A multimodal approach to biomarker discovery for spinal cord injury

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And finally to you, dear reader, whether here by choice or obligation, I hope you find something of interest, and I thank you for your time.

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List of Abbreviations

4-HNE 4-hydroxynonenal

A2M Alpha-2-Macroglobulin

AANS/CNS American Association of Neurological Surgeons and Congress of Neurological Surgeons

AIS ASIA Impairment Scale

ALS Amyotrophic lateral sclerosis

AP Alkaline phosphatase

ApoA1 Apolipoprotein A1

APP Acute Phase Proteins

APR Acute Phase Response

ASIA American Spinal Injury Association

ASK Apoptosis signal-regulating kinase

AUC Area under the curve

BASIC Brain and Spinal Injury Centre

BBB Blood brain barrier

BMI Body mass index

BSCB Blood-spinal cord barrier

CCS Central cord syndrome

CES Cauda equina syndrome

CNS Central nervous system

CRP C-reactive protein

CSF Cerebral spinal fluid

CT Computed topography

DAMP Damage associated molecular patterns

DCS Dorsal cord syndrome

DTI diffusion tensor imaging

DVT Deep venous thrombosis

ECM Extracellular matrix

EPV Events per variable

FC Fold change

FcγR Fcγ receptors

FDR False Discovery Rate

GCAP Germinal alkaline phosphatase

GFAP Glial fibrillary acidic protein

GGT Gamma-glutamyltransferase

HCD Higher energy collisional dissociation

HDL High-Density Lipoprotein

HO Heterotopic ossification

IAP Intestinal alkaline phosphatase

IAP Tissue nonspecific alkaline phosphatase

IBM International Business Machines Corporation

IgG Immunoglobulin G

IL Interleukin

IMLL intramedullary lesion length

ISNCSCI International Standards for Neurological Calssification of Spincal Cord Injury

ITP Idiopathic thrombocytopenic purpura

iTRAQ Isobaric tag for relative and absolute quantification

IVIG Intravenous immunoglobulin

KMO Kaiser-Meyer-Olkin

LC-MS/MS Liquid chromatography/tandem mass spectrometry

LDH lactate dehydrogenase

LPS Lipopolysaccharide

MAG Myelin-associated glycoprotein

MCC maximum canal compromise

MCP monocyte chemotactic protein

MCSI Midlands Centre for Spinal Injuries

ML Machine learning

MPM multi-parameter mapping

MPSS Methylprednisolone sodium succinate

MRI Magnetic resonance imaging

MRI Magnetic resonance imaging

MS Mass spectrometry

MSCC maximum spinal cord compression

MTsat magnetization transfer saturation

NF-H Neurofilament heavy chain

NFH neurofilament heavy chain

NFL neurofilament light chain

NFM neurofilament medium chain

NOGO Neurite outgrowth inhibitor

NPC Neural precursor cells

NSE Neuron specific enolase

PAMP Pathogen associated molecular patterns

PCA Principle component analysis

PLAP Placental alkaline phosphatase

PNS Peripheral nervous system

PRX-2 Peroxiredoxin 2

RBC Red blood cell

RBP4 Retinol binding protein 4

RF Random forests

RLC relative lymphocyte counts

RMSE Root mean square error

ROC Receiver operating characteristic

ROS Reactive Oxygen Species

S-100β S100 calcium-binding protein β

SA Serum Amyloid

SAA1 Serum amyloid A1

SC Schwann cell

SCI Spinal cord injury

SCIM-III Spinal Cord Independence Measure III

SCS Spinal cord stimulation

SCX Strong cation exchange

SILAC Stable isotope labelling in culture

SVM Support vector machines

TBI Traumatic brain injury

TGF-β Transforming growth factor β

TLR Toll-like-receptor

TMT Tandem Mass Tag

TNF Tumour necrosis factor

VCS Ventral cord syndrome

WBC White blood count

XIE Extracted ion chromatography

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Abstract

The extent of the devastating outcomes following spinal cord injury, particularly with respect to neurological function, are difficult to predict. This can make it difficult to plan clinical care and manage patient expectations, both of which can have profound impacts on rehabilitation and mental health. The heterogeneity in neurological recovery also make powering clinical trials challenging, stifling the development of novel therapies. Prognostic biomarkers that can aid in accurately predicting outcomes would therefore be of value in both clinical and research settings. They could also serve to uncover novel understanding as to the pathophysiological factors in heterogeneous outcomes, potentially revealing novel therapeutic targets. Whilst cerebral spinal fluid is an obvious target for identifying such biomarkers, the risk and cost associated with collecting such samples possess a challenge. Blood by contrast is easily accessible, with little risk and cost to healthcare providers, making it an ideal source for testing.

This project aims to identify novel blood biomarkers for spinal cord injury via unbiased methods. Specifically, prognostic models of discharge and 12-month post-injury neurology were developed utilising admission neurology scores, basic demographic information and routinely measured haematological markers. Here, blood markers commonly associated with liver function, including alanine transaminase and alkaline phosphatase, were found to add modest but statistically significant predictive value. This would suggest that liver health, or perhaps metabolic health more broadly, is at least partially related to variance of neurological outcomes. Further, both labelled and unlabelled shotgun proteomics of plasma from human patients revealed proteins

associated with the complement cascade to be of particular importance for both severity of injury, and differential functional recovery. Many of the proteins identified were also directly linked to the liver, once again suggesting the organ may have more significance to outcomes of spinal cord injury than previously appreciated.

Chapter 1

Introduction

Spinal cord injury (SCI) is damage to the spinal cord that causes temporary or permanent changes in its function. The global age-standardised incidence of SCI has been estimated to be 13 per 100,000, whereas the age-standardised prevalence was estimated to be 368 per 100,000.[1] With respect to the United Kingdom, it has been estimated that over 1000 new SCIs occur each year, and that 40,000 people are living with SCI.[2] The majority of SCIs have historically been traumatic in nature, most commonly as a result of vehicular accidents, falls, violence and sports, but more recently non-traumatic SCI, usually as a result of infection or cancer, has been increasing in prevalence.[3,4]

The lifetime cost of SCI in the UK is estimated to be £1.12 million (mean value) per case, with the total cost of SCI in 2016 in the UK being £1.43 billion.[5] With respect to the United States of America, a review examined the cost of SCI in 2016 for veterans and found the cost to be $30,770 to $62,563 USD per year based on 12 studies.[6] SCI can also lead to secondary conditions that increase morbidity and mortality, including deep vein thrombosis, urinary tract infections, muscle spasms, osteoporosis, pressure ulcers, risk of fracture, chronic pain, and respiratory complications. Furthermore, patients with SCI are often rendered dependent on caregivers and show substantially

higher rates of mental illness.[6]

SCI can be divided into two broad categories, complete and incomplete. Complete SCI is indicated by a loss of all functional neurology (sensory and motor) below the level of the injury, as in the case of a complete transection of the cord for example, in which the prospects of recovery are poor. More commonly the damage is compressive in nature and leads to partial loss of neurological function, whereby the injury is classed as incomplete. For incomplete SCI a patient may experience recovery up to several years after the initial injury, though the probability of this and the extent of recovery is highly variable, largely depending upon the severity of the initial injury.[7,8]

For many years, initial neurological assessment of SCI utilised the 5-point Frankel scale that was introduced in 1969.[9] In this system a patient is classed from A-E grade which corresponds to complete, sensory only, motor useless, motor useful and no neurological deficit. There are potential limitations of this scale, with regards to the subjectivity of its grades, but it is also limited in that it does not account for the level of the injury and often would not reflect subtle neurological improvements. These potential shortcomings were address by the American Spinal Injury Association (ASIA) with the introduction of the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) in 1982 which has since been widely adopted globally (described in full in section 3).[10] Neurological scoring systems certainly have prognostic value, but do not always provide reliable predictions of a given patients probability of experiencing recovery, let alone the extent or time-scale of that recovery process. This can leave SCI patients with uncertainty and clinicians less able to prescribe the most appropriate rehabilitation regime(s) at the most appropriate time.

A more robust, tailored and accurate prognosis could better inform individualised patient care and potentially lead to better outcomes. Moreover, more accurate prognoses for incomplete patients with less certain long-term outcome in the acute setting would be invaluable for advancing clinical trials in SCI, particularly those that

target incomplete patients in this setting. In addition, the efficacy of novel treatments will be easier to demonstrate and with smaller clinical cohorts, if long term outcome is more reliably predictable.[11] In recent years there has been a growing appreciation for the value of biomarkers for prognosis in many disease areas, including stroke, oncology, pneumonia, acute kidney injury and heart failure.[12–16] Likewise, there has been a growing appreciation for the value of biomarkers in SCI, which if used in combination with ASIA grade and dry biomarkers such as magnetic resonance imaging (MRI), could lead to robust prognostic models for SCI cohorts.[17] Furthermore, the identification of novel biomarkers could open new avenues of research to better understand the mechanistic nature of SCI and thus aid in the development of new therapies.

1.1 Spinal anatomy

The spine is normally made up of 33 individual vertebrae that surround and protect the spinal cord. It is divided into 5 regions which are the 7 cervical, 12 thoracic, 5 lumbar, 5 sacral (fused) and the 4 (3-5) coccyx (fused) from top to bottom (Figure 1.1). Between the vertebral column and the spinal cord, called the epidural space, is a layer of fat that further helps to protect the cord. The spinal cord itself is organised into grey matter, which contains the neuronal cell bodies, and white matter that contains the myelinated axons. The white matter can be divided into regions of afferent pathways, which convey sensory signals up the cord, and efferent pathways that convey motor impulses down the cord. These regions originate from and project to specific regions of the brain and periphery.[18]

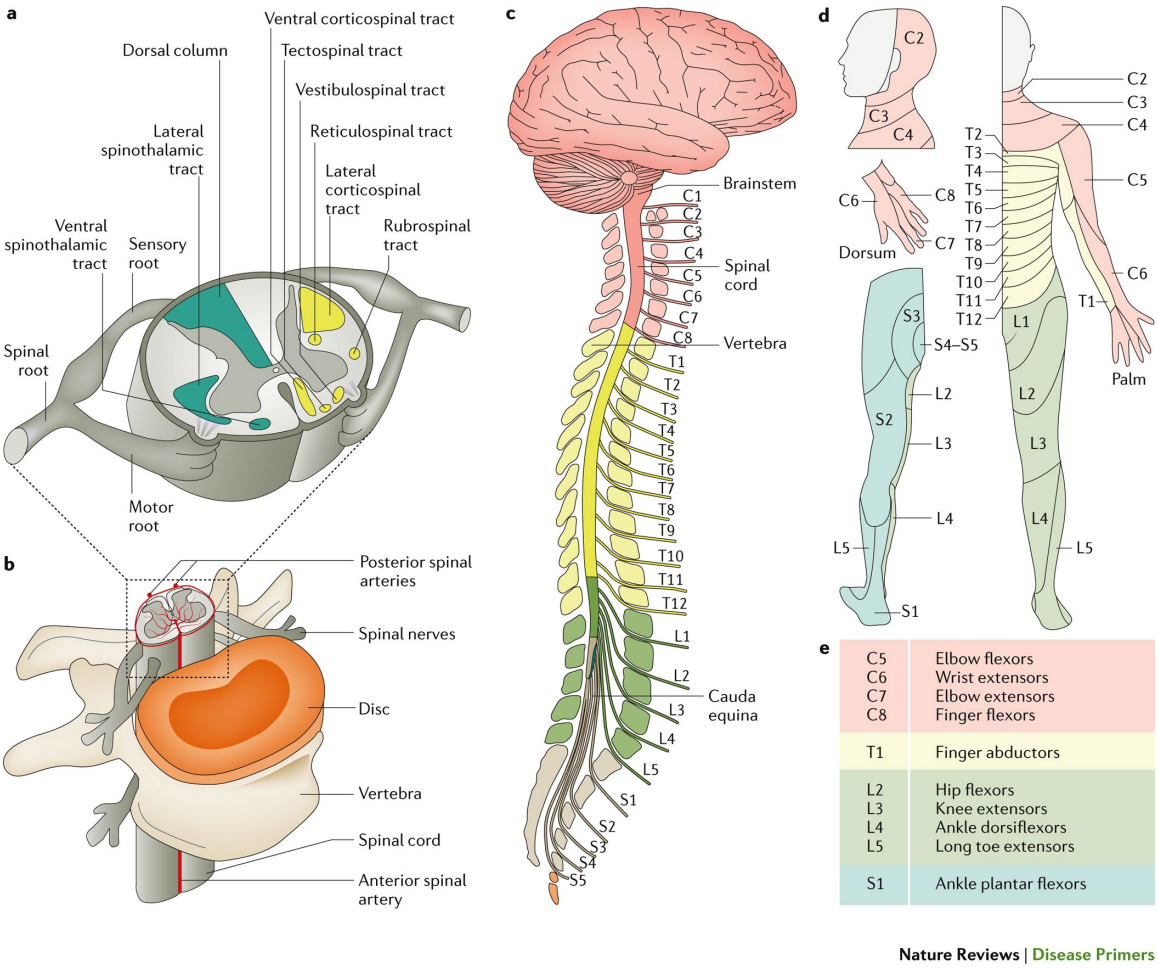


Figure 1.1: Spinal anatomy schematic. Taken from Ahuja et al. (2017).[18]

1.2 Spinal cord injury classifications

1.2.1 The ASIA impairment scale

The ASIA impairment scale consists of a 5-point scale from grade A-E and scores regions of the body based on the extent of sensory and motor function they possess (Figure 1.2). These scores are totalled to calculate the overall ASIA grade. Grade A represents complete loss of sensory and motor function in the lowest sacral segment (S4-S5). Grade B injuries demonstrate preservation of sensory but not motor function below the injury site. Whereas grade C injuries show motor function preserved below

the injury level with over half of the key muscles (those being, elbow flexors and extensors, wrist extensors, finger flexors and abductors, hip flexors, knee extensors, ankle dorsiflexors, long toe extensors and ankle plantar flexors) at a grade < 3 (which represents the ability to move against gravity without additional resistance). Grade D is motor function on key muscles with grade ≤ 3. Finally, grade E is neurologically intact patients who previously had deficiencies due to SCI.

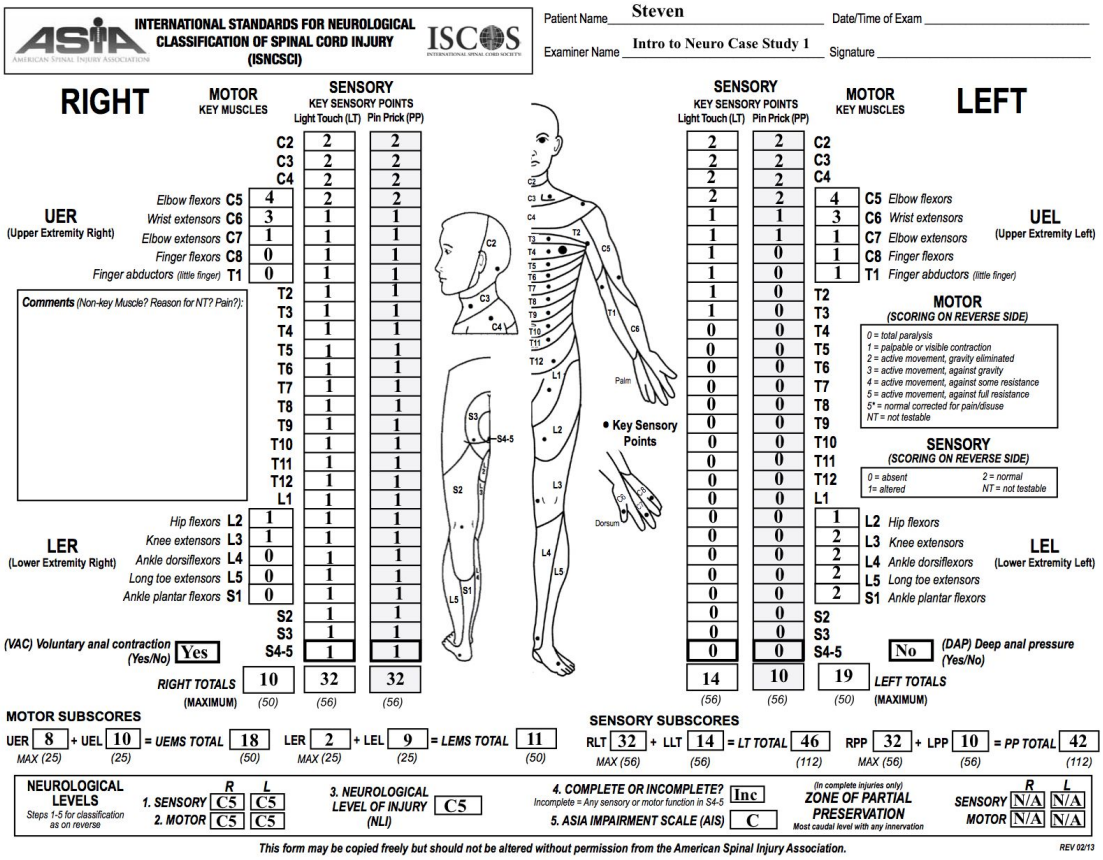


Figure 1.2: Example of modern ASIA score sheet for assessing motor and sensory function. Taken from Kirshblum et al. (2011).[19]

Sensory assessment

The sensory levels are scored on a 0 to 2 scale for each dermatome. The body is divided into two halves with 28 key sensory points to be tested. Each dermatome is tested for pinprick sensations and light touch or labelled NT(not testable) if it cannot

be tested. If the dermatome can be tested the following scores are given:

- 0: Sensation absent
- 1: Sensation present but impaired
- 2: Normal sensation

Individual scores are given for light touch and pinprick with a maximum score of 112 for each in a patient with normal sensation in all dermatomes

Motor assessment

Ten key muscles are tested in each body half, five in the upper limb and five in the lower limb. A 5-point grading scale is used:

- 0: Total paralysis
- 1: Palpable or visible contraction
- 2: Active movement with a full range of motion whilst gravity is eliminated
- 3: Active movement with a full range of motion against gravity
- 4: Active movement with a full range of motion against gravity and some resistance
- 5: Active movement with a full range of motion against gravity and normal resistance
- NT: Not testable. This can be occur if the patient is unable to reliably exert effort or if the muscle cannot be tested due to immobilization, pain on effort or contracture

Identification of neurological level

Following motor and sensory assessments neurological level is determined as motor and sensory level may differ. The neurological level is defined as the lowest segment where the motor and sensory function is normal on both sides and is most cephalad (closest to the head).

The timing with which the ASIA assessment is performed must also be considered carefully by clinicians, as studies have shown that assessments earlier than 72 hours after injury may be considered to be unreliable, particularly due to spinal shock.[20,21] Another important consideration is the specific assessor, as the ASIA assessment is requires appropriate training, and ideally the relevant certification, assessments carried out in general trauma centres may differ considerably relative to centres with access to spinal injury units and/or specialists. However, if recorded accurately and with these considerations in mind, neurological scores determining the severity of the initial SCI are currently the most useful prognostic tool for predicting long term neurological recovery. If using the ASIA scoring system, complete injuries (ASIA grade A) have a very low probability of showing even modest neurological improvement.[22] The majority of neurological recover typically occurs in grades B or C, usually in the first 6 months after injury, but patients can continue to recover up to 5 years post injury.[23–25]

Incomplete cord syndromes

Incomplete spinal cord syndromes occur when lesions encompass specific structure/functional regions of the cord, with some degree of functional preservation below the lesion site. The six common types of incomplete cord syndrome are outlined subsequently.

Dorsal Cord Syndrome Lesions focused in the posterior one-third of the cord, primarily in the posterior column can cause Dorsal cord syndrome (DCS). Patients typically present with sensory ataxia, usually in concert with unsteady gait, dizziness, and frequent falls, particularly during activities in the dark or when the eyes are closed.[26] Larger lesions can include the lateral corticospinal tract and autonomic tracts to the sacral cord potentially leading to weakness and spasticity in the case of the corticospinal tract, and erectile dysfunction, bowel and bladder incontinence, and

orthostatic hypotension in the case of the autonomic tracts.[26]

Ventral Cord Syndrome Lesions that involve two-thirds of the cord and spare the dorsal column cause ventral cord syndrome (VCS). VCS is associated with the worst prognosis of the incomplete cord syndromes, and is commonly caused by infection or spinal cord ischaemia.[27] The involvement of the corticospinal tract and anterior horn can lead to complete motor deficiency below the lesion level. Typically presentation includes loss of temperature, pain, and crude-touch sensations involving the spinothalamic tract, and bladder and/or bowel incontinence, orthostatic hypotension, and sexual dysfunction involving the autonomic centre. Conversely, sensations of proprioception, vibration and fine touch are preserved. Bilateral sensory deficits start two or three segments below the lesion as the spinothalamic tracts ascend at least two to three segments before crossing to the opposite side at the anterior commissure

Central Cord Syndrome Central cord syndrome (CCS) is the most common form of incomplete cord syndrome. It occurs in injury to the central canal, and is typically the result of trauma.[27,28] Clinical manifestations in CSS corelate to lesion size. Smaller lesions in the central cord impact the spinothalamic tract, and can cause bilateral segmental loss of temperature and pain sensations two to three segments below the lesion. Larger lesions can encompass posterior columns, corticospinal tract, anterior horn cells, and autonomic centres in the lateral horn in addition to the spinothalamic tract. Sensory and motor deficits are disproportionately severe in the upper compared with lower extremities due to the organisation of fibres in the corticospinal and spinothalamic tracts.

Brown-Séquard Syndrome Lesions that affect one half of the spinal cord are termed Brown-Séquard Syndrome or hemicord syndrome. Penetrating trauma, such as a knife or bullet injury, are common causes.[29] Clinical presentation can include an

ipsilateral upper motor neuron deficit secondary to interruption of the corticospinal tract. Posterior column damage causes ipsilateral loss of proprioception and vibration sensations, whereas injury to the spinothalamic tract results in contralateral loss of temperature, pain, and crude-touch sensation.[26,27] Again, collateral sensory deficit begins two to three segments below the lesion. At the lesion level, damage to the anterior horn cells and dorsal horn causes a band of combined ipsilateral segmental loss of motor function and total sensory deficit respectively.

Conus Medullaris Syndrome Lesions of the distal end of the spinal cord (T12-L2) can result in conus medullaris syndrome. Typical causes include disk herniation in the lower thoracic and upper lumbar spine, intramedullary tumour, infection and cord infarction.[27,30,31] Clinical features include back pain, hypoesthesia, lower-extremity weakness, impotence, and early bladder and rectal sphincter dysfunction.[27]

The lumbar and sacral nerve roots of the cauda equina also arise from this region, which can result in pathological overlap between conus meduallris syndrome and cauda equina syndrome. The key difference between the syndromes is the type of motor deficit as conus meduallris syndrome causes a mix of upper motor neuron and lower motor neuron deficits, whereas cauda equina syndrome causes purely lower motor neuron deficits.[27]

Cauda Equina Syndrome Cauda equina syndrome (CES) is caused by compression of the lower lumbar and sacral nerve roots below the level of the conus medullaris, and is not strictly an incomplete spinal cord syndrome, but is included here due to the overlap with conus medullaris syndrome. Disk herniation secondary to degenerative disease is the most common cause, with others including trauma, epidural abscess and lumbar spinal stenosis.[32] CES can be classed as incomplete or complete. Depending on the level of compression and the nerve roots involved, manifestations of incomplete CES can include back pain, unilateral or bilateral sciatica, and asymmetric unilateral or

bilateral LMN-type motor deficit in the lower extremities. In addition to these clinical features, complete CES manifests with urinary and bowel retention or incontinence.[27,32]

1.3 Longitudinal changes in spinal cord injury

1.3.1 Spinal shock and neurogenic shock

One of the primary acute clinical manifestations of SCI is spinal shock, which is a temporary state of paralysis post-SCI at or below the level of injury, making initial neurological assessment difficult. Determining when a patient is no longer in spinal shock has also been the subject of controversy.[33] Commonly, the end of spinal shock is tied to the bulbocavernosus reflex, which consists of the contraction of the bulbocavernosus muscle in response to squeezing the glans penis or clitoris, and is mediated through the pudendal nerve.[34] Spinal shock can be confused with neurogenic shock, which is a hypotensive state caused by a loss of sympathetic outflow characterised by bradycardia, wide pulse pressure and warm pink extremities. Neurogenic shock is most common in patients with injuries above T6 and is observed in around 20% of patients with cervical level injuries, as these are more likely to lead to perturbed impulses to the sympathetic splanchnic nerves located in the mid-thoracic region, and to the cardiac pacemaker.[35]

1.3.2 Acute phase

Traumatic SCI is temporally divided into the acute (<48 hours), subacute (48 hours to 14 days), intermediate (14 days to 6 months) and chronic (>6 months) phases. The initial trauma or primary injury introduces mechanical disruption to the vertebral column. This damages the central nervous system (CNS) and compromises the local vasculature, thus disrupting the blood brain barrier (BBB).[35] These events cause an immediate and sustained secondary injury cascade, which often results in greater

damage than the primary injury.[36,37] Within the first minutes of injury cellular death and dysfunction are caused by pro-apoptotic signalling, cell permeabilisation and ischemic injury due to vascular damage.[38] The blood vessel injury can allow immune cell, cytokine and vasoactive peptide infiltration, for example, the inflammatory cytokines tumour necrosis factor (TNF) and IL-1β have been found in the spinal cord within 15 minutes of SCI in a mouse model.[39] This inflammatory response can persist through the acute, subacute phases and beyond leading to sustained swelling of the spinal cord. This swelling can place the cord under further mechanical compression and can extend beyond the physical range of the initial injury, worsening the damage.

1.3.3 Subacute phase

During the acute and subacute periods, a loss of intra and extracellular ionic homeostasis due to ischaemia and excitotoxicity can lead to cell death, dysregulation of intracellular calcium concentrations, in particular, is key to the death of neurons and glia.[40,41] Additionally, inflammatory cells, including activated microglia, contribute to the inflammatory response and thus exacerbate the apoptosis of neurons and oligodendrocytes. Some of the inflammatory cells can phagocytose myelin debris at the injury site, but also release cytotoxic by-products that can cause oxidative damage due to free radical production.[42]

1.3.4 Chronic phase

Following the acute inflammatory phases, a degree of remyelination, extracellular matrix (ECM) adjustments, vasculature reorganisation and remodelling of neural circuits is attempted. Some studies have suggested this undirected plasticity may contribute to neuronal dysfunction following SCI, as it leads to the formation of aberrant neuronal circuits.[43] The damage from the previous phases promotes the formation of cystic cavities, which consist of bands of connective tissue, macrophages

and extracellular fluid. Importantly, these cystic cavities perturb directed axonal regrowth and cell migration, thus interfering with repair.[44,45] Additionally, work with animal models have found a perilesional zone surrounding the cystic cavities which contain reactive astrocytes that tightly interweave their processes, thus forming a mesh-like barrier or glial scar, further inhibiting repair.[46] Various ECM proteins contribute to the glial scar, including myelin associated debris and proteoglycans largely deposited by microglia, macrophages and astrocytes, transforming growth factor-β1 (TGF-β1) and 2 (TGF-β2) are also implicated in promoting the formation and maintenance of the glial scar.[47,48] CNS myelin was first discovered to be an inhibitor of repair, in contrast to PNS myelin, in 1985.[49]

The accumulation of myelin debris has been found to impair axonal regeneration and can act as an inflammatory stimulus that may invoke further damage.[50–53] Myelin-associated proteins, such as neurite outgrowth inhibitor A (Nogo A), myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein, bind NOGO receptors which activates the hydrolyse guanosine triphosphate (GTP)ase Rho A.[54–56] Similarly, chondroitin sulphate proteoglycans inhibit neurite outgrowth via the Rho-ROCK pathway.[57,58] Put simply, the Rho-ROCK pathway is activated by a number of different inhibitory factors via axonal surface receptors, whereupon Rho A activates Rho-associated protein kinase (ROCK) which ultimately leads to apoptosis and growth cone collapse of regenerating axons via further downstream effectors, thus inhibiting repair of the CNS.[59] Once activated, Rho-kinases can phosphorylate collapsin response mediator protein 2 which prevents it from binding tubulin and numb.[60] Tau, neurofilament and microtubule associated protein 2 are also phosphorylated by Rho-kinases, which inhibits their microtubule polymerising activity and neurofilament assembly and thus prevent neurite elongation by destabilising microtubules and intermediate filaments.[61,62]

1.4 Endogenous repair

SCI patients can experience functional recovery for several years after injury.[7,8] The probability and extent of recovery appears to depend largely on the severity and level of the initial injury sustained, but post-injury medical/surgical care and rehabilitative interventions are also relevant.[63–65] The discovery of neural stem cells as a life-long source of neurons challenged the dogma that the nervous system lacked regenerative capacity, and their potential has been particularly pertinent in SCI research.[66,67] Ependymal cells are ciliated cells which line the ventricular system of the brain and the central canal of the spinal cord (Figure 1.3). They are responsible for the propulsion of cerebral spinal fluid (CSF) and serve as a barrier to the brain and spinal cord parenchyma.[67]

In the absence of trauma, ependymal cells do not divide often, but in cell culture or following a SCI, they divide rapidly and give rise to astrocytes, oligodendrocytes, and neurons.[68] They also contribute the majority of the astrocytes that form the glial scar and some of the oligodendrocytes that myelinate axons.[68] By contrast, oligodendrocyte progenitors self-renew and more mature oligodendrocytes in the uninjured spine, and in spinal injury the production of remyelinating mature oligodendrocytes is accelerated.[68] Astrocytes maintain a stable population in the uninjured spine, but similarly to ependymal cells, they divide rapidly after injury and form the border of the glial scar.[68,69] The glial scar has been thought of a hinderance to axonal repair historically, but studies have found that ablating astrocytes in the glial scar leads to increased neuronal death, larger lesion volume and inferior functional outcomes.[68–70]

There is further nuance here as astrocytes are commonly distinguished into A1 and A2 types, where A1 astrocytes have been found to upregulate elements of the complement cascade and secrete a soluble toxin that exclusively but rapidly kills a subset of CNS neurons and mature oligodendrocytes.[71,72] By contrast, A2 astrocytes have been shown to upregulate neurotrophic factors which are thought to be protective,

including TGF-β.[73,74] Currently, it is thought that activated microglial are of particular import in triggering A1 polarisation of astrocytes via release of Il-1α, TNFα, and C1q.[75] Ultimately, glial scar formation is complex and further work is needed to elucidate which aspects of the scar and/or the cell types that produce it are providing detrimental and/or beneficial effects on recovery.

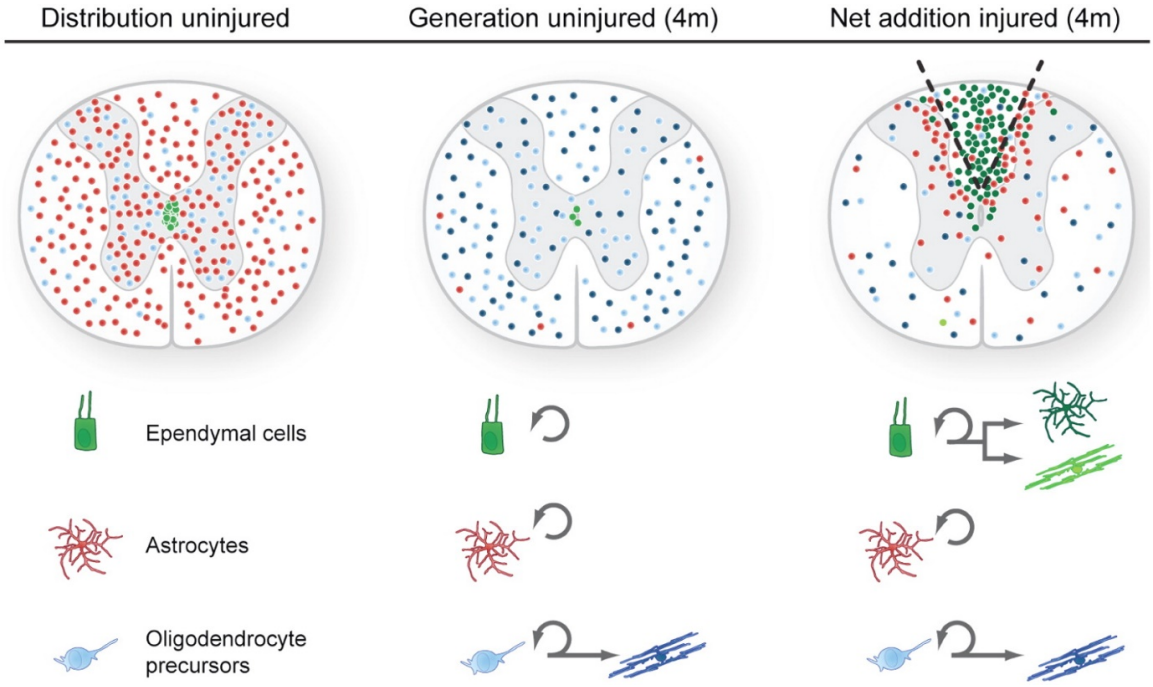


Figure 1.3: Distribution and generation of cell types in the uninjured and injured spinal cord. Taken from Barnabé-Heider et al. (2010)[68]

1.5 Current management and care

The current dogma of SCI management if often encapsulated with 'Time is spine', to illustrate the importance of speed in the treatment of any CNS insult. If appropriate care can be delivered in a sufficient time scale, the long-term functional recovery of the patient can be substantially improved. Thus, guidelines suggest the first priority is to minimise the loss of neurological tissue, and in the case of SCI this entails first immobilising the spine and head to prevent further damage due to movement.[76]

1.5.1 Conservative management and surgical decompression

Management of SCI is somewhat controversial as some advocate for surgical intervention, whereas others prefer conservative care. Those who argue for conservative management suggest the acutely injured cord is susceptible to further injury from changes in physiological parameters during anaesthesia and surgery, multiple patient transfers and direct damage from surgical intervention.[77,78] Surgical interventions are often utilised to realign and stabilise the spinal column, and for decompression of the cord. Compression of the cord has long been thought to exacerbate secondary injury via ischaemia.[79] Several animal studies utilising rats and dogs have demonstrated benefits from decompression early in the injury, and that pressure at the site of injury can be useful in determining the urgency of decompression.[80,81]

There has also been controversy over the timing of decompressive surgery in patients with CCS but without instability in the spine. Initial studies advised against surgery as the patient may experience spontaneous recovery and there is a chance of surgical intervention worsening the injury.[82] Furthermore, several non-randomised trials, case studies and retrospective studies that found early decompression does not improve neurological outcomes in human SCI.[83–86] More recent literature has suggested that surgery for such patients is safe and effective, although the optimal precise timing of the intervention is still debated.[87] The disparity between the animal studies that found surgical decompression to improve neurological outcome, and human studies that found the opposite, may be due to the variability of medical management in hospital settings.[88]

1.5.2 Methylprednisolone sodium succinate

Perhaps the most controversial aspect of SCI care has been the intravenous administration of methylprednisolone sodium succinate (MPSS) in the acute phase of the injury. MPSS is a corticosteroid used in a variety of conditions for its potent anti-

inflammatory effects and historically, MPSS was considered to have potential as a potent neuroprotective agent.[89,90] Several clinical trials were conducted investigating MPSS, the first of which found no significant improvement.[91] However, a later trial comparing a high dose (5.4 mg/kg per hour) 24-hour or 48-hour infusion of MPSS with a placebo or naloxone, ultimately found no significant difference in neurological recovery within the first 2 weeks, but did find a significant improvement at 6 weeks and 6 months post-injury, which corroborated the results of another previous trial.[92,93] Ultimately this led to many spinal surgeries administering high-dose MPSS to SCI patients who arrived at the hospital within 8 hours of their injury.[94] However, critics of these clinical trials pointed out that only a small effect (a 4 point increase in ASIA motor scores) was observed and the use of subgroup analyses to prove effect is far from ideal, which resulted in some experts recommending against the routine use of MPSS.[95] Ultimately, the 2013 American Association of Neurological Surgeons and Congress of Neurological Surgeons (AANS/CNS) guidelines advice against the routine use of MPSS and suggest the evidence of harmful side effects is more of a concern than the indication of any marginal benefit.[96] This stance was in turn criticised as the previous AANS/CNS guidelines from 2002 had said MPSS was a valid treatment option, and as no substantial randomised trials had been conducted in the interim, it has been argued to be unjustified and troubling to some clinicians.[97,98] More recent 2017 guidelines from AO Spine have stated that MPSS should be a treatment option for SCI patients, and so the controversy continues to persist in the field without a conclusive end, necessitating further randomised control trials.[99]

1.5.3 Anticoagulation Prophylaxis

SCI patients are at an increased risk of developing deep venous thrombosis (DVT) which can embolise to the pulmonary system and cause heart failure, meaning that anticoagulation strategies are required.[100] There is still debate over the optimal type

and timing for prophylaxis with respect to preventing venous thromboembolic events (VTE), but guidelines from the Paralysed Veterans of America recommend mechanical compression devices be used early, to not administer anticoagulation whilst the patient is actively bleeding, to apply low-molecular-weight heparin and intermittent pneumatic compression following primary haemostasis; and that vena cava filters be used for patients who are expected to have active bleeding for more than 72 hours.[100] Guidelines from AANS/CNS are similar and do recommend the use of prophylactic treatment for DVT in SCI patients.[101] Nevertheless, the use of anticoagulants also carries risks that must also be considered, such as bleeding, spinal cord contusion enlargement, symptomatic hematoma formation, worsening of neurologic deficits and mortality.[102]

1.6 Animal models

Animal models have been used extensively in SCI research for many years, particularly rodents, with over 2000 studies conducted and over 72% of them utilising a rat model.[103–105] Rodents are of particular use due to their availability, relative low-cost and similarity to humans, in terms of electrophysiological and morphological outcomes following SCI.[106] However, non-human primates are more representative of human SCI than rodents and are particularly useful in assessing restorative and rehabilitative therapies.[107] The majority of animal studies have examined the thoracic-level of injury, mainly due to the fact that caring for these animals is easier, but some studies have begun to investigate more severe cervical injuries due to their clinical relevance as a SCI sub-category.[108] Animal studies can be classified by mechanism of injury into contusion, compression, distraction, dislocation, transection or chemical. Contusion and compression are most commonly used and considered to be the most representative of the majority of human traumatic SCIs. Contusion models often use weight-drop, air-pressure and electromagnetic devices to apply a transient force that displaces

and/or damages the spinal cord.[109] Contusion models are also one of the oldest SCI models as the first weight-drop contusion model was developed in 1911.[110]

Compression models by contrast, apply a compressive force over an extended period. There are a variety of methods for investigating compression models some of which could be considered a contusion-compression model as they apply an acute impact which is followed by extended compression. Techniques include clip compression which was first described in rats with a modified aneurism clip in 1978.[111] Clip compression involves exposing the cord via a laminectomy and then placing the clip, which is set at a specific force, on the cord and left on the cord for a given period of time (Figure 1.4A). Calibrated forceps compression models are very similar to clip compressions and were first described in guinea pigs in 1991 (Figure.1.4B)[112] Calibrated forceps have been used in mice to produce a graded SCI model.[113] Balloon compression is another model first used in 1953 in dogs.[114] Typically balloon compression involves the insertion of a catheter with a small, inflatable balloon into the epidural or subdural space (Figure.1.4C)[115] The balloon is then filled with air or saline of a determined volume which applies the compression to the cord for a fixed duration.

