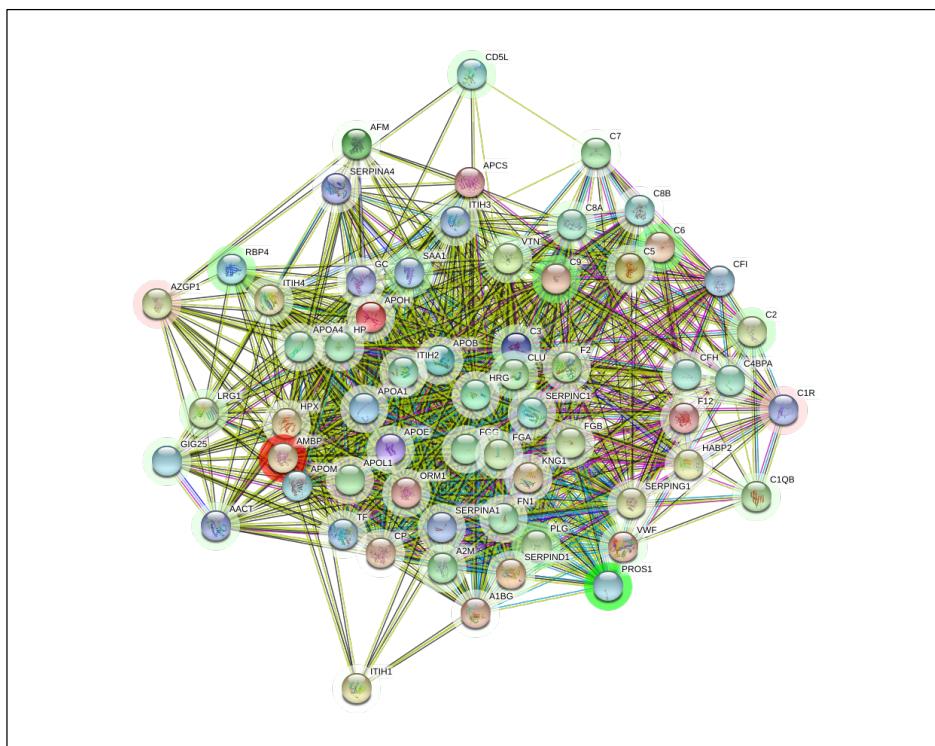


Figure S37. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

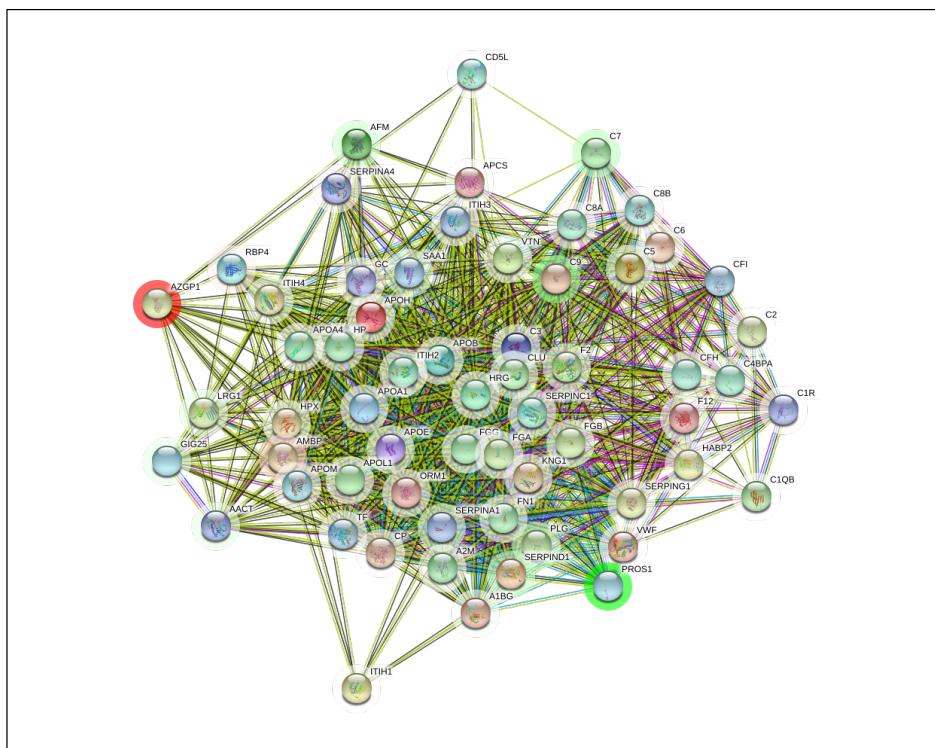


Figure S38. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

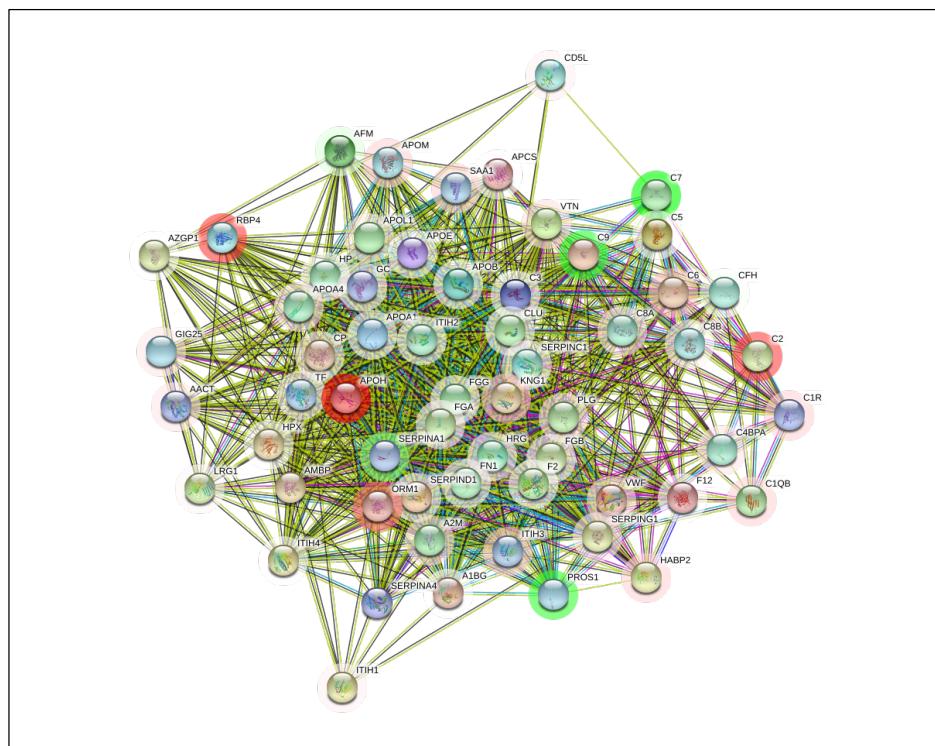


Figure S39. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

1043 5.7.2 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; gene co-expression; text-mining and protein homology.