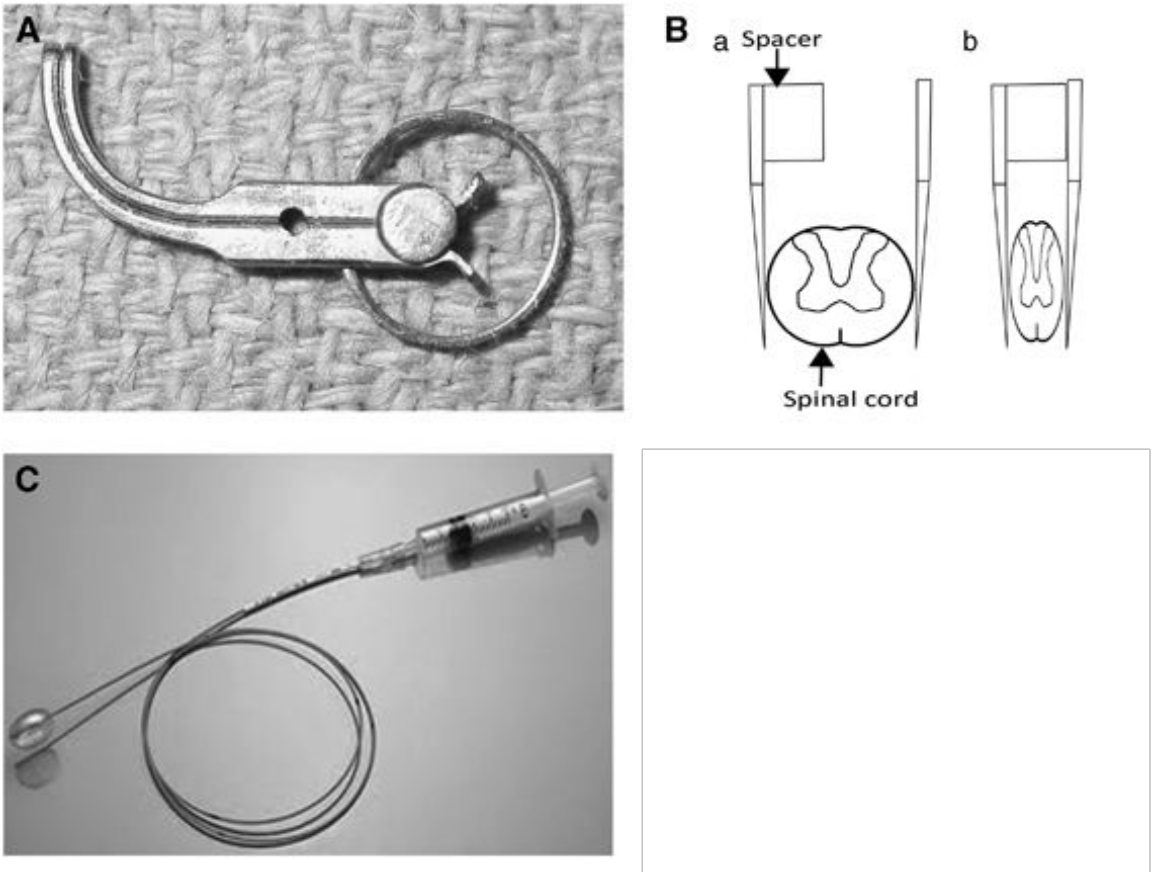


Figure 1.4: (A) Photograph of aneurysm clip used in clip compression models. (B) Diagram of compression forceps with a spacer around the spinal cord. (C) Photograph of a syringe with a balloon affixed to the end to be passed through a laminectomy or laminotomy caudal to the injury site. Adapted from Cheriyan et al. (2014).[116]

Transection models either partially or completely sever the spinal cord at a particular level.[117] A full transection model involves a complete dissociation between the caudal the rostral segments. They are typically used to study neuronal regenerations, often in a tissue engineering context, but transection of the spinal cord in human injury is extremely rare and so this type of model is not usually used for traumatic SCI.[118] Partial and unilateral transection and hemisection of the spinal cord all involve selectively lesioning part of the cord. These transection models are clinically relevant and hemisection models have been used to investigate nerve grafting, which is an experimental surgical technique that aims to treat SCI.[119] Whilst full transection

models are very reproducible, it can be difficult to inflict the same lesion in the partial models so techniques such as retrograde labelling or somatosensory evoked potentials are recommended.[120]

Dislocation models induce injury by displacement of the vertebra, whereas distraction models use opposing traction forces to stretch the cord. In 2004 a device was developed to replicate the column dislocation that can be seen in some traumatic SCI.[121] Finally, chemical models utilise chemical agents to investigate the secondary injury cascade by mimicking an aspect of the cascade. For example, photochemically induced ischemia models involve injection or direct application of a nontoxic photosensitive dye followed by irradiation of the area to be injured for a controlled period.[122] Microdialysis has also been used with glutamate, aspartate, N-methyl-D-aspartate or kainite to approximate the excitotoxicity secondary pathway at a desired level on the spinal cord.[123] To mimic oxidative damage to neurons reagents that generate reactive oxygen species such as superoxide, hydroxyl radical and peroxynitrate have been administered.[124] Injection of phospholipase A2 has also been used to model the inflammatory response to SCI.[125] There are also a number of demyelination models which involve injecting ethidium bromide, lysolethicin, murine hepatitis virus, a cocktail of myelin-specific antibodies and complement, or by adding cuprizone to the animals diet.[126] In addition, there are chemical models that recreate syringomyelia, which is the formation of a fluid filled cavity in the cord that occurs in approximately 20% of SCI, that involve the injection of kaolin suspension or quisqualic acid in rats.[127]

1.7 Novel therapies in development and in clinical trials

1.7.1 Neuroprotective strategies

Many current neuroprotective strategies aim to dampen acute inflammatory responses to injury and alter key targets of the secondary injury cascade. With respect to non-pharmacological therapies, therapeutic hypothermia can be used to lower basal metabolic rate of the CNS and decrease inflammatory response by reducing temperature to between 24°C and 33°C.[128] It has been successfully applied in neonatal hypoxic-ischemic encephalopathy and after in-hospital cardiac arrest.[129,130] Pre-clinical models demonstrated therapeutic hypothermia enhanced tissue sparring and promoted recovery, which prompted a pilot study of 14 patients with ASIA A injuries that found no increased rate of complications and a improved neurological recovery (43% vs 21%).[131,132]

In recent years there has been a growing interest in duroplasty, a surgical procedure whereby the dura is opened and a patch of artificial dura is fixed to, thereby expanding the dura with the aim of expanding the space around the swollen cord. Several ongoing clinical trials are investigating the effect of duroplasty and the impact of other pressure relieving procedures at differing time points post-injury ('Duroplasty for Injured Cervical Spinal Cord With Uncontrolled Swelling (DISCUS)', NCT04936620; 'Surgical Treatment for Spinal Cord Injury (SCI-POEM)', NCT01674764 & 'Injured Spinal Cord Pressure Evaluation (ISCoPE)', NCT02721615).

CSF drainage is another therapy that aims to prevent cord hypoperfusion in the critical postinjury period by relieving pressure analogous to external ventricular drainage for raised intracranial pressure. A 2009 study on 22 patients found no significant difference in outcomes with drainage, but the study lacked the statistical power to demonstrate efficacy.[133] A more recent study in pigs found that combining CSF

drainage with increasing mean arterial pressure resulted in improved cord blood flow that either done alone.[134] Regarding pharmacological neuroprotective therapies, several drugs have also been proposed and are in development or clinical trial including Riluzole, minocycline and monosialotetrahexosylganglioside (GM-1) Ganglioside. Riluzole is a benzothiazole sodium channel blocker approved by the US Food and Drug Administration, and the European Medicines Agency to treat amyotrophic lateral sclerosis.[135,136] Riluzole acts to reduce cell death by blocking sodium influx in injured neurons and limiting the release of presynaptic glutamate.[137] Rat studies have found Riluzole can rescue motorneurons, not only when administered just after injury, but also with a 10-day delay, and improved sensorimotor and electrophysiological outcomes.[138,139] A randomised control trial ('Riluzole in Spinal Cord Injury Study', NCT01597518) is currently recruiting patients with acute C4-8 ASIA grade A/B/C injuries and will use ASIA Impairment Scale (AIS), Brief Pain Inventory, and Spinal Cord Independence Measure as outcome measures.

Minocycline is a bacteriostatic tetracycline antibiotic found to have neuroprotective properties in disorders of the CNS, including mouse studies of multiple sclerosis and Huntington's disease.[140,141] This is thought to be due to an anti-inflammatory effect caused by inhibiting microglia activation and thus reducing deposition of interleukin (IL)-1β, IL-6 and tumour necrosis factor.[142,143] In rodent models minocycline has been shown to reduce lesion size and improve functional outcomes.[144,145] A phase II trial found patients with acute incomplete cervical SCI given minocycline intravenously for 7 days had a 14-point ASIA motor score improvement compared to placebo (P= 0.05, n=25).[146] This result has led to a phase III trial which will assess 7-day intravenous minocycline for acute SCI ('Minocycline in Acute Spinal Cord Injury'; NCT01828203).

1.7.2 Neuroregenerative strategies

Whilst neuroprotective therapies can be beneficial for patients in the acute phase of injury to minimise the loss of neurofunction, neuroregenerative therapies often target the sub-acute or chronic stages injury, which represents a larger population of patients and aims to restore neurological function that has been lost. Spinal cord stimulation (SCS), where electrical stimulation is applied to generate contractions in paralysed muscles which can improve limb mobility and facilitate restoration of bodily function (e.g. bladder and bowel function) is one example of a neuroregenerative therapy.[147] A human study with 4 participants used SCS to recover relatively fine motor control in paralysed muscle groups and restore a degree of sensory capacity even years after injury.[148] Larger clinical trials are currently being conducted (NCT02592668, NCT02313194) to further investigate the efficacy of SCS for SCI. Cellular therapies are another neuroregenerative approach being developed and trialled for SCI.

Multipotent neural precursor cells (NPCs) derived from stem cells are a popular focus as they can differentiate into CNS-specific neurons.[149] A thoracic mouse study found NPCs were able to integrate into the host spinal cord and promote functional recovery by remyelination of axons.[150] Other cell therapies utilise oligodendrocyte precursor cells (OPCs) which preferentially differentiate to functional oligodendrocytes, in order to target post-injury demyelination. Asterias Biotherapeutics Inc. completed a phase I/II dose-escalation trial in 2018 which investigated the safety of their OCP-1 cell line on 35 SCI patients (NCT02302157). Preliminary data suggests no adverse side effects were observed over a 12-month period, and MRI images showed evidence of OCP-1 engraftment into the injury site. Furthermore, motor function scores showed modest improvement, but no result have been formally published yet.[151]

Mesenchymal stem cells (MSCs) are self-renewing, multipotent progenitor cells, capable of repairing connective tissue by differentiating into chondrocytes, osteoblasts, myocytes and adipocytes.[152–154] MSCs have also been found to modulate both local

and systemic inflammation, and rat studies have shown MSCs to significantly improve clinical outcome following SCI via pro-angiogenic and neurotrophic actions.[155–158] The more robust regeneration observed in the peripheral nervous system (PNS) is thought to be due in part to the presence of Schwann cells (SCs), as axons do not extend in fluid, but require a structured path to grow along, which SCs can provide.[159,160] In rat contusion and transection models, SCs transplanted into the injury site were found to promote axonal remyelination and regeneration, reduce cyst formation and modestly improved limb movement.[161] A US based phase 1 clinical trial due to be completed in 2019 is investigating the safety of autologous transplantation of SCs into chronic SCI patients (n=10, NCT02354625). The difficulty in regenerating adult mammalian CNS due to the tissues limited plasticity is well established.[162] More recent research however has shown the CNS to have a greater capacity for repair than initially thought, though lesser in comparison to the PNS, and this capacity is reduced with age.[163–165]

1.8 An introduction to biomarker research

Biological markers, or biomarkers, are objectively measurable indicators of specific biological states (i.e. "healthy" or "diseased"), pathogenic processes (i.e. diseases stages), or pharmacological responses to therapies (Figure 1.5).[166] Biomarkers have proven to be an excellent resource to inform diagnosis, prognosis, treatment and clinical trials in a wide variety of diseases, including osteoarthritis, cardiovascular disease and, perhaps most successfully, in cancer.[167–169] Biomarkers studies often examine samples taken from a diseased tissue of interest, or from surrounding fluids such as blood or lymph. More recently, there has been a growing appreciation for routine measures, such as blood alkaline phosphatase levels, providing insight into a given patient's status. Routinely measured markers have been of particular interest in predicting patient outcomes, and/or assessing the efficacy of novel therapies in clinical trials in

breast cancer, osteoporosis, cardiac arrest and liver diseases.[170–173] As data sharing becomes more ubiquitous amongst both researchers and healthcare providers, the opportunity to leverage vast datasets of thousands or even millions of patients, often referred to as 'big data', can allow for powerful predictive modelling in many disease states.[174] In this context, omics are often used as an example, due to the large volume of data these techniques produce, but these processes are time-consuming and costly. By contrast, the data already available in a patient's medical records could be easily leveraged at low cost, and still provide powerful insight into a patients' disease state. International Business Machines Corporation (IBM) has illustrated this potential with the development of Watson for Oncology, a platform which analyses medical records to assist clinicians' treatment decisions.[175,176]

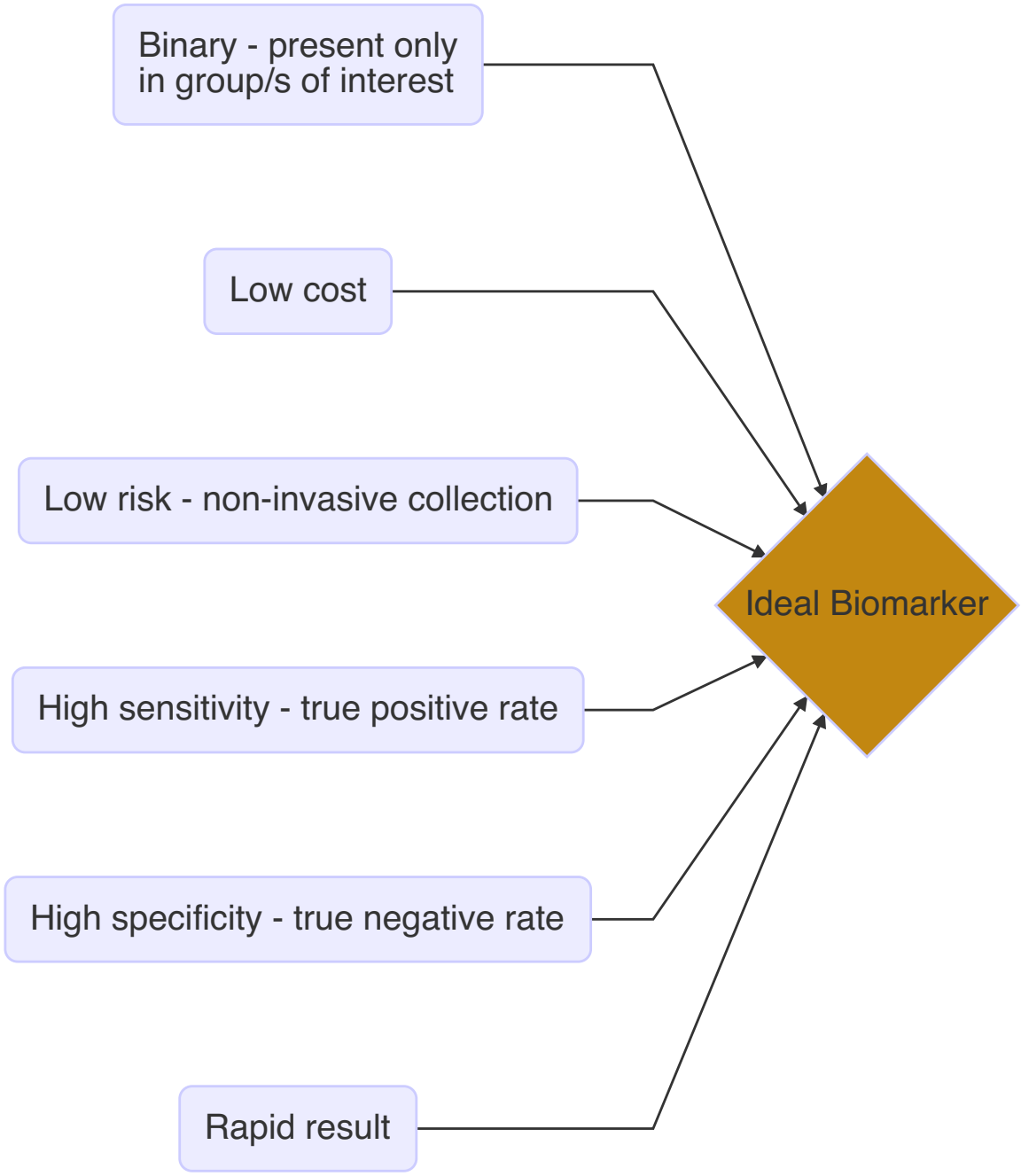


Figure 1.5: Schematic illustrating the components of an ideal biomarker.

There are two broad approaches in biomarker discovery, the first and more common is targeted approach whereby a specific molecule/s are selected based on presumed relevance to the disease in question. The second approach, which has been gaining more traction in recent years, is an unbiased investigation where an entire category of

biomarkers is assessed (proteins, lipids, RNA, etc.), usually utilising omics technology. This change has been facilitated by the advent of increasingly more affordable high-throughput screening techniques, and is particularly useful in cases where relatively little is known about the mechanistic nature of a disease, in more obscure genetic conditions such as Duchenne muscular dystrophy for example.[177] Unbiased biomarker discovery has also been employed in better studied conditions such as neurodegenerative and cardiovascular disease, to successfully identify novel biomarkers of interest.[178,179] Furthermore, it has been suggested unbiased techniques may be useful in enhancing the efficiency of drug discovery and assessment.[127]

1.8.1 Fluid biomarkers

Whilst there have not been many studies utilising unbiased approaches to biomarker analysis in SCI, there have been several that target specific biomarkers that are suspected to be relevant (Table 1.1). For example, the rupturing of neuronal or glial cells, which one would expect in a typical SCI, would release a plethora of potential biomarkers. An important consideration with respect to SCI biomarkers is the relation between CSF and blood biomarker levels. It seems intuitive that CSF biomarkers concentration would be higher than that of blood due to its closer proximity to the injury site. Whilst no study has extensively compared CSF and blood biomarkers in SCI, some studies have found concentrations of IL-6, IL-8, MCP-1, Tau, S100β and Glial fibrillary acidic protein (GFAP) to be approximately 10-times higher in CSF compared to blood within 48 hours of injury.[180,181]

Table 1.1: Summary of spinal cord injury serum/CSF biomarkers. Adapted from Schading *et al.* 2021.[182]

Biomarker	Pathophysiological process/origin	Main findings and diagnostic/prognostic utility	Reference
GFAP	Glial cell injury	Discrimination between AIS sub-groups by concentrations during early phase after SCI. Lower levels associated with better neurological recovery	Dalkilic *et al.* (2018)[183]; Kwon *et al.* (2017)[184]; Ahadi *et al.* (2015)[185]; Yang *et al.* (2018)[186]
S-100β	Glial cell injury	Discrimination between AIS sub-groups by concentrations during early phase after SCI. Lower levels associated with better neurological recovery	Dalkilic *et al.* (2018)[183]; Kwon *et al.* (2017)[184]; Du *et al.* (2018)[187]; Yang *et al.* (2018)[186]
NF-L; NF-H	Axonal injury	Higher levels in SCI patients compared to controls during acute phase. NF-L was correlated with long-term outcome; while NF-H showed no significant associations	Ahadi *et al.* (2015)[185]; Yang *et al.* (2018)[186]; Kuhle *et al.* (2015)[188]; Casha *et al.* (2018)[190]
Tau	Neuronal injury	Elevated levels during acute phase after SCI and discrimination between AIS sub-groups. Levels correlated with long-term functional outcome	Dalkilic *et al.* (2018)[183]; Kwon *et al.* (2017)[184]; Tang *et al.* (2019)[191];Caprelli *et al.* (2018)[192]
NSE	Neuronal cell body injury	Elevated levels in SCI patients without differences between AIS sub-groups. Inconsistent findings regarding predictive utility	Ahadi *et al.* (2015)[185]; Du *et al.* (2018)[187]; Li *et al.* (2014)[193]; de Mello Rieder *et al.* (2019)[194]
IL-6; IL-8	Inflammation	Increased levels during acute and subacute phase. Predictive of AIS conversion in patients	Dalkilic *et al.* (2018)[183]; Kwon *et al.* (2017)[184]; Yang *et al.* (2018)[186]; de Mello Rieder *et al.* (2019)[194]
MCP-1	Inflammation	Elevated early after SCI. Results about prognostic utility are inconsistent	Dalkilic *et al.* (2018)[183]; Kwon *et al.* (2017)[184]; Casha *et al.* (2018)[190]; Heller *et al.* (2017)[195]
Albumin	Hepatic synthesis	Hypoalbuminemia after SCI was associated with poor long-term neurological outcome	Tong *et al.* (2018)[189]; Vo *et al.* (2021)[196]
TNF-α	Inflammation	Improvers were found to have lower TNF-α at 9h c.f. non-improvers.	Biglari *et al.* (2015)[197]; Davies *et al.* (2007)[198]

A study found levels of neuron specific enolase (NSE), S100 calcium-binding protein β (S-100β), tau and neurofilament heavy chain (NF-H) in the CSF are distinct between patients with complete SCI and those with incomplete SCI.[199] Interestingly, another study ($n = 15$) with 12-18 month follow-up found phosphorylated NFH could determine SCI severity with CSF collected at 6 hours post-injury, but not with CSF collected 24 hours post-injury.[200] NF proteins are abundant in the neuronal cytoskeleton so their extracellular presence is indicative of axonal damage and accumulation of NF is associated with neurological diseases.[201] NF proteins are also known to be resistant to breakdown by proteases and therefore have a relatively long half-life of over 3 weeks.[202]

These properties make NF proteins an attractive potential biomarker, and NF-H has been found to be highly elevated in the CSF of patients with complete SCI (n=9) as opposed to incomplete injuries (n=7).[199] Another study found significant differences between serum NF-H of traumatic SCI patients (n=26) and patients with spinal fracture but no spinal cord trauma (n=9) at 24 and 48 hours post injury.[185] Similarly, NF-light chain (NF-L) was also found to be elevated in the CSF of SCI patients as comparted to uninjured and whiplash-injured patients.[203] Another later study also found NF-L to correlate with acute SCI severity and outcome.[188] These papers suggest NF may have value as a prognostic biomarker for patients who will recover in SCI.

Like NF, tau proteins are also abundant in neurons and implicated in the cytoskeleton, particularly in axonal transport, by stabilising microtubules.[204,205] Tau is generally not secreted from cells so its presence in CSF is thought to be indicative of neuronal damage.[206] There has been little work in humans investigating Tau as a biomarker for SCI, but a study in dogs with intervertebral disc herniation found an increase in CSF Tau levels correlated with delayed functional recovery.[207] With regards to humans, one study found no significant difference in CSF Tau levels between motor-complete and motor-incomplete SCI patients.[199] However, another two studies comparing complete and incomplete SCI found CSF Tau did significantly differ in a

severity-dependent manner.[180,184] This discrepancy may be due the difference in time points measured, as Pouw et al. took samples at 3- and 24-hours post-injury, whereas Kwon et al. only collected sample 24-hours post-injury. These studies suggest Tau may have value in predicting ASIA improvement, though it is important to consider the time points measurements are made following injuries.

NSE is the neuronal form of the glycolytic enzyme enolase and is a marker of ischemic brain damage.[208] Importantly, NSE has a short half-life of less than 24 hours, so it is likely that it would only have value as a biomarker in the acute injury setting.[209] In rats NSE levels are elevated in CSF, serum and plasma during the acute phase of SCI.[210,211] Furthermore, NSE levels have been correlated with severity of injury in a rat contusion model.[211] Regarding human studies, higher CSF NSE levels have been found in complete injured patients as compared to incomplete injuries.[199] However another study found there to be no difference in serum NSE between patients with vertebral fracture with and without neurological deficit in the first 24 hours of injury, which suggests NSE may be inappropriate as a SCI severity biomarker.[212]

S-100β is specific to glial cells and is involved in intracellular processes such as cell proliferation, migration, apoptosis and differentiation, and has functions in extracellular signalling.[213] S-100β has been shown to be released into CSF and blood in acute brain injury.[214] Similarly to NSE, S-100β was found to be elevated in the blood and CSF of rats at 6 hours post-injury, as opposed to sham injured animals.[210,211,215] In humans S-100β was found to elevated in the serum of patients with vertebral spine fracture as compared to uninjured patients within the first 48 hours of injury.[212] S-100β is also elevated in the CSF of patients with ASIA A injuries as compared to ASIA B or C.[184] Further, CSF levels of S-100β were shown to be higher at 24 hours post injury in those whose ASIA score did not improve at 6- and 12-months post injury, which suggests acute-phase assessment of S-100β could predict future neurological improvement.[199]

GFAP may also serve as an indicator of neurological improvement. GFAP is an intermediate filament protein found in astroglia and is used as a biomarker for severe brain damage as serum and CSF levels of GFAP have been shown to be higher in patients with traumatic brain injury as compared to injured controls.[216] While less work has been done on the relevance of GFAP in SCI, CSF GFAP has been shown to be elevated in the acute-phase of SCI in rats, as compared to sham-injured controls.[181] In humans, serum levels of GFAP were demonstrated to be increased in acute SCI relative to uninjured controls.[185] Additionally, CSF GFAP was found to correlate with SCI severity as it was higher in complete vs incomplete patients.[183,184,186,187,199]

With respect to predicting neurological outcome, Kwon et al. reported a model that predicted ASIA score improvement with 83% accuracy based on IL-6, IL-8, monocyte chemotactic protein 1, tau, S100β, and GFAP, though the small sample size (n=50) and "baseline AIS grade" determination by nurses based on initial examination of the patient before operation should leave doubt as to the robustness of this particular prognostic model.[184] The release of pro-inflammatory cytokines across the BBB following SCI has prompted research into their relevance in predicting neurological outcome. TNF-α is an inflammatory cytokine associated with the acute phase of injury and in the sub-acute phase it was found to be increased in the serum of SCI patients relative to uninjured patients.[198] This result was corroborated in a rat study that also found serum TNF-α was increased in SCI as opposed to sham-injured animals.[217] Another study found TNF- α levels were lower at 9 hours post-injury in patients who showed neurological improvement vs those who did not.[197]

IL-1β is another cytokine that modulates inflammation and is thought to be crucial for glial scar formation.[218] A rat study found serum IL-1β to be increased in SCI injured animals, at 24- and 48-hours post-injury as compared to sham injured animals.[217] In humans, baseline serum assessment of IL-1β showed no difference between those who did and did not improve ASIA score, but patients who maintained a high level of

IL-1β between 1 and 4 weeks post-injury did show an improvement, whereas those whose serum IL-1β dropped did not.[217] The aforementioned Kwon et al. study also concluded that CSF levels of IL-6 and IL-8 in the acute-phase of injury could be used to predict injury severity and ASIA grade improvement at 6 months, though again this model was based on a very small sample size (n=50).[184]

Routine haematological biomarkers

There has been limited exploration of the potential of routine clinical measures as biomarkers for SCI, despite success in other fields, including cancer, traumatic brain injury, and Alzheimer's disease.[219–221] One small study ($n = 18$) identified correlations between various cytokines, and subpopulations of monocytes with neurological outcomes post-SCI.[222]

A study found associations between baseline peripheral haematological biomarkers and clinical outcome of advanced melanoma patients treated with iplilimumab.[223] After assessing serum from 209 patients, a baseline signature of low absolute monocyte count, lactate dehydrogenase (LDH) and myeloid-derived suppressor cells as well as high regulatory T cells, absolute eosinophil counts and relative lymphocyte counts (RLC) was associated with favourable outcomes following iplilimumab treatment. A similar study by the same group looked at baseline biomarkers for melanoma patients treated with pembrolizumab.[224] Serum from 616 patients was used in this study and they associated high relative eosinophil count and RLC, low LDH and absence of metastasis with favourable outcomes.

A systematic review of CSF and haematological biomarkers for the diagnosis of Alzheimer's disease found total tau, phosphorylated tau, amyloid β42, NFL in CSF and plasma total tau were strongly associated with the disease.[225] Similarly, another study found plasma NFL increased with age and was predictive of dementia status but found no relationship with long-standing epilepsy or premorbid ability.[226] Yet

another study found the secondary structure of amyloid β in plasma to perform as well as a prognostic biomarker as the more established, but more invasive and costly, CSF and PET scan biomarkers.[227]

A study of 132 inpatients with bipolar disorder found mean white blood count (WBC), median neutrophil-lymphocyte ratio, platelet-lymphocyte ratio, lymphocyte, neutrophil, mean platelet volume and plateletcrit were significantly higher in patients with bipolar disorder compared to 135 health controls, whereas median haemoglobin, red blood cell (RBC), haematocrit and mean corpuscular haemoglobin concentration were significantly higher in the control group.[228]

Another study of 493 acute exacerbation of chronic obstructive pulmonary disease patients found lower absolute blood eosinophils count to be associated with poorer clinical outcomes, though this study did convert the continuous data into categories which is not ideal due to loss of information and consequently statistical power.[229,230] Two studies form another group found the prevalence rates of alcohol consumption in early pregnancy could be assessed with blood carbohydrate deficient transferrin and gamma-glutamyltransferase (GGT).[231,232]

Both low and high levels of adiponectin, a protein synthesised and secreted predominately by adipocytes into peripheral blood, have been associated with a wide range of obesity-related diseases.[233]

1.9 Building prognostic models

Prognosis means to predict or estimate the probability of a future condition, for example weather or economic forecasts are a form of prognosis. In the context of clinical medicine, prognosis refers to the risk of future health outcomes in people with a given disease or condition. A prognostic model therefore, is an equation/algorithm that attempts to predict a patient's health outcome based on a start point, which is

typically some metrics of baseline health such as age, gender or increasingly biomarker measures. Prognostic research has become increasingly important in recent years, as population demographics have shifted and more individuals are living with one or more diseases, many of which are chronic in nature.[234] A robust prognostic model is also of interest to healthcare providers and researchers as an accurate model can provide insight in the quality of patient care in different settings and into the efficacy of novel therapies, thus assisting the translation of research into treatment. Prognostic models also tie in with the movement towards stratified medicine, where patient care is tailored to the unique health status of individual patients, rather than the current 'one size fits all' methodology.[235] Unfortunately, whilst many prognostic models are published, a high proportion of them are poorly designed and/or reported.[236] This is often due to inappropriately small sample sizes relative to the number of predictors investigated, but it can also be due to poor data analysis techniques such as dichotomising continuous predictors or univariable screening.[230,237,238]

This led to the creation of the PROGRESS papers, which aimed to outline good practice for prognostic modelling and the TRIPOD paper which outlines appropriate reporting of models to allow for robust external validation.[239–243] With respect to the types of models used, they can be divided into two broad categories, the more traditional statistical modelling techniques which often employ regression, such as logistic regression or linear regression, and the machine learning (ML) algorithms such as random forests (RF), artificial neural networks and support vector machines (SVM).[244,245] ML is often sub-grouped into supervised, where the outcome of interested is already known and included in the training process, and unsupervised techniques, which lack outcome data. However, the exact definition of what constitutes ML is still debated, with some arguing the more traditional statistical methods and ML should be seen as parts of a continuum as opposed to fundamentally different approaches.[246–248]

Regardless, interest in ML methods has grown with the rise of increasingly large

datasets or 'Big Data', such as electronic health records, have become available.[246,249] Some have argued ML outperforms traditional techniques, as its flexibility better handles a large number of predictors.[246,249–252] Other research has suggested that ML requires larger data sets than regression based on receiver operating characteristic (ROC) area under the curve (AUC) analyses, and that the "black-box" nature of many ML techniques precludes an understanding of how the model works.[253] Others still argue that each type of models should be treated differently, suggesting that some techniques may be more or less suited to explanatory, descriptive or predictive investigations, and imply traditional techniques are more appropriate from a descriptive perspective, whereas algorithmic methods may better suit predictive modelling.[254]

1.9.1 Regularisation - Ridge, Lasso and Elastic net regression

Regularisation is the process of reducing the variance of a model at the cost of a slight increase to the bias. Here bias refers to the difference between the true population parameter and the expected estimator. Bias measures the accuracy of estimates. The variance is a measure of spread, or uncertainty, in the estimates (Figure 1.6). Ridge regression is an example of a regularisation technique which aims to penalise the effect of predictors with weak correlations by shrinking their coefficients toward 0, but never reach exactly 0, so they do not have a large impact on the model.[255] This allows the complexity of the model to be reduced without completely removing predictors that may be weak but could still add some value to predictions. Lasso, or Least Absolute Shrinkage and Selection Operator, regression is very similar to ridge regression, but the penalisation is adjusted such that predictor coefficients can be reduced to exactly 0, and so Lasso can remove predictors entirely.[256]

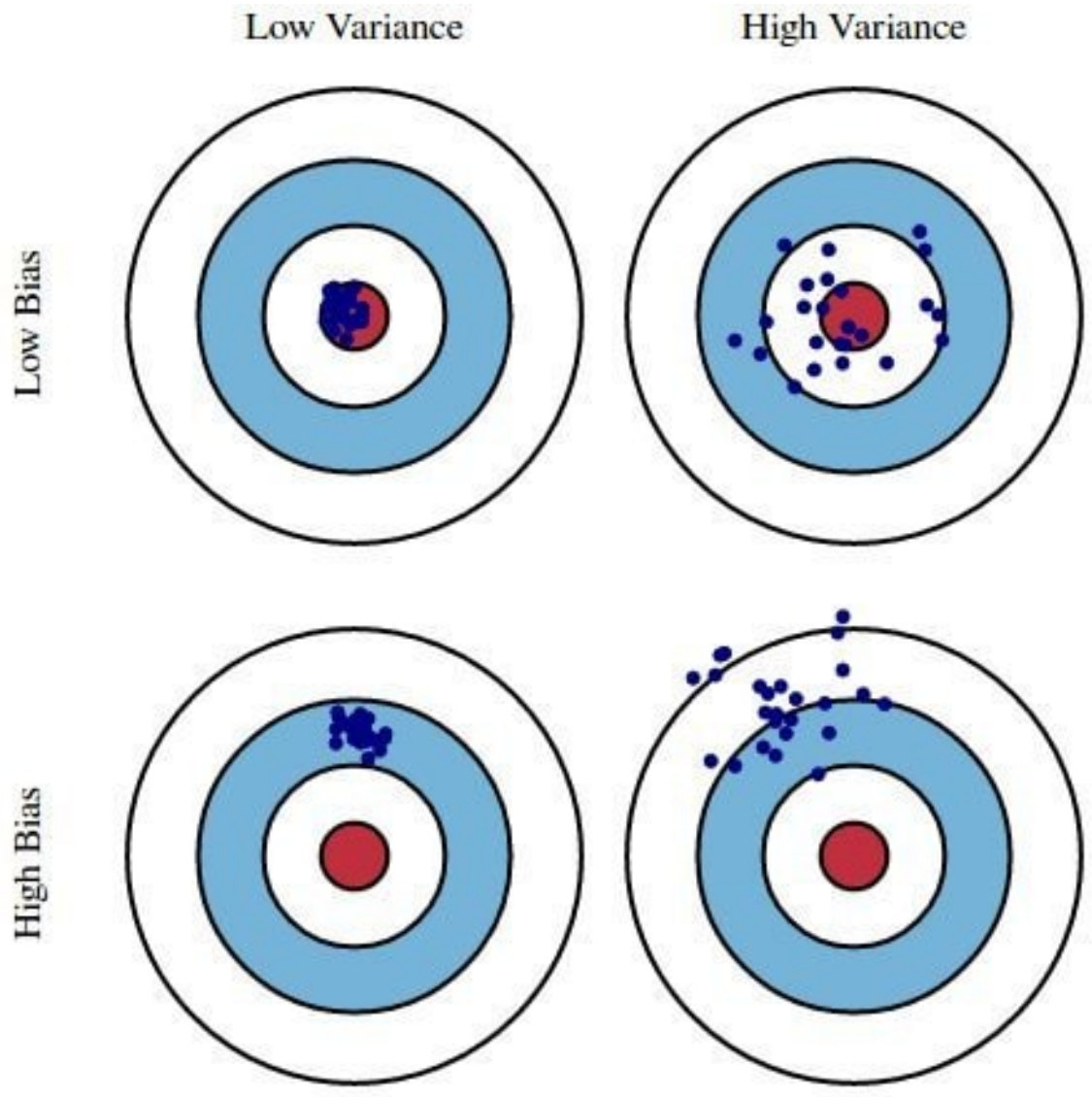


Figure 1.6: Schematic representation of variance and bias

Assuming one knows the true parameter values, ridge regression tends to work well when most of the parameters impact the outcome, whereas lasso tends to work better when a smaller number of predictors have a large impact on the outcome. In practice however, there is no point in modelling a system if the true parameter values are known, so it is typical to cross validate on both to assess which works best for each given data set. Elastic net regression combines the penalisations of both lasso and ridge regression and thus is able to remove useless predictors entirely, whilst still

retaining weak but useful predictors.[257]

1.9.2 Pitfalls in model design

An important issue of consideration in modelling, and part of the reason for so many poor-quality published models, is the degrees of freedom afforded to data analysts. Degrees of freedom here refers to the number of choices the analyst can make whilst building the model. A high degree of choice leaves more opportunity for conscious or unconscious bias to emerge, particularly 'fishing for significance', whereby the analyst tried many approaches and only presents the approach that yields the most favourable result.[258] This issue has demonstrated by using small data sets that are high-dimensional.[259,260]

Variable selection is example of this as it can have a huge impact on the final model but there are many approaches that can be taken, such as stepwise, forward or backward selection. The best way to avoid such bias is to have robust validation. Some ML techniques such as RF can be said to have intrinsic variable selection and are controlled by tuning parameters, whereas logistic regression requires manual selection. Methods for internal validation such as cross-validation, bootstrapping or data splitting can provide evidence the model is stable, but to truly validate a model appropriate external validation must be conducted. This is where the model is assessed in an independent dataset, ideally by a different group, from a different population.

1.9.3 Regression VS Machine learning

Logistic regression is a traditional statistical modelling technique for predicting binary outcomes, developed by David Cox in 1958.[261] Put simply, logistic regression applies a logistic function to predict a binary dependent variable. In the logistic model, the log-odds of the event (the logarithm of the odds) is a linear combination of one or more

independent variables ('predictors'), which themselves can be categorical or continuous. Logistic regression requires a fully and correctly specified model, otherwise estimates can be biased. This can mean the model ignores interactions between independent variables or ignoring nonlinear influences on independent variables. These issues are inherent to parametric regression modelling. ML can be seen as nonparametric modelling methods which avoids these pitfalls. Traditionally ML applications have been limited to classification, as it was thought that nonparametric probability estimation was too difficult.[262] More recently, it has been suggested that reformulation can allow the nonparametric probability estimation, and if they perform well on nonparametric regression problems, then they will also perform well on probability estimation problems.[263]

An advantage of regression models is that they have much greater 'computational transportability' than ML techniques, which is distinct from how applicable the model is in other settings (a different hospital or country for example), though this is also an important consideration. In the case of logistic regression, it is easy for another researcher to test the model as long as the coefficients of all covariates are reported (thus it is important these values be included in publications). This makes it easy to input new data to test predictions and is also very easy for the original author/s to report. By contrast, ML methods can typically only be applied by other researchers if they have access to the corresponding software objects the model generates, or to the training data used to build the model. Theoretically, it would be possible to report ML methods such as RF in a paper, but it would take so many pages, and be so laborious for any other researchers to copy, that it is highly impractical. Therefore, the software object should be made available via some means (the journals/authors website, a data exchange platform, directly from the author/s on request). However, this can be challenging to do in the long term, especially if the particular software lacks stable infrastructure, as updates may impair the model application. There are some potential

solutions to this problem, such as using gradient-boosting with component-wise linear base-learners which results in essentially the same interpretation as classic regression models.[264] Even so, regression models are much more interpretable, allowing for an accessible understanding of the relevance of any given variable, which the "black-box" nature of many ML techniques precludes.

Reproducibility in modelling can be challenging, largely due to the aforementioned poor reporting, but in ML modelling this is often worse, as different software, that supposedly use the same method, will often produce different results, typically due to differing underlying assumptions the software makes. Therefore, it is important that the software/code used in the modelling process be provided in publications to enable reproducible validation.[265]

1.10 Conclusions

It is currently difficult to know whether, and to what extent, a given SCI patient will experience meaningful neurological recovery, making it difficult to plan treatment. The ability to predict neurological recovery following a SCI would therefore help inform patient care, particularly with respect to rehabilitation, and thus could potentially improve patient outcomes. It would also be valuable in the translation of research by assessing the efficacy of new therapies in clinical trials. If such a model could be built using simple demographic data and/or routine blood data, the model would be very low cost to healthcare providers as it would not require any additional work on the part of clinical staff. Proteomic data could improve the accuracy of such a model, albeit at a higher cost of implementation. With respect to modelling techniques, it is important that care is taken to use an appropriate technique, and is reported such that it can be reproduced and externally validated.

1.11 Aims of the project

- This project will aim to find novel blood biomarkers of SCI functional outcomes to better understand the underlying pathophysiology, potentially identify additional therapeutic targets and aid in powering further clinical trials.
  - Firstly, by building prognostic models of neurological recovery following SCI using simple demographic data, initial neurological assessment and routine blood data.
    - For the demographic and routine blood data, electronic health records from 500 patients will be used.
    - Statistically significant proteins would indicate physiological systems relevant to improved functional recovery.
  - Additionally, the plasma proteome of human SCI patient will be characterised by two unbiased techniques: labelled and label-free.
    - For the labelled work, two 4-plex iTRAQ experiments will be conducted, comparing samples pooled by AIS grade, neurological improvement, and at different time points post-injury.
    - Data processing between propriety vendor-provided and free open-source software will be compared to ascertain the viability of a more reproducible workflow.
    - For the label-free work, Proteominer™ beads will be used for dynamic range reduction in order to identify less abundant proteins.
    - Proteins differentially abundant across groups of varying recovery, and their associated pathways are of particular interest.

Chapter 2

Materials and Methods

2.1 Neurological assessment

A full ASIA grade assessment is conducted on patients on admission to the ward, once spinal shock is seen to have ended by testing of the bulbocavernous reflex. Please see section 1.2.1 for details of the ASIA scoring system. Follow-up ASIA assessments are conducted regularly, with one done prior to discharge to the ward and at approximately 12-months post-injury wherever possible.

A Spinal Cord Independence Measure III (SCIM-III) assessment is also conducted concurrently where applicable. SCIM-III is a disability scale developed to quantify the ability of SCI patients to perform basic activities of independent daily living, focused on three areas: self-care (feeding, bathing and dressing), respiration and sphincter management, and mobility .[266,267]

2.2 Patient cohorts

The patients samples/data used here were from a cohort who had previously consented for their patient records to be accessed as part of two other ethically approved studies (National Research Ethics Service [NRES] Committee North West Liverpool East

[11/NW/0876] and NRES Committee West Midlands, Staffordshire [13/WM/0158]). Please see the Annex (section A.4) for the ethical approval letter.

The following sections provide a summary of the cohort demographics, and other measures relevant to the subsequent modelling of functional outcomes.

2.2.1 Preliminary study of routine blood modelling

Retrospective analysis of records from 99 patients who had been admitted to the Midlands Centre for Spinal Injuries (MCSI) between 1980 and 2017 was conducted. Seventeen patients were excluded: one patient because of previous acute myeloid leukaemia, the remaining because of incomplete data on initial and 3 month follow-up AIS scores, or their injuries being non-traumatic.

Eighty-two SCI patients (age range 17-81 years) whose initial blood samples were taken on average at 7 ± 4 days following traumatic injury were included in the statistical analyses (Table 2.1). The blood data were reviewed, and information regarding full blood counts, urea/electrolytes, liver function, bone profile measures including magnesium, C-reactive protein (CRP), and other parameters such as prothrombin time were recorded (Table 2.2).

Table 2.1: Summary of patient demographics

	n
Age (mean±SD) 44.4±17.2 y	82
Males	60
Females	22
Level of injury	
Cervical	47

Table 2.1: Summary of patient demographics *(continued)*

	n
Thoracic	27
Lumbar	8
Injury level	
Above L1	72
At L1	6
Below L1	2
Neurologically intact	2
Initial AIS grade	
A	34
B	9
C	26
D	11
E	2
Outcome AIS grade	
A	29
B	7
C	15
D	29
E	2
Complete	34
Incomplete	46
Neurologically intact	2
Tetraplegic	46
Paraplegic	34

Table 2.1: Summary of patient demographics *(continued)*

	n
Improver	
A - B	3
A - C	2
B - C	2
B - D	3
C - D	15
CCS patients	11
Vertebral fracture	67
Surgery required following injury	34
Initial infection	7
Diabetes	8
Pressure sores	9
Co-morbidities	
None	60
One	11
Two	7
Three	3
Smoker	
No	47
Previous	13
Alcohol	
Yes	20
No	16

Table 2.1: Summary of patient demographics *(continued)*

	n
Medications with potential to impact blood analytes	
Yes	64
No	34
Yes, but no known impact	18
Impact of existing ailments on bloods	
Yes & potential to impact	30
None	46
One ailment	19
Two or more ailments	17
*Polytrauma	47
Non-polytrauma	34

Table 2.2: Summary of initial neurology scores and blood analyte values (mean ± SD (and range)) for patients on admission to the Midlands Centre for Spinal Injuries

Parameter	n	Mean ± SD (range)	Normal Range
Initial Total Motor Score (all muscle groups)	75	114.0 ± 56.6 (2 - 213)	
Initial Touch Score	82	68.9 ± 30.3 (12 - 112)	Max 112
Initial Pain Score	82	68.0 ± 30.3 (12 -112)	Max 112
Initial ISNCSCI AIS Motor Score	82	46.8 ± 24.5 (0 -100)	Max 100
Initial ISNCSCI AIS Sensory Score	82	137.0 ± 60.2 (24 -224)	Max 224
Haematological Indices			

Table 2.2: Summary of initial neurology scores and blood analyte values (mean ± SD (and range)) for patients on admission to the Midlands Centre for Spinal Injuries *(continued)*

Parameter	n	Mean ± SD (range)	Normal Range
White blood cells (x10^9/L)	82	10.1 ± 3.4 (2.9 -23.1)	3.8 - 11.0
Red blood cells (x10^12/L)	61	4.1 ± 0.7 (2.83 -5.63)	4.6 - 6.2
Haemoglobin (g/L)	82	124.1 ± 19.7 (82 -166)	130 - 180
Haematocrit (L/L)	82	36.6 ± 5.9 (24.9 -48.6)	42.0 - 52.0
Mean Cell Volume (fL)	82	90.4 ± 5.2 (68.6 -107.0)	82.0 - 102.0
Mean Cell Haemoglobin (pg)	82	30.6 ± 2.0 (22.0 -36.3)	27.0 - 31.0
Red Cell distance width (%)	82	12.9 ± 1.1 (11.1 -17.3)	
Platelets (x10^9/L)	82	266.4 ± 120.1 (83 -747)	150 - 500
Neutrophils (x10^9/L)	78	7.7 ± 3.1 (1.9 -18.4)	2.0 - 8.0
Lymphocytes (x109/L)	81	1.4 ± 0.6 (0.5 -2.9)	1.0 - 4.0
Mononuclear cells (x10^9/L)	78	0.7 ± 0.3 (0.2 -2.0)	0.1 - 0.9
Biochemical Markers			
Eosinophils (x10^9/L)	60	0.13 ± 0.12 (0.0 -0.5)	0.1 - 0.4
Basophils (x10^9/L)	60	0.04 ± 0.09 (0.0 -0.5)	0.0 -0.1
Sodium (mmol/L)	78	137.2 ± 4.1 (119 -144)	133 - 146
Potassium (mmol/L)	78	4.3 ± 0.4 (3.6 -5.5)	3.5 - 5.3
Urea (mmol/L)	81	6.2 ± 2.3 (2.9 -16.4)	2.5 - 7.8
Creatinine (umol/L)	81	67.0 ± 14.7 (42 -102)	60 - 110
Albumin (g/L)	77	32.7 ± 4.6 (22 -44)	35 - 50
Alkaline Phosphatase (u/L)	77	83.1 ± 35.4 (40 -211)	40 - 120
Alanine Transaminase (u/L)	77	69.7 ± 52.0 (19 -328)	0 - 45
Total bilirubin (umol/L)	77	14.1 ± 6.7 (4 -46)	0 - 21
Gamma-glutamyl transpeptidase (yGT)	77	78.0 ± 84.4 (11 -540)	0 - 75

Table 2.2: Summary of initial neurology scores and blood analyte values (mean ± SD (and range)) for patients on admission to the Midlands Centre for Spinal Injuries *(continued)*

Parameter	n	Mean ± SD (range)	Normal Range
C-Reactive Protein (mg/L)	62	58.3 ± 68.9 (1 -345)	0 - 5
Total protein (g/L)	68	59.5 ± 7.0 (44 -87)	60 - 80
Calcium (mmol/L)	68	2.2 ± 0.1 (1.9 -2.4)	2.1 - 2.6
Phosphate (mmol/L)	68	1.3 ± 0.3 (0.7 -2.1)	0.8 - 1.5
Adj Calcium (mmol/L)	68	2.3 ± 0.1 (2.1 -2.5)	
Magnesium (mmol/L)	49	0.8 ± 0.1 (0.6 -1.1)	0.7 -1.0
Prothrombin time (sec)	50	14.0 ± 3.9 (11 -35)	10-114
International normalised ratio (INR)	49	1.2 ± 0.4 (0.9 -3.2)	Max 1.1

The five outcome measures used were total AIS motor score (the sum of all muscle group scores), AIS Motor score (the sum of key muscle groups scores), AIS total sensor score and a breakdown of sensor score into touch and pain scores, at approximately 3- and 12-months post-injury (Figure 2.1). The number of co-morbidities, presence of vertebral fractures, poly-trauma at injury, whether surgical intervention was conducted, infection, diabetes, pressure sores, smoker status and alcohol consumption were all recorded. Additionally, the number of any medications, beyond the typical painkillers and anti-stomach acid and anticoagulant preparations received following a SCI, that may have influenced the relevant haematological biomarkers were also recorded and grouped into those that might influence haematological biomarker levels such as steroids, statins, antidepressants, and certain antibiotics, and those that would likely not.

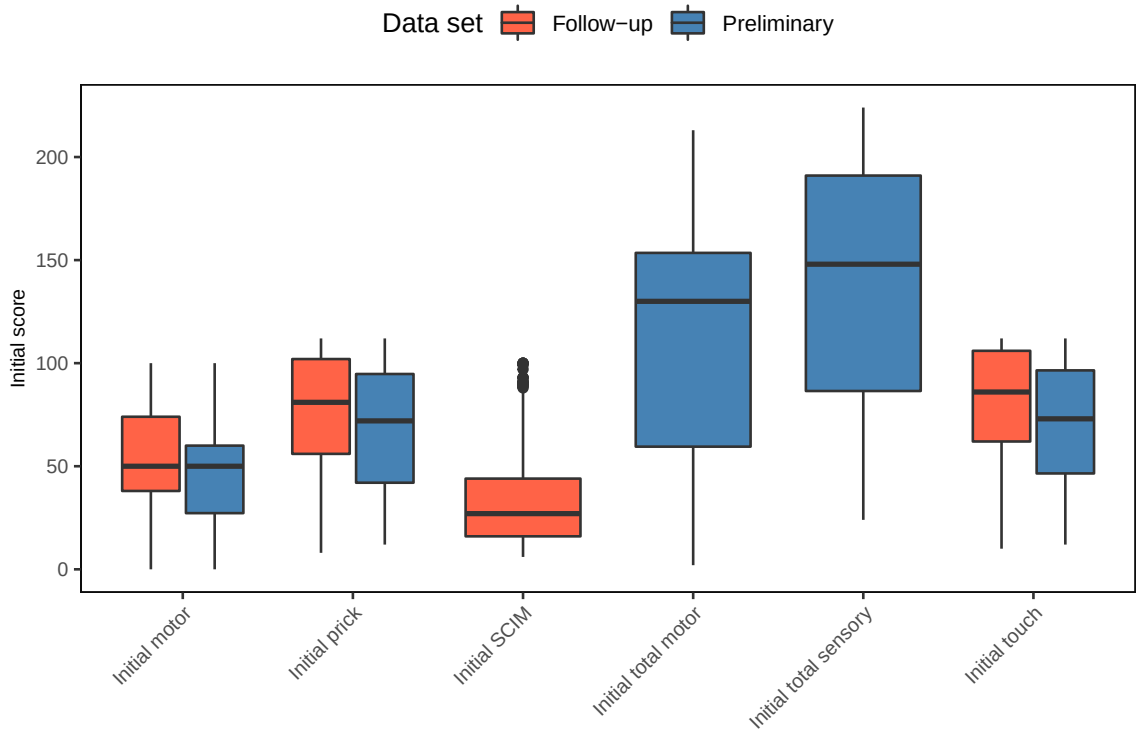


Figure 2.1: Box plots of AIS neurological scores at admission across both cohorts of the preliminary and follow-up studies

2.2.2 Follow-up study of routine blood modelling

We retrospectively studied the electronic health records of 500 patients who had been admitted to the MCSI in the last 10 years (Table 2.3). Following the exclusion of patients that had been admitted over 6 months post-injury, 83 individuals were removed from further analysis.

The remaining 417 patients had their initial blood sample taken at a mean of 31 ± 30 (SD) days post-injury. Blood measures that less than 50% of the patient cohort had had assessed were regarded as not part of "routine care" and excluded. Excluded blood measures included tests for malaria and illegal drug use. The remaining blood measures were adjusted calcium estimate, alkaline phosphatase, CRP, haematocrit, haemoglobin, mean cell haemoglobin, mean cell volume, mononucleocytes, platelets, potassium, red blood count, red blood distance width and WBC.

Table 2.3: Patient demographics.

	Number of SCI patients (n out of 417)	Percent
Age at injury (Median years)	56±28	
Length of stay (Median days)	100±66	
Fracture	225	53.0
Surgery	217	51.0
Traumatic injury	319	75.0
Type 1 diabetes	5	1.0
Type 2 diabetes	44	10.0
Smoker		
No	281	66.0
Yes	52	12.0
Unknown	84	20.0

Table 2.3: Patient demographics. *(continued)*

	Number of SCI patients (n out of 417)	Percent
Alcohol consumption		
No	181	42.0
Yes	152	36.0
Unknown	84	20.0
Sex		
Male	283	66.0
Female	134	31.0
First blood test	22±35	
Time from injury (Median±IQR days)		
Admission	20±34	
Discharge	128±82	
Month-12 assessment	390±103	
Neurological level of injury		
Cervical	244	57.0
Lumbar	30	7.0
Sacral	1	0.0
Thoracic	142	33.0
Admission AIS grade		
A	108	25.0
B	48	11.0
C	151	35.0
D	110	26.0
AIS conversion from admission to 12-Months		

Table 2.3: Patient demographics. *(continued)*

	Number of SCI patients (n out of 417)	Percent
A-B	4	0.9
A-C	4	0.9
A-D	1	0.2
B-C	11	2.6
B-D	4	0.9
C-D	47	11.0
C-E	1	0.2
D-E	1	0.2
AIS conversion from admission to discharge		
A-B	4	0.9
A-C	4	0.9
B-C	13	3.0
B-D	4	0.9
C-D	47	11.0
D-E	3	0.7

In addition to AIS overall grade, AIS motor, sensory touch and sensory pin prick scores were recorded at admission, discharge (mean 138 days post-injury ± 71) and approximately 12 months post-injury (mean 422 days post-injury ± 148). SCIM-III assessments were also recorded at these same time points (Figure 2.2).[268]

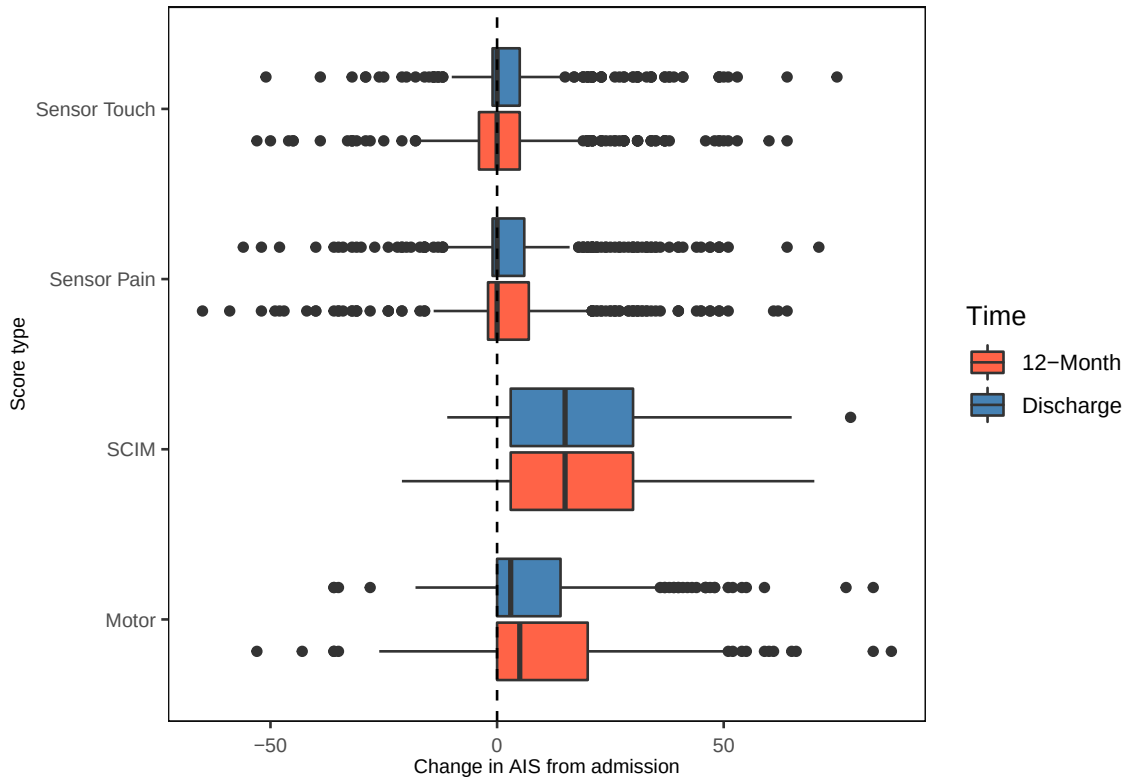


Figure 2.2: Box plots of AIS score change from admission

Additional information that may impact neurological recovery and/or the assessed blood measures were included. The incidence of diabetes (types I and II), smoker and alcohol drinking status were recorded as binary. The neurological level of the injury was recorded as cervical, thoracic, lumbar or sacral. Details were recorded as to whether the injury was traumatic, and whether there were any fractures at the injury site. Age at injury in years, gender and the time between injury and the first blood tests in days were also included. Medications that patients were prescribed were also collected, however after filtering to drugs that at least 50% of patients were given, the remaining drugs were either painkillers or anti-spasm medications. As the inclusion

of this drug data would have added a large number of variables to the model, thus decreasing statistical power, and they correlated strongly with initial injury severity, this data was not included in the modelling process.

2.2.3 Proteomics cohort

Plasma samples were taken from 39 patients (Table 2.4). "Improvers" were defined as individuals who experienced an AIS grade improvement from admission to a year post-injury, whereas "non-improvers" were defined as patients who saw no change in AIS grade in the same period.

Table 2.4: Patient demographics. ± denotes interquartile range. Polytrauma denotes cases of other broken bones, including additional fractured vertebrae, at injury

	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

Table 2.4: Patient demographics. ± denotes interquartile range. Polytrauma denotes cases of other broken bones, including additional fractured vertebrae, at injury *(continued)*

	n	Percent
AIS change		
A	11	28
C	7	18
C->D	10	26
D	11	28
Age at injury (Median years±IQR)	53±26	-

2.3 Blood sample analysis

Routine blood analyses for both studies were conducted in the Haematology and Biochemistry department located at the Robert Jones and Agnes Hunt Orthopaedic Hospital. Haematology analyses were performed on either a Beckman Coulter LH-500 (Beckman Coulter, High Wycombe) or a Sysmex XN-1000 (Sysmex America, IL). Biochemical analyses used VITROS slides (dry multi-layered chemistry slides) in conjunction with the VITROS 5,1 FS Chemistry System (Ortho Clinical Diagnostics, New Jersey, USA) to measure albumin, ALT, calcium, creatinine, GGT, potassium, magnesium, sodium, total bilirubin, total protein, and urea.

Over the period of historical data gather, haemoglobin was initially measured in g/dL, but was later changed to g/L. These older measurements were converted to be in g/L for consistency during modelling.

2.3.1 Plasma collection and storage

Samples were collected within 2 weeks of injury (acute) and at approximately 3 months post-injury (subacute). Upon collections samples were spun at $600g$ in EDTA-coated tubes for 15 minutes, to pellet erythrocytes and the resultant plasma fraction was aspirated and divided into aliquots for long-term storage in liquid nitrogen.

2.3.2 Sample preparation and analysis using iTRAQ proteomics

Protein assay and plasma pooling

$2\mu l$ of thawed plasma were diluted with $98\mu l$distilled water. Total protein was quantified using a Pierce™ $660nm$ Protein Assay (Thermo Fisher Scientific, Hemel Hempstead, UK)[269]. In brief, $10\mu l$ of the diluted sample was added to $150\mu l$ of Thermo Pierce 660nm protein assay reagent in triplicate and the optical density was read at 660nm using an FLUOstar® Omega plate reader. A total of $100\mu g$ of plasma protein was taken from each sample and pooled equally to form a patient test group. For example, the AIS C improver group was pooled from 10 separate patient samples, 10mg of protein per patient.

Sample preparation

The pooled plasma samples were then precipitated by incubation of the sample in 6 times the volume of chilled acetone for 1 hour at -20°C. Prior to incubation the pooled samples was mixed with the chilled acetone by inverting the tube 3 times. The samples were then centrifuged at 6,000G for 10 minutes at 4°C, and re-suspended in $200\mu l$ of triethylammonium bicarbonate buffer. Sequencing Grade Modified Trypsin ($10\mu g/85\mu g$ of protein; Promega, Madison, WI, USA) was then added to the samples for overnight digestion at 37°C. For each group $85\mu g$ of protein underwent reduction

and alkylation (according to the manufacturer's instructions; Applied Biosystems, Bleiswijk, The Netherlands). Tryptic digests were labelled with iTRAQ tags (again according to the manufacturer's instructions for the iTRAQ kit), before being pooled into test groups and dried in a vacuum centrifuge (Figure 2.3). The following tags were used for each group of patient samples 114 tag - acute improvers, 115 tag - sub-acute improvers, 116 tag - acute non-improvers and 117 tag - sub-acute non-improvers for run 1 and 114 tag - acute improvers, 115 tag - acute non-improvers, 116 tag - AIS grade A and 117 tag - AIS grade D for run 2.

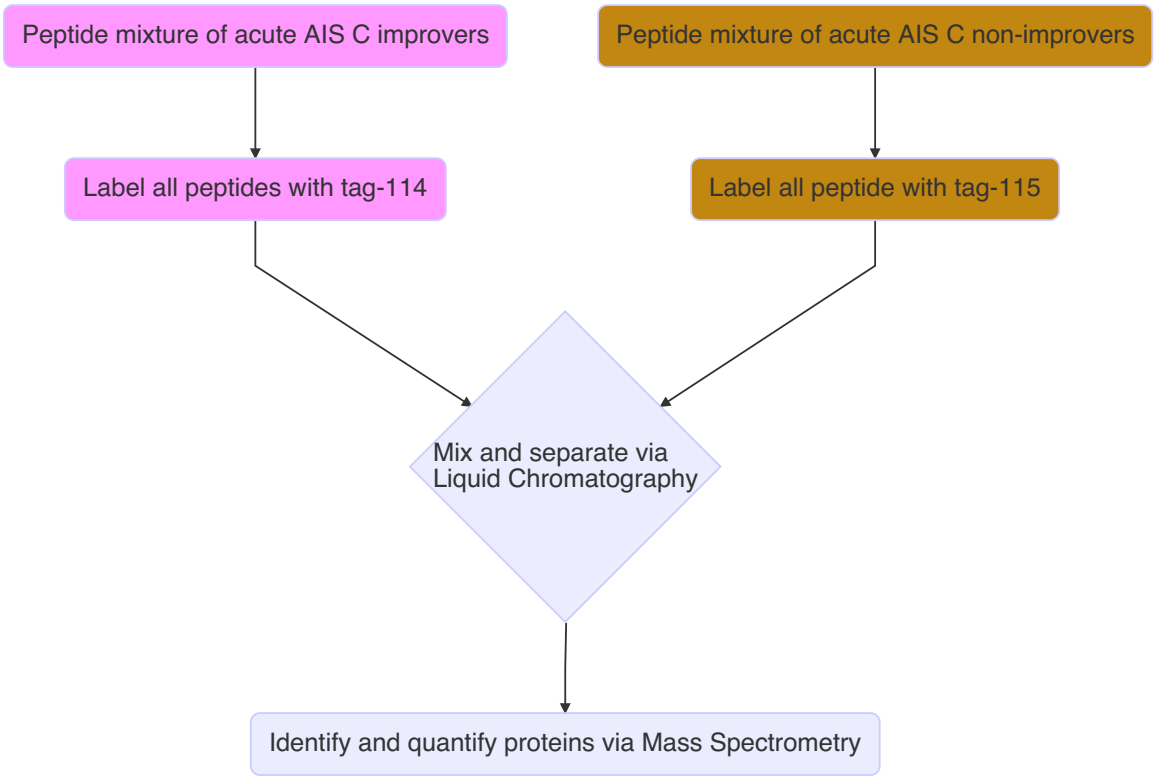


Figure 2.3: Flow diagram illustrating part of the iTRAQ sample processing.

Cation exchange fractionation

The samples were analysed at the Biomedical Sciences Research Complex, St. Andrews University Mass Spectrometry and Proteomics Facility. iTRAQ-labelled peptides were re-suspended in $0.6ml$ of loading buffer ($10mM$ monopotassium phosphate [KH_2PO_4],

20% acetonitrile [MeCN], pH 3.0), followed by sonication. The pH was adjusted to 3.0 with $0.5M$ orthophosphoric acid (H_3PO_4). The peptides were separated by strong cation exchange chromatography (SCX) on a PolySulfoethyl A column ($200mm$ x $2.1mm$, $5\mu l$, $200nm$ pore size, PolyLC, Columbia, MD, USA). The column was washed with 100% buffer Ascx at $1ml$ min^{-1} for 22 minutes so the optical density on the ultraviolet chromatogram would return to baseline. A gradient of 0-50% Bscx (10 mM [KH_2PO_4], 20% MeCN, $500mM$ KCl, pH 3.0) was applied for 20 minutes, 50-100% Bscx for 3 minutes, followed by 100% Bscx for another 3 minutes to wash the column, prior to re-equilibration in 100% Ascx for a further 11 minutes. Fractions ($0.5mL$) were collected every 30 seconds for total of 12 fractions, which were then desalted on C18 spin columns (PepClean C18 spin columns, ThermoFisher Scientific, Waltham, MA, USA) using the manufacturer's instructions, eluting in $20\mu l$ of 70% MeCN. The elution solvent was removed by vacuum concentration and the fractions re-suspended in $20\mu l$ of 0.1% formic acid (FA) prior to mass spectrometric analysis.

Mass spectromety analysis

A total of 12 SCX fractions were analysed by nano-electrospray ionisation-liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a TripleTOF 5600 tandem mass spectrometer (AB Sciex, Framingham, MA, USA) as described previously.[270]

Each fraction ($10\mu l$) was then analysed by nanoflow LC-ESI-MSMS. The peptides were separated using a nanoLC Ultra 2D plus loading pump and nanoLC AS-2 autosampler chromatography system (Eksigent, Redwood City, CA, USA), using a PepMap RSLC column ($75\mu l$ x $15cm$) and an Acclaim PepMap100 trap ($100\mu m$ x $2cm$) (ThermoFisher Scientific, Waltham, MA, USA). After washing the peptides on the trap column for 20 minutes at $5\mu L$ min^{-1}, the trap was switched in line with the column and the peptides eluted with a gradient of increasing MeCN from 95% buffer A (98% H_2O, 2% MeCN, 0.1% FA), 5% buffer B (2% H_2O, 98% MeCN, 0.1% FA)

to 65% buffer A, 35% buffer B over 60 minutes, then to 50% buffer A, 50% buffer B over a further 20 minutes, before increasing the concentration of buffer B to 95% over a further 10 minutes. The column was then washed with 95% buffer B before re-equilibration in 95% buffer A. A flow rate of $300nL\ \text{min}^{-1}$ was employed. The eluent was sprayed into a TripleTOF 5600 tandem mass spectrometer (ABSciex, Foster City, CA, USA), using a NANOSpray III source, and analyzed in Information Dependent Acquisition (IDA) mode, performing $250ms$ of MS followed by $100ms$ MSMS analyses on the 20 most intense peaks with a charge state of +2 to +5. Parent (MS) ions were accepted with a mass tolerance of 50 mDa and MSMS was conducted with a rolling collision energy (CE) inclusive of preset iTRAQ CE adjustments. Analyzed parent ions were then excluded from analysis for 13 s after 3 occurrences.

2.3.3 Sample preparation and analysis using label-free proteomics

No sample pooling was used, and so each of the 73 samples were maintained separately throughout protein equalisation, mass spectrometry, and label-free quantification steps. Thus, protein abundance was quantified for each sample, whereupon mean protein abundance across experimental groups was calculated to assess protein changes.

Plasma preparation and protein equalisation using ProteoMiner™

To reduce the dynamic range of proteins, ProteoMiner™ beads (BioRad, Hemel Hempstead, UK) were used.

To begin, plasma was treated with 1 $\mu g/mL$ of hyaluronidase. Digestion was confirmed with Coomassie stained 1D-SDS PAGE gel. The supernatant was centrifuged through a 0.22 μm cellulose acetate membrane (Costar Spin-X, Corning, Tokyo, Japan) tube filter ($5000g$ for 15 minutes) to remove insoluble material. Total protein was quantitated with a Pierce™ $660nm$ Protein Assay (Thermo Fisher Scientific, Hemel

Hempstead, UK), whereupon 5 mg of total protein was applied to ProteoMiner™ beads.[269] After a washing step, bead-bound proteins 0.05% (w/v) RapiGest (Waters, Manchester, UK) was applied in 25 mM ammonium bicarbonate for 10 min at 80°C precessing reduction, alkylation, and in-situ protein digestion without removing the beads to ensure the complete proteome is preserved. Finally, completion of digestion was achieved via LoBind protein tubes (Eppendorf, Stevenage, UK), followed by acidification of trifluoroacetic acid for a final concentration of 0.5% (v/v). This inactivates and precipitates the Rapigest detergent, to be removed by centrifugation. The supernatant fractions were frozen at -80°C before LC-MC/MC.

LC separation and Quadrupole-Orbitrap instrument

The tryptic peptide were subjected to LC-MC/MC via a 2-h gradient on a NanoAcquity™ ultraperformance LC (Waters, Manchester, UK) connected to a Q-Exactive Quadrupole-Orbitrap instrument (Thermo-Fisher Scientific Hemel Hempstead, UK). The Q-Exactive was operated in a data dependent positive electrospray ionisation mode, automatically switching between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300-2000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) following accumulation of ions to 1×10^6 target value based on the predictive automatic gain control values from the previous full scan. Dynamic exclusion was set to 20s, the 10 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by higher energy collisional dissociation (HCD), with a fixed injection time of 100ms and 35,000 resolution. The following mass spectrometric conditions were used: spray voltage, 1.9kV, no sheath or axillary gas flow; normalised HCD collision energy 30%; heated capillary temperature, 250°C. MS/MS ion selection threshold was set to 1×10^4 count and 2Da isolation width was set.

2.4 Statistical analysis

2.4.1 Preliminary study of routine blood modelling

Statistical analysis in the preliminary analysis (objective 1) was first conduced with IBMI SPSS version 24.0 (SPSS Inc., Chicago, United States).[271] The following is the methodology used in the preliminary paper:

Normality was assessed with both the Kolmogorov-Smirnov and Shapiro-Wilk tests. The majority of the data was not normally distributed, therefore non-parametric tests were carried out. Binary attributes that could influence haematological biomarkers, such as gender and infection, were assessed via Mann-Whitney U test (exact, 2-tailed) whereas categorical attributes with more than two levels, such as injury level, were assessed via Kruskall-Wallis. All variables were assessed for significant associations using Kendall's tau for non-parametric rank correlations. Only complete cases (patients with no missing values) were included.

Due to the small number of patients relative to variables available, principle component analysis (PCA) was used to identify variables that could be grouped and those that could be exuded from model building. Factor analysis with PCA was conducted on blood analytes and initial neurology scores, where any variables with high ($r > 0.8$) or low ($r < 0.3$) correlations via oblique rotation (Direct Oblimin) were removed. The PCA conducted on the individual initial AIS scores (total motor, pain and touch) were called PCA-I, whereas the PCA which utilised the combined scores (total motor and sensor scores) was termed PCA-C. Sampling adequacy was assessed by Kaiser-Meyer-Olkin (KMO), where analytes that did not achieve a KMO over 0.5 were removed.[272] The KMO of both PCA-I and PCA-C was 0.69. Bartlett's test of sphericity was $\chi^2(120) = 451.1, p < 0.001$ and $\chi^2(66) = 273, p < 0.001$ for PCA-I and -C respectively, indicating that the use of factor analysis was appropriate. The names of the subsequently generated factors were ascribed based on the biological function

of the factors constituents.

The factors generated from PCA were used with the aforementioned clinical attributes for multiple regression analysis with forward variable selection to determine their potential to predict outcome neurology at 3- and 12-months post-injury. A model was also built which included all blood analytes. Values of $p < 0.05$ were considered statistically significant. Statistical analyses and p-values were not adjusted for multiple testing and should be interpreted accordingly.

As outlined in the chapter introduction 3.1, the statistical method used in the follow-up study, described below, was subsequently applied to the data from the preliminary paper, which is what that chapter will focus on.

2.4.2 Follow up study of routine blood modelling

Data analyses were performed with the statistical programming language R version 4.1.3 (2022-03-10). Substantial cleaning of the data were conducted, including but not limited to reformatting of inconsistent dates, identification and exclusion of patients with missing initial neurological assessments, reclassification of poorly recorded categorical variables (such as smoking status) to binary measures and reordering of rows and columns to adhere to the principles of tidy data.[273] Missing blood measures were median imputed, then scaled and centred. Less than 21% of the initial and discharge AIS/SCIM scores were missing, whereas 50-60% of the 12-month scores were missing (Table 2.5). These missing AIS grades or scores were imputed with either last observation carried forwards (LOCF) or next observation carried backwards (NOCB) where relevant.[274] LOCF and NOCB were used as it is unusual for AIS or SCIM scores to have decreased over time in SCI patients.[275,276] These scores typically only either remain largely unchanged, or improve with time.[277] Therefore, the use of this imputation effectively assumes that in cases of missing score data, the patients' score did not change. This assumption can only worsen model performance, as opposed to

Table 2.5: Missing AIS and SCIM scores (out of 417 total patients).

	Total number of missing values	Percent missing
Initial motor	15	4
Initial sensor prick	16	4
Initial sensor touch	17	4
Discharge scim	66	16
Discharge motor	74	18
Initial scim	78	19
Discharge sensor prick	83	20
Discharge sensor touch	83	20
Month-12 scim	202	48
Month-12 motor	214	51
Month-12 sensor touch	219	53
Month-12 sensor prick	220	53

giving rise to the overly optimistic models that could be generated by more complex multiple imputation techniques. Additionally, we have been advised that most cases where neurological assessment was missing at admission or discharge is due to failures in data entry. In the case of missing 12-month assessments, this is most commonly due to a given patient not attending their appointment or having received follow-up from a different hospital. As the number of model features was relatively high compared to the number of observations (45 features and 417 observations), linear regression with elastic net penalisation was performed in addition to linear regression without any penalisation. Elastic net penalisation is a hybrid of ridge regression, whereby the penalty term shrinks predictor effect equally and never to 0, and least absolute shrinkage & selection operator (LASSO), whereby the penalty term shrinks each predictor differently and allows variables to be removed entirely by shrinking coefficients to 0.[256,257] Put simply, elastic net reduces the impact of less important model features and can effectively eliminate such features entirely, thus performing variable selection during the model building process, as opposed to other methods such as backward variable selection, which are conducted before model building and

eliminate features based on co-linearity. To better understand this, imagine two theoretical variables which are perfectly correlated. If you knew the value of one these variables you would be able to exactly calculate the other, so there is no value in knowing both. In this way, variables that are highly correlated add little value to model predictions but do increase model complexity, and so penalisation techniques aim to identify these variables and either reduce their influence on the model by reducing the respective coefficient, or remove the variable entirely by setting the coefficient to 0. Elastic net penalisation has been previously found to perform well in models with numerous predictors and in the presence of correlated predictors.[278]

Eight independent models were generated, with and without elastic net penalisation, to determine if the features could predict four outcome measures: AIS motor, AIS sensor touch, AIS sensor prick and SCIM, at two time points: discharge and 12-months post-injury. The data was randomly split 80-20%, whereupon 80% was used for training the model and the remaining 20% was used to test the models performance. To reduce model optimism, internal validation was performed by 10-fold cross validation.[279]

2.4.3 iTRAQ data processing

Data analyses were performed with the statistical programming language R version 4.1.3 (2022-03-10), please see table A.1 for a list of packages used and the respective version numbers.[280–330]

The mass spectrometry data files were combined and analysed using the ProteinPilot 4.5 software with the Paragon^{TM} and ProGroup^{TM} algorithms (ABSciex) against human protein sequences in the SwissProt database. Searches were performed with the default iTRAQ settings in ProteinPilot. The cleavage enzyme was set to Trypsin and MMTS modification of cysteines with a "Thorough ID" search effort. ProteinPilot's Bias correcting setting was used, which assumes no change in protein abundance between groups. Detected proteins were reported with a Protein Threshold

[Unused ProtScore (confidence)] >0.05 and included in the quantitative analysis if identified with two or more peptides with >95% confidence. False Discovery Rate (FDR) analysis was also performed using the ProteinPilot. ProteinPilot calculated P-values for the iTRAQ ratios and those with $P > 0.05$ were considered statistically significant. Proteins with iTRAQ ratios $\geq \pm 1.2$ fold change (FC) were used for network analysis.

2.4.4 OpenMS analysis - iTRAQ

The TripleTOF 5600 tandem mass spectrometer output files are in the ABSciex proprietary `.wiff` file format. For analysis with OpenMS (version 2.6.0) conversion to an open file format, `.mzML` in this case, was needed and the docker image of ProteoWizard version 3.0.20287 was used for conversion, and peak picking was applied on conversion.[331]

OpenMS version 2.6.0 was used for further analysis.[332] Unless otherwise stated, default arguments were used. The 12 fraction files were merged and sorted by retention time. A decoy database was generated with `DecoyDatabase` and the `-enzyme` flag set to `Trypsin`, the human reference proteome was taken from Uniprot (Proteome ID: UP000005640, downloaded: 2020-10-01), as was the `.fasta` for porcine trypsin (Entry: P00761, downloaded: 2020-10-01).[333]

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

To annotate the search results `PeptideIndexer` and `PSMFeatureExtractor` were

used. For peptide level score estimation and filtering `PercolatorAdapter` was used with the following arguments: `-score_type q-value -enzyme trypsinp`. `IDFilter` was used to filter to a peptide score of 0.05 with `-score:pep 0.05`

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

Below is some sample code of the options used for analysis:

```
## conversion of file from .wiff to .mzML and peakpicking
## path to .wiff file
wiff1="/home/mateus/proteomic_data:/proteomic_data proteowizard/"
wiff2="pwiz-skyline-i-agree-to-the-vendor-licenses wine input_file.wiff"
sudo docker run -it --rm -v ${wiff1}${wiff2} --filter "peakPicking true 1-" \
     -o output_directory

## merge fractions
FileMerger -in *run1*.mzML* \
           -out ../fractions_merged_run1_2020_11_02.mzML -threads 4

## sort by retention time
FileFilter -in input_file.mzML -sort -out output_file.mzML -threads 4
```

```
## create the decoy database for the search
DecoyDatabase -enzyme Trypsin -in human_reference.fasta \
              pig_trypsin.fasta -out decoy_database.fasta

## run MSGF search
MSGFPlusAdapter -in input_file.mzML -out output_file.idXML \
  -executable ~/Development/OpenMS/THIRDPARTY/All/MSGFPlus/MSGFPlus.jar \
  -database decoy_database.fasta -fixed_modifications \
  "Methylthio (C)" "iTRAQ4plex (N-term)" \
  -variable_modifications "Oxidation (M)" \
  -precursor_mass_tolerance 20 -enzyme Trypsin/P \
  -protocol iTRAQ -instrument high_res \
  -java_memory 16000 -threads 8

## annotate results
PeptideIndexer -in input_file.idXML -out output_file.idXML

## feature extraction
PSMFeatureExtractor -in input_file.idXML -out output_file.idXML

## peptide level score estimation and filtereing
PercolatorAdapter -in input_file.idXML -out output_file.idXML \
  -percolator_executable \
  ~/Development/OpenMS/THIRDPARTY/Linux/64bit/Percolator/percolator \
  -score_type q-value -enzyme trypsinp -threads 8 -verbose 5
```

```
## filter to peptide score of 0.05
IDFilter -in input_file.idXML -out output_file.idXML -score:pep 0.05 \
         -threads 4

## add isobaric tag information
IsobaricAnalyzer -type itraq4plex -in input_file.mzML \
                 -out output_file.consensusXML -threads 6

## assign protein/peptide identification to features
IDMapper -id input_file.idXML -in input_file.consensusXML -out \
         output_file.consensusXML -rt_tolerance 0.1 -mz_reference \
         precursor -feature:use_centroid_mz false -threads 6

## score estimation and protein inference
Epifany -in input_file.consensusXML -out output_file.consensusXML \
        -greedy_group_resolution remove_proteins_wo_evidence \
        -algorithm:keep_best_PSM_only false
## remove decoys and protein group level FDR - 0.05
IDFilter -in input_file.consensusXML -out output_file.consensusXML \
         -score:protgroup 0.05 -remove_decoys -threads 4
IDConflictResolver -in \
  openms_cmd_outputs/idfilter_prot/file_merge_2020-11-02.consensusXML \
  -out openms_cmd_outputs/id_conflict_resolver/file_merge_2020-11-02.consensusXML
  -threads 4

## get protein quantification
ProteinQuantifier -in input_file.consensusXML -design \
```

```
                    experimental_design_file.tsv -out \
                    protein_qunatifier_output.csv -ratios
```

2.4.5 OpenMS analysis - label-free

For quantification, the raw spectra files were analysed via OpenMS (version 2.6.0) command line tools, with the workflow from the prior section (2.4.4) adapted to suit a label-free analysis. The files were first converted from the proprietary .Raw format to the open .mzML standard with the `FileConverter` tool via the open-source `ThermoRawFileParser`.[332,334] Unless otherwise stated, default arguments were used throughout.

The decoy database generated in the prior chapter was reused here, see section 2.4.4 for details. The `CometAdapter` was used to run the search.[335] Fixed modifications were set to "Carbamidomethyl (C)" and "Oxidation (M)" was set as a variable modification. To reflect the instrument the following flags were also set: `-precursor_mass_tolerance 20 -isotope_error 0/1`.

To annotate the identified peptides with proteins the `PeptideIndexer` tool was used. As in section 2.4.4, `PeptideIndexer` and `PSMFeatureExtractor` were used for annotation. For peptide level score estimation and filtering `PercolatorAdapter` was used with the following flags: `-score_type q-value -enzyme trypsin`. `IDFilter` was used to filter to a peptide score of 0.01 with `-score:pep 0.01` followed by `IDScoreSwitcher` with the following flags: `-new_score "MS:1001493" -new_score_orientation lower_better -new_score_type "pep" -old_score "q-value"`. The `ProteomicsLFQ` was used for subsequent processing with the flags: `-proteinFDR 0.05 -targeted_only true`. The `-out_msstats` flag was also used to produce quantitative data for downstream statistical analysis with the R package `MSstats`.[307]

2.5 Validation of iTRAQ results using enzyme-linked immunosorbent assay

Four proteins identified by the iTRAQ analysis were measured by enzyme-linked immunoabsorbent assay (ELISA) from non-pooled samples to validate the iTRAQ findings (Figure 2.4). These proteins were alpha-2-macroglobulin (A2M), retinol binding protein 4 (RBP4), serum amyloid A1 (SAA1) and apolipoprotein A1 (ApoA1). They were selected for their biological relevance and differential abundance between AIS C improvers and non-improvers, implying potential as biomarkers of neurological outcome prediction. A2M, RBP4 and SAA1 were assessed using a human DuoSet® ELISAs (R&D Systems, Abingdon, UK). ApoA1 was assessed using a human Quantikine® ELISA (R&D Systems, Abingdon, UK). Samples were diluted 1:600,000 for A2M and RBP4, 1:100 for SAA1 and 1:20,000 for ApoA1 in the respective assay kit diluent. Samples that were above the assay detection limit were rerun at 1:300 and 1:40,000 for SAA1 and ApoA1 respectively. All ELISAs were carried out according to the manufacturer's protocol. Protein concentrations were normalised to the sample dilution factor. Statistical analysis was perfomed using the statistical programming language R version 4.1.3 (2022-03-10). Pairwise t tests with bonferroni adjusted P-values with the R `rstatix` package were used to assess differential abundance.

Figure 2.4: Flow diagram illustrating ELISA process.