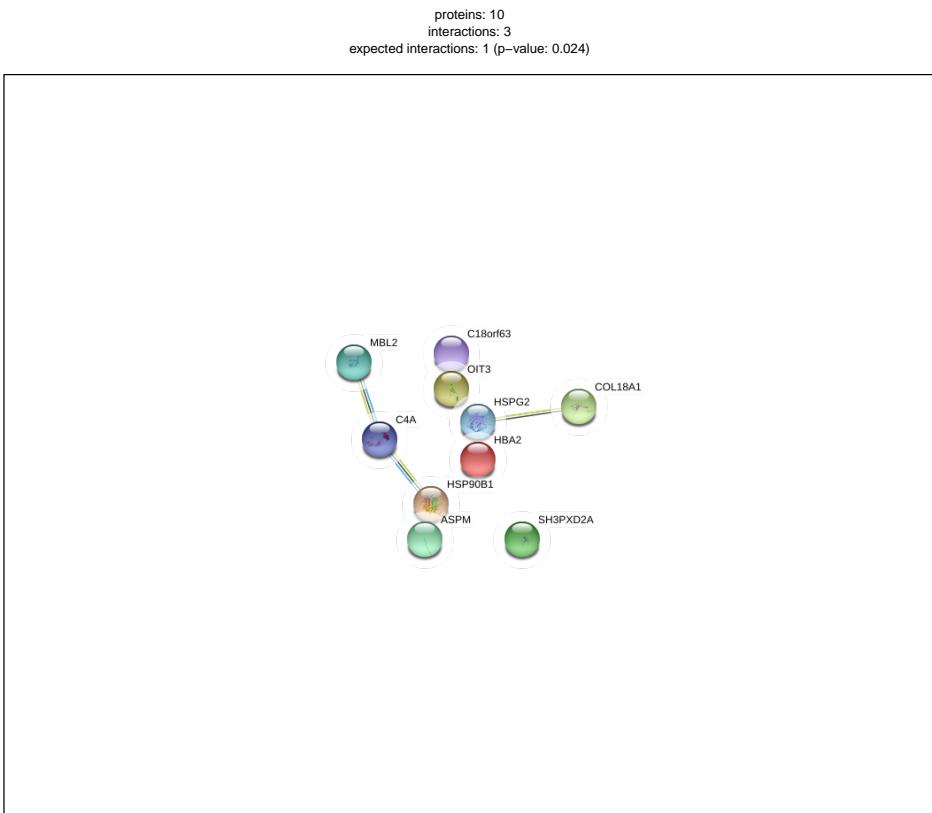


Figure S41. Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there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

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

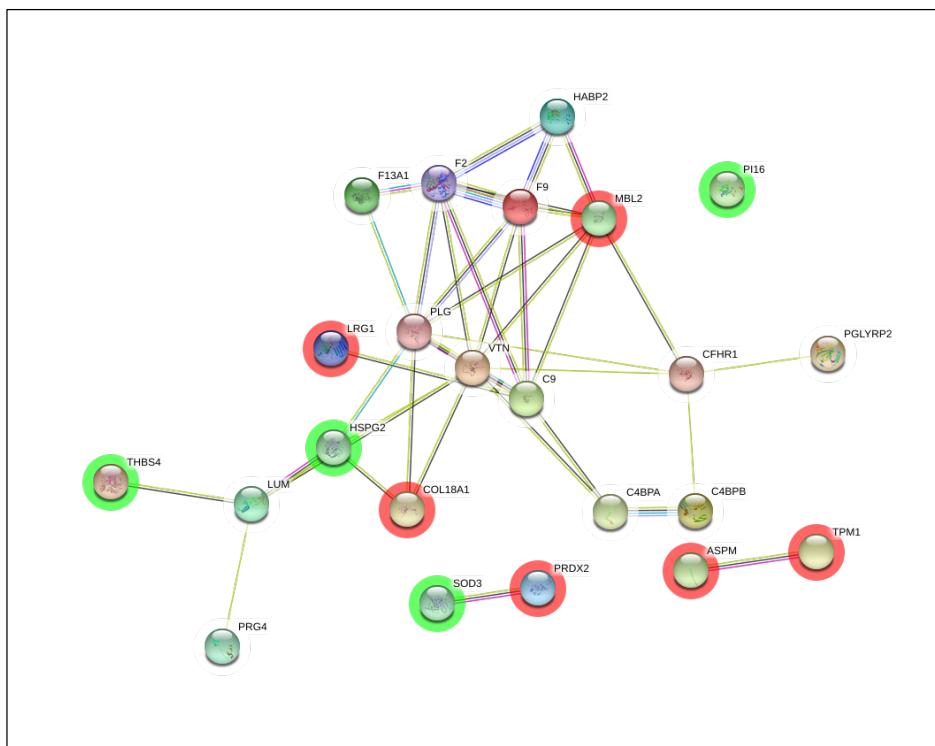


Figure S42. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance.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

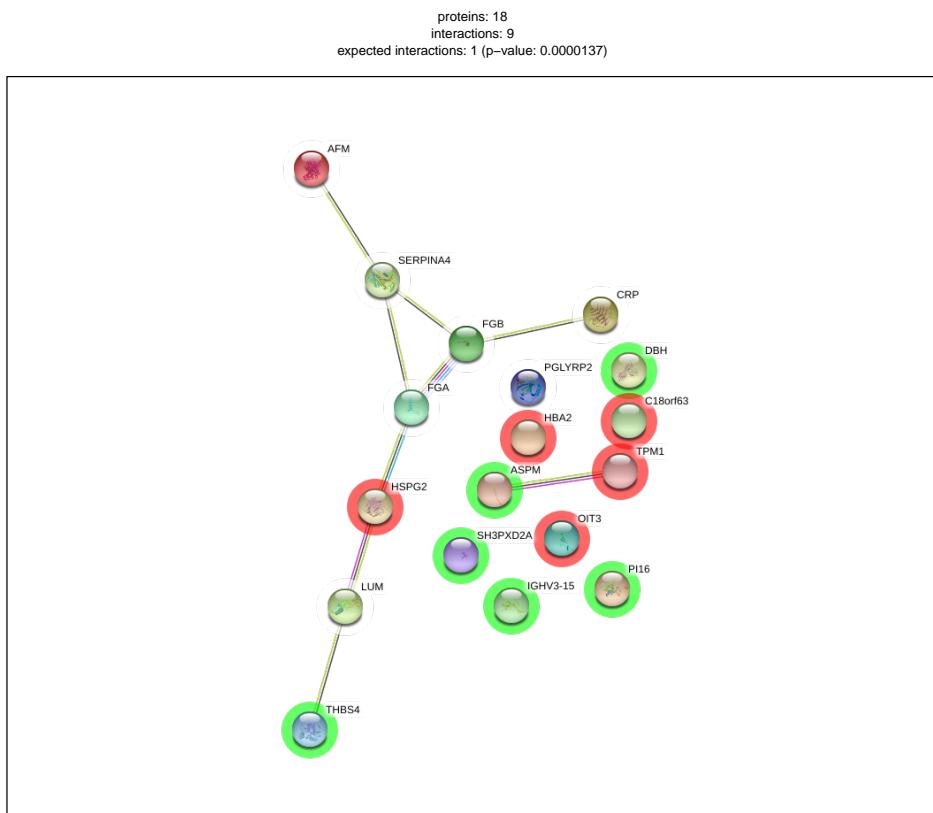


Figure S43. Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater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

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

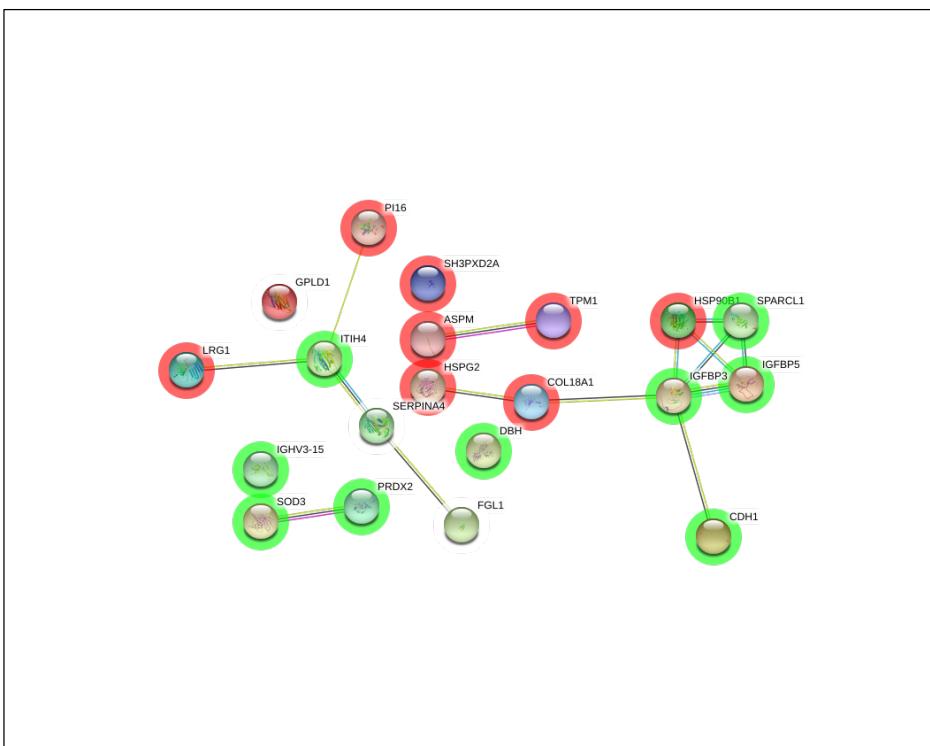


Figure S44. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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