2.6 Network and pathway analysis

Protein interation networks were created using the Bioconductor package `STRINGdb` which provides an R interface to STRING version 11.[336] Instantiation of the STRINGdb reference class was done with `species` and `score_threshold` set to `9606`, for *Homo sapiens* , and `400` respectively. Clustering of networks with `STRINGdb` used the "fastgreedy" algorithm from the `iGraph` package.

The Bioconductor package ReactomePA, which employs the open-source, open access, manually curated and peer-reviewed pathway database Reactome was used for network analysis.[312,337]

Chapter 3

Prognostic modelling of SCI outcomes with routine blood measures

3.1 Introduction

As discussed in the General Introduction (1) there is a need to identify prognostic biomarkers for SCI because the SCI population is inherently heterogeneous and experience a highly variable degree of "natural" recovery.[275] Currently, the best predictor of neurological outcome is the initial measure of neurologic impairment, as assessed with the ISNCSCI examination.[338] However, the ISNCSCI exam was not intended to be predictive of functional recovery, and it has been found that changes in AIS grade do not necessarily indicate meaningful changes to daily living for patients.[339]

Robust SCI biomarkers could help stratify patients if their baseline functional recovery could be more accurately predicted. This is particularly important in the acute setting, when long-term outcome is less certain, allowing for any potential novel therapies to be properly assessed, thus accelerating research and clinical trials in

particular via covariate adjustment.[340] A reliable prognostic model of SCI would also allow healthcare providers to better plan patient care, relieve patients of potentially damaging psychological uncertainty, and could highlight new avenues of research.[11]

3.1.1 Biomarkers of neurological injury

With respect to biomarkers of neurological injury more broadly, traumatic brain injury (TBI) has received increasing attention in the past decade as the prevalence of TBI in high-contact sports and military veterans has been more widely appreciated.[341,342] As with SCI, CSF is often regarded as the optimal source of biomarkers as it is in direct contact with the extracellular matrix in the brain, and its composition often reflects biochemical changes in the CNS.[343] The CSF:serum albumin ratio is a commonly used biomarker of BBB function, as albumin is primarily synthesised in the liver and most albumin in CSF is derived from the blood via passage across the BBB.[344] An increase in this ratio indicates compromise of the BBB and is observed in various CNS disorders, such as inflammatory diseases, infections, cerebrovascular diseases or brain tumours.[343] The ratio has also been found to be elevated in severe TBI but not mild cases, suggesting BBB integrity is persevered in all but the most severe cases of TBI.[345–348]

Neuroinflammation and acute axonal injury

Several studies report increased levels of inflammatory proteins such as IL-6, IL-8 and chemokine CC ligand-2 in CSF following severe TBI, but there has not been as much investigation of inflammatory markers for mild TBI.[345,346,349–351] Regarding axonal injury, two well established biomarkers are total tau and neurofilament light chain (NFL), which are regionally distributed in thin, nonmyelinated axons of cortical interneurons and in the large calibre myelinated axons that project into deeper brain layers and the spinal cord respectively.[352,353] Total tau protein levels in ventricular

CSF correlate with lesion size and clinical outcome and so higher levels of total tau are associated with more severe TBI.[354–356] Neurofilaments are comprised of intermediate filaments, where each filament is composed of one light subunit (NFL) and either one medium subunit (NFM) or one heavy subunit (NFH), arranged head-to-tail.[357] High levels of phosphorylated NFH have been observed in ventricular CSF in cases of severe TBI and in the CSF of amateur boxers following mild TBI via lumbar puncture.[358,359]

3.1.2 The state of biomarker research for SCI

Whilst relatively few studies have sought to identify prognostic biomarkers for SCI, recent years have seen some early/discovery phase publications.[17,360–362] These studies have largely focused on biomarkers in CSF during the acute phase of injury, with little information regarding the chronic or recovery phase. Please refer to the General Introduction (Section 1.8.1) for more details on the state of research into fluid biomarkers for SCI.

Neuroimaging biomarkers

Radiological assessments are often incorporated in the diagnosis evaluation of SCI, and can be particularly useful in understanding the level and severity of the trauma. Scans are typically computed topography (CT) or magnetic resonance imaging (MRI). CT allows for fast image acquisition and high quality visualisation of osseous anatomy and fractures.[363] However, some have argued CT's lack of soft tissue contrast and the insensitivity for visualisation of neural damage could lead to underestimation of canal compromise and ultimately to a misdiagnosis in the clinical setting.[364,365] By contrast, MRI can aid in evaluation of damage to both neural and discoligamentous structures post-injury, potentially assisting with early clinical decision-making.[366]

One standard protocol entails T1-weighted and T2-weighted MRI images of the lesion level, allowing for swift patient screening, and serving as a powerful prognos-

tic indicator in routine care.[363,367] These images enable key lesion measures to be assessed, including the intramedullary lesion length (IMLL), maximum spinal cord compression (MSCC) and maximum canal compromise (MCC) (Figure 3.1).[366] Some studies have report high intra- and inter-observer reliability of both MCC and MSCC measurements, and a correlation with neurological outcomes and injury severity.[364,365] However, subsequent studies have observed this prognostic capability faded when initial neurological status was included in modelling.[368,369]

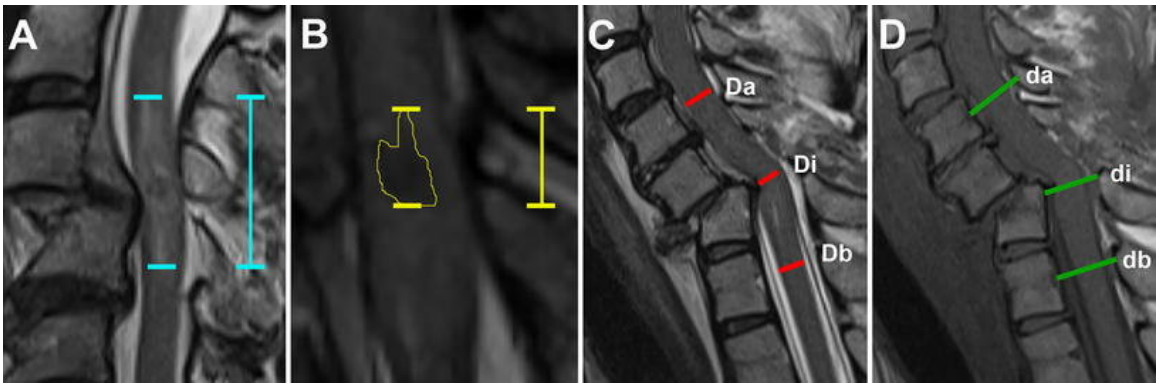


Figure 3.1: Example lesion characteristics via MRI imaging. (**A**) Cord edema. Vertical distance measured between most cranial and most distal point of the edema in the mid-sagittal plain on T2-weighted. (**B**) Haematoma. Vertical length measured as distance between the most cranial and most caudal border of the haematoma in the mid-sagittalplane on T2-weighted. (**C**) Components of the maximum spinal cord compromise (MSCC), computed as $MSCC = 1 - (\frac{D_i}{D_a + D_b/2})$ where spinal cord diameter at the level of injury (Di), first unaffected level above the injury (Da), and first unaffected level below the injury (Db) are measured on mid-sagittal T2-weighted. (**D**) Components of the maximum canal compromise (MCC), computed as $MCC = 1 - (\frac{d_i}{d_a + d_b/2})$ where spinal canal diameter at the level of injury (di), first unaffected level above the injury (da), and first unaffected level below the injury (db) are measured on mid-sagittal T1-weighted. Taken from Rutges et al. (2017).[370]

The IMLL is measured as the length from the most rostral to the most caudal apices of hyperintensive signal changes within the spinal cord on mid-sagittal T2-weighted images. It has also been found to be superior in predictions of outcomes as compared to MCC and MSCC.[371] Similarly to those measurements, IMLL was also found to add

little value when initial AIS grade is included.[369]

An alternative to these measures was proposed to improve the assessment of injury severity prognostication in the form of the Brain and Spinal Injury Centre (BASIC) score.[372] This system utilises a 5-point classification scheme to qualitatively grade the extent of intramedullary T2-weighted signal abnormalities in the axial plane. Follow-up studies reported BASIC score outperforming MCC, MSCC and IMLL in predictions of AIS grade, and importantly that inclusion of both clinical status and BASIC score could improve prognostication.[368,373,374]

The degree of preserved dorsal and ventral tissue has been linked to patient recovery, electrophysiological recordings and patient motility.[375–377] Furthermore, the width of tissue bridges at 1-month has been significantly associated with neurological outcomes, and so could serve as a prognostic measure.[375,376,378,379] Moreover, ventral and dorsal tissue bridges were significantly related to motor and sensory function respectively, allowing distinction between tract-specific impairment.[378] Additionally, a small scale study provided modest evidence that assessment of tract-specific damage via axial MRI slices may add prognostic value.[380]

Importantly, T2-weighted images do not provide information pertaining to the underlying pathophysiology, and so signal changes could represent both irreversible and transient pathologies. Variation across time and patients is also high, and quantification can suffer from subjectivity of the examiner.[369,381] This highlights the importance of robust validation for MRI derived neuroimaging biomarkers for diagnosis and prognosis.

Novel neuroimaging markers Whilst conventional MRI sequences allow for macrostructural assessment of neural tissue, novel protocols seek to provide parameters to assess microstructural changes.[382,383] One modality that may offer diagnostic and prognostic value for SCI is diffusion tensor imaging (DTI). DTI has a higher

sensitivity to early structural changes by assessing microstructural integrity of fibre tracts.[367,384] Comparing post-mortem histological samples and DTI measurements of spread white matter revealed a strong correlation between the techniques, indicating DTI could have value as a non-invasive assessment of neuronal tract integrity.[385] Further, comparisons between animal models and human SCI patients revealed differences in DTI measurements relative to healthy controls. Specifically, at the level of injury SCI patients were found to have increased values of mean diffusivity and decreased values of fractional anisotropy, suggesting disorganisation with fibre tracts and reduced axonal/myelin content respectively.[384,386,387] These parameters have also been shown to be different between controls and SCI patients above and below the lesion site.[388–390] Several studies have also reported significant association between DTI measures and functional outcomes of SCI, suggesting their value as prognostic markers.[384,387–392] Conversely, another study failed to find any superiority via DTI measures relative to conventional MRI parameters in predictions of neurological outcomes, though this study and several of the prior cited studies have a small sample size.[393]

Measures for quantification of microstructural changes are magnetisation transfer saturation (MTsat) and longitudinal and effective transverse relaxation rates as outlined in the multi-parameter mapping protocol (MPM).[394] These parameters are associated with iron and myelin content providing alternate pathophysiologic insight following trauma.[395–397] A primate SCI study reported MTsat to be a robust in measuring loss of macromolecules and demyelination post-injury.[398] MPM-based results could highlight increased iron content and reductions in myelination in areas of atrophy.[399–402] These changes are not restricted to the lesion site and can occur in remote regions of the spinal cord and brain. Some studies have reported correlations between these measures and longitudinal neurological outcomes, indicating potential value as biomarkers for SCI.[399–401]

3.1.3 "Big data" in biomedical research

Recent years have seen an increased interest in using health data that is recorded as part of routine medical care for prognosis. As more data is recorded electronically it has become easier to acquire larger datasets than was previously practical.[403] "Big Data" has had significant impact in many areas of biomedical research, particularly in pathology where malignancy has undergone reclassification.[404] It is worth reflecting on how "Big Data" can be defined. Big Data has no arbitrary size threshold and is often described as being composed of the four "V"s, which are Volume, Variety, Velocity and Veracity (Figure 3.2).

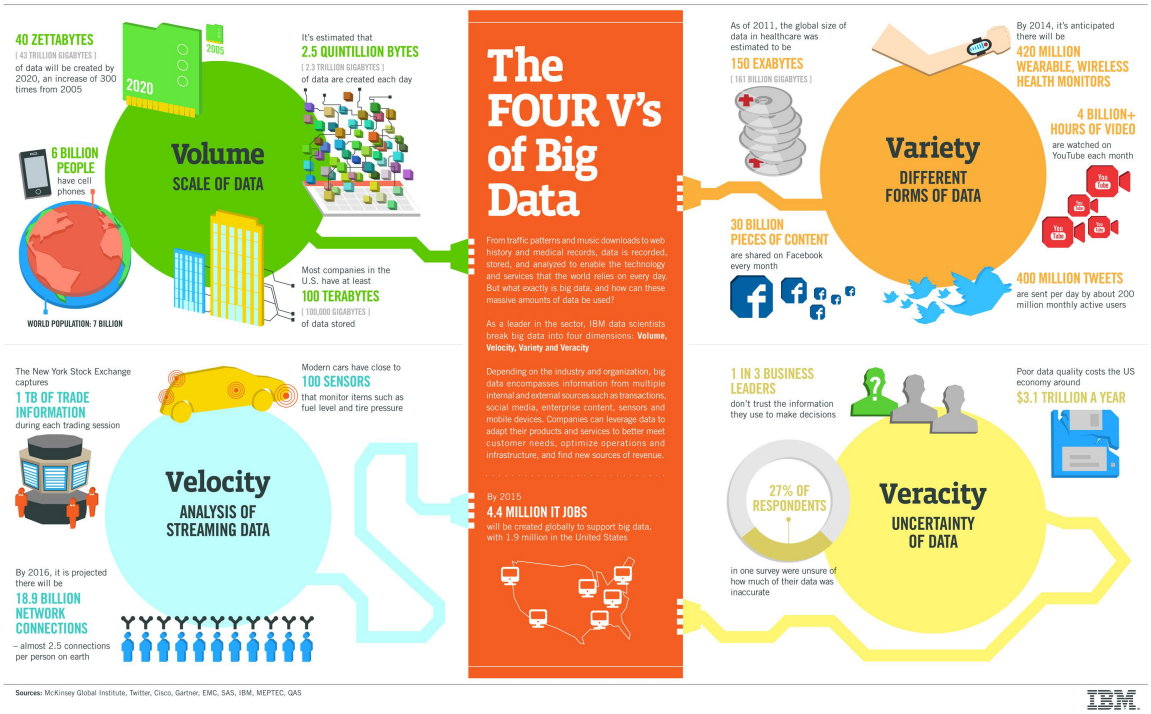


Figure 3.2: Infographic explaining the characteristics of "Big Data". Taken from ibmbigdatahub.com

Volume refers to the absolute size of the dataset, but historic statistical theory is built on the notion of datasets increasing in depth (more rows) whereas new theory is actively being developed for situation where data sets are getting wider (more columns).[244] Variety refers to the different forms of data in addition to the traditional use of numerical data such as videos, images and text. Whilst most current papers still mainly utilize traditional numeric or categorical data, more papers may begin to try and take advantage of the text in clinical notes in model building.[405] Velocity refers to the speed with which data can accumulate. Depending on the size of the country, there could be in excess of 1 million blood tests taken each day.[406] This can lead to terabytes or even petabytes of data which can present challenges for storage and access. Veracity refers to the fact that data may be subject to errors, missing values or other inadequacies.[407] In the context of biomedicine, errors in data recording can be considered less of an issue as the quality of laboratories used for clinical diagnostic

testing are typically rigorously maintained. However, missing values due to a given test not being requested or censored data such as cases where levels are outside the detection limits of laboratory equipment are a common occurrence.

This chapter will explore the value of routinely measured blood parameters to predict neurological outcome in SCI. First, a preliminary assessment to gauge whether routine bloods have any prognostic value was conducted, which was published.[271] Once this viability was established another model with built with an independent, larger cohort, and using the more robust modelling techniques such a cohort allows for. These methods were then applied to the same data in the preliminary paper, as they were found to be simpler, more reliable and easier to reproduce.[408]

3.2 Methods

Please see the Material and Methods chapter sections 2.2, 2.2.1 and 2.2.2, and 2.3 for details of the ethics/patient cohort used, and routine haematological analysis respectively. Sections 2.4.1 and 2.4.2 outline statistical methods.

3.3 Results

3.3.1 Preliminary study

All 82 patients had undergone a traumatic SCI: 33 patients had sustained a fall from a height or step; 29 patients had been involved in a motor vehicle incident (car, motorbike, go-karting); 15 patients were injured during sporting activities (horse riding, climbing, skateboarding, skiing, air sports, cycling, rugby, fairground ride); 4 patients had been struck by a falling weight; and 1 patient had been assaulted.

There were 34 patients classed as ISNCSCI AIS A, 9 patients classed as AIS B, 26 patients classed as AIS C, 11 patients classed as AIS D, and 2 patients classed as

neurologically intact following their initial neurological examination following injury. All patients had follow-up neurological scores; 79 patients at ~3 months and 72 patients at 1 year. Of the 82 patients, 25 were termed "improvers," as their ISNCSCI AIS score improved by at least one level (Table 2.1).

Seventy-five percent of patients included in the study were males, with most injuries occurring at the cervical level (57%) and the least number of injuries occurring in the lumbar region (10%). An AIS A complete injury was sustained in 41% of patients (for the purposes of this study AIS A improvers were described as complete). The second most common injury was AIS C (32%). The majority of patients (56%) had an incomplete injury and 56% were tetraplegic. Although 6% of AIS A patients improved to become AIS B (4%) or C (2%), and 2% of AIS B patients became AIS C, the greatest percentage of improvers occurred in the AIS C group, with 18% of patients becoming AIS D.

CCS occurred in 13% of patients, whereas vertebral fractures were detected in 82% of patients. Surgery was required in 41% of cases whereas initial infections (9%), diabetes (10%), and pressure sore incidences (11%) were relatively uncommon. The majority of patients had no comorbidities (74%), whereas the remaining had one (14%), two (9%), or three (4%) comorbidities. Of these existing medical conditions, most had no effect on blood analytes (56%), whereas medications that could impact on these measures were being taken by just over one third of patients (37%). More than half of patients (58%) had sustained polytrauma, and the majority had never smoked, whereas 80% regularly drank alcohol.

Table 2.2 provides a summary of the neurological scores and various analytes routinely measured in SCI patients on admission. RBC measures had a tendency to be lower than the normal range whereas white blood measures tended to be higher. Most electrolytes fell within the normal range, but liver function and bone profile measures tended to be higher, with albumin and total protein levels being lower than

normal values. The inflammatory marker, CRP, was also higher in the majority of SCI patients, in comparison with normal levels.

Multiple regression models were utilised to produce predictive models of neurological outcome following SCI at ~3 and 12 months post-injury. These models included many of the clinical variables described in Table 1 (all clinical features not in italics); for example: age, gender, injury level, polytrauma, smoking, the existence of additional conditions, and medications that could affect blood analytes. Standard linear regression models (LRM) with no variable selection produced no statistically significant (P-Value < 0.05) variables, low R^2 values (range 0.001 - 0.532) and high root mean square error (RMSE) (range 83 - 2846) Generalised linear models (GLM) (linear regression with elastic net penalisation) performed better for all outcomes excluding month-12 pain, motor and AISA sensory with higher R^2 (range 0.32 - 0.904) and lower RMSE (range 11 - 45) (Figures 3.3 and 3.4).

R^2, also known as the coefficient of determination, is a measure of the proportion of the variance for a dependent variable that is explained by an independent variable or variables in a regression model, put more simply it is a measure of how well the data fit the model, where an R^2 of 1 indicates a perfect fit. RMSE is a measure of the mean distance between the predicted values of the model and the actual values in the dataset, and is formally defined as follows: $\sqrt{\sum_{i=1}^{n} \frac{(\hat{y}_i - y_i)^2}{n}}$.

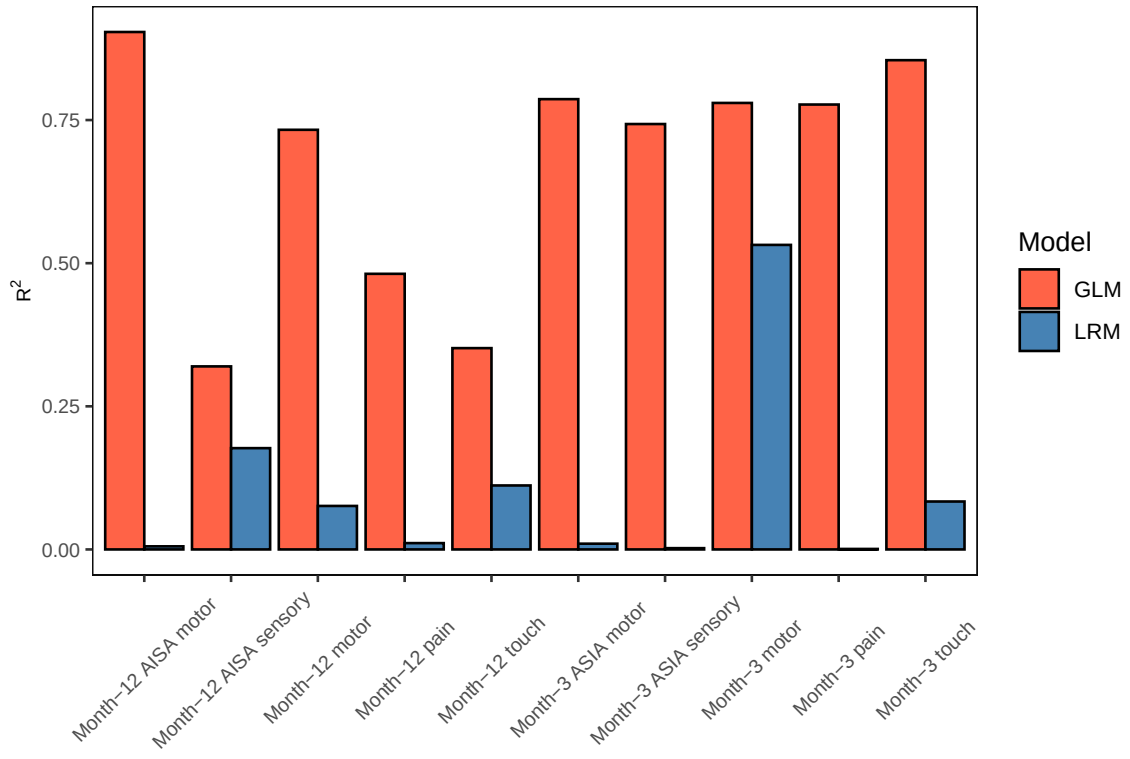


Figure 3.3: R^2 for models of neurological outcome at discharge and 12 months post-injury for preliminary study.

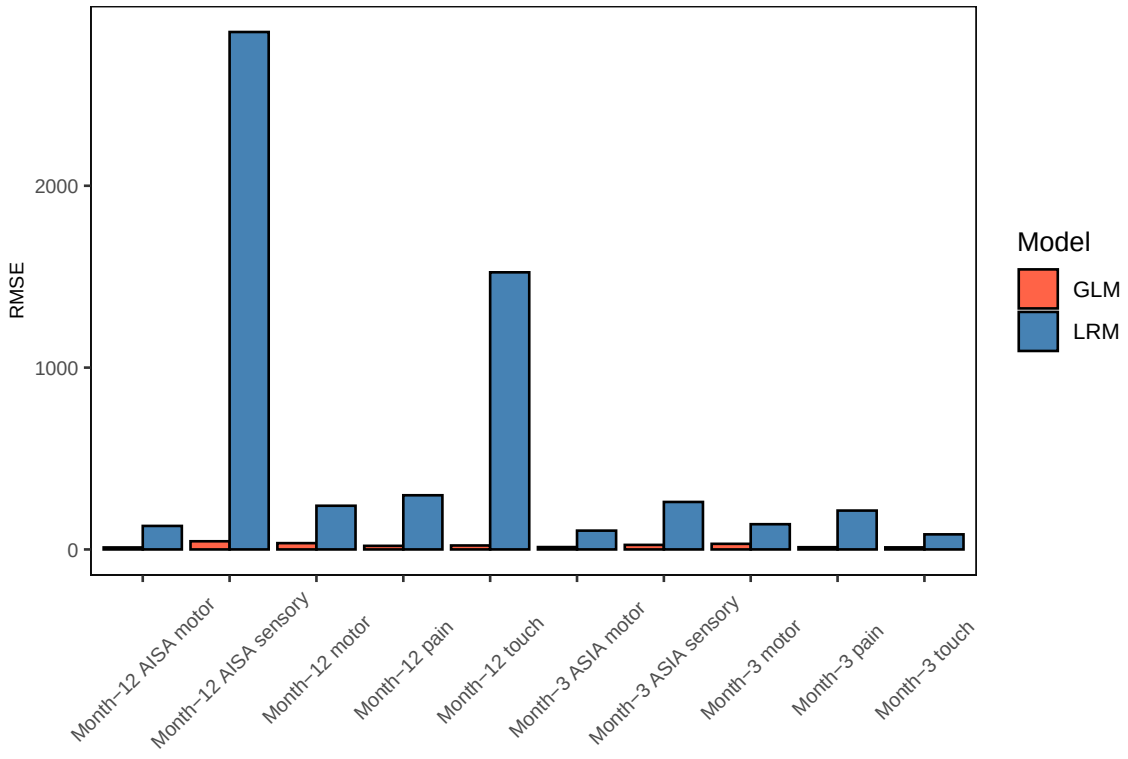


Figure 3.4: Root mean square error (RMSE) for linear regression models with and without elastic net penalisation (GLM and LRM respectively) of neurological outcome at discharge and 12 months post-injury for preliminary study.

3.3.2 Follow-up study

Multiple regression models of the AIS motor and sensory scores, and of SCIM, at discharge (mean 138 ± 71 days post-injury) and approximately 12 months post injury (mean 422 ± 148 days post-injury) were built (Tables @ref(tab:glm-model-coeffs-table, A.5 & A.6). Both LRM and GLM models were also applied to this data in the same manner. The modelling techniques performed similarly (GLM R^2 range 0.67-0.82 and RMSE range 12-16, LRM R^2 range 0.64-0.81 and RMSE range 12-16) (Figures 3.5 and 3.6)

Please see the appendix section A.2 for model coefficients.

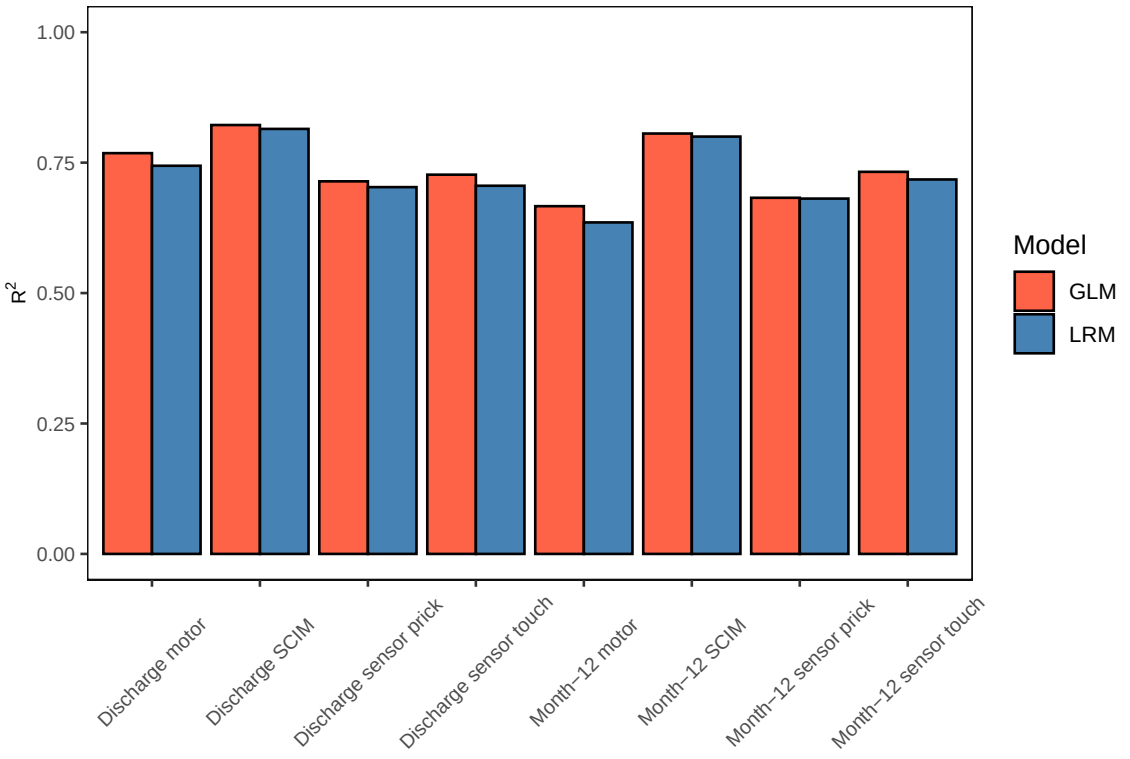


Figure 3.5: R^2 for models of neurological outcome at discharge and 12 months post-injury for follow-up study.

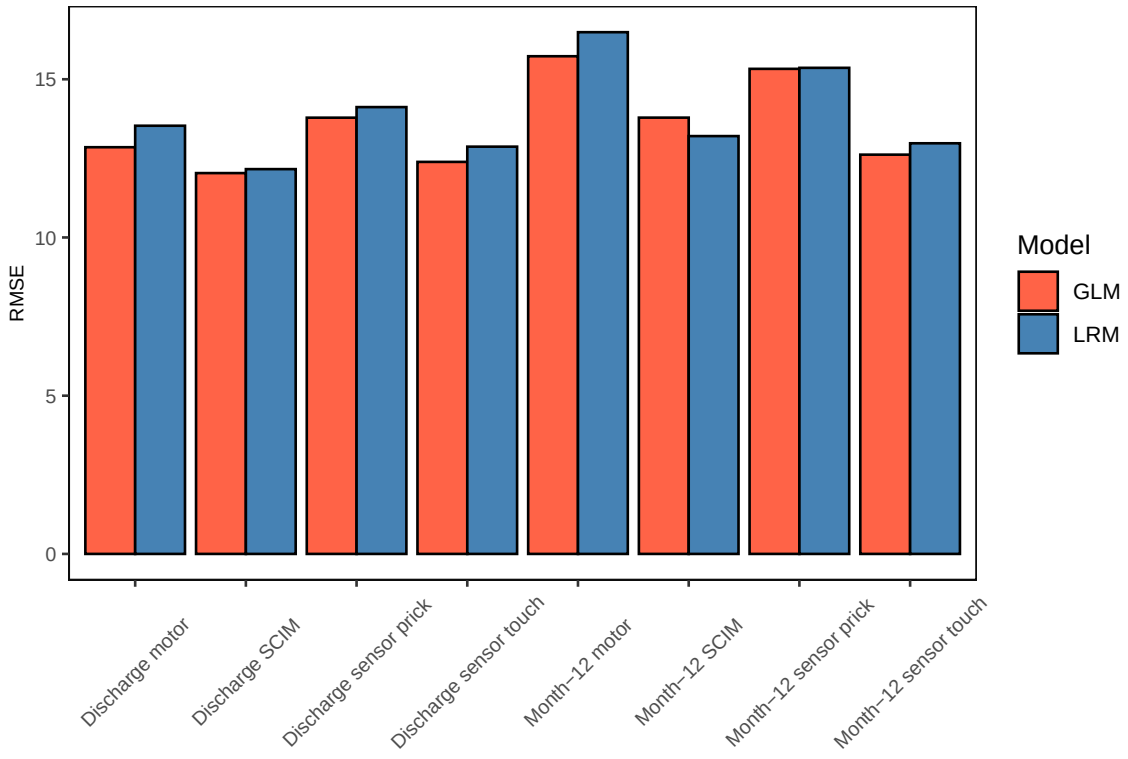


Figure 3.6: Root mean square error (RMSE) for linear regression models with and without elastic net penalisation (GLM and LRM respectively) of neurological outcome at discharge and 12 months post-injury for follow-up study.

3.3.3 Model features

With respect to model features, AIS measures of initial neurological function were the most consistently conserved features and the most powerful predictors of outcome measures for the generalised models in both the preliminary data and follow-up cohort (Table 3.1). Initial SCIM was also included for all the models of SCIM outcome for both techniques, and in models of AIS motor score for GLM models in the follow-up cohort. The blood markers, AP, albumin, CRP, haematocrit, haemoglobin, mean cell haemoglobin, mean cell volume, monocytes, platelets, WBC, ALT, creatinine, GGT, potassium, sodium, total bilirubin, and urea were significant (P-Value < 0.05)/included in one or more models.

For the linear regression models, the AIS grade on admission was the only feature that was statistically significant (P-Value < 0.05) in all models except 12-month SCIM (Table 3.1). The initial measure of the model target, so the initial AIS motor score for the models of discharge and 12 month AIS motor for example, was also significant in all models (Table 3.1). Other significant features that were not blood measures included diabetes and smoker status, age at injury, time until first blood test from injury, the neurological level of injury, gender, and the presence of fracture at the injury site (Table 3.1). Regarding blood measures, ATL, albumin, AP, CRP, creatinine, GGT, haematocrit, haemoglobin, magnesium, mean cell haemoglobin, mean cell volume, monocytes, phosphate, platelets, potassium, prothrombin time, RBC, total bilirubin, total protein, urea and WBC were all significant in one or more of the models (Table 3.1).

Table 3.1: Counts of model feature occurrence. For unpenalised linear regression (LRM) statistically significant (P-value < 0.05) features are included. For penalised models (GLM) features that were not penalised to 0 are induced

Model feature	GLM	LRM	GLM-Prelim
(Intercept)	8	8	10
Admission AIS grade B	2	2	1
Admission AIS grade C	6	6	6
Admission AIS grade D	6	6	5
Admission AIS grade E	0	0	1
Age at injury	2	2	4
Alanine Transaminase (u/L)	2	0	0
Albumin (g/L)	1	0	0
Alkaline Phosphatase (u/L)	1	0	0
C-Reactive Protein (mg/L)	1	0	0

Table 3.1: Counts of model feature occurrence. For unpenalised linear regression (LRM) statistically significant (P-value < 0.05) features are included. For penalised models (GLM) features that were not penalised to 0 are induced *(continued)*

Model feature	GLM	LRM	GLM-Prelim
Central cord syndrome	0	0	6
Comorbidities	0	0	5
Creatinine (umol/L)	4	2	0
Diabetes (either type)	0	0	2
Drinking yes	5	1	0
Fracture	1	1	0
Gamma GT (u/L)	1	0	0
Haematocrit (L/L)	4	0	1
Haemoglobin (g/L)	5	0	0
Initial motor	8	6	4
Initial scim	4	2	0
Initial sensor prick	8	2	5
Initial sensor total	0	0	6
Initial sensor touch	5	3	9
Initial total motor	0	0	2
International Normalized Ratio	0	0	1
Lumbar injury	2	0	1
Magnesium (mmol/L)	0	0	1
Mean Cell Hb (pg)	4	0	0
Mean Cell Volume (fL)	6	0	1
Medications	0	0	1

Table 3.1: Counts of model feature occurrence. For unpenalised linear regression (LRM) statistically significant (P-value < 0.05) features are included. For penalised models (GLM) features that were not penalised to 0 are induced *(continued)*

Model feature	GLM	LRM	GLM-Prelim
Monocytes (10*9/L)	7	0	0
Neuro level T	1	0	0
Paraplegia	0	0	1
Phosphate (mmol/L)	0	0	1
Platelets (10*9/L)	1	0	0
Potassium (mmol/L)	1	0	0
Previous ailments	0	0	1
Prothrombin Time (s)	0	0	1
Red blood count (10*12/L)	0	0	1
SexMale	2	1	0
Smoker status known	1	0	0
Smoker status unknown	0	1	0
Surgery	1	0	0
Time from injury to 12-month neurology	0	0	3
Time to first blood test (Days)	0	2	0
Total Bilirubin (umol/L)	5	3	0
Total Protein (g/L)	1	0	0
Type 1 diabetes	2	0	0
Type 2 diabetes	3	1	0
Urea (mmol/L)	1	1	1
White blood count (10*9/L)	1	0	0

3.3.4 Model performance

With respect to model predictions, both modelling techniques performed similarly when predicting against the test data (Figures 3.7).

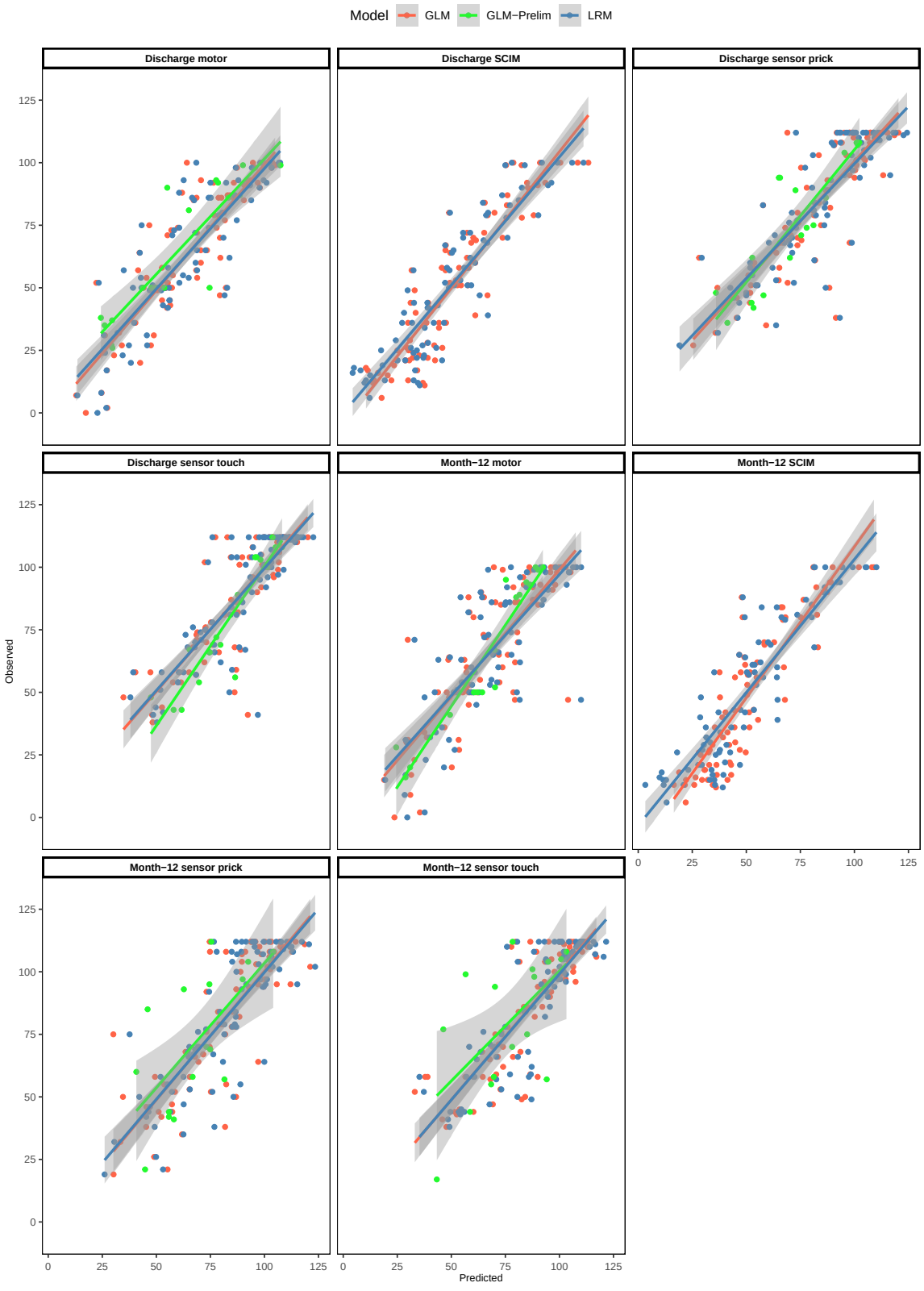


Figure 3.7: Predicted scores as compared to the observed scores in the testing data split.

3.4 Discussion

In these studies, we have analysed a plethora of routinely gathered blood analyte data in a cohort of SCI patients and compared this to longitudinal neurological outcome measures following SCI. If these routine measures can offer prognostic value they would add value to the monitoring of patient response to care and/or therapies at no additional cost to healthcare providers. They could also offer insight into the underlying pathophysiology of SCI, potentially directing further research into the relevant biochemistry.

3.4.1 Preliminary study

With respect to correlations between measures and outcomes, previous studies have linked hypokalemia with trauma.[409] Therefore, the observed correlation in the preliminary study between potassium and total motor score at three months is not unexpected. In a previous study involving 591 SCI patients, albumin measured at 1, 2 and 4 weeks post-injury was suggested to be an independent marker of longer-term (1 year post-injury) AIS motor score, which may explain the correlation with 3 month outcome measures (except for pain scores).[189] Other literature has also reported notable alterations to RBC, haemoglobin, and haematocrit values in non-traumatic SCI, and has demonstrated that lymphocytes decreased over time in traumatic SCI.[410]

The predictive model generated in our preliminary study based on the PCA analysis found that the "liver function" factor, which is made up of AP, ALT, and GGT, and the "acute inflammation and liver function" factor, composed of CRP and total bilirubin, added prognostic value to this model. However, it needs to be noted that although these factors added significant value to the models, the greatest R^2 achieved was only 4%. Nonetheless, it appears that blood analytes related to liver function may have the potential to significantly predict neurological outcome for SCI patients. In particular,

when we ran the model with the individual analytes, one of the contributing analytes in the "liver function" factor, ALT, appeared to be the most predictive (R^2=15%) of the blood measures, although it must be stressed that this analysis is not statistically robust, because of the small cohort sample size. It may be possible that the lack of patients in this study, and hence the use of PCA, may have reduced the effects of individual analytes. In fact, the PCA resulted in some blood analytes not being considered in the predictive model.

Interestingly, none of the components of the "liver function" and "acute inflammation and liver function" factors, which were the only factors to add predictive value to the outcome models in this study, directly correlated with any outcome measures. This highlights the importance of taking into account clinical factors that might impact the patient's outcome neurology and blood measures in generating a predictive model. An ideal predictive model for outcome neurology in SCI patients would also take into account the patient's body mass index (BMI) and perhaps other information such as "dry" biomarker data garnered from clinical imaging.

3.4.2 Follow-up study

With regards to the follow-up study, most of the significant blood measures in the linear regression models (creatinine, total bilirubin, total protein, and urea) are typically associated with liver and kidney function. Urea for example is commonly used as an indicator of kidney function, but may be altered due to hydration status as well.

With the exception of smoker status and time to first blood test post-injury, all of the same features were included in the GLM models and the linear regression models, but other related bloods were also included (ATL, albumin, AP, CRP, GGT, haematocrit, haemoglobin, mean cell haemoglobin, mean cell volume, monocytes, platelets, potassium and WBC), such as platelets and WBC, form part of the complete

blood count, which is likely related to the initial injury severity via blood loss due to bony soft tissue or visceral injury, gastrointestinal bleeding, and/or surgery.[411] Monocytes were included in SCIM and AIS sensory touch score GLM models at both time points. Similar to the components of the complete blood count, monocytes levels may be indicative of anaemia (if low), but have also been associated with hepatitis and inflammatory diseases (if high).[412,413] Estimated serum creatinine, based on glomerular filtration rates, are typically used in the evaluation of renal function.[414,415] SCI patients have also been found to have an increased risk of renal deterioration and are recommended to receive lifelong, regular renal and upper urinary tract examinations after injury.[416,417] SCI has been found to lead to systemic inflammation which can in turn cause secondary organ complications, including in the liver, kidneys and lungs, which may explain why these blood measures are useful in predicting outcomes.[418–422] Whether these secondary complications are the result of a more deleterious physiological response to injury, pre-existing health status or some combination thereof is still unclear however.

Importantly, the blood measures alanine transaminase, alkaline phosphatase, CRP, GGT and total bilirubin, highlighted in the follow-up study were also found to be significantly predictive of AIS scores in our preliminary study. Two factors "liver function", consisting of alanine transaminase, alkaline phosphatase and GGT and "liver function and inflammation" consisting of CRP and total bilirubin added statistically significant value to models of AIS touch and pain scores at 3-months post injury, and AIS motor and pain scores at 12-months.[271,423,424] Total bilirubin in particular was included in 5 out of 8 penalised models and was significant in 3 of the non-penalised models. This provides further evidence that liver function is relevant to neurological recovery in SCI. Interestingly, alanine transaminase, alkaline phosphatase, GGT and albumin were only retained in the models of SCIM. This could be because these markers indicate liver status, which in turn typically reflects general metabolic

health.[425] Therefore, aberrant ALT and GGT values may be a proxy measure of poor metabolic health or systemic inflammation.

Type 1 and type 2 diabetes was also significant/included in 2 and 4 of the 16 models built in this study respectively, which may also reflect the relevance of general metabolic health with regards to recovery. Serum albumin has also been previously found to be significantly predictive of AIS grade improvement up to 52 weeks.[189] Platelets and gender were also only retained in models of SCIM, suggesting they do not add predictive value to AIS measures directly, but do for the SCIM assessment. Previous studies have suggested that gender does not significantly correlate with functional neurology or independence, which may appear to contradict this results.[426,427] However, it may be that some elements of the SCIM questionnaire have an implicit gender bias; self-catheterisation for instance may be easier for males, and so they are able to obtain slightly higher scores than females, even at a similar level of neurological function (as determined by AIS scores). In future revisions of the SCIM assessment further attention should be paid to this aspect , and potentially consider adjusting scores based on gender, if not change the weighting of questions themselves.

Surgery was also only found to be a significant predictor of SCIM at discharge in the GLM models. This suggests surgery does not have a substantial influence on AIS outcomes. It should be stressed that the MCSI favours a conservative approach to care of SCI patients, only choosing to operate in the most extreme cases and so both the rate and type of surgery given to this cohort may differ from other spinal centres.[428] Interestingly, whether the injury was traumatic or not was retained or significant in any model. Despite the very distinct pathophysiology of non-traumatic injuries, this data suggests trauma is not a strong predictor of AIS motor or sensor score outcomes, and the prediction of SCIM may be due to secondary injuries sustained with the initial trauma.[429] Prior studies have also observed similar functional outcomes between traumatic and non-traumatic injuries.[430]

3.4.3 Exploring the link between SCI and the liver

ALT is an enzyme released from damaged hepatocytes, and is a key clinical marker of fatty liver disease.[431] The preliminary study found it to be the only routine blood measure to predict neurological outcome in the form of AIS motor score at both 3 and 12 months (Table A.2). Further, when grouped by PCA, "liver function" markers (AP, ALT, GGT) were predictive of neurological outcome, as were total bilirubin and CRP in the second predictive group "acute inflammation and liver function." SCI is known to lead to a systemic inflammatory response that can result in secondary organ complications, particularly in the liver, lungs, and kidneys.[418–420] In addition, a rat contusion model study found ALT to be significantly raised in the 21 days following injury, coupled with excess lipid accumulation and increased expression of pro-inflammatory genes.[432] Interestingly, this study also detected liver inflammation following lumbar SCI, whereas previous studies in rats had shown that liver inflammation can occur within 30min of SCI, and that its severity correlates with level of injury.[420,432,433]

AP, another analyte of the predictive "liver function" factor, is an enzyme that plays a significant role in bone mineralisation, and has four isoenzymes, which are intestinal (IAP), placental (PLAP), germinal (GCAP), and tissue nonspecific (TNAP).[434–438] TNAP is the most abundant isoenzyme in the blood and is primarily derived from the bone, kidneys, and liver, but is also found in neuronal tissue.[439] Higher levels of AP have been associated with the presence of heterotopic ossification (HO) following SCI, although other, smaller studies have found no association.[440–442] HO is the abnormal formation of lamellar bone within extraskeletal soft tissues, where bone does not typically exist.[443] HO occurs in ~20% of SCI patients, and is a deleterious complication which may impact on both rehabilitation and outcome measures for the patient.[444] No note of this clinical feature was made during this study, but HO potentially needs to be recorded for future haematological biomarker studies. TNAP has been

shown to be elevated in Alzheimer's disease and in brain injury patients.[445,446] A TBI study using a rat blast and weight drop model found that the injury resulted in a decrease in TNAP expression and activity in the brain and plasma at 6 and 24h post-injury.[447] Further, exogenous administration of AP has been shown to be beneficial, including improvements to renal function, for inflammatory disorders, such as sepsis, in humans and in murine models, and hence is worthy of further investigation in SCI patients.[448,449]

GGT, the final component of the predictive "liver function" factor, is a liver enzyme found on the plasma membrane of most cells and organ tissues, and it is frequently used as a marker for liver disease and more recently, many other conditions such as cardiovascular disease.[450–452] Little is known of the possible role of GGT following SCI, but positive correlations between GGT and age, BMI, smoking, and alcohol consumption have been identified.[453,454] *In vivo* and *in vitro* studies have also found overexpression of GGT to have a harmful effect on bone metabolism by accelerating bone reabsorption and causing osteoporosis.[455,456] Another study analysed the serum of 2415 Finnish men with good cognitive function, and found GGT to be positively associated with future risk of dementia.[457] It has been proposed that GGT may contribute to dementia risk and poorer bone metabolism because of its pro-inflammatory and pro-oxidative properties.[458] Future studies could compare GGT levels before and after SCI to establish whether elevated GGT before injury is associated with worse recovery.

The second factor found to have prognostic value from the PCA analysis was the "acute inflammation and liver function" factor, which consists of total bilirubin and CRP. Bilirubin is the breakdown product of heme and circulates in plasma conjugated to albumin until it is processed by hepatocytes and ultimately excreted from the body. The aforementioned systemic inflammation that follows traumatic SCI and impacts liver function is likely to influence blood bilirubin levels.[418–420]

CRP is one of the key proteins in the acute inflammatory response.[459] However, CRP is more specifically known to be indicative of acute infection, as opposed to the chronic low-grade inflammation associated with atherosclerosis, for example.[460–462] CRP levels have been shown to be elevated in SCI patients in the absence of infection and regardless of injury level or duration, when compared with able-bodied controls.[463] Other pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) have been found to be elevated in the serum of SCI patients compared with uninjured controls during the subacute phase of injury (2-52 weeks post-injury).[198] Further, patients who had lower TNF-α at 9h post-injury experienced improved neurological function compared with SCI patients with higher TNF-α.[197] These results suggest that inflammatory markers may hold prognostic value for the neurological progression of SCI.

The low-grade systemic inflammation associated with obesity in particular, may be an important confounding variable for additional prognostic models.[464–466] Some studies have found SCI to induce hepatic lipid deposition and inflammation, within 3 months of injury in rats, which is symptomatic of non-alcoholic steatohepatitis (NASH), the hepatic presentation of metabolic syndrome.[432,467] Metabolic syndrome is also more common in SCI patients than the general population and, SCI patients consequently have an increased risk of diabetes, stroke and heart disease.[462,467–469]

As four out of the five analytes that make up the two PCA components that were found to be predictive of neurological outcome are markers of liver function, and the remaining analyte is reflective of inflammatory status, this suggests that the liver may play a greater role in recovery following SCI than has been previously appreciated. Further research is needed to establish the role of the liver in SCI, particularly whether the liver is causally implicated in functional recovery, or if it is merely a proxy indicator of systemic inflammation inhibiting healing. Once this association is established, clinicians could consider monitoring the liver function of

SCI patients more closely, perhaps attempting to restore and/or maintain healthy parameters in the interim. Example could include minimising the use of hepatotoxic drugs where possible, adjustments to diet or the use of supplements.

Larger cohort studies, should focus on improving neurological outcome prediction, which is likely to be most useful for patients with AIS B and C SCI, because of the decreased power of the initial AIS score in predicting outcome in these subsets of SCI patients.[470] Further, there is scope to assess whether adjusting some of these routine measures improves outcomes for SCI patients. However, whether a targeted approach to alter the levels of these molecules can cause a concurrent change in a SCI patient's neurological outcome remains to be assessed, in appropriate randomised controlled clinical trials.

3.4.4 Statistical considerations

Whilst there has long been a dogma that 10 events per variable (EPV) is sufficient to avoid "overfitting" of the data when modelling, more recent studies have argued that there is no rationale for this.[471,472] As there were 30 blood analytes in the preliminary study, and only 82 patients (ideally ≥ 300 patients would have been used), PCA was conducted to see whether the blood and neurology measures could be reduced into factors enabling a more robust predictive model to be generated from the data. This meant that the contributions of some blood analytes to the factors were not included, and their individual contribution to the predictive model was not fully addressed.

The follow-up study included 417 patients and 45 variables, we also investigated the impact of modelling with and without variable selection in the form of elastic net penalisation. The follow-up study aims to address this somewhat, with both a larger cohort to better elucidate the impact of all the individual blood analytes routinely measured in SCI patients. The purpose of the preliminary study was to provide an indication as to which blood analytes may have the most potential for future use in

predictive neurological outcome models.

In the follow-up study a standard linear regression model with no variable selection performed very similarly to GLM with elastic net penalisation with respect to R^2 and RMSE, though the R^2 of GLM was slightly higher and RMSE slightly lower for all model targets (Figures 3.5 and 3.6). This suggests that elastic net penalisation does not provide a substantial boost to overall model performance at this sample size relative to linear regression. However, there was a difference in the variables each model utilised, demonstrating that appropriate care must to afforded to variable selection methodology.

An important limitation of both studies here is the volume and veracity of the data used in model building to borrow the parlance of the four V's of big data (Figure 3.2). A larger and more diverse patient cohort will always lead to a more robust and widely applicable model, and whilst there was enough to build linear regression models, a larger dataset (>5000) could allow for robust logistic regression models to predict a change in AIS grade. Furthermore, the data used here contained missing values, and whilst these were imputed to have minimal effect on model performance, it is still preferable to have a complete dataset. Relatedly, medication details were not included in the follow-up study due to the significant number of variables this would have added, undermining the power of the study. Whilst only a small subset of the cohort received medications that could impact the blood measured used, and they correlated strongly with initial injury severity, suggesting they'd add limited predictive value, they are still a potential confounder, and a larger validation study with adequate power should include medication data to account for this. Finally, an independent external validation of these models on separate data would be desirable, particularly for the GLMs as it is difficult to obtain robust estimates of bias in penalised regression, making standard errors and confidence intervals inappropriate.[473]

3.5 Conclusion

The results from the preliminary study suggest that routine blood analytes when statistically incorporated into "linked" factors can provide prognostic value for AIS motor and sensory score in SCI patients at 3 and 12 months post-injury. In particular, markers of liver function were found to add the most predictive value. The follow-up study corroborates these findings and highlights the relevance to routine bloods in predicting SCIM-III outcomes, and a potential implicit gender bias in the SCIM-III questionnaire. This indicates that maintaining a healthy liver function acutely following SCI may be a key rehabilitative target in order to achieve optimal neurological recovery in the long term. Clinicians could consider possibly limiting use of hepatotoxic drugs in treatment where possible as it may be relevant to neurologic functional recovery. More research is needed to establish whether or not the relationship between SCI recovery and liver function is causal. Ultimately these finding need to be validated on a larger independent cohort before any clinical recommendations can be made.

Chapter 4

Characterisation of the human plasma proteome following Spinal Cord Injury

4.1 Introduction

As discussed in prior chapters, the precise nuances of pathophysiology post-SCI are not fully understood, particularly with respect to heterogeneous neurological recovery. To further investigate potential blood biomarkers we characterised the plasma proteome of SCI patients. We use an unbiased shotgun proteomic approach to investigate differentially expressed proteins, seeking to both identify further biomarkers, and assess whether the proteomes reflect the results of metabolic involvement with neurological outcomes from the prior chapter (3.3) and respective publications.[271,408]

4.1.1 Unbiased biomarker discovery approaches

Proteomics and metabolomics

Proteomics is the analysis of the entire protein complement of a biological sample, which could be a population of cells, a fluid such as blood, lymph or saliva; or a whole tissue/organism. Mass spectrometry (MS) is incorporated into most proteomic techniques. Generally, the proteome of a sample is separated as it is difficult to analyse a pure sample due to the shear complexity of the proteome; gel chromatography and liquid chromatography-based separations are often utilised to this end. Following this extensive separation, proteins are characterised by MS analysis of either enzymatically digested proteins (bottom-down) or of intact proteins (top-down). Proteins are then identified based on their mass as compared to masses calculated from genome data.[474] Proteomic techniques can be divided into labelled and unlabelled methods. In theory, relative protein abundancies could be found by comparing MS signal intensities across different samples. In practice however, prior handling of the samples can introduce variation and there can be fluctuations in MS signalling causing run-to-run errors. Thus, labelled techniques were conceived to overcome these issues.

Labelled proteomic techniques Isobaric tag for relative and absolute quantification (iTRAQ) is an example of a chemical labelling technique which is widely used in discovery-based proteomics (Figure 4.1).[475,476] In iTRAQ, peptides are labelled at the N-terminus and at the ϵ side chain of lysines, and it is a multiplex system (4-plex and 8-plex) where every peptide ion selected for fragmentation generates sequence and abundance data for proteins from up to 8 samples.[477,478] As the tags in iTRAQ are isobaric, the MS signal is the sum of the peptide contribution from all samples, which improves sensitivity.

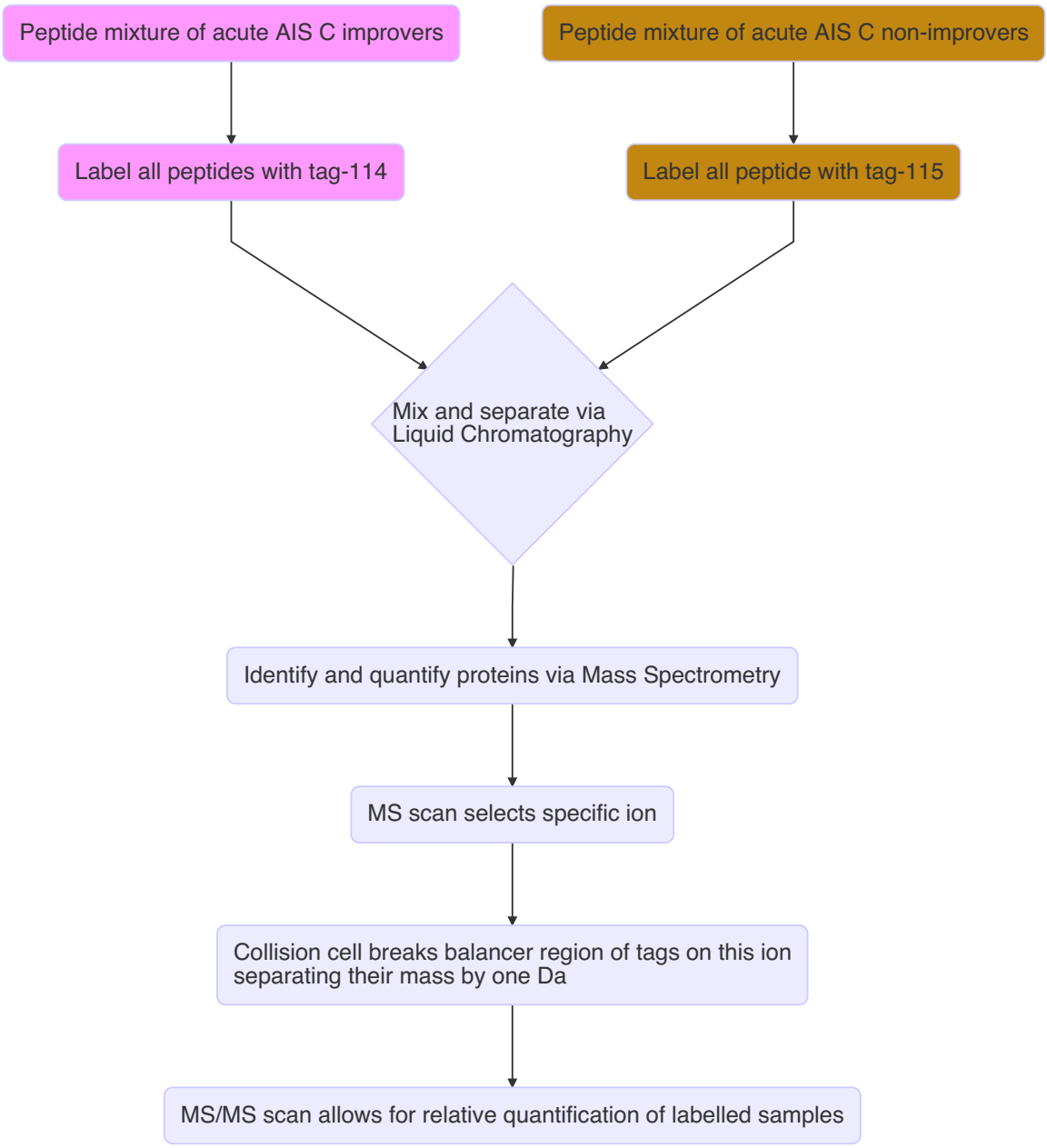


Figure 4.1: Flow diagram illustrating part of the iTRAQ sample processing and subsequent scans in the mass spectrometer.

In the initial scan (MS level 1, or MS) the mass spectrometer scans all the ions injected into the system. It will then select a specific ion from this scan, which will correspond to a particular peptide, for a further scan (MS level 2, or MS-MS). As the tags are isobaric, this peak will represent that peptide from all samples. Prior to the second scan the specified ion is passed through a discrete part of the instrument

called a collision cell. Collision cells vary between instruments, but typically they will contain a inert gas such as nitrogen at a higher relative pressure. As the ions pass through the cell they collide with the gas and are further fragmented. Crucially, the balancer region of the iTRAQ tag is lost here, so the tags are no longer isobaric and instead separate by exactly 1 Da (Figure 4.2). Therefore when the newly fragmented mixture is scanned, the relative abundance of that peptide for each sample can be measured.

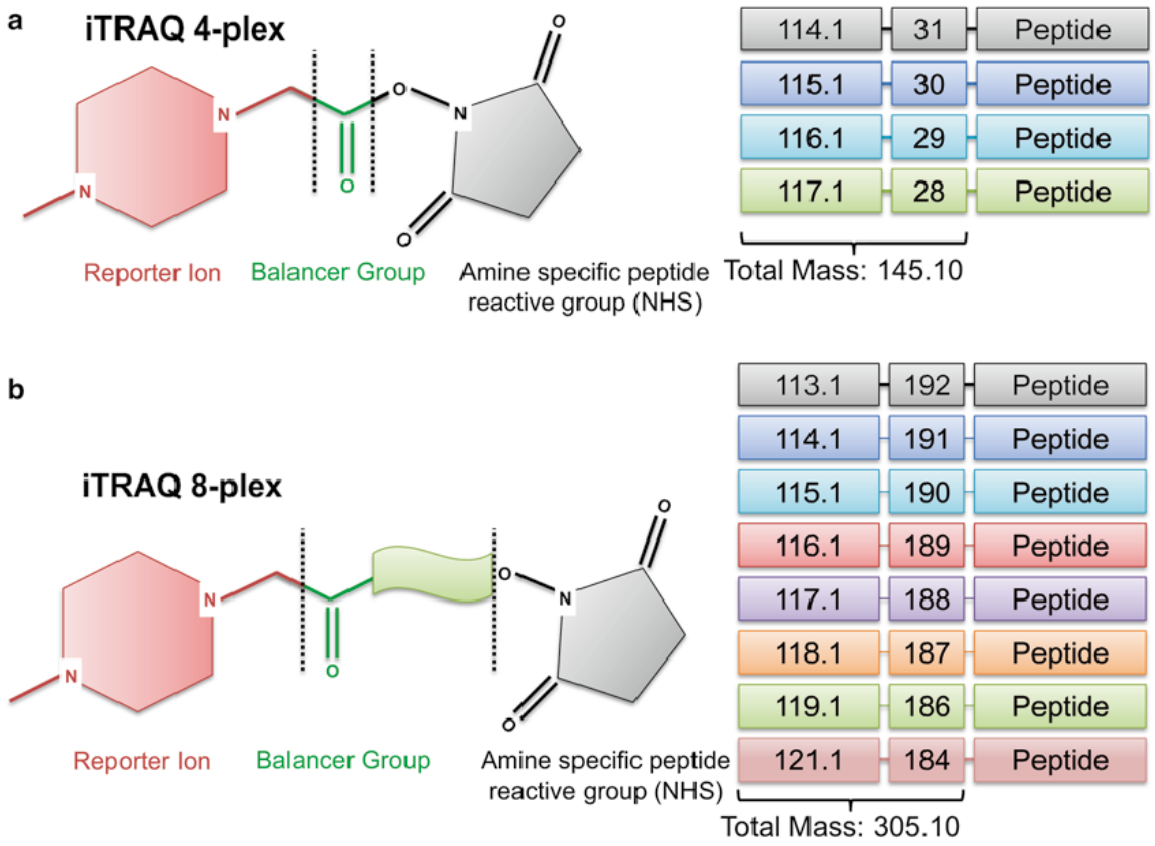


Figure 4.2: Cartoon of iTRAQ tags. The tags contain three regions: a reporter ion, balancer group and the amine specific peptide reactive group. The mass of the reporter ions vary by 1 Da and the balancer group compensates such that the total mass is of each tag is 145.10 Da for 4-plex and 305.10 Da for 8-plex, thus the tags are isobaric. Taken from Creative Proteomics Blog

Alternative isobaric tags In addition to iTRAQ, Tandem Mass Tag(TMT) are another popular commercially available isobaric tag. Originally introduced and

later improved by Thompson *et al*, TMT is currently able to encode up to 11 different conditions with suitable mass spectrometers.[479] Both tag systems work in the same fundamental way, all isobaric tags contain a functional group that enables covalent peptide attachment, typically via the amines on a peptide's N-terminus or lysine side-chains. Furthermore, all isobaric tags contain a reporter group and a mass balancer group. The total number of heavy isotopes in the tag is constant, thus they are isobaric in the MS1 spectrum, though the distribution of these isotopes between the reporter and balancing group differs for the different conditions. A comparison of 4-plex iTRAQ, 8-plex iTRAQ and 6-plex TMT found 4-plex iTRAQ to provide a higher peptide identification, concluding that 8-plex iTRAQ may suffer due to the appearance of fragment ions form the larger tag in the MS2.[480] There are also several non-commercial alternatives isobaric tags including N, N-Dimethly Leucine tags, Combinatorial isobaric Mass Tags, SulfOxide Tag and Easily Abstractable Sulfoxide-based Isobaric tag.[481]

Stable isotope labelling in culture (SILAC) is an example of a metabolic labelling technique first adapted for proteomic analysis in 1999 by labelling bacterial proteins with nitrogen.[482] SILAC typically requires that cells be grown in the media that contains the isotope labelled amino acids for at least 5 divisions so that the labels can be incorporated into the proteins (Figure 4.3). At this point any differential experimental conditions would usually be applied. One of the strengths of SILAC is the ability to analyse posttranslational modifications in a site specific manner, which can give insight into the network of cell signalling.[483]

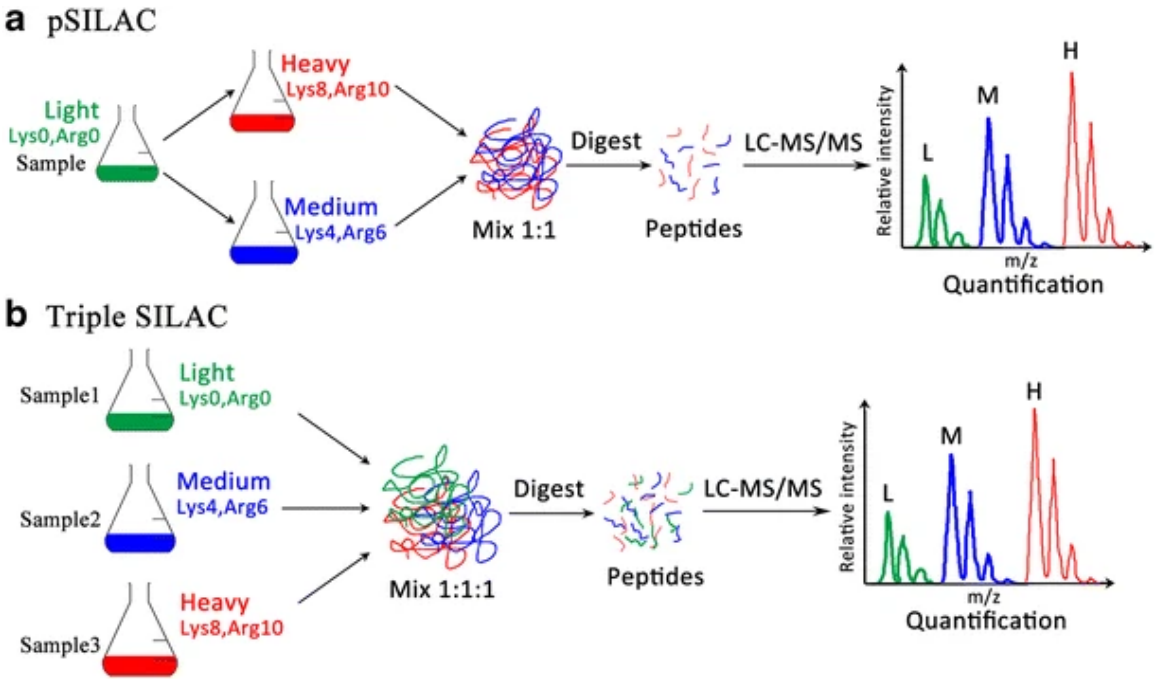


Figure 4.3: Outline of SILAC technique. Cells are cultured with coded amino acids (controls are cultured with low nitrogen media and the experimental group is cultured with nitrogen-enriched media). The cells are then combined, lysed and proteolysis is done after denaturation and reduction. MS is then performed on the samples. Taken from Chen et al. (2017).[484]

Label-free proteomic techniques Label-free techniques have the inherent advantages of a simpler experimental setup compared to labelled techniques. Spectral counting (SC) is one of the two major label-free techniques. SC provides relative quantification by the number of spectra produced by a protein in multiple liquid chromatography MS/MS runs.[485] The second technique is based on precursor ion intensity from extracted ion chromatography (XIE), which is the plot of intensity versus retention time of a particular m/z value.

Pooling of samples The time and financial costs of proteomic analysis is currently high enough to potentially limit the number of biological replicates needed to appropriately power a study.[486] Available material per biological sample can also be a limiting factor, particularly for cases where samples have low protein concentrations,

such as CSF. Conversely, a high number of biological replicates has been found to decrease global protein spot matching efficiency in 2-D electrophoresis experiments.[487] Pooling of samples may address these limitations. Theoretically, the reduction in measured biological variance should increase statistical power, and if pools represent average protein abundance, the primary differences and similarity between groups may be easier to identify. This has been successfully tested in microarray studies.[488,489] Mathematically, this is defined as Jensen's inequality whereby: if the pool value equates to the average value of the individual sample that comprises the pool, then the assumption of biological averaging holds true and pooling is both suitable and beneficial.[490]

However, pooling may introduce a loss of information, particular through dilution effects on low abundance proteins.[491] Some have shown sample pooling to work well so long as appropriate experimental design is used.[492] By contrast, a more recent study examining top-down proteomic techniques concluded that sample pooling can decrease statistical power, and increase false positives.[493] To summarise, pooling of samples can reduce both cost and sample volume required, and, under ideal conditions, highlight trends between groups rather than individual differences. Conversely, pooling results in a loss of information with respect to biological variation, any unknown subpopulations within a group/s may be lost and proteins identified will only represent the largest changes, with any smaller differences also being lost. Ultimately, pooling of samples necessitates validation, typically via immunoassays, to address these limitations, but it can be a valuable technique, particularly in early discover work, and/or in cases where sample volumes are limited.

Here, two 4-plex iTRAQ experiments are used to access relative levels of proteins in plasma from ASIA grade C SCI patients (total $n = 17$) contrasting those who experienced an AISA grade conversion ($n = 10$), and those who did not ($n = 7$) collected within 2 weeks, and at approximately 3 months post-injury (Improvers

$n = 9$ vs Non-improvers $n = 6$). Relative protein expression in AIS grade A ($n = 11$) and grade D ($n = 11$) patients was also examined. To address the limitations of pooling samples, key biomarkers of interest were validated with ELISA immunoassays. This unbiased, high-throughput approach was used to identify novel biomarkers, and molecular pathways associated neurological recovery post-SCI.

4.2 Methods

Please refer to Material and Methods chapter sections 2.2 and 2.2.3, and 2.3 for details of the ethics/cohort and blood collection respectively. For sample preparation and data analysis methods, please see sections 2.3.2 and 2.4.3 respectively.

4.3 Results

In the interest of brevity, only the plots of acute and subactue AIS C improvers VS non-improvers are included here, please see the appendix for the other comparisons (section A.3.1).

4.3.1 Comparing OpenMS and ProteinPilot

A total of 79 and 64 unique, largely overlapping, proteins were identified across both runs for OpenMS and ProteinPilot respectively, though OpenMS consistently produced more proteins for each group (Tables 4.1, 4.2 and Figure 4.4). AIS C improvers had 18 more abundant proteins, and 49 less abundant proteins at the acute phase with OpenMS, as opposed to 8 and 20 with ProteinPilot (Figure 4.5). At the subacute phase, AIS C improvers had 34 and 9 proteins of increased abundance were found, whereas 34 and 6 proteins were less abundant, for OpenMS and ProteinPilot respectively (Figure 4.5).

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

Table 4.1: OpenMS $\log_2$ 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	-0.9032	-0.1018	-0.6088	0.1926	0.2253	0.7937	-0.3498	0.4440	-0.5750	0.2187
A2M	-1.0386	-0.2464	-0.6761	0.1161	-1.2301	1.4248	-1.6030	-0.1782	-0.3729	1.0519
AFM	-0.3788	-1.2249	0.4815	-0.3645	0.5518	1.1924	-1.2566	-0.0642	-1.8084	-0.6160
AHSG	1.1795		-0.5545							
AMBP	0.6562	-0.3433	0.8607	-0.1389	-0.9023		1.2038		2.1061	
APCS	0.1498	0.2109	-0.0114	0.0497		0.3557			-0.0495	0.3063
APOA1	-0.1817	-0.6924	-0.2338	-0.7444	-0.7677	0.6941	-1.3173	-0.6232	-0.5496	0.1446
APOA2	0.0900	-1.1461	-0.6668	-1.9029						
APOA4	0.1296	0.9637	-1.2313	-0.3972	-1.3254	0.7876	-1.3347	-0.5471	-0.0093	0.7783
APOB	0.1379	-0.0164	-0.6333	-0.7876	-0.8570	0.5260	-1.2346	-0.7086	-0.3775	0.1485
APOE	-1.2134	0.2931	-0.6884	0.8180	-0.9078	0.7747	-1.5477	-0.7731	-0.6399	0.1347
APOH	-0.3602	-0.7025	-0.6445	-0.9867	-0.9997	2.8144	-1.0092	1.8052	-0.0095	2.8048
APOL1	-1.1791	-0.5194	-1.0440	-0.3843	-0.1153	0.5653	0.1299	0.6952	0.2452	0.8105
APOM	-1.2168	-0.6820	0.6935	1.2283		0.6562			0.6665	1.3227
ATRN			-1.0063							
AZGP1	1.2192	1.0252	0.0811	-0.1129	-3.3890	-3.6441	0.3703	-3.2738	3.7592	0.1152
C1QB	-0.8410	-2.0020	0.7071	-0.4539	-1.9729	1.3563	-2.0066	-0.6503	-0.0337	1.3226
C1R	-0.4335	-0.7632	0.0366	-0.2931	-0.1467	0.7976	0.3564	1.1540	0.5032	1.3008
C1S	0.0295	-0.8194	0.1680	-0.6809						
C2					-2.5581	2.5641	-2.5953	-0.0312	-0.0372	2.5269
C3	-0.7441	-0.6969	0.0652	0.1124	-1.0731	1.2388	-2.1616	-0.9228	-1.0886	0.1503
C4BPA	-0.1810	-2.4455	1.6628	-0.6017	-1.2379	1.5490	-1.8449	-0.2959	-0.6070	0.9420
C5	-0.5448	-0.2031	0.9230	1.2647	-0.7200	1.2710	-1.6769	-0.4058	-0.9569	0.3142
C6	-1.3936	1.7817	-1.3097	1.8656	-3.0452	1.7642	-3.2550	-1.4908	-0.2098	1.5544
C7	-0.9642	0.8848	-0.7827	1.0663	0.9970	0.0709	-1.1136	-1.0428	-2.1107	-2.0398

Table 4.1: OpenMS $\log_2$ 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
C8A	-0.5118	0.2737	-0.7630	0.0224	-2.8108	0.1731	-2.1285	-1.9554	0.6823	0.8554
C8B	-2.1950	0.2789	-1.5955	0.8785	-1.8944	-0.4803	-0.9598	-1.4400	0.9346	0.4544
C8G			-1.6305							
C9	-2.2199	0.4534	-1.9250	0.7483	-0.7346	0.6496	-3.2424	-2.5928	-2.5078	-1.8583
CD5L	-0.9293	-0.6205	-0.7146	-0.4057	-2.4643	0.4483	-2.3260	-1.8778	0.1383	0.5865
CFH	-1.1240	0.7407	-1.6481	0.2166	-1.0359	0.1380	-1.3260	-1.1880	-0.2902	-0.1522
CFI		0.5360		1.2578						
CLU	-1.1959	-0.8682	-0.1722	0.1555	-1.3664	0.8252	-2.1976	-1.3724	-0.8312	-0.0060
CP	-0.3892	0.2565	-0.4537	0.1920	-0.6658	0.4235	-0.2696	0.1540	0.3962	0.8197
F12	0.4852	-0.9398	0.6703	-0.7547	-0.8534	0.5550	-1.3146	-0.7596	-0.4612	0.0938
F2	-0.7493	-0.7564	0.0983	0.0912	-0.5409	1.1677	-1.5476	-0.3799	-1.0067	0.1610
FCN3		0.9645								
FGA	-0.9591	-0.5109	0.4842	0.9324	-1.0156	1.0487	-1.4708	-0.4221	-0.4552	0.5934
FGB	-0.8339	-0.1254	0.0684	0.7770	-0.8343	1.0951	-1.4647	-0.3695	-0.6303	0.4648
FGG	-1.1433	-0.0247	-0.2978	0.8208	-0.7191	0.7607	-1.0780	-0.3173	-0.3589	0.4018
FN1	-0.2796	-0.3153	0.2899	0.2541	-0.5778	1.1463	-1.2551	-0.1088	-0.6773	0.4690
GC	-0.5583	0.4051	-0.7950	0.1684	-1.8700	-0.2961	-1.2641	-1.5602	0.6059	0.3098
GSN	0.0705	0.0479	-0.6710	-0.6935						
HABP2					-0.5367	1.4446	-0.7071	0.7375	-0.1704	1.2742
HP	-1.2469	0.5276	-0.3488	1.4257	-0.6394	0.9683	-1.2963	-0.3280	-0.6570	0.3114
HPX	-0.4105	-0.2881	-0.7115	-0.5891	-0.3598	0.9360	-1.1034	-0.1674	-0.7437	0.1924
HRG	0.5979	1.0673	0.0322	0.5015	-0.7301	0.6894	-0.8232	-0.1338	-0.0931	0.5963
IGHA1	1.7636	1.3477	0.3629	-0.0530	-2.0152	0.4328	-2.2081	-1.7753	-0.1929	0.2399
IGHD					-2.4500	0.4182	-3.4285	-3.0102	-0.9785	-0.5603
IGHG1	-0.0855	0.9292	-0.4963	0.5184	-0.0970	-1.8091	0.4814	-1.3277	0.5785	-1.2306

Table 4.1: OpenMS $\log_2$ 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
IGHG2	0.9720	0.3502	0.4608	-0.1611	-0.6249	-1.5107	0.2705	-1.2401	0.8955	-0.6152
IGHG3	-0.1942	1.4323	-0.9310	0.6955	-1.8544	-0.3927	-1.8870	-2.2798	-0.0327	-0.4254
IGHM	-0.6318	-0.8967	-0.4175	-0.6824	-1.1742	1.7916	-2.3509	-0.5593	-1.1767	0.6149
IGKC	-0.0697	0.0420	-0.1150	-0.0032	-1.1868	-0.2875	-1.1765	-1.4641	0.0103	-0.2772
IGKV3D-20					-0.3699	-0.0537	0.2115	0.1578	0.5814	0.5277
ITIH1	-0.9767	0.7057	-0.5212	1.1612	-0.6149	0.5496	-0.5039	0.0456	0.1110	0.6605
ITIH2	-0.3143	-0.5283	-0.2363	-0.4504	-0.7432	0.6757	-1.2137	-0.5379	-0.4705	0.2052
ITIH3	-0.5456	0.6139	0.3513	1.5108	-2.0564	1.2902	-1.8743	-0.5841	0.1821	1.4724
ITIH4	-0.0670	-0.2189	0.3809	0.2289	-1.0844	0.9773	-1.8198	-0.8425	-0.7355	0.2418
KLKB1		-2.2093		-0.2714						
KNG1	-0.6198	-0.0025	-0.0676	0.5497	-0.6644	0.8053	0.0312	0.8365	0.6956	1.5009
LRG1	-0.7988	0.2565	0.1402	1.1955	-0.9516	1.7018	-2.1951	-0.4933	-1.2435	0.4583
LUM	0.0832	0.6580	-1.2636	-0.6888						
ORM1	-0.1975	1.1178	-0.2240	1.0913	-1.9126	1.6761	-1.3026	0.3735	0.6100	2.2862
PGLYRP2										
PLG	-0.3680	0.0881	-0.8410	-0.3850	-1.0702	2.7112	-2.8493	-0.1381	-1.7792	0.9321
PROS1	-0.3301	0.0624	-0.7963	-0.4039	-0.5090	1.5350	-3.8745	-2.3396	-3.3656	-1.8306
RBP4	0.4506	0.4186	-0.0212	-0.0532	-4.0971	1.4352	-2.9877	-1.5525	1.1094	2.5446
SAA1	-2.7778	2.3464	-0.5152	4.6090	-1.3859	2.4855	-2.5594	-0.0739	-1.1735	1.3120
SERPINA1	0.6826	0.0482	1.7824	1.1481	-0.0999	-0.1559	-1.3635	-1.5194	-1.2636	-1.4195
SERPINA3	-0.7582	-0.1618	0.1837	0.7802	-0.7418	2.2311	-2.0353	0.1958	-1.2936	0.9375
SERPINA4	0.0099		-1.0180		-1.4474		-0.6572		0.7902	
SERPINA5				0.2757						
SERPINC1	-0.5553	-0.2339	-0.5421	-0.2207	-0.7720	1.1067	-1.3465	-0.2398	-0.5744	0.5322

Table 4.1: OpenMS $\log_2$ 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
SERPIND1	0.2536		0.0459		0.3050	2.3844	-1.6469	0.7375	-1.9519	0.4325
SERPING1	-1.1615	0.1192	-1.3511	-0.0705	-0.9302	1.0767	-1.0905	-0.0138	-0.1603	0.9164
TF	-0.2824	-0.1105	-0.4844	-0.3125	-0.7682	0.5876	-0.9946	-0.4070	-0.2264	0.3612
VTN	-0.6186	-0.0324	-0.2690	0.3172	-1.7235	1.4919	-2.1518	-0.6599	-0.4283	1.0636
VWF		1.0586		1.3918	-2.5663	0.5162	-1.9774	-1.4612	0.5889	1.1051

Table 4.2: 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 A vs D
A1BG	-1.644	-1.472								
A2M	-6.138	-9.908		1.380	-5.861	-3.467		1.660	5.861	3.564
AFM		2.512		-4.055						-3.499
AHSG				-2.249						
APCS		1.871				4.207	1.722			
APOA1	-11.803	-3.698		-3.251	-2.884	-2.884	-3.802		-1.406	
APOA2	-14.322			-4.966						
APOA4	-11.588	-5.916		-2.109	-2.965	-1.556	-2.489	1.871		-1.629
APOB	-2.443	3.020		-6.026	3.732	-1.282	1.368	-4.742	-2.805	1.722
APOC1				-4.529						
APOC4						1.318		4.920		-4.529
APOE			-1.528	-1.754		-1.837	-3.020	-1.803	-3.020	
AZGP1	2.270	2.630	3.598		1.820	4.446				-4.130
C1QB						-1.514				
C1R						-4.446				
C3	2.754	-1.941		3.981	-2.399	-4.365	1.614	-1.977	3.598	6.546
C4B	2.270	-2.148	-1.941	2.655						
C4BPA		-1.419					1.660	-2.014		3.251
C5	1.738			2.228		-2.333		-1.770		2.168
C6	1.888					-2.070	-2.805			
C9		-2.421		9.908		-4.055		-1.500	7.178	9.376
CD5L		-2.831	-3.281		-1.820	-1.820				

Table 4.2: 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 A vs D
CFB		-1.675	2.535	4.285		-2.128	2.032	-1.690	2.512	4.055
CFH				2.559					2.333	1.803
CFI										2.270
CLU								-2.582		
CP			2.582	3.020			2.188		2.780	
F2							1.675			1.528
FGA	3.467	-1.644		12.134	-3.532	-2.655			5.200	4.093
FGB	3.281		2.443	9.204	-2.188	-1.331	2.655		5.248	3.133
FGG	2.032	-1.959		9.638	-2.312	-1.644	4.325		9.204	6.368
FN1	2.582	2.228			1.941	-2.466	1.472	-4.875		3.404
GC							1.542		2.606	2.399
GSN	-2.312			-4.055	-3.020		-4.365			
HBA1		3.133		-4.018					-2.655	-2.535
HBB		10.000		-15.996	5.058	2.168			-6.138	-2.559
HP	3.499		2.512	13.428		-2.965			4.093	4.786
HPX		-2.148					1.995		2.208	
HRG						3.532		3.908		
IGHM		-5.152	-3.664		-5.200	-4.656			3.221	2.938
IGKC						1.754	5.649	1.786	5.808	
ITIH1								-3.598		
ITIH2				-1.629		-2.089	-2.208	-2.070	-2.208	
ITIH3		-2.051		2.466					2.109	2.630

Table 4.2: 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 A vs D
ITIH4	1.820	-2.312		3.105	-1.837	-3.105		-1.738	2.377	4.093
JCHAIN			-4.130		-5.012					
KNG1							2.754			
LPA			10.765	14.723						
LRG1		-2.168		3.048	-6.368	-9.727		-1.629		3.311
LUM	-4.405			-3.251						
ORM1			16.904				3.631			2.992
PLG	1.556				2.312	1.871	2.938			
RBP4		5.495								
SAA1			28.054	51.523						
SAA4						-2.805				1.905
SERPINA1		-2.333		7.586	-2.754	-5.598		-2.188	3.221	7.112
SERPINA3	2.109	-1.738	3.837	12.706	-1.977	-5.916		-3.251	4.325	12.246
SERPINC1								-2.070		
SERPIND1	1.770				2.032					
SERPINF1					-4.365	-5.248				
SERPINF2						-4.207		-3.467		
SERPING1		-2.535		2.965	-1.837	-4.365		-2.489	2.188	5.248
TF	-2.729		-1.528	-5.445			1.722			
TTN					-1.706	-2.208	-1.770			1.259

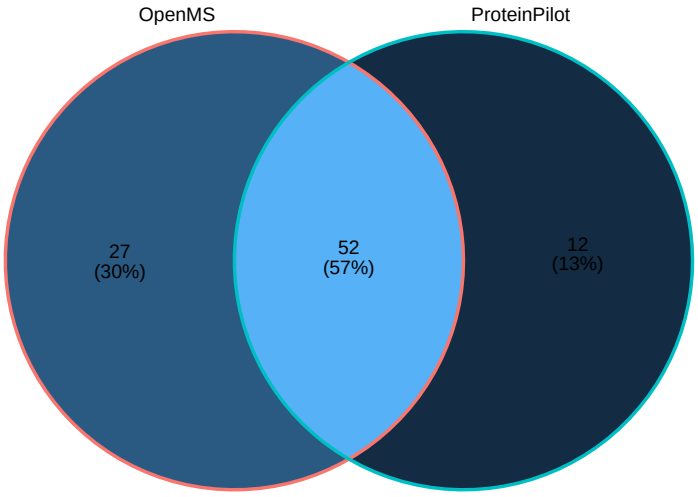


Figure 4.4: Venn diagram of the overlap in unique proteins identified from analysis with ProteinPilot and OpenMS respectively on data from 2 4-plex iTRAQ experiments.