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

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

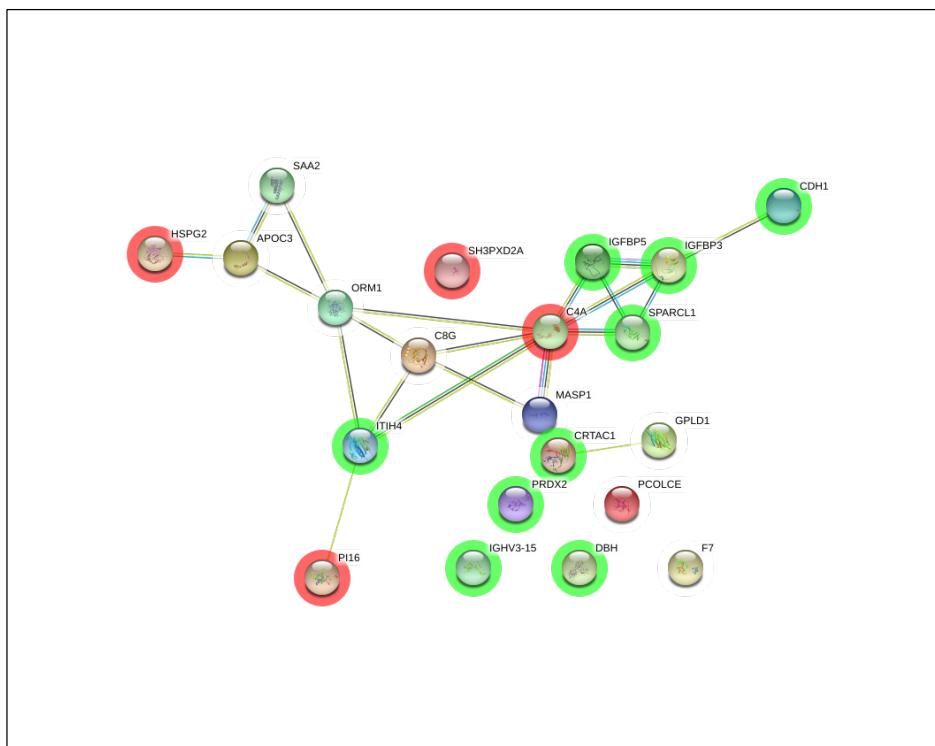


Figure S46. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations;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

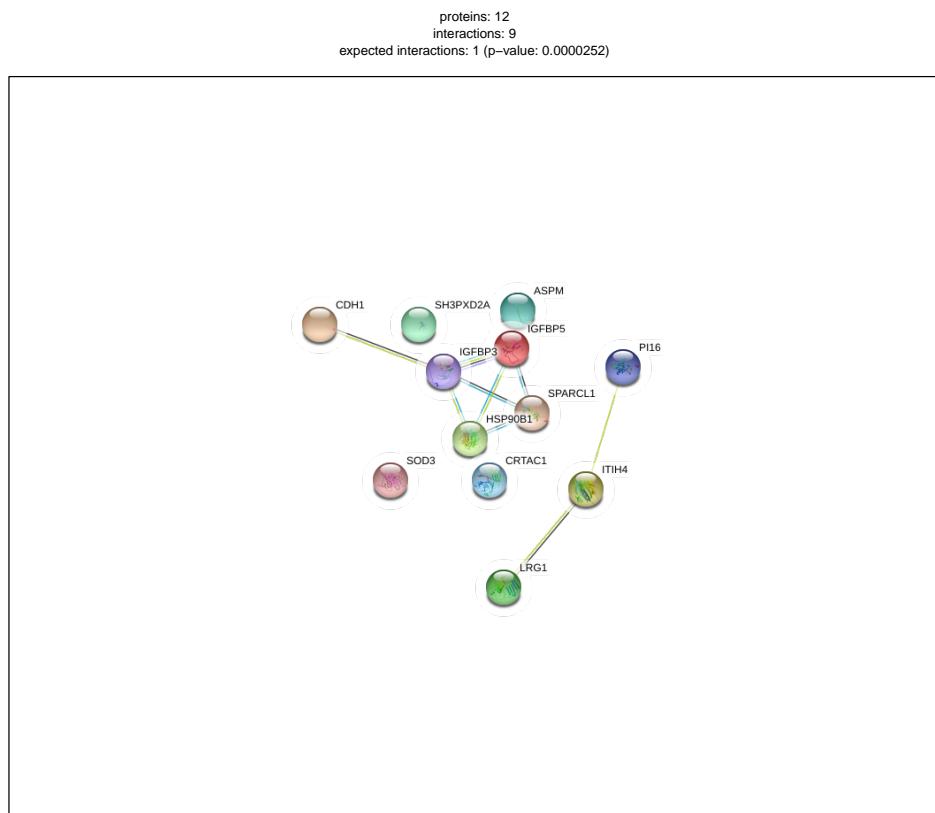


Figure S47. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

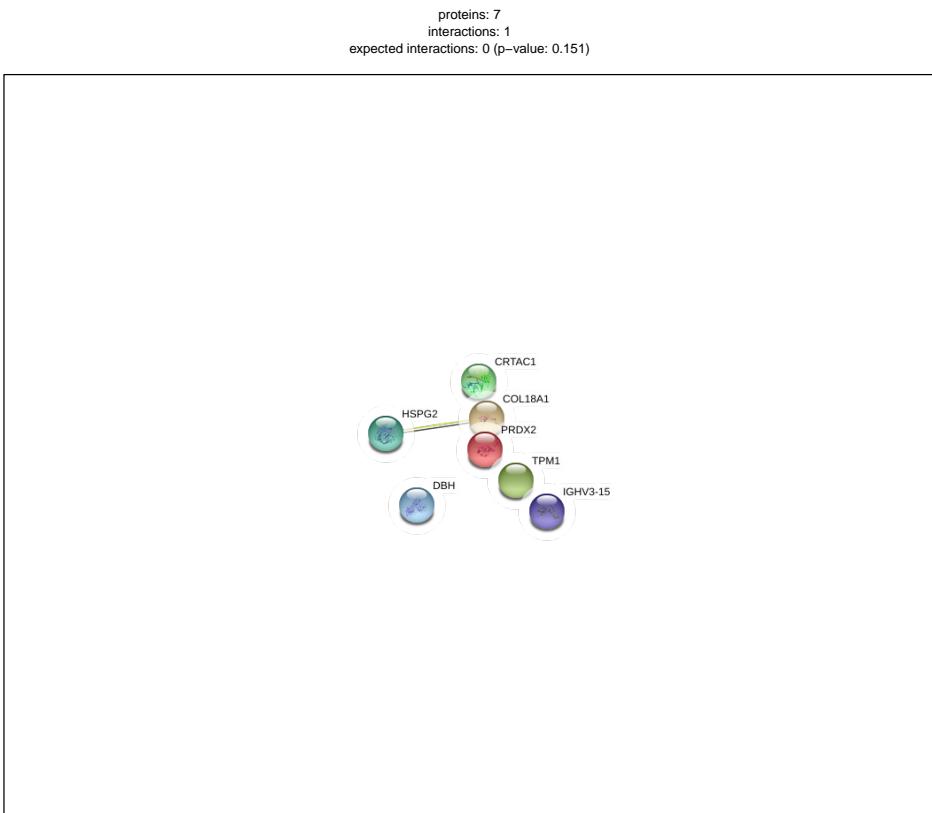


Figure S48. Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured “halo” denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein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