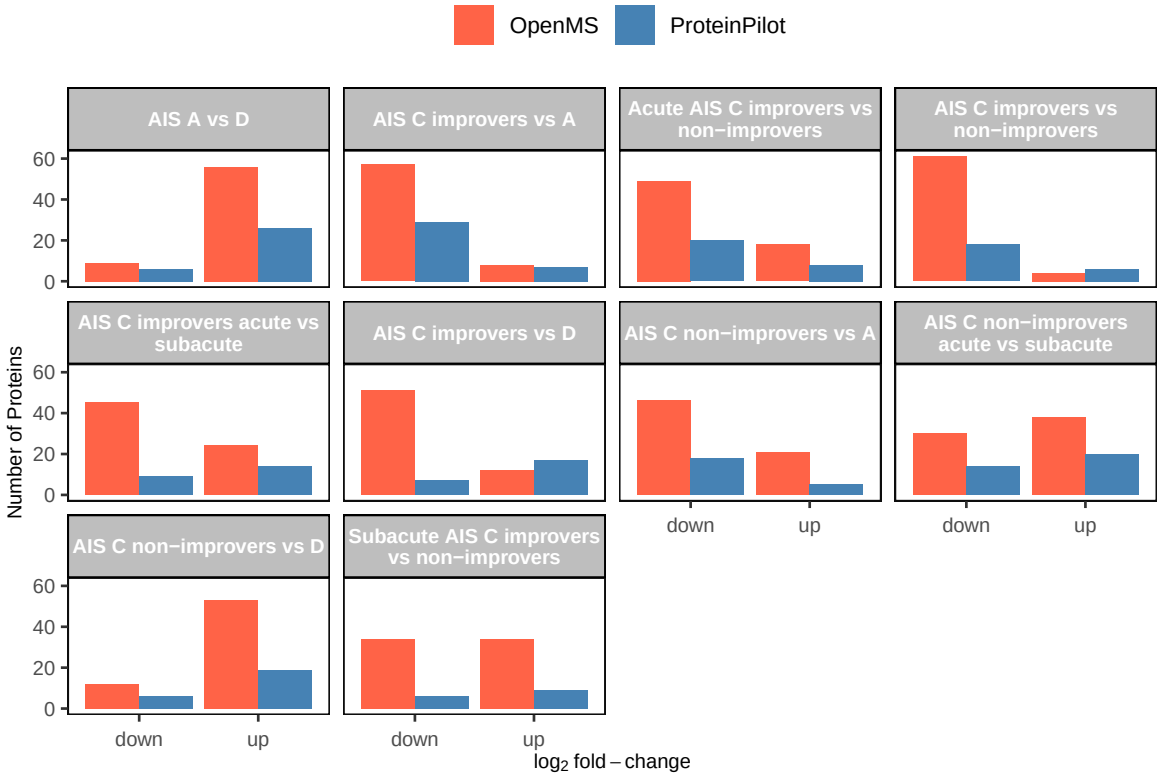


Figure 4.5: The number of proteins found to be up- or down-regulated via ProteinPilot and OpenMS.

4.3.2 STRINGdb network plots

Network interaction plots generated from the OpenMS processed data via `STRINGdb` revealed that all test groups contained similar proteins, albeit with different abundances, with no distinct group-specific networks observed (Figures 4.6, 4.7, A.3, A.5, A.7, A.9, A.11, A.13 and A.15). Clustering of these networks produced three groups of differentially expressed proteins in acute AIS C improvers compared to non-improvers (Figure A.1). The first cluster is comprised of proteins from the compliment and coagulation cascade (Figure A.1 (a)). The second cluster is comprised of proteins associated with cholesterol metabolism (Figure A.1 (b)). The final cluster is a single protein; ATRN, AKA attractin, which is implicated in immune cell clustering during inflammatory responses and may regulate the chemotactic activity of chemokines (Figure A.1 (c)).

These three clusters are also represented in the subacute AIS C improvers compared to non-improvers, with an additional fourth small cluster made up of proteins predominately associated with the complement cascade (Figure A.2 (a, b, c)). This pattern of clusters is replicated in all of the remaining comparison groups (Figures A.4, A.6, A.8, A.10, A.12, A.14 and A.16).

Please see appendix section A.3.1 for additional plots.

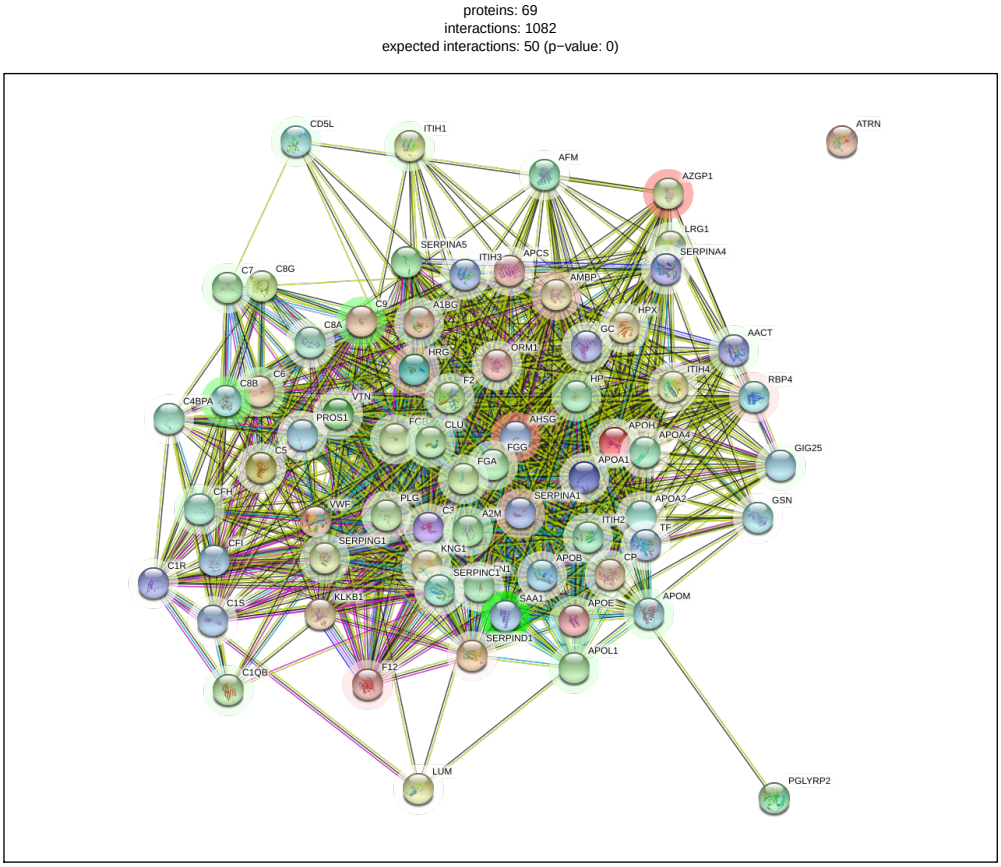


Figure 4.6: The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates 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-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

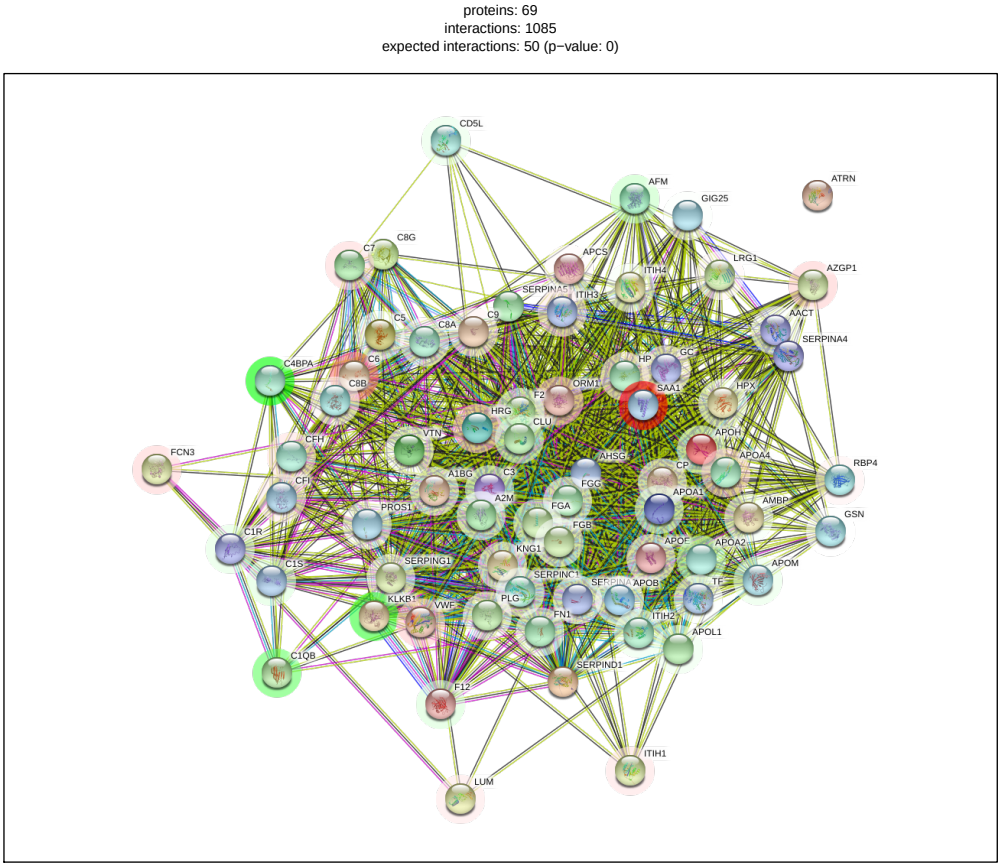


Figure 4.7: Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there 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-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

4.3.3 Heatmaps

The majority of the pathways associated with the proteins identified by these iTRAQ experiments are related to the complement cascade and clotting via platelets (Figures

4.8, 4.9, A.17, A.18, A.19, A.20, A.21, A.22, A.23, A.24). There are also several pathways implicated in metabolic processes, particularly with apolipoproteins and retinoids.

Please see appendix section A.3.1 for additional plots.

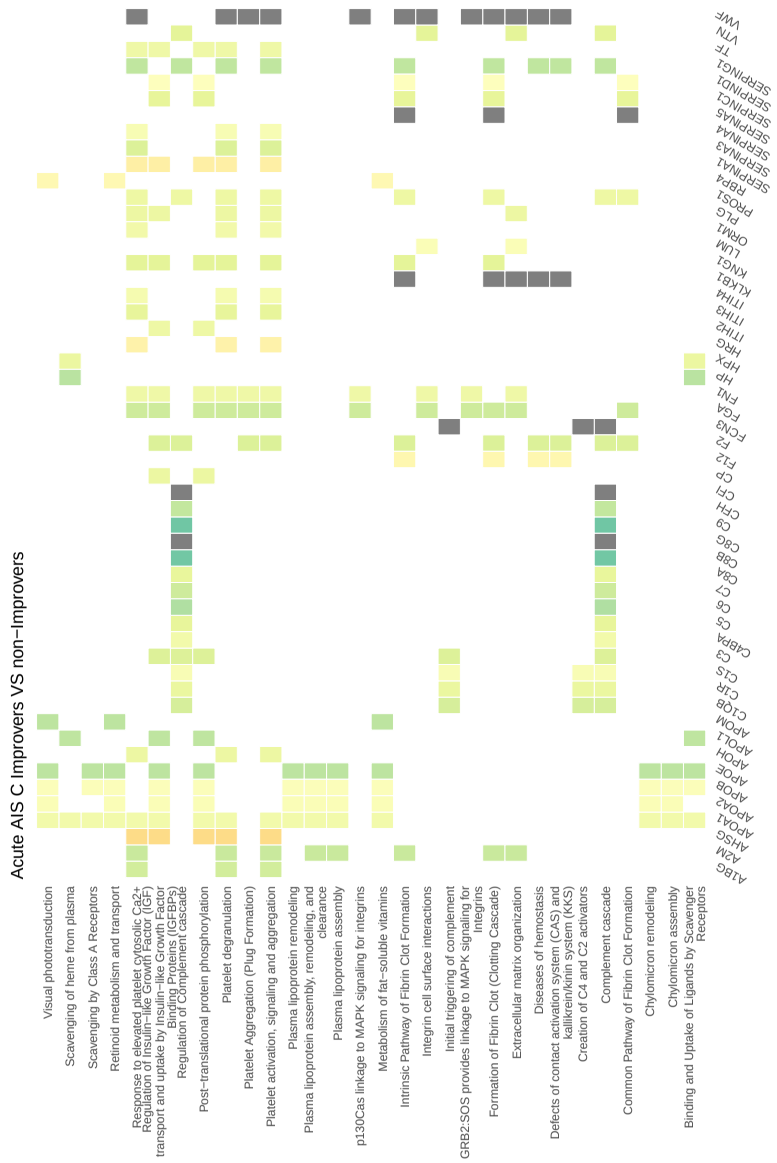


Figure 4.8: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

Figure 4.9: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

4.3.4 Cnetplots

Similar to the heatmaps, network plots highlight the majority of proteins are associated with the complement cascade and pathways linked to platelets (Figures 4.10, 4.11, A.25, A.26, A.27, A.28, A.29, A.30, A.31, A.32). Several proteins are also associated with the regulation of insulin-like growth factor.

Please see appendix section A.3.1 for additional plots.

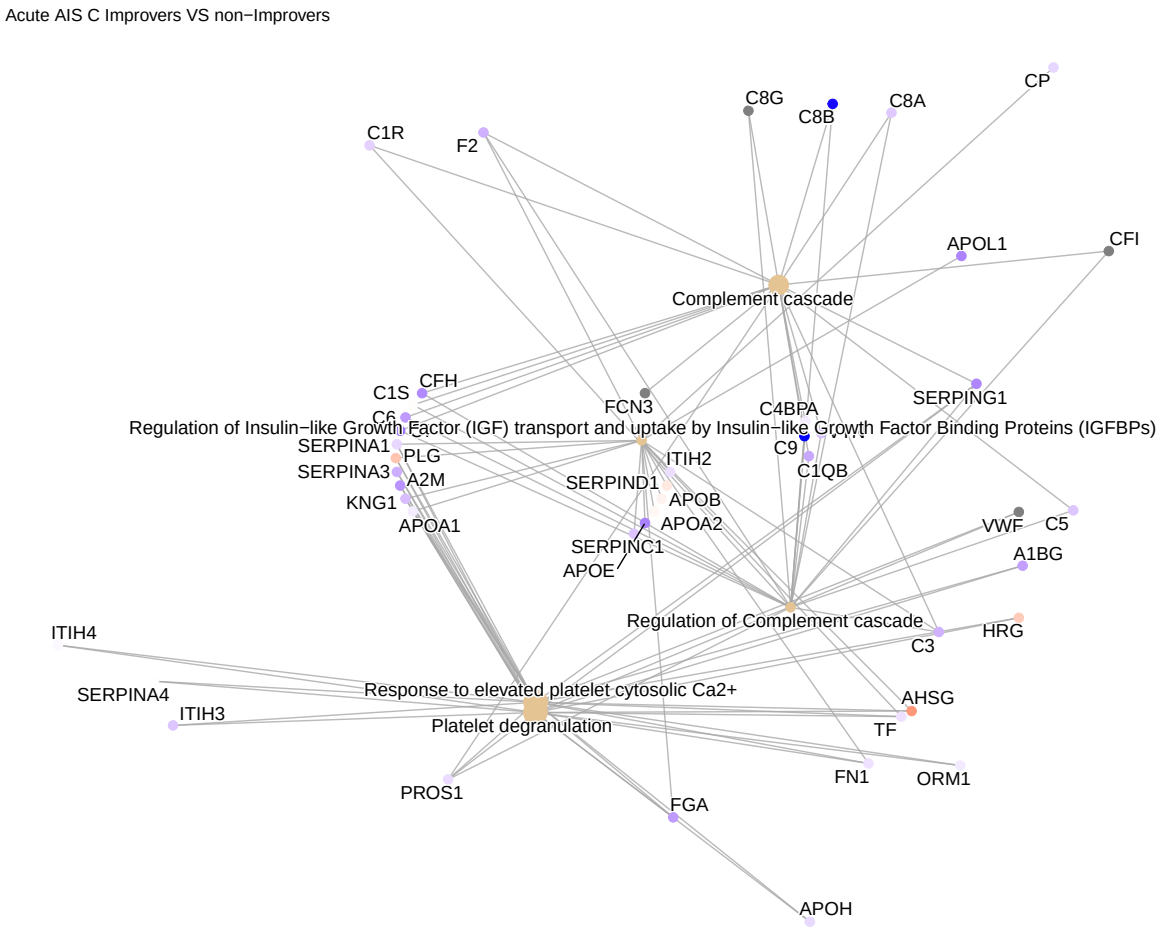


Figure 4.10: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

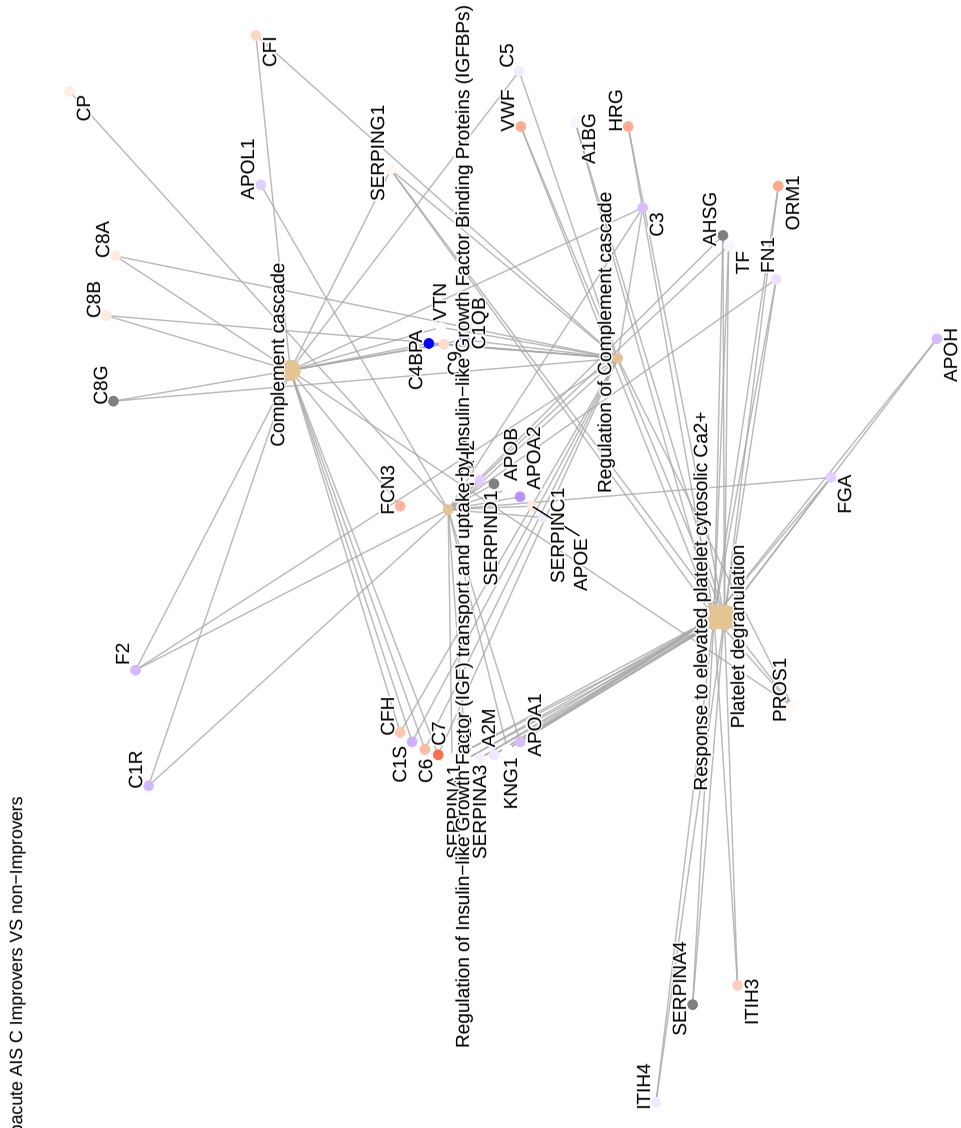


Figure 4.11: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

4.3.5 ELISAs

No statistically significant difference between groups for A2M abundance in plasma via DuoSet® ELISAs, though there were outliers in the AIS A and D groups, and particularly in the AIS C patients at 3-months who did not experience an AIS grade conversion (Figure 4.12). A significant difference was found between AIS C non-improvers at 2-weeks and AIS D for SAA1, with outliers in AIS C non-improvers at 2-weeks, and both AIS C improvers and non-improvers at 3-months post-injury (Figure 4.13). For ApoA1 plasma abundance estimated via Quantikine® ELISAs, statistically significant differences were found between AIS C improvers at 2-weeks and both AIS C improvers and non-improvers at 3-months, AIS C 3-month improvers and AIS A and D, and AIS C 3-month non-improvers and AIS A and D (Figure 4.14). A statistically significant difference was also found between AIS C improvers and non-improvers at 2-weeks post-injury for RBP4 (Figure 4.15).

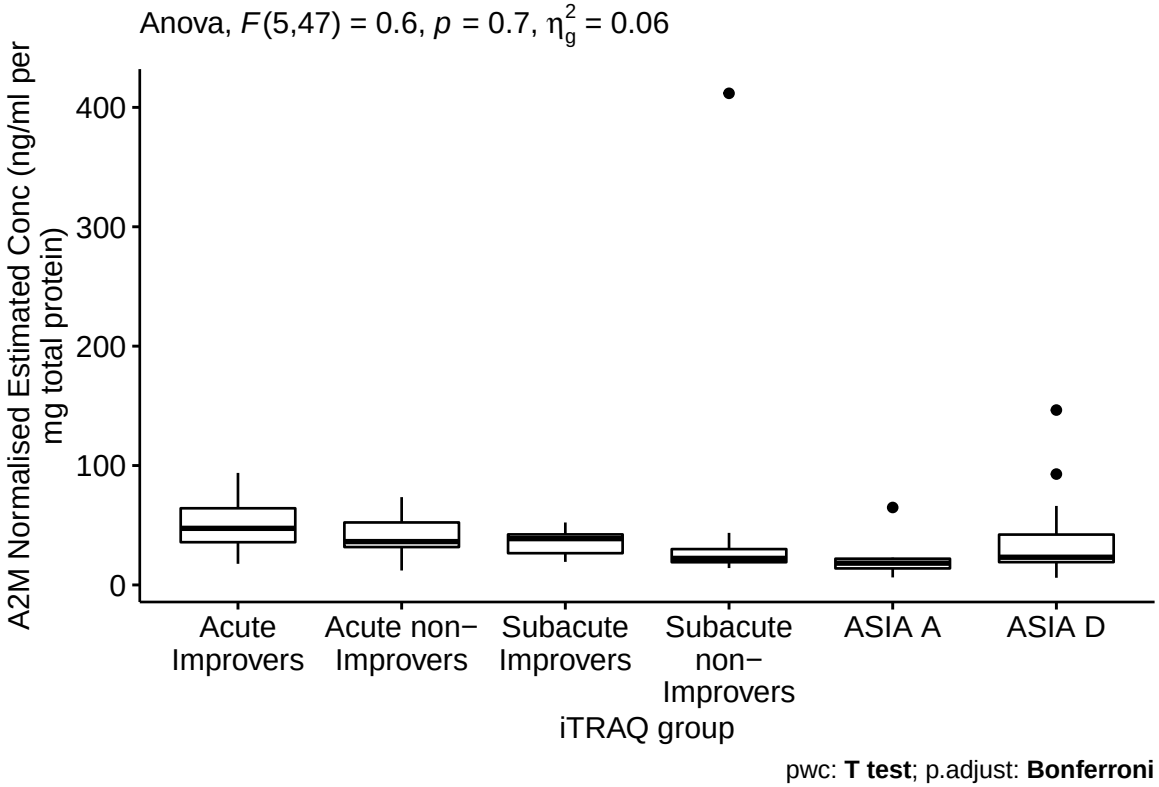


Figure 4.12: Normalised estimated concentration of α-2-macroglobulin. Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled iTRAQ samples was assayed and pairwise t-tests with bonferroni adjusted P-values were preformed to assess differential abundance.

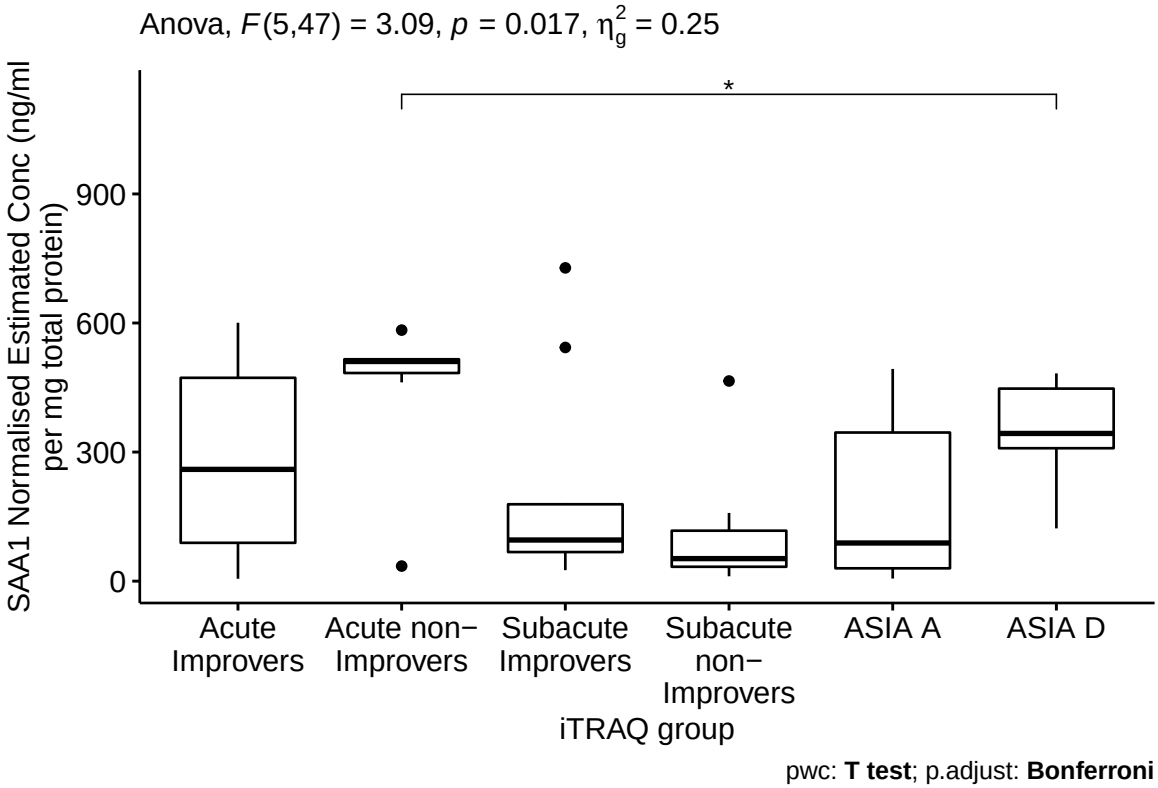


Figure 4.13: Normalised estimated concentration of serum amyloid A1. Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled iTRAQ samples was assayed and pairwise t-tests with bonferroni adjusted P-values were preformed to assess differential abundance.

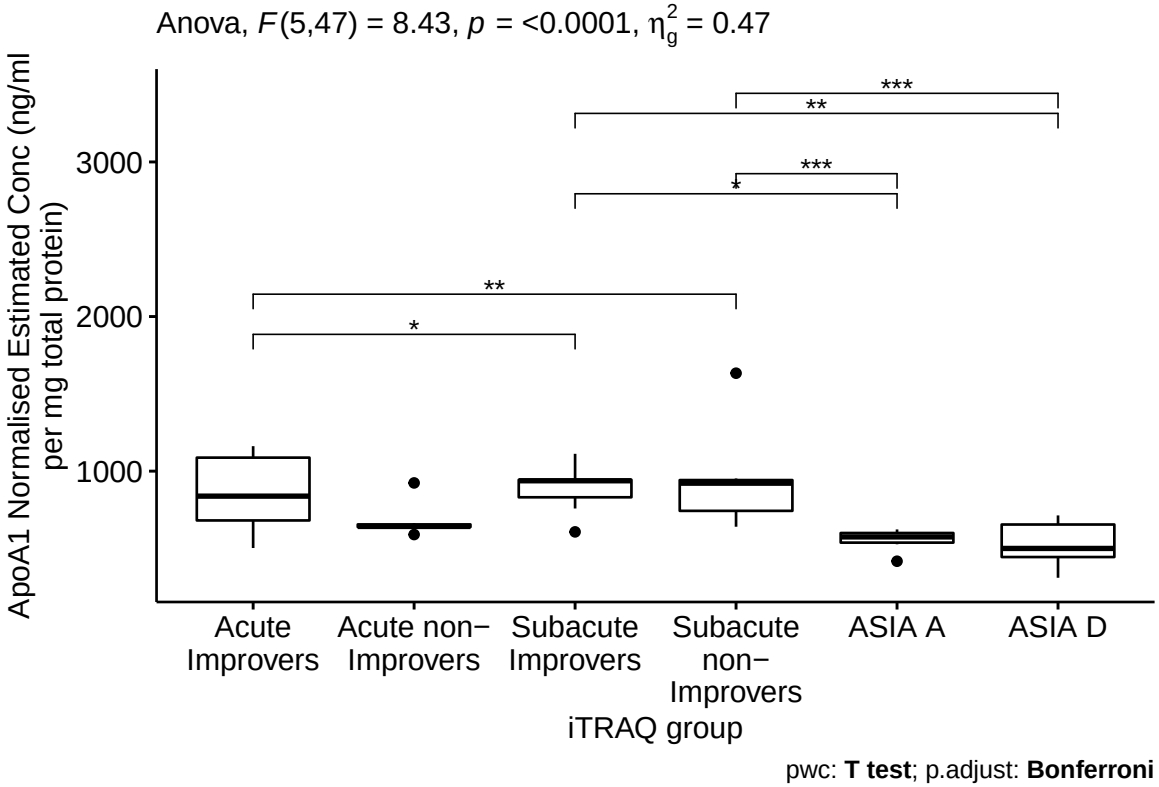


Figure 4.14: Normalised estimated concentration of apolipoprotein A1. Estimates were calculated from the optical density of a standard curve produced via a Quantikine® ELISA. Plasma from each patient that made up the pooled iTRAQ samples was assayed and pairwise t-tests with bonferroni adjusted P-values were preformed to assess differential abundance.

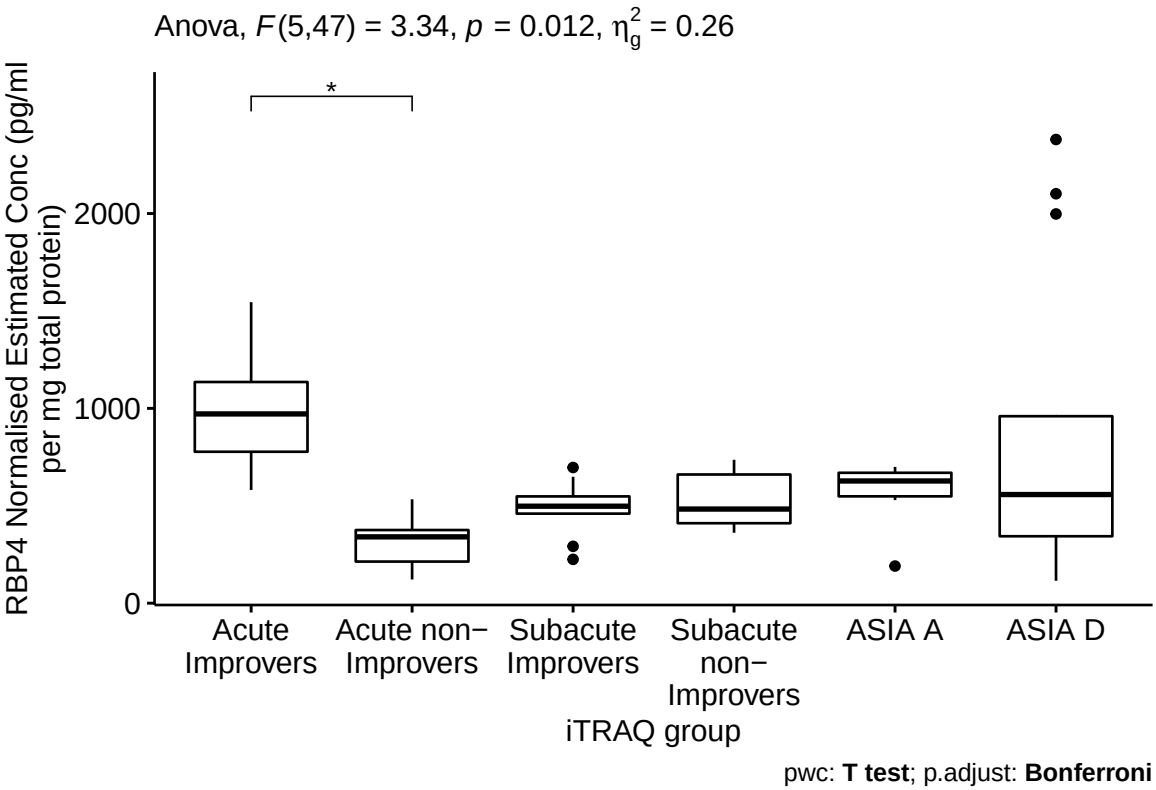


Figure 4.15: Normalised estimated concentration of retinol binding protein 4. Estimates were calculated from the optical density of a standard curve produced via a DuoSet® ELISA. Plasma from each patient that made up the pooled iTRAQ samples was assayed and pairwise t-tests with bonferroni adjusted P-values were preformed to assess differential abundance.

4.3.6 Pathway analysis

Pathway analysis via the `pathview` R package returned the complement and coagulation cascade to be on the sole significant KEGG pathway to derive from the OpenMS analysed data. The majority of the proteins present in this pathway were less abundant in the 2-week post-injury plasma of AIS C patients who experienced an AIS grade conversion and those who did not (Figure 4.16).

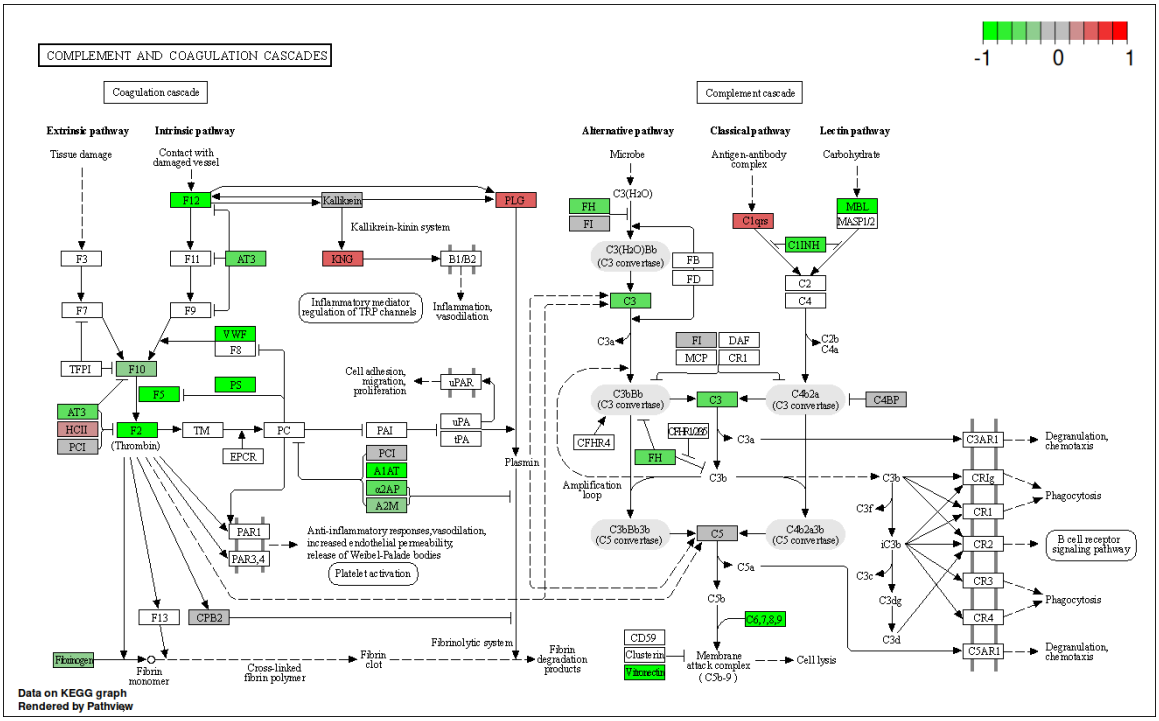


Figure 4.16: KEGG complement cascade pathway annotated with $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

4.4 Discussion

This work builds on the previous chapters (3.1) modelling of routine bloods by analysing the plasma proteome of SCI patients grouped by injury severity and improver status. In addition to continuing the pursuit of novel biomarkers of SCI, the link between the liver and neurological recovery hinted at in the aforementioned chapter is examined here.

4.4.1 ProteinPilot and OpenMS

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

The manufacturers of mass spectrometers often offer software tailored to their instruments which is often used in the literature. However, the source code for these software suits is not publicly available, and indeed manufactures often boast of their particular inscrutable proprietary algorithms, often related to peak picking. This combination of complexity and opacity in analysis methodology can make it extremely difficult to reproduce results from other labs, or even analysis from one's own lab.[494]

To address this issue many open-source (meaning the source code is publicly

available) software packages which may perform one or several steps of a complex analysis workflow have been developed. This issue here is that incorporating multiple software packages together can be both time-consuming and error-prone, and require significant maintenance and documentation to maintain reproducibility.

The OpenMS project aims to address these challenges by providing a flexible software environment, with both pre-assembled workflows that aim to provide best-practices, and allow for more granular control with both command line and Python scripting interfaces. OpenMS is also integrated with graphical workflow systems such as KNIME and Galaxy, increasing the accessibility of the platform.[495,496]

Here we used both the vendor provided proprietary ProteinPilot and OpenMS to analysis two 4-plex iTRAQ experiments. We observe that both approaches produce similar results, with a similar number of total proteins identified, a large degree of overlap in the specific proteins identified, and similar fold changes (Figures 4.4 and 4.5). As the results are similar we choose to focus on the OpenMS results due to aforementioned superior reproducibility.

4.4.2 Proteins identified

A total of 79 proteins were identified across both runs for OpenMS, many of which are related in function. (Figure 4.4). Here we explore the potential these proteins have a biomarkers of SCI.

Alpha-2-macroglobulin

A2M is an inhibitor of an unusually diverse array of proteinases by a unique 'trapping' mechanism. The protein achieves this with a peptide stretch, called the "bait region", which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced whereby A2M traps the proteinase. The entrapped enzyme retains active against low molecular weight

substrates, whereas activity against high molecular weight substrates is greatly reduced. Following cleavage in the bait region, a thioester bond is hydrolysed and mediates the covalent binding of the protein to the proteinase.[497,498] A2M is unique in it's ability to inhibit virtually any protease regardless of it's specificity, origin or catalytic mechanism.[499,500]

Alpha macroglobulins are an integral part of innate immunity and thus are evolutionarily conserved.[501] Alpha macroglobulins have significant primary sequence homology with complement components C3, C4 and C5. The A2M-proteinase complex is cleared from circulation primarily by receptors on hepatocytes.[502,503] The mammalian receptor for proteinase-reacted A2M is a low-density lipoprotein receptor related protein.[504–506]

A2Ms definitive function is the delivery of proteinase to an endocytotic proteinase clearance pathway. A2Ms trap the proteinases released by granulocytes and other cells during inflammation and also regulate the extracellular proteolytic activity resulting from clotting and fibrinolysis. A2M can also help protect against pathogens as it can trap proteinases from non-human origins as well. A2M can be recognised and phagocytosed by macrophages and hepatocytes, and it has been proposed to aid in the clearance of defensins and other peptide mediators in inflamed tissues, thus contributing to the regulation and containment of inflammation.[507] Myelin basic protein is released into the circulation following traumatic injury and A2M has been seen to be the only major myelin basic protein-binding protein in human plasma, suggesting A2M protects the immunogenic protein from degradation by proteases and help in its clearance from circulation.[508]

We observe A2M to be less abundant in AIS C improvers, within 2-weeks post injury and at 3-months, albeit to a lesser extent (Tables 4.1 and 4.2). Similarly, A2M was more abundant is AIS As relative to all groups, and whilst A2M was less abundant in AIS C improvers at 2-weeks compared to AIS Ds, AIS C non-improvers

had more A2M than AIS Ds. (Table 4.1). With less A2M there would be more protease activity in these individuals, which may aid in the clearance of damaged tissue, and in particular may lessen the development of an astroglial scar, thus aiding repair. However, glial scaring is not entirely negative, the primary benefit it offerers is minimising the extent of secondary damage to neighbouring areas by functioning as a barrier around the injury site. Animal studies have demonstrated that prevention of astroglial scar formation following CNS injury leads to greater lesion size and poorer function outcomes.[509,510] Interestingly, a rat study using quantitative liquid chromatography-mass spectrometry with CSF, found A2M to be more abundant in moderately injured animals compared to more severe injuries.[511]

Apolipoproteins

We found ApoA1, ApoA2, ApoH, ApoL1 and ApoM to be less abundant in AIC improvers at both time points, whereas ApoA4 was more abundant at both time points (Tables 4.1 and 4.2). ApoA1 is the main protein component of high-density lipoproteins (HDL). Plasma HDL include two main apolipoproteins, these being ApoA1 and ApoA2 (~70% and ~20% of total HDL protein content respectively), but some HDL particles can also contain small amounts of other apolipoproteins, including ApoA4, ApoA5, ApoC, ApoD, ApoE, ApoJ and ApoL. The primary function of HDL in plasma is the transport of cholesterol, which can have dietary origins, but also be produced endogenously in the liver.

HDL Activity HDLs have serve a wide range of functions, including contributing to anti-inflammatory activity. They can limit chemokine secretion from multiple cells types including endothelial cells and monocytes.[512–514] Rats injected with ApoA1 showed significant reduction in expression of CCR2 and CX_3CR1, the receptors for chemokines of the same name, which play a role in leukocyte migration.[514]

HDL is also associated with protection from oxidative damage, also inhibiting the potentially atherogenic oxidised LDL formation.[515] The exact mechanisms of these antioxidant effect is still actively researched, the enzyme paraoxonase-1, which is present on HDL particles are likely important.[516] Apolipoproteins, including ApoA4 and ApoAE also have antioxidant properties, for example phospholipid hydroperoxidase can be reduced by methionine residues of ApoA1, forming redox-inactive phospholipid hydroxides.[517,518]

HDLs can also suppress proliferation of haematopoietic stem cells, thus reducing leucocytosis and monocytosis.[519] Furthermore, HDLs are implicated in the transport of microRNAs, though the mechanisms of loading the microRNAs and their biological significance is still under study.[520]

ApoE was less abundant in AIS C improvers within 2-weeks and more abundant at 3-months, and more abundant in more severe injury, such as AIS A relative to D or C and in AIS C relative to D (Table 4.1). ApoE is primarily produced by hepatocytes in the liver, but second-most in the brain, synthesised in and secreted by astrocytes, and has been found to an important determinant in response to types of CNS injuries in both animal and human studies.[521,522] A key function of ApoE is as a ligand for the LDL receptor family of proteins, which mediate trafficking of cholesterol to neurons, which is vital for axonal growth, and for synapse formation and remodelling.[523] Additionally, ApoE is implicated in the clearance of neuronal apoptotic bodies.[524] In humans there are three variants/alleles of ApoE: ApoE2, ApoE3 and ApoE4, which have a frequency of 8.4%, 77.9% and 13.7% globally.[525] The variant proteins differ by one or two amino acids and have been found to result in substantial physiological alterations.[526,527] The presence of the ApoE4 variant has been linked to worse outcomes in SCI and TBI.[527–530] More specifically, the SCI study reported significantly lower change in the median AIS motor score compared the individuals without the ApoE4 allele during rehabilitation.[527]

Prior *in vivo* rodent studies have demonstrated up-regulation of ApoE following SCI and TBI, though ApoE is not observed in neurons of rodents under normal neuropathology, and they only posses a single ApoE allele.[531–533] A separate rodent study reported ApoE levels decreased for the first 3 days post-injury, and then increased peek expression at 7 days post-injury, a similar pattern to our results.[534] Furthermore, mouse studies have demonstrated replacement of ApoE in neurons with human ApoE4 have impaired neurite outgrowth compared to replacement with ApoE2 or ApoE3, suggesting ApoE4 interferes with neuroplasticity.[532,535] The underlying mechanism/s by which ApoE and its alleles effect neuroplasticity is not currently known, but proposals have been made. One possibility is reduced lipid transport from astrocytes to neurons, potentially impeding the membrane generation required to support axon growth or dendrite sprouting. ApoE has anti-oxidant properties, so others have suggested impaired anti-oxidant activity may contribute. ApoE4 has been found to be both secreted less than ApoE2 or ApoE3, and to have inferior anti-oxidant abilities, lending some credence to this idea.[536,537] Knowing this, whilst ApoE may make for a useful biomarker for SCI, it will be important that particular variants of ApoE a given patient has could be just as important, if not more so, than simple abundance.

Serum Amyloid A1

SAA1 was less abundant in AIS C improvers at 2-weeks relative to non-improvers, but more abundance in plasma at 3-months (Table 4.1). SAA1 was also more abundant is AIS A relative to less severe injuries, and in AIS Cs relative to Ds (Table 4.1). SAA1 is a major acute-phase protein mainly produced in the liver by hepatocytes in response to infection, tissue injury and malignancy.[538] SAA1 is a precursor of amyloid A (AA), the aberrant deposition of which leads to inflammatory amyloidosis.[539] There are 5 known SAA1 variants, though currently, no indication of substantial functional

differences have been identified.[540] However, some alleles have been linked to disease, including increased amyloidogenesis and tumour suppression.[541,542]

During the APR, plasma levels of SAA increase up to 1000-fold, and so serves as a well-established clinical biomarker for inflammatory disorders.[543] SAA isoforms produced by hepatocytes during an APR are swiftly released into the blood where they associate with HDL, displacing ApoA1 and becoming an apolipoprotein of HDL.[544,545] Reverse cholesterol transport, whereby cholesterol in non-hepatic tissues is transported back to the liver, is conducted via plasma components such as HDL, ABCA1 and ABCG1. ApoA1 acts as an acceptor for cholesterol in this process, and studies have found that SAA in lipid-free form can similarly function as a cholesterol acceptor for ABCA1. Whilst SAA is though to be an important facet of lipid metabolism, its role is likely complex as mice knockout studies which eliminate SAA1 and SAA2 have shown little effect on cholesterol transport, HDL levels and ApoA1 clearance.[546,547] These studies indicate that the *in vivo* functions of SAA related to lipid metabolism are more complex than prior *in vitro* studies implied.

SAA1 can both induce anti-inflammatory interleukin 10 (IL-10)-secreting neutrophils, but also promotes the interaction of invariant natural killer T cells with those neutrophils, which limits their suppressive activity by diminishing the production of IL-10 and enhancing the production of IL-12, indicating that SAA1 can have both pro- and anti-inflammatory effects.[548] There has however been conflicting results reported of SAA's cytokine induction abilities, and some studies have suggested that recombinant human SAA1 provided by some vendors may have additional cytokine-inducing activity due the altered amino acid sequence.[549]

Macrophages are a major source of SAA in inflammatory tissues, and elevated SAA production has been observed in rheumatoid arthritis, Crohn's disease, Type 2 diabetes and atherosclerosis.[550–554] SAA binding to HDL was reported to increase affinity for macrophages whilst decreasing affinity for hepatocytes.[555] This change

is thought to favour the removal of cholesterol from site of inflammation.[556] SAA inhibits the binding of the scavenger receptor SR-BI and cholesterol efflux is enhanced in a SR-BI-dependent manner.[557,558] It has been suggested that the SR-BI-mediated re-uptake of cholesterol underpins the role of SAA in cholesterol recycling during tissue repair, where a great deal of cholesterol is required.[559]

In blood circulation SAA1 may also function as a immune opsonin for increased neutrophil uptake of Gram-negative bacteria.[560] Both human and mouse SAA proteins have been found to bind retinol with nanomolar affinity that limits bacterial burden in tissues after acute infection.[561] Retinol is important to the body's response to microbial infection, so SAA may also have a role in limiting bacterial burden, particularly in the liver, spleen and intestine. The aforementioned study demonstrated that mice lacking in both SAA1 and SAA2 have a higher bacterial burden in the liver and spleen following infection.[561] All 3 SAA isoforms are found in intestinal epithelium, which is exposed to the gut microbiome, in mice. The anti-bacterial properties of SAA isoforms may therefore explain the role of SAA as an acute-phase protein that protects the host in tissues and organs exposed to bacteria.

Retinol-binding protein 4 (RBP4)

In plasma within 2-weeks post-injury, RBP4 was less abundant in AIS C improvers relative to AIS D and A, and more abundant in AIS C non-improvers again, relative to AIS D and A (Table 4.1. Similarly, AIS A plasma had more RBP4 compared to AIS D, and AIS C improvers were also more abundant in RBP4 compared to non-improvers at both 2-weeks and 3-months post-injury (Table 4.1.

Vitamin A is a collective term for a group of fat-soluble compounds with a range of essential biological activities including aspects of growth, vision and metabolism.[562] Following dietary absorption, vitamin A is ferried from the intestine, with chylomicrons as retinyl esters, to tissues for immediate use or the liver for storage in hepatic stellate

cells. A subsequent dietary deficiency of vitamin A will result in these liver stores being mobilised by hydrolysing the retinyl esters to release retinol. The retinol is then bound by RBP4, which is also mainly synthesised in the liver, and secreted into circulation from hepatocytes, whereupon it is bound by an additional transport protein, transthyretin.[563] The membrane plasma protein STRA6 facilitates retinol transport from RBPs across the cell membrane.[564] Once delivered to target cells, retinol can either be converted to retinaldehyde, which is required for functional vision, or oxidised to retinoic acid, which is a ligand for nuclear receptors, thus regulating gene expression.[565,566]

RBPs are localised in the ventral region, associated with motor neurons, in the mammalian developing neural tube.[567,568] The role of retinoid signalling in spinal cord and motor neuron differentiation, including development of regions of the spinal cord has been outlined, and implies a possible involvement in maintaining motor neuron integrity.[569,570]

The mRNA of a rodent homologue of RBP, named cytosolic retinol binding protein, was found to be up-regulated at 24 hours post-SCI and may promote cell proliferation and regeneration by increasing retinoid metabolism.[571,572] Another study of amyotrophic lateral sclerosis (ALS), a neurodegenerative disease, comparing gene expression between post-mortem spinal cord samples of ALS and controls also observed up-regulation of RBP1 in ALS spinal cord.[573] Furthermore, a transgenic mouse study reported retinoid signalling may contribute to the retained plasticity and regenerative potential of the mature spinal cord.[574]

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

4.4.3 Metabolism and SCI

Acute phase response

The bodies first response to injury or infections, including SCI, is often referred to as the "acute phase response" (APR), which is non-specific, innate reaction that precedes more specific and situational immune reactions.[575,576] This systemic response is largely coordinated by factors released from the liver, but the APRs effects extend to multiple peripheral organs including the kidneys, lungs and spleen.[418–421] This hepatic response is typically transient and quickly fades, but prolonged liver inflammation and pathology has been observed in rodent SCI models.[432,577]

Basic liver functions are chronically impaired by SCI, including metabolising carbohydrates, fats and proteins, storage of minerals vitamins and glycogen and filtering blood from the digestive tract.[432,578–581] This is likely related to the elevated incidence of metabolic disease in the SCI cohort, including insulin resistance, impaired glucose tolerance and cardiovascular disease.[582–585] 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.[586,587] More recently, a longitudinal study found SCI patients had a significantly higher incidence of acute pancreatitis relative to a matched healthy cohort.[588]

The acute (1-7 days) liver response to SCI is well documented; the inflammatory cytokines including TNFα, IL-1α, IL-1β and IL-6, released at the injury site, reach the liver through the bloodstream.[420,433] This provokes the liver to enter the APR and produce acute phase proteins (APPs) thus stimulating a greater immune response.[420,589] 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.[590–593] 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.[594,595]

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.[543,596–598] 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.[432]

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.[589,593] For example, a rodent study demonstrated depletion of Kupffer cells prior to injury resulted in few neutrophils infiltrating the injury site.[419,599]

4.4.4 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.[600] This will reach the liver through the portal vein where Kupffer cells function as a "first line of defence".[601,602] 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.[600,603] 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.[604–606] A rodent study found transcription factors for tight junctions down-regulated following SCI, and that application of probiotics improved neurological outcomes.[607,608] Human studies of the microbiome post-SCI have also demonstrated dysbiosis, both chronically and more acutely post-injury.[609–611]

4.4.5 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.[432] The liver takes up circulating fatty acids, and when levels exceed the oxidative and secretory limits of the liver, hepatocytes store the excess as triglycerides.[612] Adipose tissue lipolysis during elevated sympathetic activity leading to spikes in circulating fatty acids has been reported in human subjects following SCI.[613]

De novo lipogenesis occurring within the liver can also drive hepatic steatosis.[614] Ceramides are lipid signalling molecules and regulators of apoptosis and inflammation; they can contribute to insulin resistance, oxidative stress and inflammation-induce liver adiposity through sustained Toll-like-receptor(TRL)-4 activation.[615–617] If released into the circulatory system, ceramides can cause CNS toxicity, including oxidative damage and changes to the aggregation of proteins associated with diseases such as Parkinson's, Huntington's and Alzheimer's.[617–619] Mature and precursors of hepatic ceramides and enzymes which contribute to ceramide synthesis are elevated by 1 day post-injury.[432] Endotoxins can also stimulated the synthesis of ceramides and so the aforementioned "leaky gut" may also contribute to this elevation.[620] Ceramide synthesis and lipogenesis genes are also stimulated by TNF-α, which, as touched on in the general introduction (1.8.1), has been found to be elevated post-SCI, and associated with differential neurological recovery.[197,198,217,432,621]

4.4.6 Chronic liver inflammation in SCI

The hepatic APR and associated inflammation that typically follows bodily trauma, subsequently rapidly subsides, whereas post-SCI this hepatic inflammation persists chronically. This chronic phase may be due in part to long-term changes in intestinal permeability via fewer tight junctions in intestinal epithelial cells, resulting in gut dysbiosis.[603,605,607,608] Bacterial translocation and gut dysbiosis can be the result of non-mechanical intestinal obstruction, impaired intestinal motility and systemic immune suppression, all of which are potential complications of SCI.[622] Specifically, butyrate-producing bacteria have been found to be reduced in SCI relative to a healthy cohort.[610] Butyrate is known to modulate epithelial differentiation and cell growth, and suppress macrophages, including CNS inflammation, thus the reduction in butyrate from bacteria may contribute to recovery post-SCI, though links to the liver specifically have not yet been studied.[623–626]

LPS is another potential modulator of post-SCI chronic liver physiology. Kupffer cells, hepatic endothelial cells and hepatocytes all participate in the clearance of LPS via CD14- and TLR4-dependent mechanisms.[627–629] LPS induces the release of factors such as TNF-α.

4.4.7 Longitudinal metabolic health

Prior work has found at least 25% of acute SCI patients to be obese, which is well known to induce low-level systemic inflammation, and that this cohort has significantly worse outcomes compared to non-obese SCI patients.[630] Alcohol abuse has also been associated with poorer SCI neurological outcomes.[631] Furthermore, advancing age is associated with increased liver inflammation and the SCI population has followed the broader trend of the general population.[632,633] Taken together, it is not unreasonable to assume that a large number of SCI patients may have pre-existing liver inflammation at injury. This may be an important differentiator that contributes to the degree of

neurological recovery a given patient may experience. Future experiments investigating neurological outcomes of SCI may benefit from establishing parameters of metabolic health, including the composition of the microbiome, as close to injury as possible, and potentially monitoring changes in these parameters longitudinally.

4.4.8 Validation of results

The ELISAs used to validate the proteomic data often did not demonstrate significant differences between the groups (Figures 4.12, 4.13, 4.14 and 4.15). This may be in part to the individual variability of the samples. However, the trends of the data do largely reflect those found in the iTRAQ data, suggesting that with greater statistical power there may be a more robust validation. Furthermore, the ApoA1 ELISAs resulted in the most significant differences, and was the only Quantikine® kit used (Figure 4.14). As the Quantikine® kits are highly optimised, including for use with plasma, whereas the DuoSet®s, which were used for the other proteins, are not. Future studies should therefore consider either simply using Quantikine® kits, or ensure good optimisation of the DuoSet® kits in advance. These results are also corroborated by a recent label-free proteomic SCI study, using a rodent model, which reported similar proteins associated with complement cascade, including A2M and C3.[634]

4.5 Conclusion

This work shows that proteins associated with the complement cascade, and apolipoproteins in particular, have potential as prognostic biomarkers for SCI. For some of these biomarkers, ApoE in particular, it may not be pure abundance, but also the particular allele of the patient that may provide valuable insight. However, the relatively small number of proteins identified here is a limitation, likely due to highly abundant proteins impacting the dynamic range of the samples, which cannot be fully mitigated with

SCX fractionation. Relatedly, the pooling of samples obscures individual variability in protein abundance, and may therefore also effect the number of proteins identified. Subsequent proteomics experiments using label-free techniques, and depletion of highly abundant proteins would address these limitations and serve as a validation should this return similar results. These results, in concert with the prior chapters findings (3.5), provide further evidence of a link between metabolic function and functional neurological recovery post-SCI. Further work is needed elucidate the precise biochemistry at play, and perhaps more importantly, whether modulation of these pathways has the potential to improve outcomes. Experiments that closely monitor the liver, modify diet and analyse metabolites, particularly longitudinally post-injury, would all give further insight into this relationship.

Chapter 5

Characterisation of the human plasma proteome following Spinal Cord Injury continued

5.1 Introduction

The previous chapter (4.1) sought to characterise the plasma proteome of SCI using iTRAQ. However, as was outlined (4.5), a relatively small number of differentially abundant proteins were identified. There are several factors that can impact the number of proteins identified in proteomics including the number of fractions, the instrument, the column length, the length of gradient and most importantly the sample type and preparation. Plasma can be particularly challenging due to the enormous dynamic range of protein abundances. Haptoglobin, immunoglobulins, serotransferrin and albumin make up more than 99% of the total proteins in serum and plasma, interfering with the detection of less abundant proteins and thus limiting the efficacy of LC-MS/MS.[635,636]

5.1.1 Techniques to address dynamic range for proteomics

Depletion

One solution to this is to employ techniques to deplete the most abundant proteins, thereby reducing the dynamic range and allowing detection of lower abundance molecules such as lipoproteins, chemokines, cytokines and peptide hormones. This can is not without issue itself, as lower abundance proteins can also be inadvertently lost through some of these techniques. Furthermore, depletion introduces another potential source of variation, as the same proteins may not be depleted to the same extent in all samples. A common method for depletion is via a column containing immobilised antibodies against the top 1-20 proteins. However, these antibodies never have perfect specificity, and so can bind several other proteins, thus potentially distorting the original proteome.[637,638]

Combinatorial peptide ligand libraries

Other techniques employ combinatorial peptide ligand libraries to simultaneously deplete high abundance proteins and enrich low abundance proteins.[639] The library comprises millions of spherical gel porous beads, each covalently bound to multiple copies of a single hexapeptide structure. The library is made with a combinatorial synthesis process, using single amino acids grafted one after another, also known as a "split-and-pool" procedure.[640] Each bead has an affinity for either a single protein, or a group of proteins with a shared affinity for the same peptide structure.

Once the sample is loaded, the high-abundance species saturate the corresponding affinity beads, with the excess remaining free in solution. By contrast, the low-abundance species bind their specific beads with no excess, thus concentrating them. Upon washing, the excess proteins, largely consisting of the high abundance proteins, are eliminated. The remaining proteins can then be dissociated from the beads,

resulting in a greatly compressed dynamic range.

A large number of physicochemical parameters must be carefully controlled to ensure this technique can be applied reproducibly.[641] This includes the temperature, pH, ionic strength of the buffer, presence/concentration of competitors and the extent of overloading. Two large factors to success with this technique are the enrichment of low-abundance proteins, which is dependent on the size of the sample, and the ability to desorb the bead-captured proteins.

Fractionation

Another approach to mitigate dynamic range is to fractionate the samples, which can be done in various ways at both the proteins and peptide level. SDS-PAGE and strong cation exchange (SCX) can be likened to immunodepletion, and reverse-phase nanoLC-MS/MS can be used for intermediate protein and peptide fractionation, respectively.[642] The key drawback here is that by increasing the number of samples run through the mass spectrometry, cost is increased, but a risk of compounding error in downstream analysis of the spectra is also introduced. Using an FDR of 1% is standard in many MS-based proteomics studies, though many studies using plasma use a less stringent statistical approach.[643]

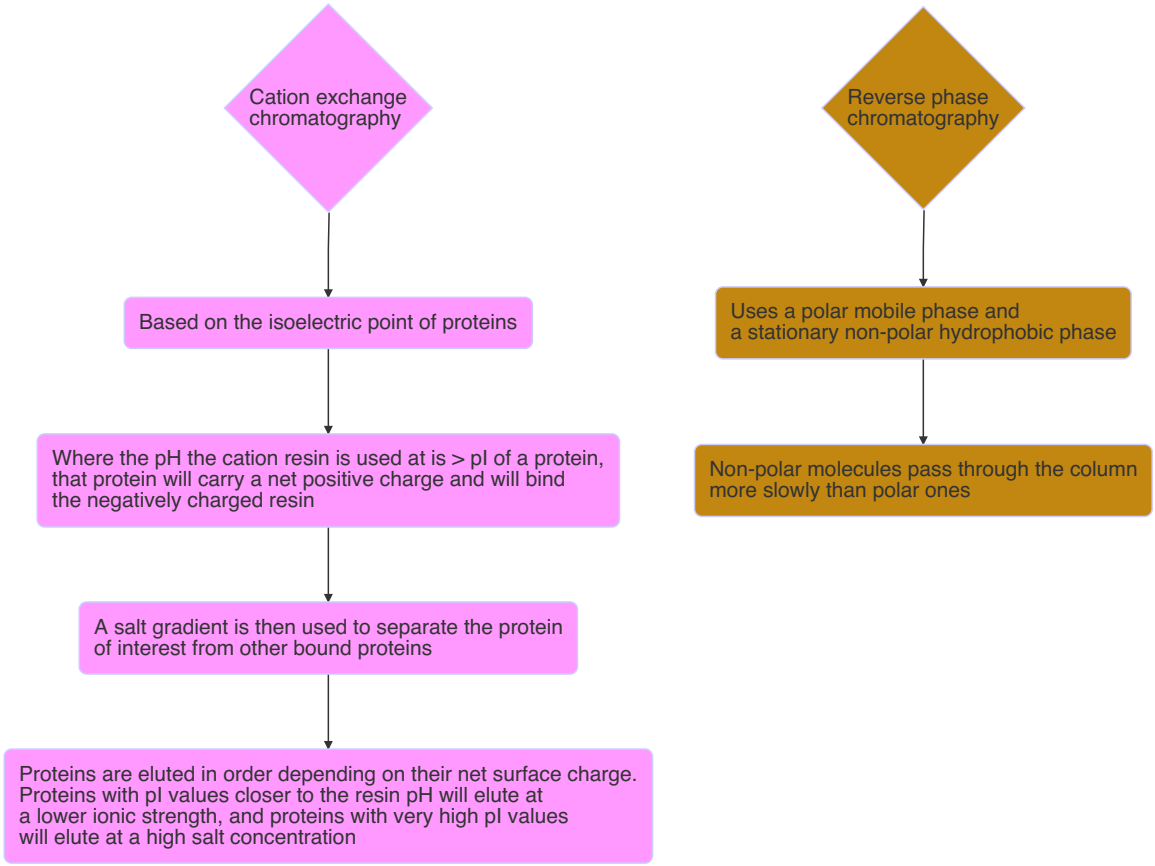


Figure 5.1: Flow diagram illustrating commonly used fractionation techniques for proteomics.

5.2 Methods

Please refer to Material and Methods chapter sections 2.2 and 2.2.3, and 2.3 for details of the ethics/cohort and blood collection respectively. The same samples were used for the iTRAQ experiments were used here, with the addition of the subacute AIS A and D samples. For sample preparation and data analysis methods, please see sections 2.3.3 and 2.4.5 respectively.

5.3 Results

In the interest of brevity, only the plots of acute and subactue AIS C improvers VS non-improvers are included here, please see the appendix for the other comparisons (section A.3.2).

The data processed by `MSstats` was filtered to proteins with a adjusted P-value of < 0.05 and a $\log_2$ FC of $> \pm 1.2$. The total number of significant proteins is 486.

5.3.1 Volcano plots

The mean number of down-regulated and up-regulated significant proteins in each group is 10.5714, and 6.7857. Between AIS C improvers and non-improvers, 8 and 4 proteins were up- and down-regulated acutely, whereas 6 and 6 were up- and down-regulated subacutely (Figures 5.2 and 5.3). Longitudinally, AIS C acute improvers had 10 up-regulated and 7 down-regulated proteins relative to subacute improvers, while for non-improvers 6 and 12 were up- and down-regulated respectively (Figures A.33 and A.34).

Please see appendix section A.3.2 for additional plots.

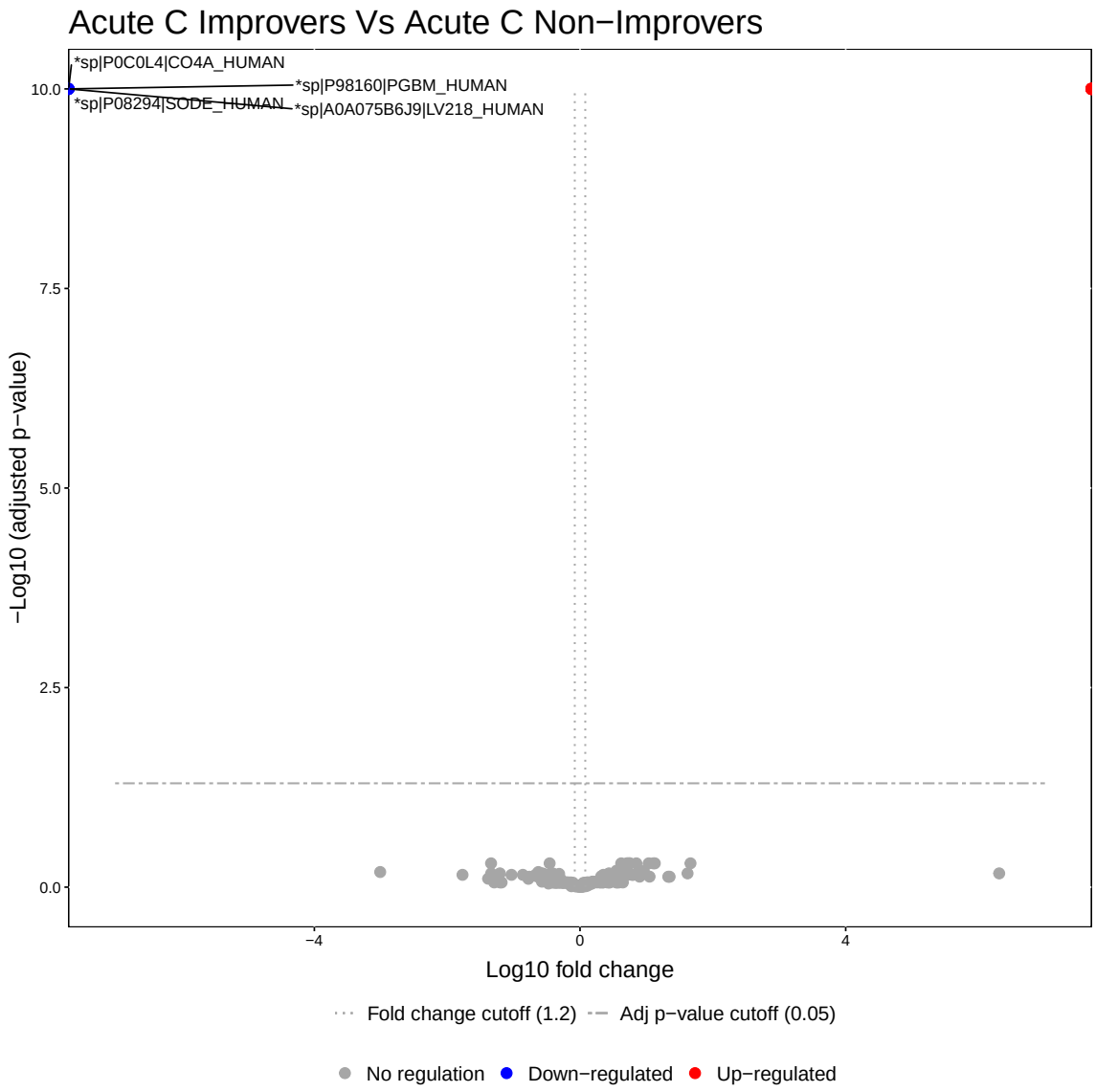


Figure 5.2: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

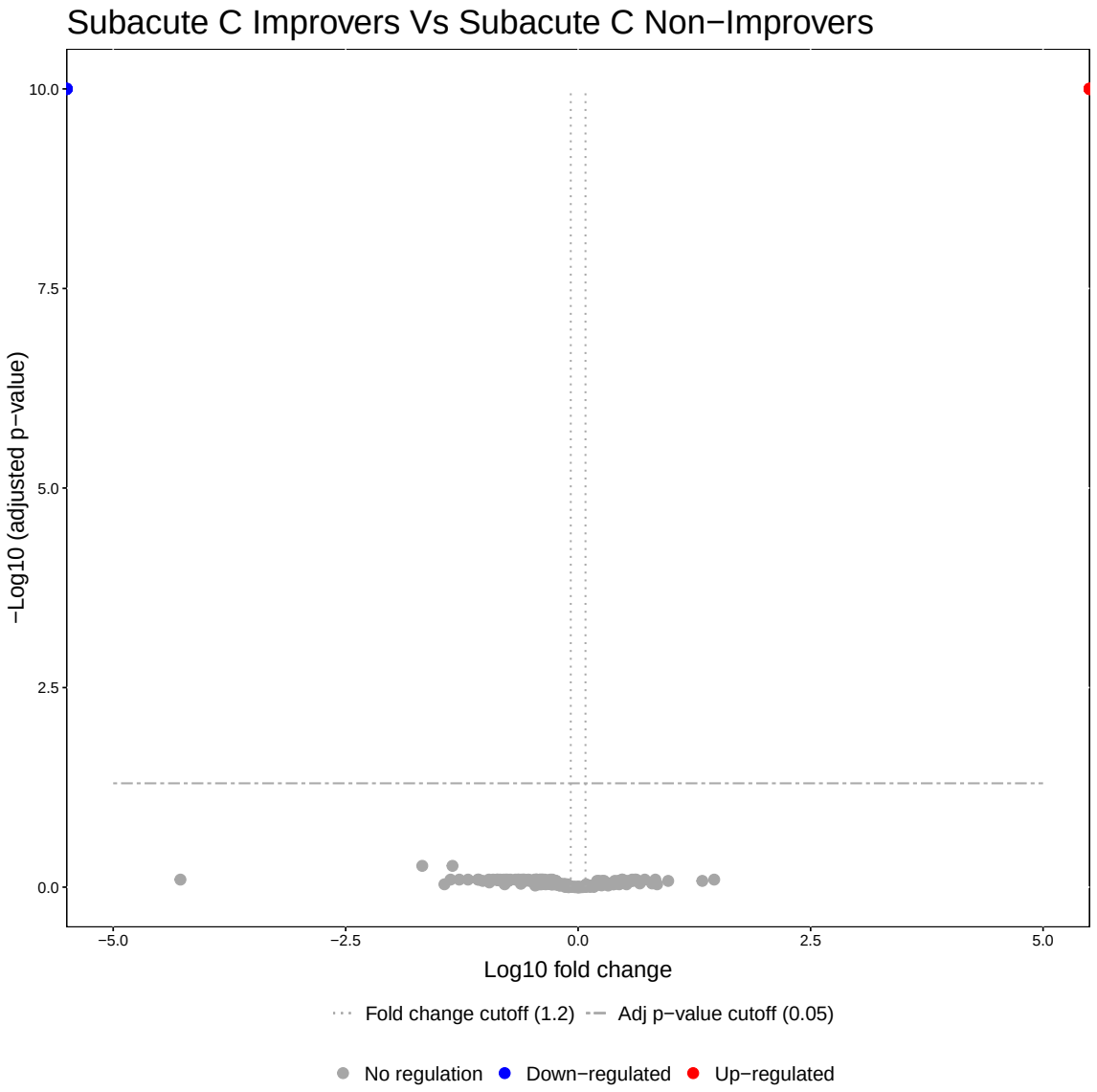


Figure 5.3: Volcano plot of $\log_10$ fold change and $\log_10$ adjusted p-value for plasma proteins from 3-months post-injury between AIS C patients who experienced an AIS grade conversion and those who did not. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

5.3.2 STRINGdb network plots

Network interaction plots generated of the significant proteins via `STRINGdb` revealed that all groups contained similarly smaller networks, with many proteins with no know interactions in the STRING database (Figures 5.4, 5.5, A.44, A.46, A.48, A.50, A.52,

A.54, A.56.

Clustering of these plots further highlights the smaller groups of interacting proteins, many of which are linear networks of proteins interacting in a chain (Figures A.42, A.43, A.45, A.47, A.49, A.53, A.51, A.55, A.57). Please note that only clusters containing more than one protein are shown.

Please see appendix section A.3.2 for additional plots.

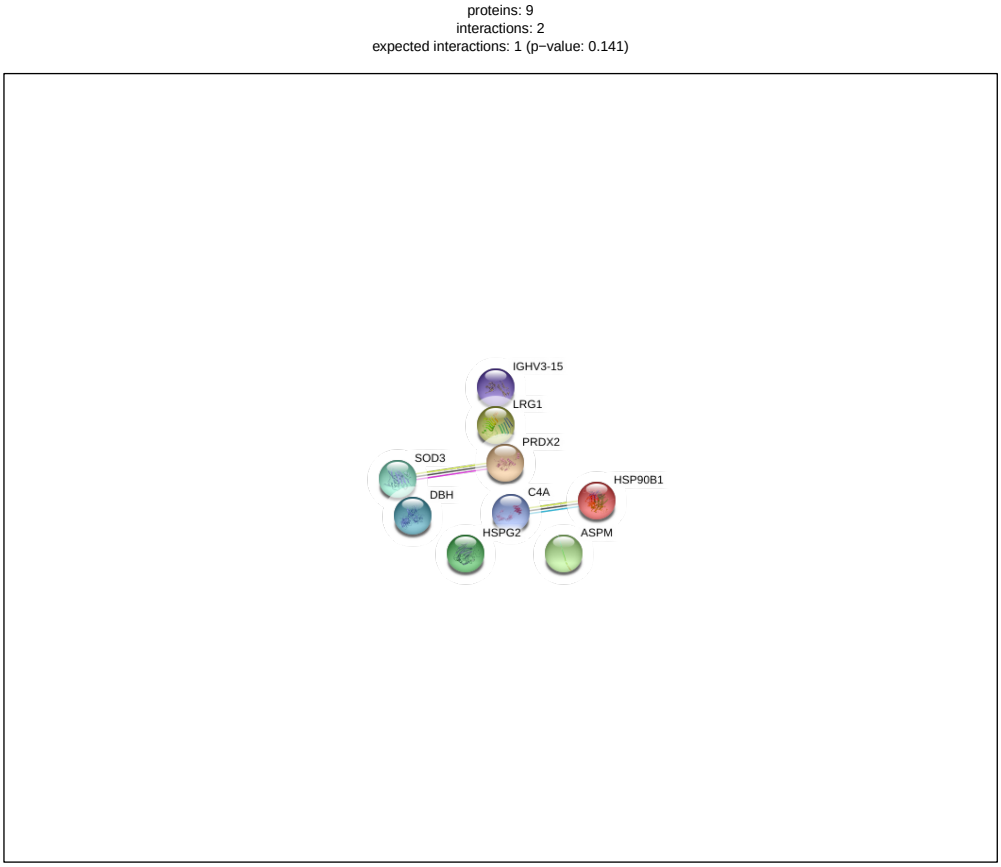


Figure 5.4: The interaction network of differentially abundant proteins found in plasma 2-weeks post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red indicates greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurrence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

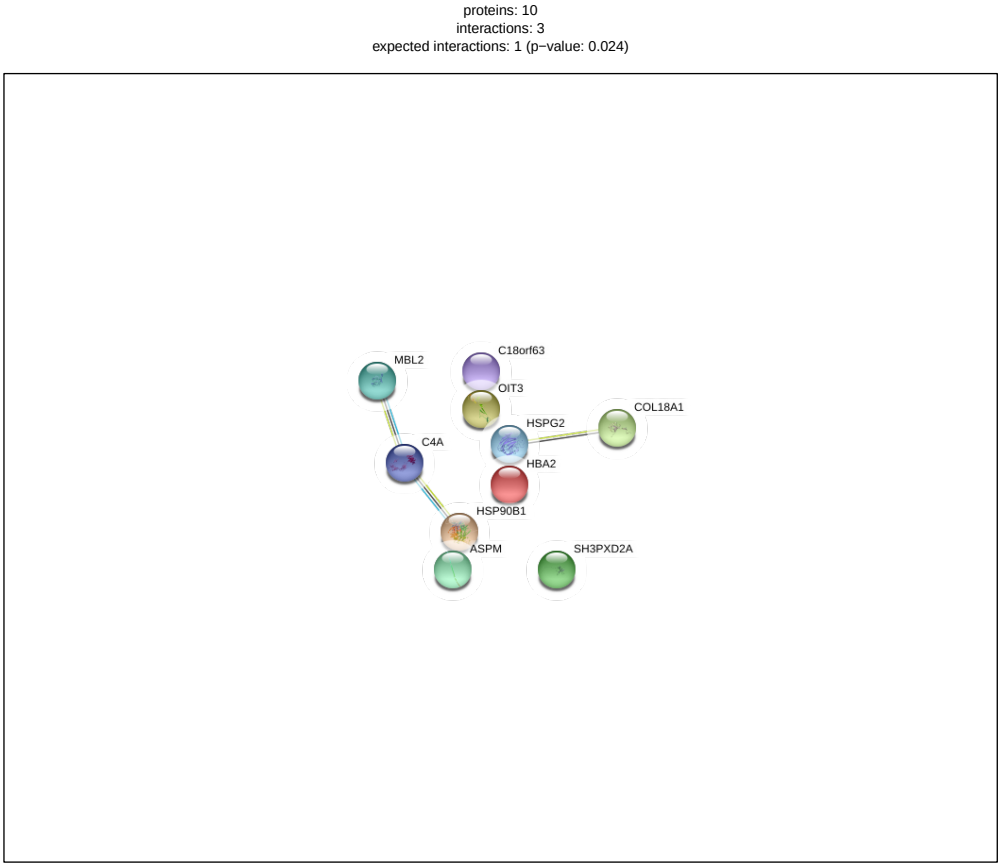


Figure 5.5: Interaction network of differentially abundant proteins from plasma 3-months post-injury, between AIS C patients who experienced an AIS grade conversion and those who did not. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there 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 .