Acute C Improvers Vs Acute C Non-Improvers

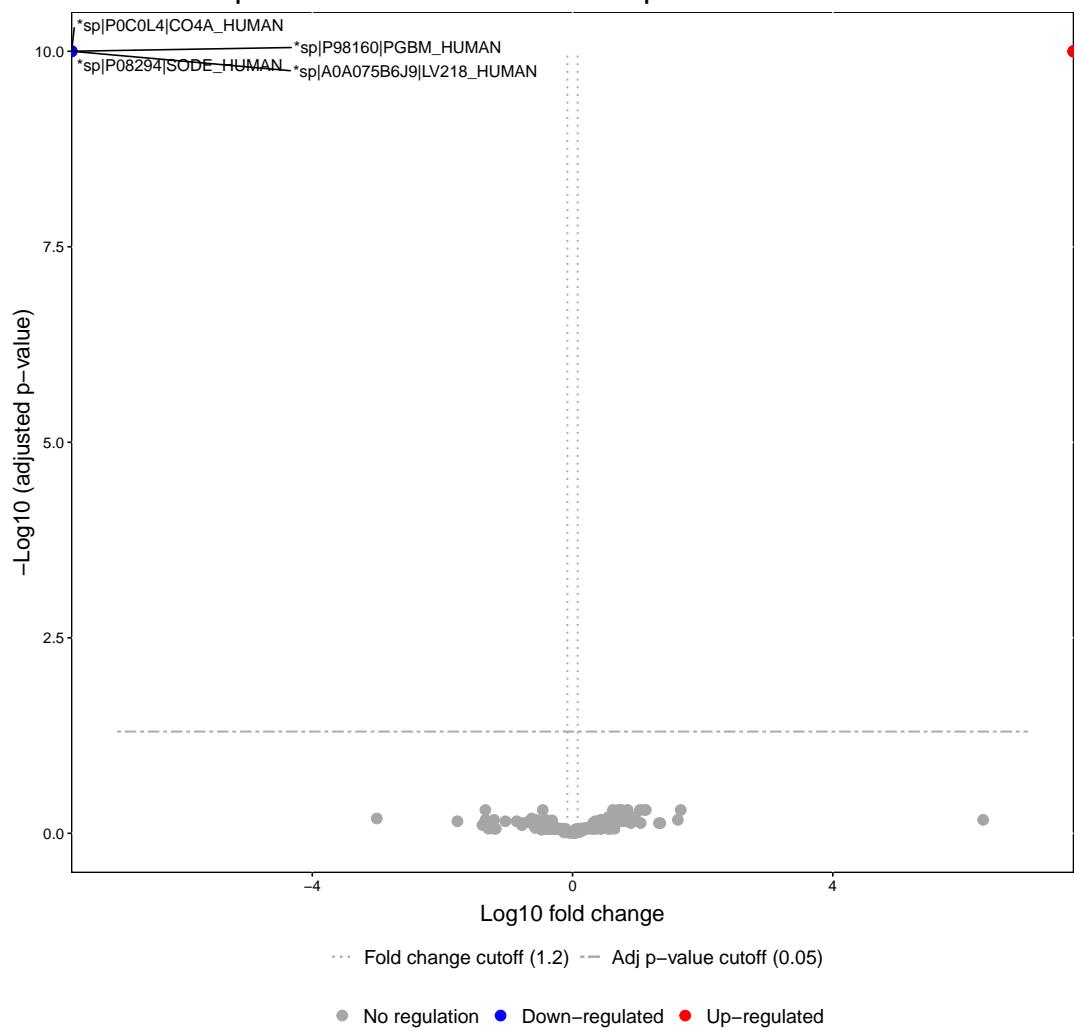


Figure S49. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Subacute C Improvers Vs Subacute C Non-Improvers

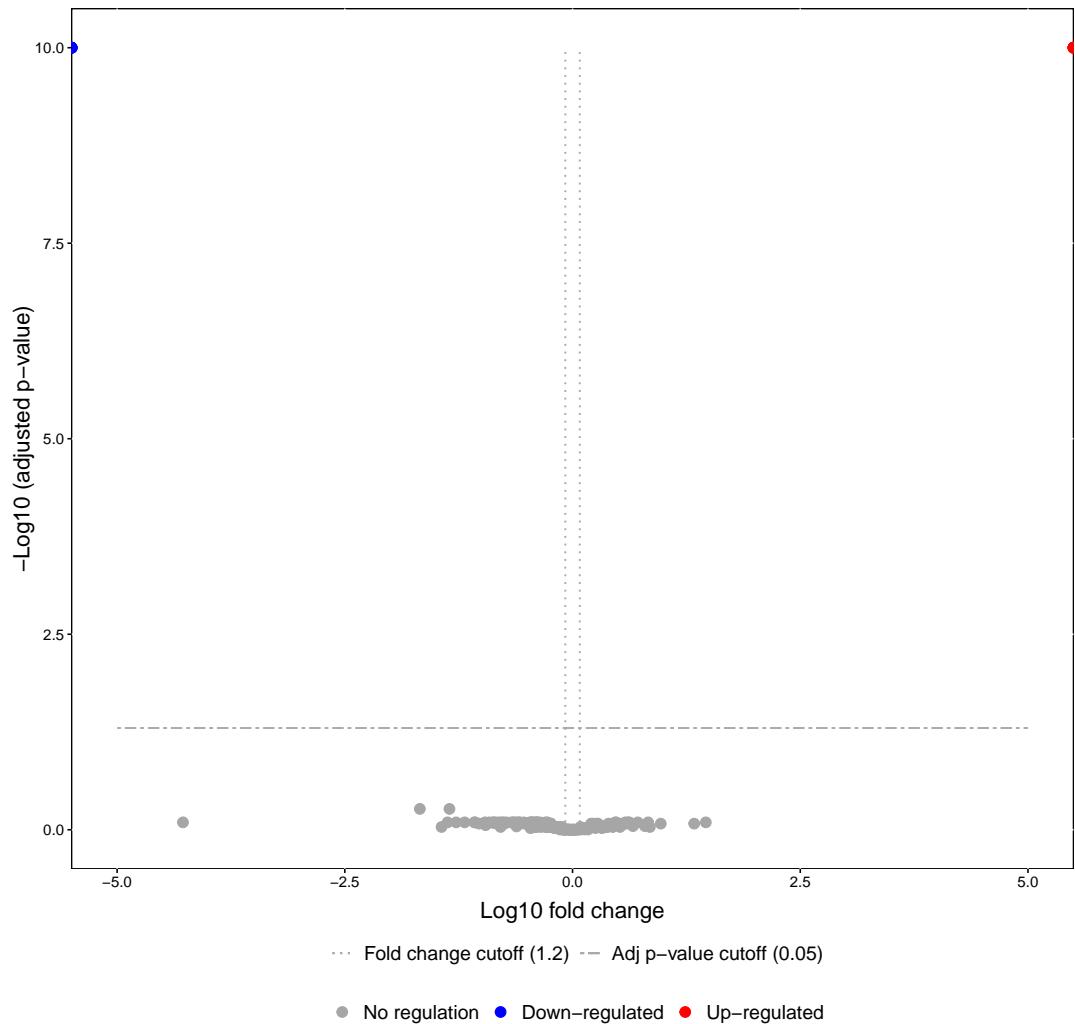


Figure S50. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 3-months post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Subacute C Improvers

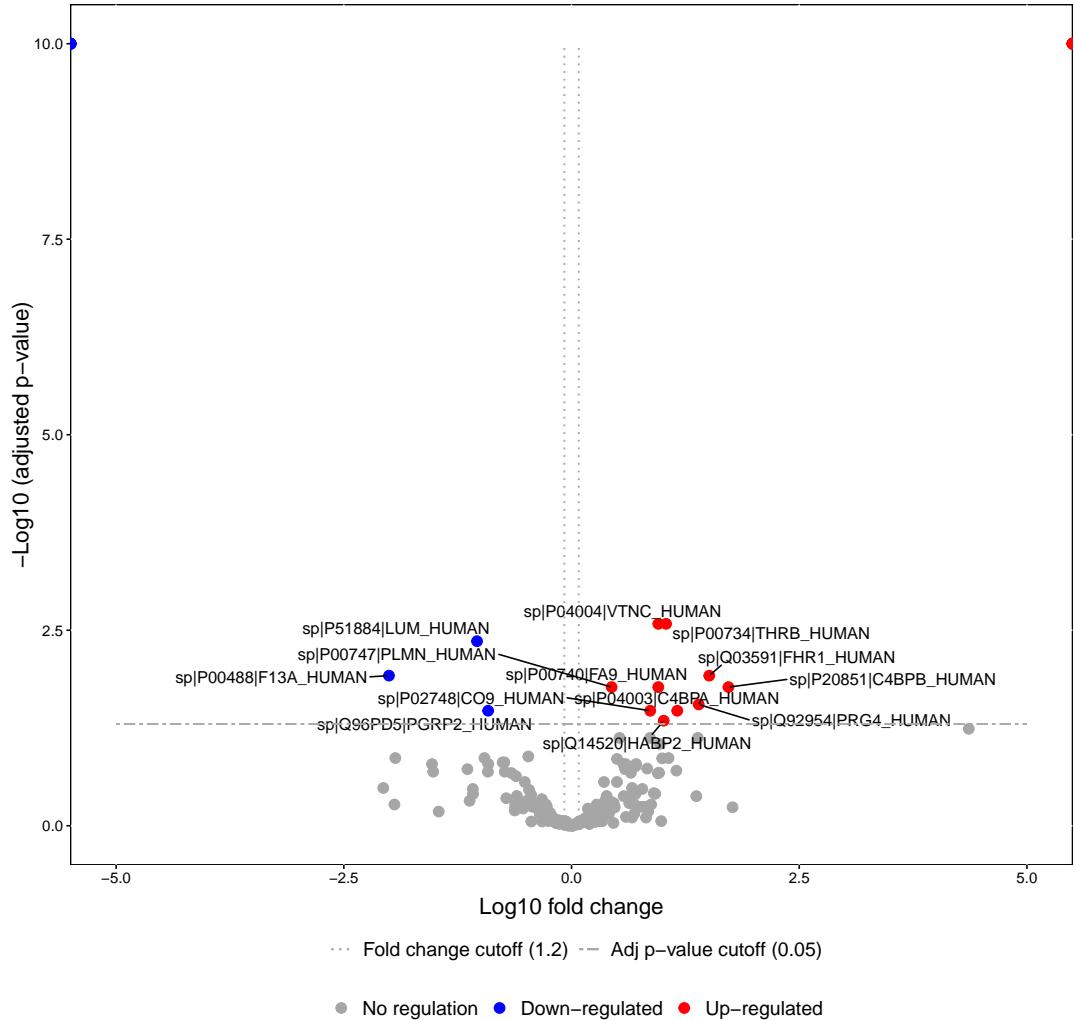


Figure S51. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Non-Improvers Vs Subacute C Non-Improvers

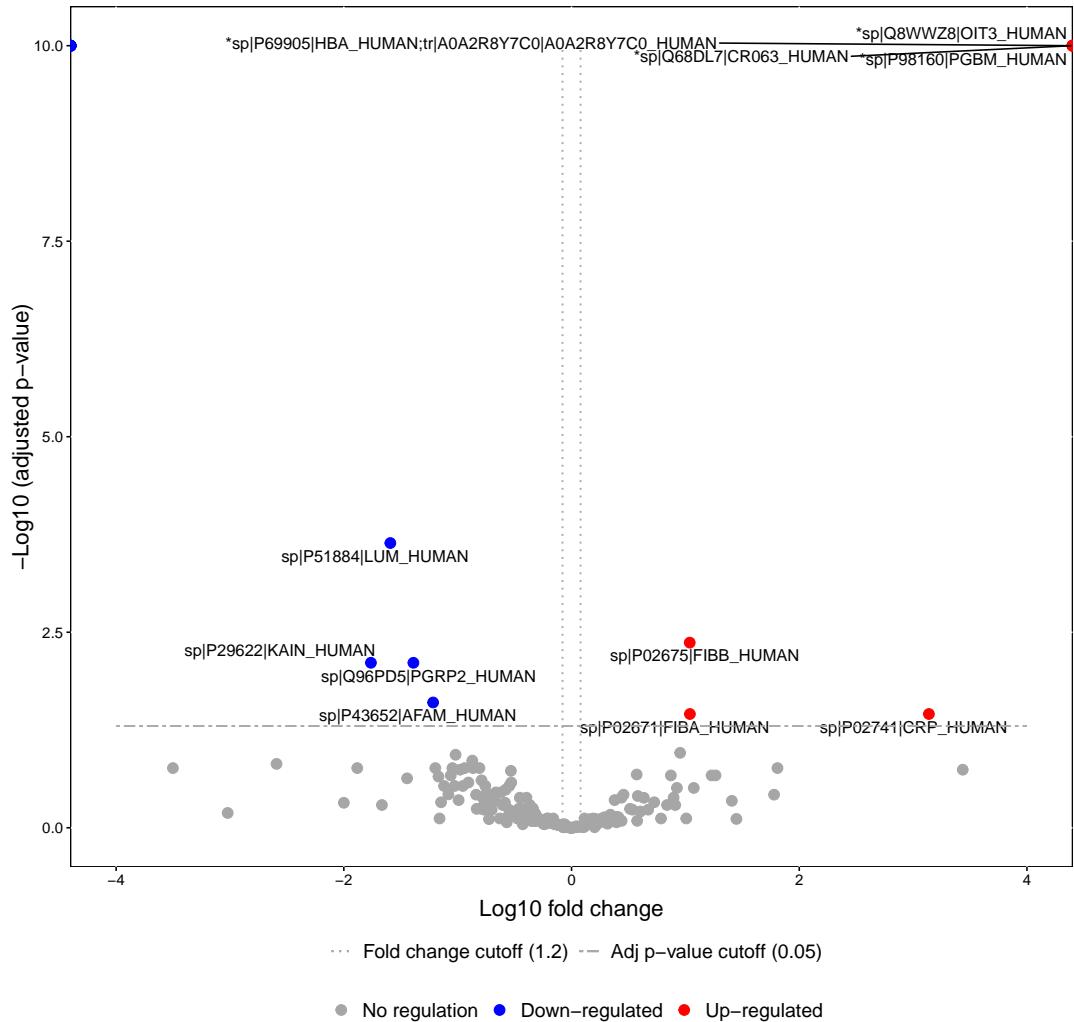


Figure S52. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute D

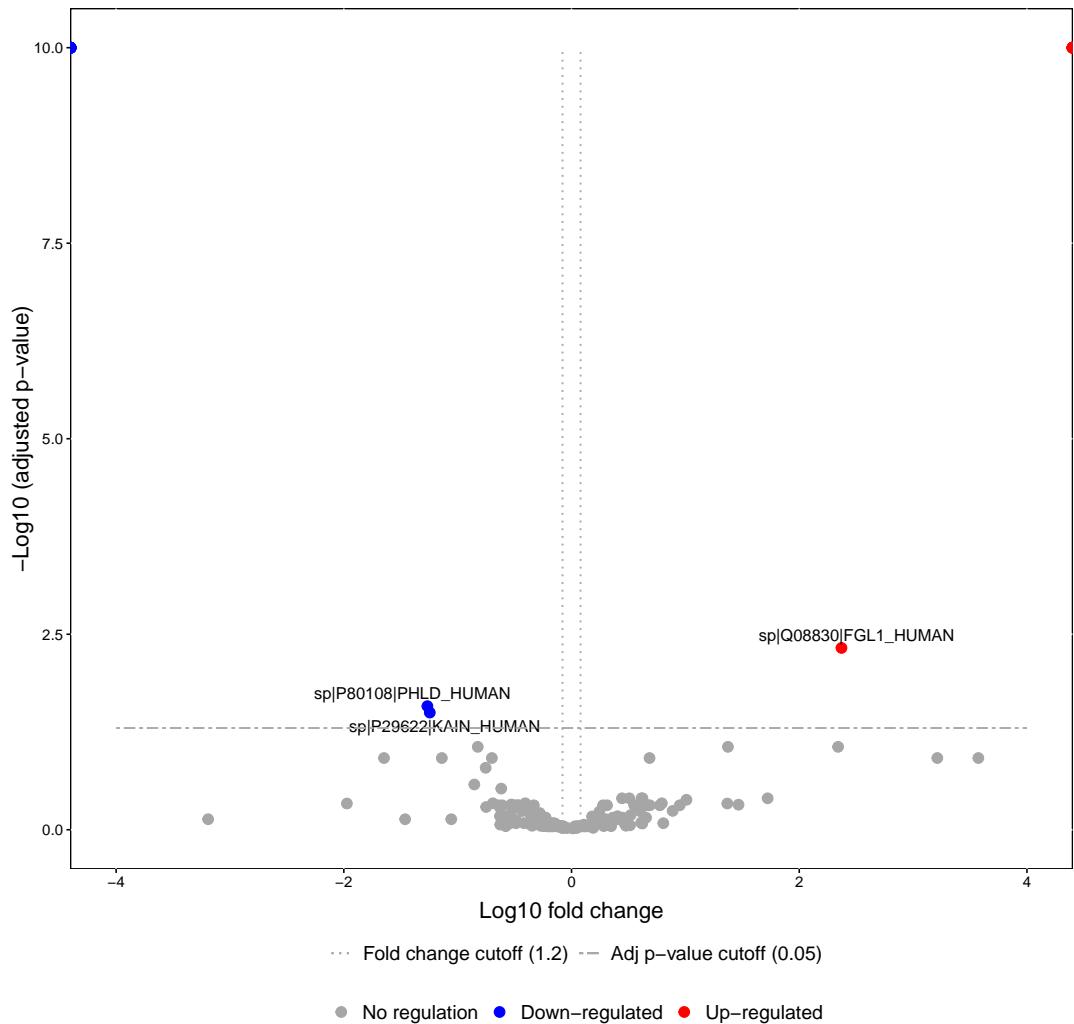


Figure S53. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Subacute A

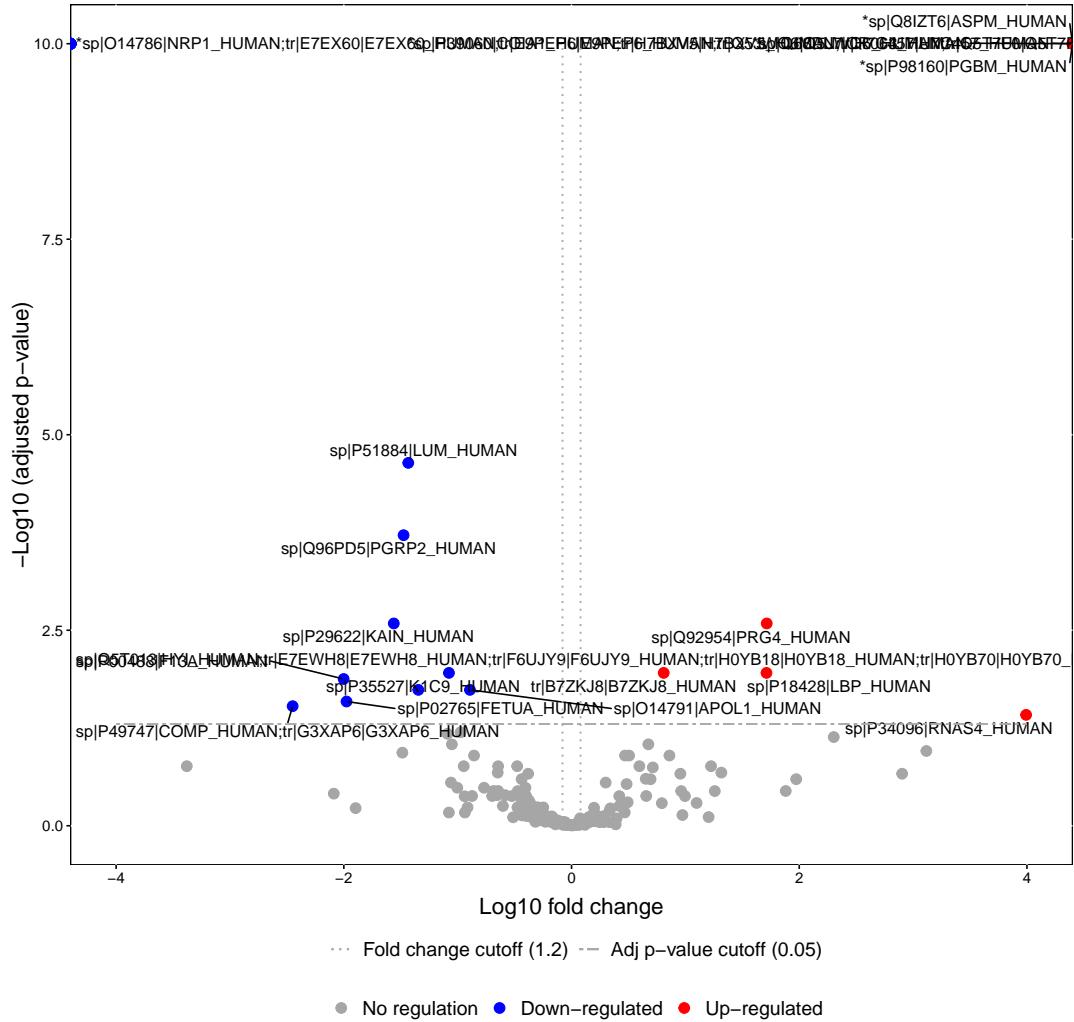


Figure S54. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS A patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute D Vs Subacute D

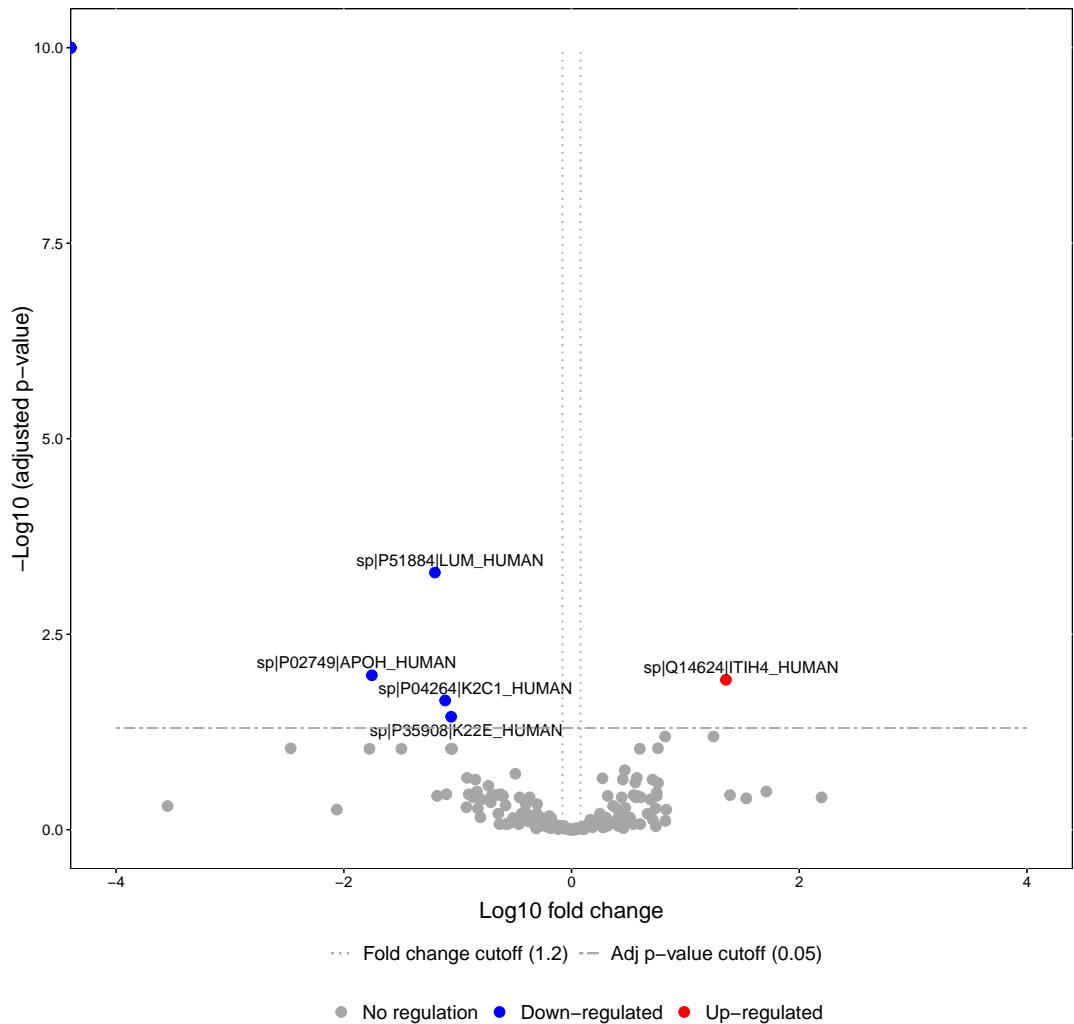


Figure S55. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS D patients. Proteins with a fold changes beyond >1.2 and an adjusted p-value less than 0.05 are labelled.

Acute C Improvers Vs Acute D

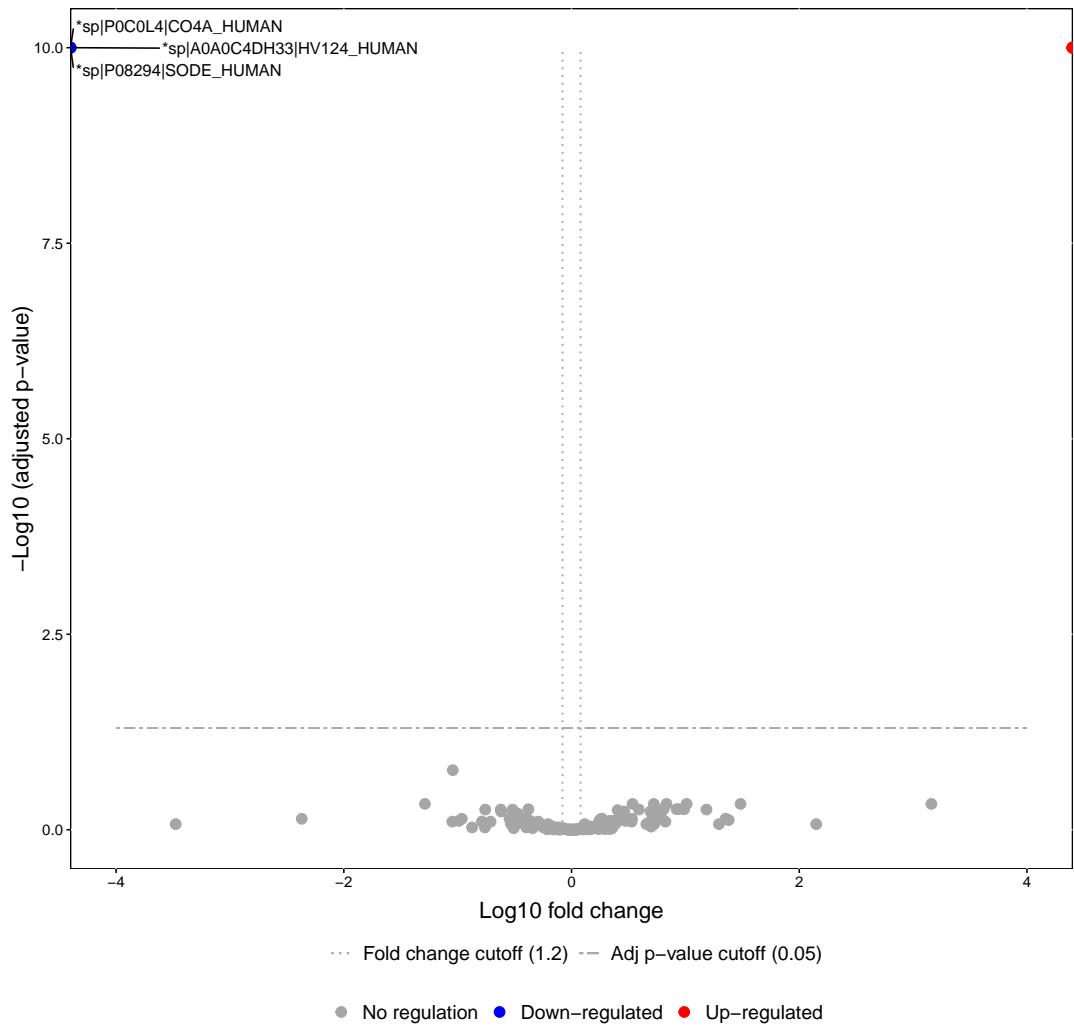


Figure S56. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute C Improvers

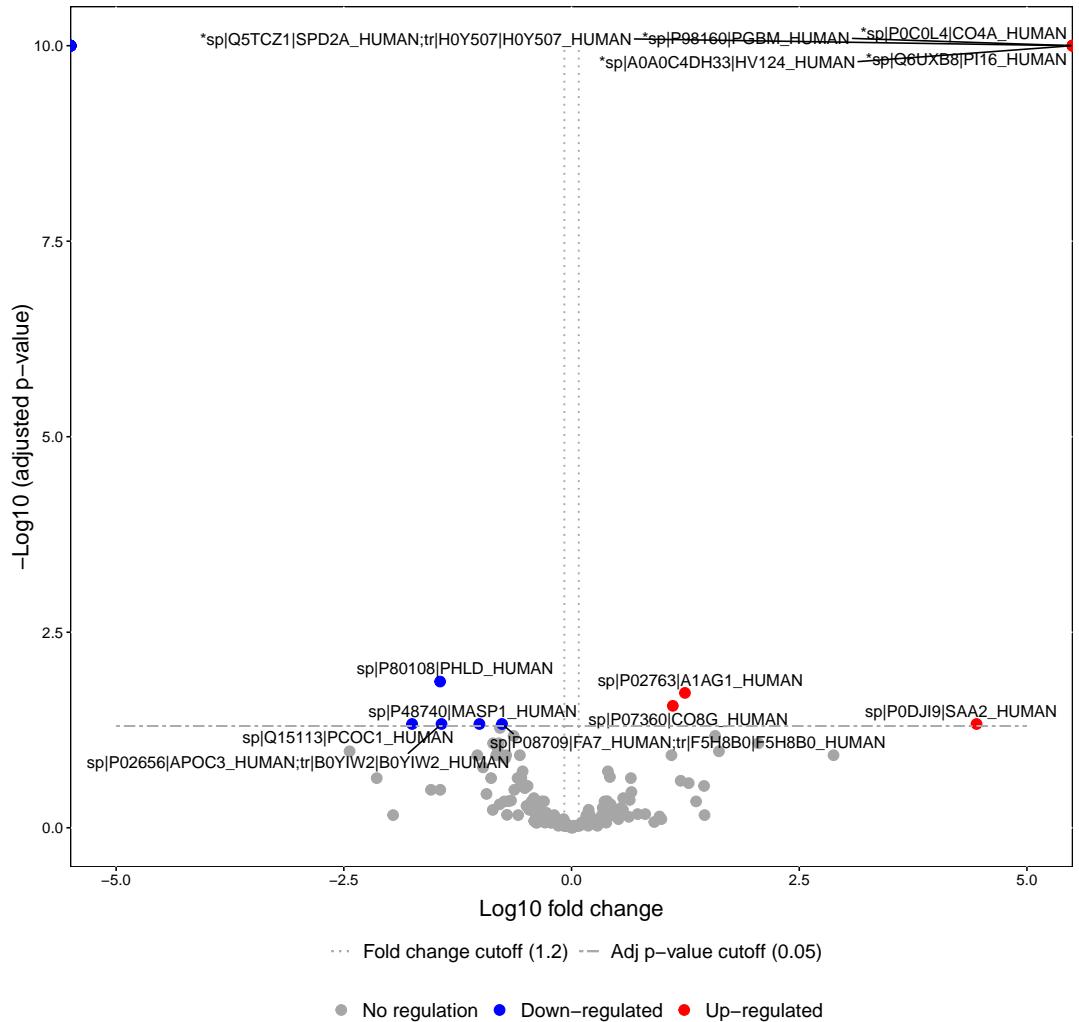


Figure S57. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond 1.2 and an adjusted p-value less than 0.05 are labelled.

Acute A Vs Acute C Non-Improvers

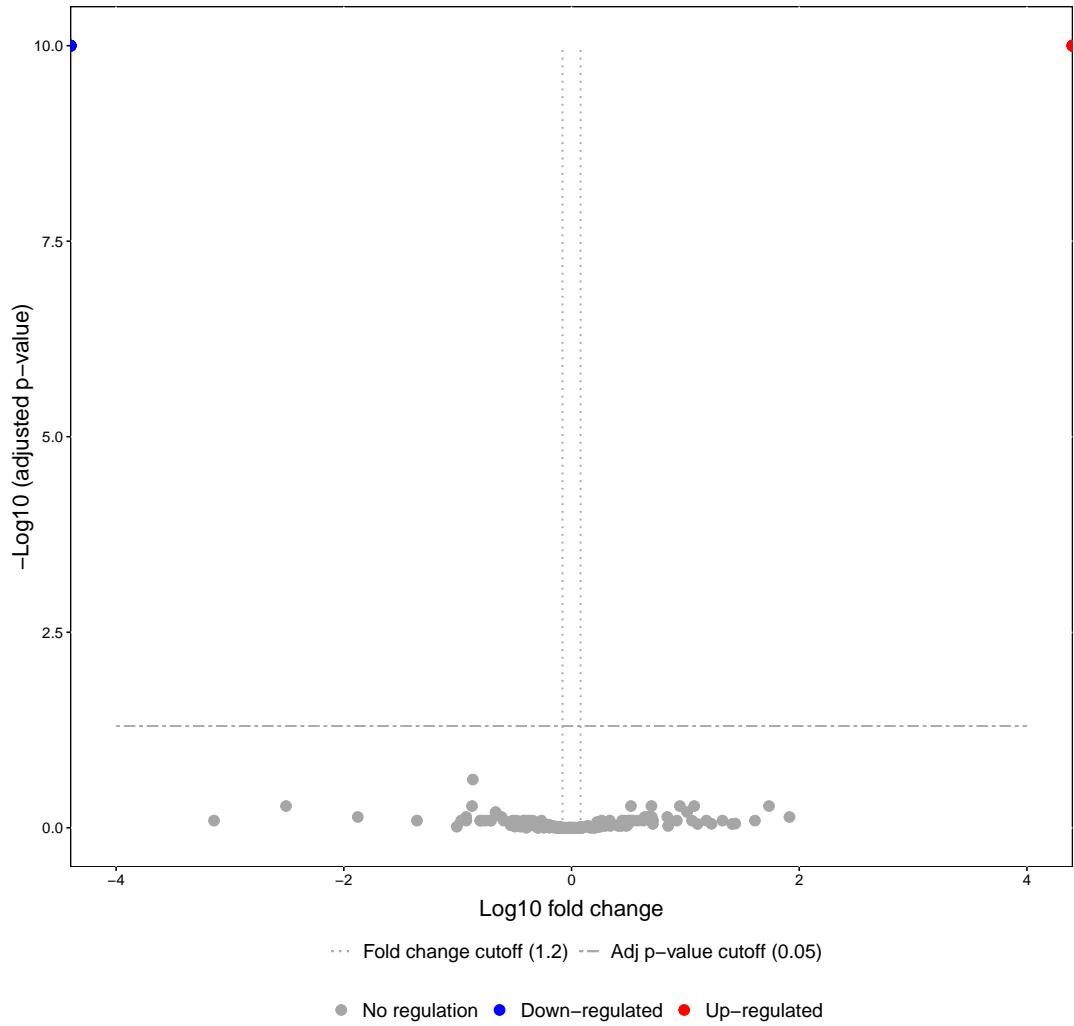


Figure S58. Volcano plot of \log_{10} fold change and \log_{10} adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

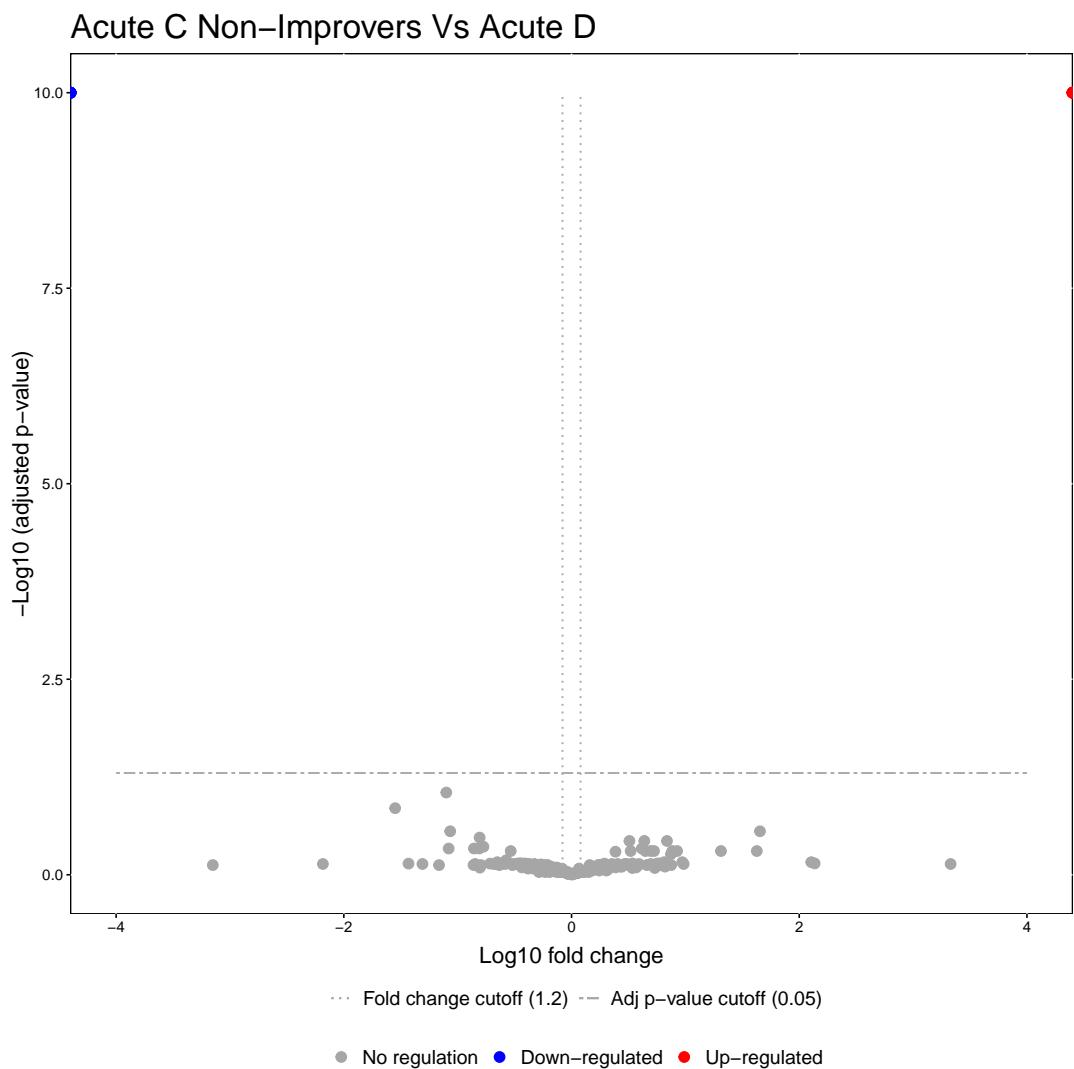


Figure S59. Volcano plot of log₁₀ fold change and log₁₀ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who did not experience an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond ,1.2 and an adjusted p-value less than 0.05 are labelled.

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