5.3.3 Heatmaps

Similarly to the iTRAQ data (4.3.3), many of the Reactome pathways are associated with the complement cascade, platelets and clotting (Figures 5.6, 5.7, A.58, A.59,

A.60, A.61, A.62, A.63, A.64).

Please see appendix section A.3.2 for additional plots.

Figure 5.6: 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.

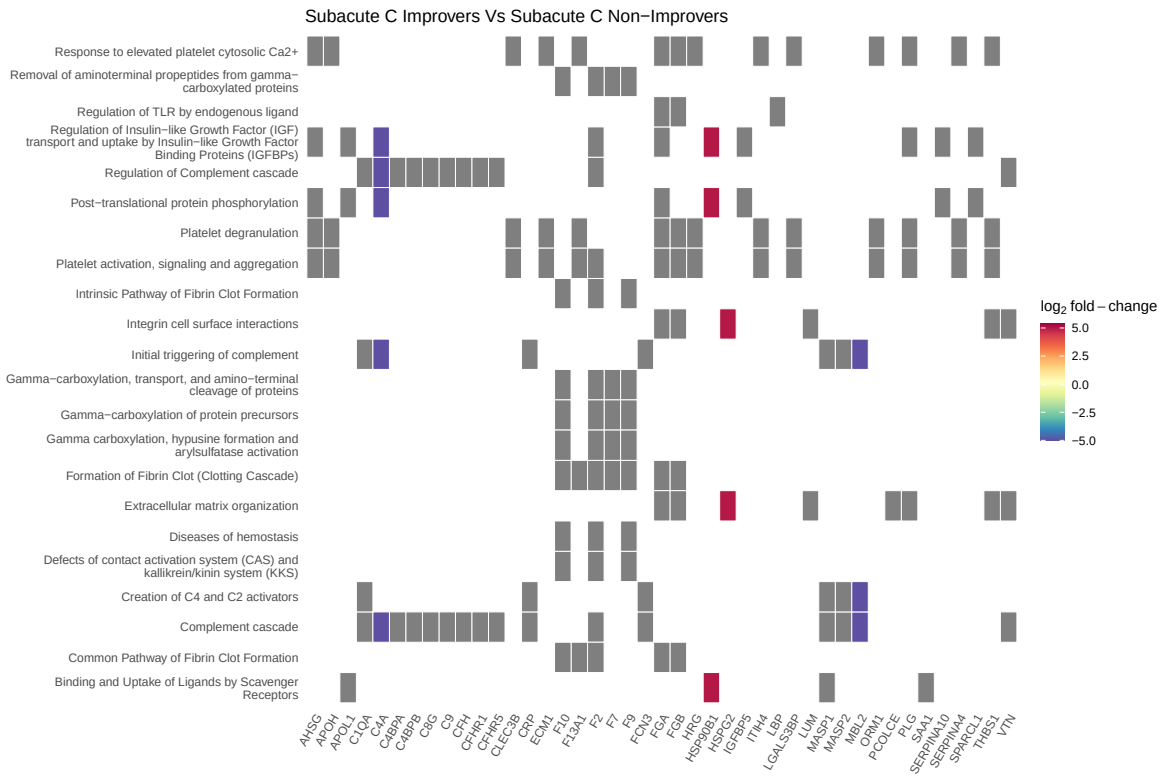


Figure 5.7: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not. Grey blocks denote proteins not present in the comparison.

5.3.4 Cnetplots

Similarly to the heatmaps and the iTRAQ data (4.3.4), network plots highlight the majority of proteins are associated with the complement cascade and pathways linked to platelets (Figures 5.8, 5.9, A.65, A.66, A.67, A.68, A.69, A.70, A.71).

Please see appendix section A.3.2 for additional plots.

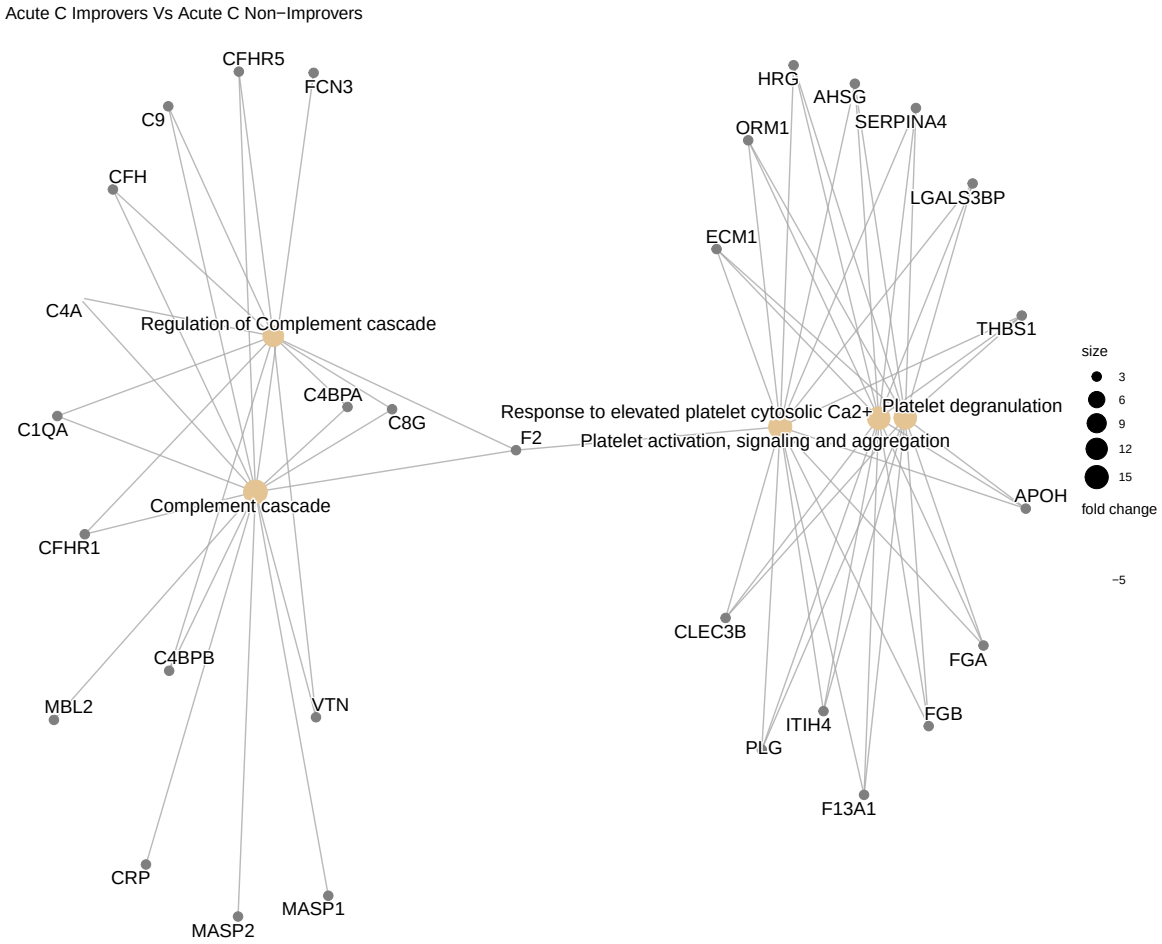


Figure 5.8: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

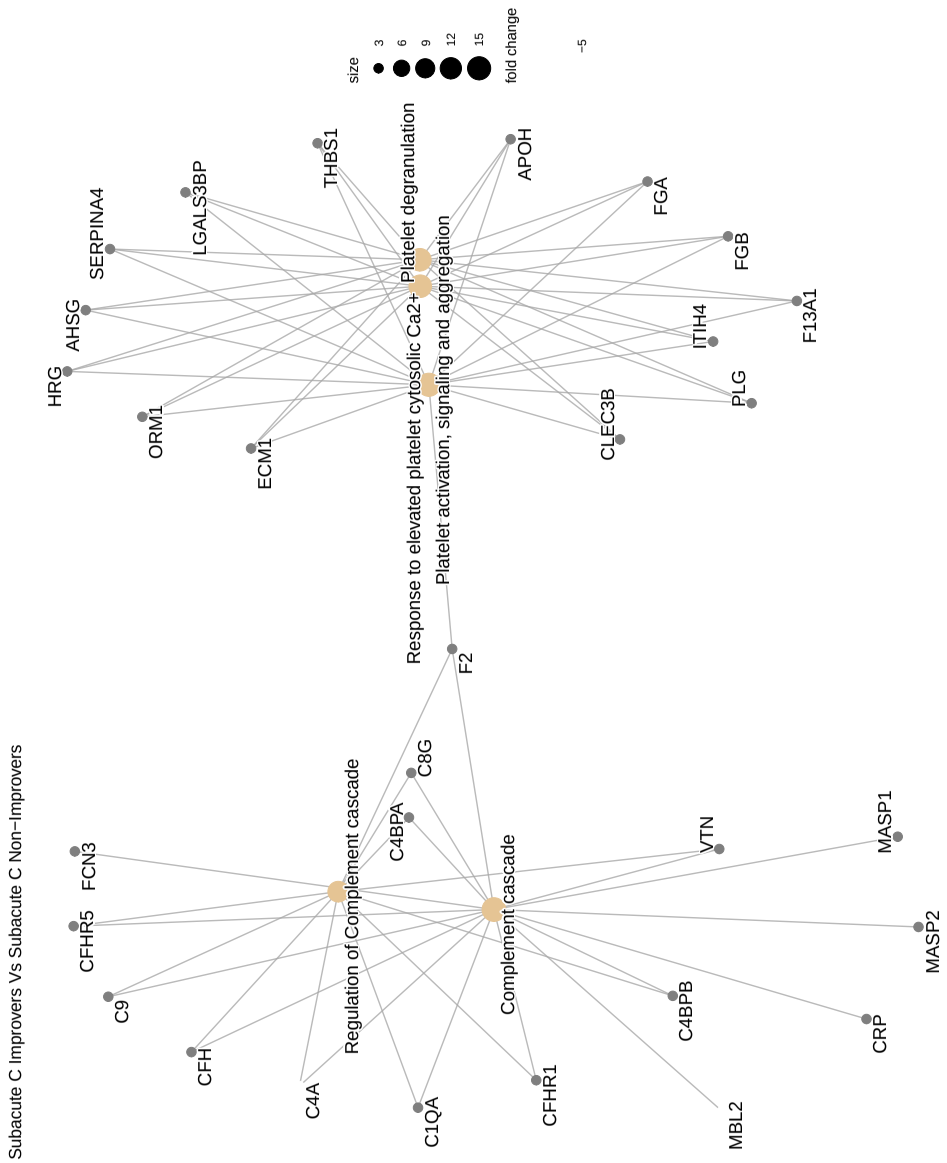


Figure 5.9: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

5.3.5 Pathway analysis

As with the iTRAQ data (section 4.3.6), pathway analysis via the `pathview` R package returned the complement and coagulation cascade to be on the sole significant KEGG pathway. The majority of the proteins present in this pathway were less abundant in the 2-week post-injury plasma of AIS C patients who experienced an AIS grade conversion and those who did not, much the same as the iTRAQ data (Figure 5.10 and section 4.3.6).

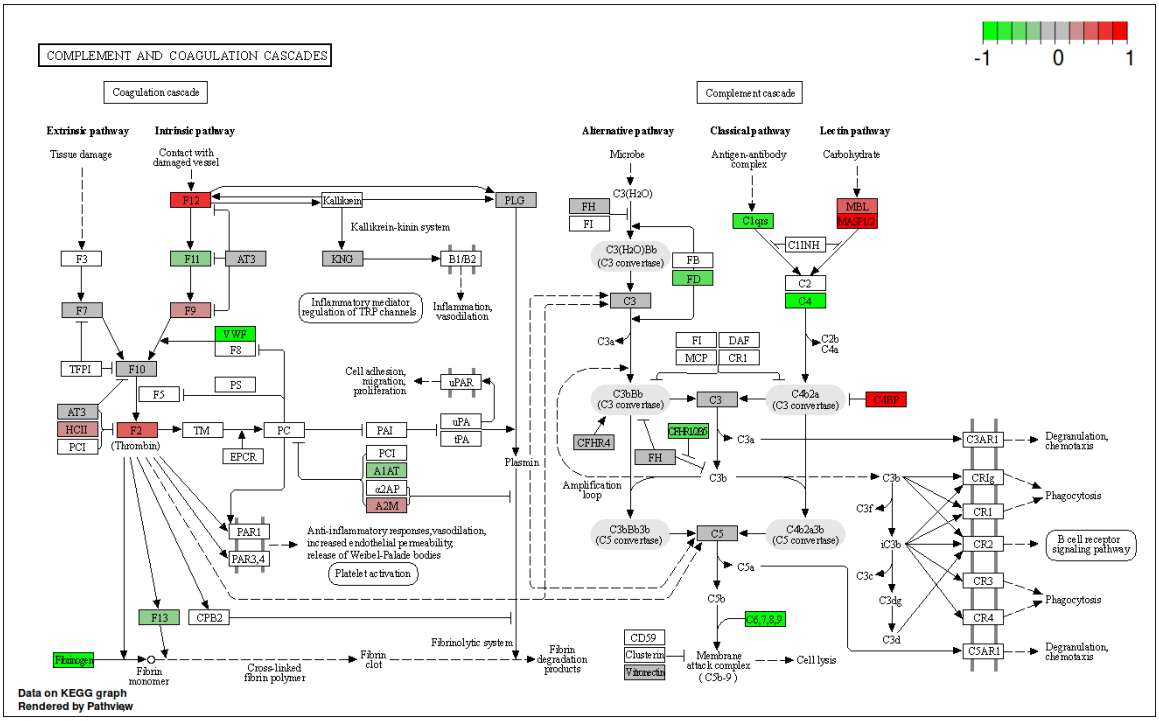


Figure 5.10: KEGG complement cascade pathway annotated with $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not.

5.3.6 Comparing iTRAQ and label-free proteins

A total of 87 and 79 unique proteins were identified across the label-free and iTRAQ experiments respectively, with modest overlap in those identified (Figure 5.11).

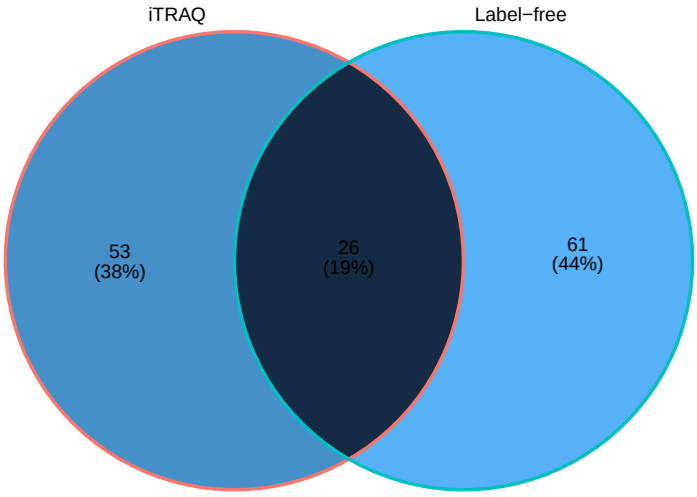


Figure 5.11: Venn diagram of the overlap in unique proteins identified from iTRAQ and label-free proteomic experiments analysed via OpenMS.

5.4 Discussion

As outlined previously (4.5), two key limitations of the iTRAQ experiments were the pooling of samples, which prevents statistically robust group-wise comparisons, and the high dynamic range of protein abundances in plasma potentially obscuring less abundant proteins. This work seeks to address these factors by a combination of Proteominer™ beads to shrink the dynamic range of protein abundances, and by not pooling samples.

5.4.1 Proteins identified

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

Oxidative stress

Oxidative stress is an important mediator of secondary injury following trauma to the brain or spinal cord. Oxygen radical-induced lipid peroxidation is a predominant source of oxidative damage.[644] Post-traumatic rapid elevation of intracellular Ca^{2++} triggers a cascade of oxygen radical reactions with a single electron (e^-) reduction of an oxygen molecule (O_2) resulting in a superocide radical ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is only modestly reactive in that in can either act as an oxidant by stealing an electron from another oxidisable molecule, thus spawning more potentially damaging radical species, or it can act as a reductant by donating it's unpaired electron to another radical species.

Whilst $O_2^{\cdot-}$ itself is less reactive than •OH, its reaction with nitric oxide (•NO) forms a highly reactive oxidising agent in the form of peroxynitrite (PN, ONOO−). ONOO− can subsequently either undergo protonation to form peroxynitrous acid (ONOOH) or react with carbon dioxide to form nitrosoperoxocarbonate ($ONOOCO_2$). These products can break down to form the highly reactive nitrogen dioxide ($•NO_2$) and •OH in the case or ONOOH, or into $•NO_2$ and carbonate radical ($•CO_3$). The elevation of reactive free radicals is often termed "oxidative stress", and can lead to "oxidative damage" whereby lipids and proteins suffer functional compromise, potentially leading to cell death.

One manifestation of oxidative damage involves oxidative attack on cell membrane polyunsaturated fatty acids triggering lipid peroxidation, which can be broken down into 3 distinct phases: initiation, propagation and termination. The initiation is triggered when a highly reactive (electron seeking) oxygen radical (such as the aforementioned •OH, $•NO_2$ or $•CO_3$) reacts with membrane polyunsaturated fatty acids (e.g., eicosapentaenoic acid, arachidonic acid, docosahexaenoic acid) thus disrupting membrane integrity. The propagation stage follows when the resulting lipid radical (L•) reacts with O_2 to form a lipid peroxyl radical (LOO•). LOO• then acquires a hydrogen atom from an adjacent polyunsaturated fatty acid yielding lipid hydroperoxide

(LOOH) and another L•, thus starting a propagating chain reaction. This propagation yields in the termination step when the peroxidisable substrate is depleted and/or a lipid radical reacts with another radical or radical scavenger to produce a relatively stable non-radical molecule/s. The end products resulting from this termination can be highly toxic. Two such aldehydic products of lipid peroxidation are 4-hydroxynonenal (4-HNE) or 2-propenal (acrolein), both of which have to characterised in experimental models of both brain and spinal injury.[645,646] Each of them can covalently bind several amino acids in cellular proteins, altering structure and function.

Another major mechanism of oxidative damage involves carbonylation by reaction of free radicals with amino acids. Free radicals can also cause nitration of some amino acids, either of which can retard protein function. For example, $\bullet NO_2$ can nitrate the 3 position of tyrosine residues, forming 3-nitrotyrosine (3-NT).[647]

Mitochondrial oxidative stress and the resulting dysfunction is particularly relevant to the post-traumatic cell death present in the injured brain or spinal cord.[648–651] The mitochondria attempt to sequester the aforementioned post-traumatic rise in CA^{++} ions, which results in mitochondrial respiratory dysfunction, lessened CA^{++} buffering capacity and oxidative phosphorylation, and ultimately mitochondrial failure due to mitochondrial permeability transition.[648,649,652,653] The possible activation of mitochondrial caspase-dependent and caspase-independent cell death cascades is not the only problematic outcome as this mitochondrial failure can also cause disruptions to synaptic function, with synaptic mitochondria being more susceptible than non-synaptic mitochondria.[652]

The disruption of respiratory function is either preceded by, or perhaps coincides with, an increase in mitochondrial free radicals and/or free radical-triggered lipid peroxidation-mediated oxidative damage.[649–651] Increasing evidence has emerged suggesting that the formation of the oxidant peroxynitrite is of particularly importance in the injured spinal cord and brain.[651,654–658] Studies have reported an increase in

peroxynitrite-induced 4-HNE (i.e., lipid peroxidation), 3-NT (i.e., protein nitration) and 4-HNE and protein carbonyl content in mitochondrial proteins preceding post-SCI mitochondrial dysfunction.[659,660] Mitochondria exposed to Ca^{++}, which causes dysfunction in them, generates peroxynitrite which in turn triggers the release of mitochondrial Ca^{++}.[661] Experiments introducing three forms of peroxynitrite, $ONOOCO_2$, ONOOH and $ONOO^-$, *in vitro*, were found to cause protein nitration and depletion of mitochondrial antioxidant stores, both indicative of peroxynitrite oxidative damage.[662] Additional *in vivo* experiments found early post-trauma treatment with the peroxynitrite radical scavenger tempol, reduced markers of oxidative damage and loss of mitochondrial function, in both TBI and SCI.[656,657] Furthermore, acrolein was found to be 10 times more potent than 4-HNE in a study on the concentration-related attenuation of the respiratory function of normal brain or spinal cord mitochondria.[663] This study also found spinal cord mitochondria to be more susceptible than mitochondria isolated from the brain. Relatedly, the carbonyl scavenging compound phenelzine was reported to reduce both mitochondrial respiratory depression and levels of aldehyde-modified mitochondrial proteins.[664]

Peroxiredoxins Peroxiredoxins are a large and highly conserved family of enzymes that reduce peroxides. Peroxiredoxin 2 (PRX-2) is highly abundant in RBCs and intracellularly serves as an important anti-oxidant role in various cells types, including neurons.[665] By contrast, extracellular PRX-2 has been suggested to act as an inflammatory DAMP, leading microglia and macrophages to release a plethora of pro-inflammatory factors.[666–668] An *in vitro* primary neurons and microglia co-culture study reported PRX-2 activating microglia via TLR-4, potentially leading to neuronal apoptosis.[669] A mouse study found over-expression of PRX-2 attenuated oxidative stress and neuronal apoptosis following subarachnoid haemorrhage.[670] Over-expression of PRX-2 is speculated to protect again ischaemic neuronal injury by

modulating the redox-sensitive thioredoxin-apoptosis signal-regulating kinase (ASK) 1 signalling complex.[671] Several molecular chaperones can interact with ASK1, including thioredoxin and TNF receptor-associated factor 6.[672] The dissociation of the thioredoxin-ASK1 complex activates ASK1. PRX-2 is oxidised after scavenging free radicals, whereupon its antioxidantive activity is reduced. This inactivation can be reversed by the thioredoxin-thioredoxin reductase system, whereby oxidised PRX-2 can regain its activity by reducing thioredoxin, leading to the dissociation of the thioredoxin-ASK1 complex.[673] Additionally, oxidised PRX-1 can inhibit ASK1-induced apoptosis via the thioredoxin-binding domain on ASK1.[674]

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

Neuroinflammation post-SCI

The neuro-inflammatory response begins immediately post-trauma, and involves a complex series of events that can persist well into the chronic phase. The sudden emergence of necrotic cell debris and associated DAMPs lead surviving CNS-resident cells to produce cytokines, complement factors and ROS. Within minutes, CNS cells at the lesion site have been found to secrete several pro-inflammatory mediators, including TNF-α and interleukins, in both rodent models and human patients with SCI.[39,675–677] The resulting inflammatory response occurs in parallel to the mechanical destruction of the blood-spinal cord barrier, and the development of tissue oedema and ischaemia combine to propagate damage to parts of the cord spared by the initial trauma.[18,678]

The microglial population at the lesion site have been observed to be significantly depleted immediately post-injury, due to death via both the apoptosis and mechanical injury in a rodent model.[679] Surviving microglia change in shape and migration patterns, and begin to produce ROS, oxidative metabolites and pro-inflammatory cytokines.[39,680] These cells can associate with damaged axons rapidly post-injury, but are thought to not actively phagocytose these cells until approximately 4 days post-trauma.[39,679,681]

The following hours and days post-injury are characterised by a substantive complement system activation and sequential leukocyte migration from the periphery into the injured neural parenchyma.[682–684] Curiously, though the breakdown of the BSCB would presumably allow unrestricted access of circulating leukocytes into the injured cord segment, recruitment of these cells remains a highly controlled process.[685,686] A mouse study reported lymphocytes, which account for approximately 80% of circulating leukocytes, only enter the cord in substantial numbers at least several weeks to months post-injury.[685] Early infiltrate is instead largely comprised of myeloid cells, predominantly neutrophils, which are a minority of circulating cells

but are the swiftest peripheral responders to SCI, with studies detecting them at the lesion site within 4 hours of injury.[687] Neutrophil numbers have been reported to peak at 1 day post-trauma, but also to remain at the site for a minimum of 42 days post-injury.[688,689]

This neutrophil recruitment is often viewed as principally detrimental to recovery following SCI, but also wound healing more generally. A recent study found circulating neutrophil numbers in admission bloods from human SCI patients were negatively correlated with patient outcomes at discharge.[686] The same study utilising a contusive SCI mouse model, showed the extent of neutrophil presence at the lesion site inversely correlated with neurological outcomes, and depletion of said cells with an antibody against Ly6G improved recovery of motor function.[686] However, other studies have suggested neutrophil activity which potentially benefits SCI recovery.

A transgenic mouse contusion model study showed over-expression of secretory leukocyte protease inhibitor, which can arise from neutrophils and activated macrophages, improved locomotive functional outcomes, and reduced markers of secondary injury.[690] Another study, using a peripheral nerve injury mouse model, reported neutrophil infiltration and associated cytokine/chemokine production was vital for clearance of myelin debris.[691] Additionally, another study using a mouse contusion model found increased lesion sizes and impaired neurological outcomes following neutrophil depletion, though the Gr-1 antibody used also depletes inflammatory monocytes, muddying the picture somewhat.[692] Regardless, it is clear that the complexity of the role neutrophils play in the SCI response extends beyond any simple binary beneficial/harmful distinction.

Moving forward in the SCI pathology, newly proliferated and recruited microglia begin actively phagocytosing necrotic cell debris, and begin accumulating around the lesion epicentre.[39,679,681] The presence of microglia appears to be vital, particularly during the first week post-SCI, as depletion via the colony stimulating factor-1 in-

hibitor PLX5622 has been linked to substantially worsened functional outcomes.[679,693] Relatedly, another mouse SCI model study found early enhancement of microglial activation can reduce secondary pathology.[694]

Circulating inflammatory monocytes are also recruited during the first days post-trauma. Adoptive transfer experiments have shown recruitment to pick up at approximately 3 days post-injury, and peak at 7 days.[695] Whilst monocyte turnover at the lesion appears to be high, infiltrating monocyte-derived macrophages remain at the site of weeks to months post-trauma.[695,696] Interestingly, the timing of monocyte recruitment appears to be delayed relative to non-neurological tissue injury. For instance, monocytes are reported to be rapidly recruited to the heart following a myocardial infarction, as early as 1 day post-injury, and their numbers return to baseline by roughly 16 days post-injury.[697]

Owing to the diversity of monocyte subsets and macrophage phenotypes, a complete understanding of their role with respect to SCI pathology is still lacking, and requires further research.[698] Some polarisation states associated with recruited macrophages are thought to be implicated in propagating secondary injury via fibrotic scar formation and demyelination of axons.[699–701] Similarly, several studies have reported a reduction in infiltration of monocytes/macrophages is associated with better SCI outcomes.[699,701,702] Conversely, others have found depletion of circulating monocytes/macrophages significantly increased lesion size and results in worse function outcome, with restoration of blood monocyte numbers attenuating this phenotype.[696]

More recent *in vitro* studies suggested blood-derived macrophages can suppress microglial phagocytosis without reducing microglial proliferation and extension of processes.[681,703] This literature represents an ongoing controversy over the role of monocytes/macrophages in relation to recovery post-SCI. Importantly, many of these studies are based on somewhat crude depletion of cell types, with little discrimination paid toward any potential subpopulations and/or cell polarisation status. Given the

sheer complexity of the pathology at play, more nuanced approaches will likely be needed in future studies to paint a more complete picture.

B cell recruitment is yet another wave of immune cell infiltration, thought to occur several days post-injury. These cells can form follicle-like structures in combination with T cells, microglia and macrophages from roughly 28 days post-trauma, and remain present and the lesion well into the chronic phase of SCI.[704] Whilst the extent of B cell presence has been reported to vary between animals, they have been correlated with self-reactive antibodies that recognise epitopes within protein homogenates of the spinal cord.[705] Adoptive transfer experiments in a mouse model isolated antibodies from SCI mice, and found injected them into the neural parenchyma of naive animals induced significant damage, whereas mice lacking B cells have improved recovery post-SCI.[704]

Move evidence is needed to establish whether these self-reactive antibodies precede an autoimmune event, or signify a autoimmune disease. Alternatively, they may serve as a mechanism for opsonisation and debris clearance from the lesion site.[706] Naturally occurring autoantibodies with well-established role in tissue regeneration and repair have been found to be elevated following SCI.[707,708] Much like the aforementioned monocyte/macrophage controversy, it should be pointed out that any positive effects of these autoantibodies does not preclude any simultaneous negative impacts which could be modulated. For instance, another study reported naturally occurring IgM antibodies contribute to secondary injury during the more acute phase post-SCI.[709]

Neuro-inflammation is less understood at the chronic phase of SCI, as most studies focus on the first hours and days post-injury. By this stage, the glial scar has established a well-defined border between the lesion core and the health tissue flanking it.[710] Infiltrating immune cells are largely restricted to within the lesion itself, as opposed to the surrounding spared tissue. B and T cells, macrophages and neutrophils have all been detected here many months post-trauma.[685,704,711] The chronic phase

is also marked by substantial metabolic dysfunction, characterised by reduced lipid metabolites and increased oxidative stress, in addition to elevated pro-inflammatory mediators.[712]

There are fewer studies that attempt to elucidate the underlying mechanisms driving this non-resolving inflammatory response in the chronic phase of SCI. One study suggested communication with infiltrating monocytes suppresses chronic microglial activation and inflammation after SCI.[703] Interruption of this communication was linked to worsened function outcomes, implying the initial microglial response to trauma may be beneficial, whilst their protracted activation can eventually become detrimental.[679,703]

Furthermore, a rodent model study of chronic SCI, found use of the anti-inflammatory drug licofelone, applied daily for 1 month at 8 months post-injury, observed some improvement to metabolic functions, but no benefit to locomotor function.[712] To summarise, understanding of persistent inflammation during the chronic phase of SCI is lacking, and particularly complicated by the plateaus in locomotive recovery that typically occurs well before the chronic SCI phase is reached. Thus, there is a need for further studies to uncover the role of the various immune cell populations with respect to ongoing neurological dysfunction and pathology during the chronic phase of SCI.

Intravenous immunoglobulin Intravenous immunoglobulin (IVIG) is increasingly used as an immunomodulatory strategy for managing acute neurological conditions, including neurotrauma. Originally developed as an antibody replacement therapy for immunodeficiency disorders, IVIG is a product comprised primarily of immunoglobulin G (IgG) taken from the blood plasma of healthy donors.[713,714] IVIG therapy was found to increase platelet number in idiopathic thrombocytopenic purpura (ITP) patients, which lead to an interest in using it as an immunomodulatory therapy.[715] Its potent

effects and limited side effects have lead high-dose IVIG therapy to be commonly used in a plethora of inflammatory and autoimmune disorders, including ITP, arthritis, Kawasaki's syndrome and Guillian-Barré syndrome.[716,717]

Some recent research using a contusive SCI mouse model has reported promising results of high-dose IVIG as a therapeutic for SCI.[718] The study found that a clinical dose of IVIG (0.5-2g/kg body weight) lead to a 30-40% reduction in lesion size, and reductions in demyelination, central canal dilation, and axonal degeneration, though doses below 0.5g/kg were ineffective.[718] The same study also found albumin treatment did not produce the same effects as IVIG, suggesting simple protein loading is not the causative mechanism. Likewise, rodent studies utilising purified human IgG in a high-level (C7-T1) clip aneurysm model, and another lower-level (T9) contusion SCI study, reported similar improvements.[719–721] Additionally, a Phase I/IIa clinical trial aiming to explore the safety and efficacy of IVIG therapy in human SCI patients is approved and underway (ACTRN12616001385437). However, whilst there are several pre-clinical studies reporting IVIG treatment can benefit outcomes in CNS injury from a range of neurological conditions, the exact mechanism/s behind any potential neuroprotective effects of IVIG for SCI are currently unclear.[722]

In TBI mouse models, animals treated with IVIG were shown to have improved neurobehavioural outcomes, and a reduction in neuronal degeneration both acutely and chronically, relative to vehicle-treated controls in rotarod and Morris water maze experiments.[723] Further mouse studies using cerebral artery occlusion, a model of stroke, reported high-dose IVIG significantly reduced infract volumes, neurological impairment and mortality rates.[724,725] Under condition of BBB/BSCB compromise, IVIG has been found to enter the neural parenchyma within hours of injury.[718,724] SCI studies have found IVIG to localise to oligodendrocytes, astrocytes, neurons, macrophages, microglia, pericytes and blood vessels.[718,720] Additionally, reductions in immune cells, as indicated by F4/80^{+} microglia/macrophages and polymorphonuclear cells in brain

and spinal injury models respectively, have also been reported.[719,720,723] Relatedly, the aforementioned SCI IVIG mouse study found reduced $CD68^+$ macrophages at and surrounding the lesion 35 days post-injury.[718] Importantly, these studies do not differentiate between resident microglial and infiltrating monocytes/macrophages. Thus, further research is needed to understand the influence of IVIG on both recruitment and activation states of these cell subsets.

Speculative mechanisms of action for IVIG in SCI As IVIG is made from pooled antibodies taken from thousands of donors, it includes a vast repertoire of antibodies specific against millions of unique antigens, allowing for a diverse variety of effects in differing disease contexts. Whilst there is extensive research of IVIG and autoimmune disorders, such as Guillain-Barré syndrome, the immune pathology found in the acute phase of CNS injury is not typically considered to be driven by autoimmune processes.[716,717] There may be some overlap in therapeutic mechanism, but it seems more likely any benefits are coffered through modulation of the innate rather than adaptive immune responses. The potential mechanisms of IVIG can be split between those mediated via the IgG constant (Fc) fragment, which binds the Fc receptors, and the F(ab)‘$_2$ fragment, which governs antigen recognition.[714] In the context of neurological diseases, mechanisms related to F(ab)’$_2$ are thought to potentially bind and therefore neutralise cell surface receptors, complement, cytokines and autoantibodies. By contrast, Fc-dependent mechanisms are speculated to include regulation of Fc receptor expression, saturation of the neonatal Fc receptor, block activation of Fc receptors, and modulate T cells.[714,716,726] Furthermore, models of neurological injury suggest both F(ab)’$_2$ and Fc-dependent signalling cascades could be involved in the modulation of several chemokines and cytokines.[726]

Modulation via the variable F(ab)’$_2$ region Self-reactive antibodies have been found circulating in both chronic rodent SCI models and human patients 1 year

post-injury.[704,727] Whilst some studies have suggested potential relevance of naturally occurring autoantibodies (germline encoded and produced by B1 cells) in acute SCI, it remains unclear whether IVIG treatment may have any impact on them.[707,709] The impact, or lack thereof, of IVIG on chronic phase SCI autoimmunity also remains to be seen.

A separate potential F(ab)'$_2$-dependent mechanism involves the neutralisation of the cell death mediator Fas (AKA CD95). Studies of Lyell's syndrome, a disorder whereby active Fas ligand binds Fas present on keratinocytes, inducing apoptosis, reported IVIG therapy completely inhibited Fas ligand-ind cued cell death both *in vitro* and in human patients.[728,729] Importantly, IVIG blocked Fas, as opposed to Fas ligand, in these studies, as this result was only observed with cells pre-treated with IVIG. Incubation of IVIG with soluble Fas ligand did not attenuate cell death, implying IVIG contains antibodies specific to Fas.[728,729]

This modulatory effect of the Fas-Fas ligand pathway may have relevance in SCI, as a study using knock-out mice lacking Fas showed a reduction in both apoptosis at the lesion site and glial scarring, and improved motor function post-SCI.[730,731] Neurons and glial cells from post-mortem human patients were found to be more Fas- and Fas ligand-positive, but this was limited to the acute phase of SCI, and not observed chronically, suggesting this pathway is more significant immediately post-njury.[731] Therefore, acute IVIG treatment could act by attenuating secondary cell death by blocking Fas, thus disrupting this pathway.

Conversely, agonistic anti-Fas antibodies have also been reported within IVIG preparations.[729] Whilst it remains unknown how these agents may act in SCI, one could postulate a benefit if they induce apoptosis in circulating leukocytes, which could otherwise do harm.[732] Supporting this, papers have found reductions in polymorphonuclear cell populations within the lesion at 1 day post-injury in rodent models.[719–721] However, IVIG-induced apoptosis has only been observed in human leukocytes, not in

rodents, casting doubt on this idea.[729,732]

Alternatively, the reduced recruitment could be a result of IVIG regulating the expression of adhesion molecules or molecules involved in leukocytes trafficking. A feline ischaemia-reperfusion injury model study found IVIG to down-regulate expression of integrins on leukocyte cell surfaces, inhibiting adhesion and subsequent extravasation of the cells into the damaged site.[733] Again however, these finding are contradicted by an experimental stoke study where IVIG was found to increase leukocyte and platelet trafficking to the injury, leading to formation of aggregates within cerebral vasculature.[734]

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.[735,736] Multiple studies utilising various models of CNS injury have reported IVIG attenuating complement.[718,724] Specifically in SCI, IVIG was found to reduce levels of the complement activation products C3b and C5a within the damaged cord.[718]

Similarly, an experimental stroke study reported IVIG reducing C3b levels in the infract area.[724] 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.[724]

Modulation via the constant Fc region With respect to the Fc region, this portion normally binds to Fcγ receptors (FcγRs), which are present on most leukocytes and resident CNS cells. Many FcγRs act as activating receptors, such as inducing phagocytosis in response to opsonised targets, or as an inhibitory receptor

that dampens effector cell responses.[714] A given cells response to an immunoglobulin isotype is determined by the combination of which FcγRs are expressed by said cell.

Myeloid cell all express come combination of these activating FcγRs, 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.[737] The inhibitory FcγRIIb receptor is also expressed on myeloid cells, in addition to B cells, but not natural killer cells or resting T cells.[738] Whilst there is debate over the expression and function of FcγRs in neurons, *in vitro* work with neuronal cultures has detected mRNA for all FcγRs.[739] Astrocytes, microglia and oligodendrocyte precursors have also be found to express FcγR, and up-regulate them under some disease states.[739]

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 FcγRs play a key role in the mechanism of IVIG.[740–742] With respect to CNS injury, some evidence suggesting a role of FcγRs comes from a mouse study with animals lacking the common γ-chain, and thus no functional FcγRs, which were found to be protected from experimental stroke and SCI.[704,743]

Within the context of antibody-mediated autoimmune disorders, high-does IVIG may saturate Fc receptor and reduce the half-life of pathogenic endogenous IgG.[714]

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

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

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

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

subacute Ds.

5.4.2 Conclusion

Much like the iTRAQ experiments (4.5), the majority of proteins identified are functionally associated with the complement cascade. Unlike the iTRAQ however, many of the proteins where only detected in one group of the pairwise comparisons, suggesting greater suitability as biomarkers. PRX-2, a protein associated with oxidative stress, is of particular interest, both as a biomarker for improvement in acute AIS C patients, but also mechanistically in relation to functional recovery. Furthermore, several immunoglobulins were identified as differentially abundant, though further *in vitro/vivo* work is needed to elucidate the pathophysiological relevance of each precursor. The λ 2-18 and 3-10 precursors are of particular relevance to acute and subacute AIS C improvement respectively, and both are of interest longitudinally in AIS As, with 2-18 potentially being linked to severity of injury.

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

Concluding comments

5.5 Summary of key findings

The research that comprises this thesis has focused on unbiased approaches to biomarkers discovery for SCI. Firstly via prognostic modelling of neurological outcomes via baseline assessment, demographic information and routinely measured haematological markers. Secondly via two proteomic approaches, both labelled and unlabelled, of blood plasma from human SCI patients. The key findings from each of these is subsequently summarised.

5.5.1 Chapter 3 *Prognostic modelling of SCI outcomes with routine blood measures*

Routinely measured haematological markers added statistically significant value to predictions of neurological outcomes in both the preliminary and larger scale follow-up studies. In particular, markers commonly associated with liver function, including alkaline phosphatase, alanine transaminase and γGT, were found to add modest predictive value, implicating the liver in the heterogeneity of neurological outcomes post-SCI. Modulation of liver health may therefore present a clinical target for attenuating both functional recovery and secondary disease such as diabetes. Gender was also found to be significant in predictions of SCIM-III, suggesting a potential gender bias in the scoring.

5.5.2 Chapter 4 *Characterisation of the plasma proteome following Spinal Cord Injury in Humans*

iTRAQ proteomics revealed the complement cascade to be of particular importance with respect to neurological improvement in AIS C and severity of injury. Key proteins identified include several apolipoproteins, SAA1 and RBP4. All of these proteins have strong links to the liver and metabolic health more broadly, reinforcing the relevance of the organ to SCI.

5.5.3 Chapter 5 *Label-free proteomics*

A label-free proteomics approach largely corroborated result of the complement cascade relevance found from the iTRAQ experiments. However, the un-pooled biological replicates allowed for more robust statistical analysis, and compression of the sample dynamic range via ProteoMiner™ beads allowed further proteins to be identified. Proteins of particular note with respect to differential recovery and injury severity include PRX-2, C4 and several immunoglobulin precursors. These proteins are associated with oxidative stress and the complement cascade respectively and were found to be binary in their presence or absence across groups, indicating high suitability as prospective biomarkers.

5.6 Future direction

This research presents two broad avenues of further work. Firstly, more targeted *in vitro/vivo* experiments investigating the pathophysiological relevance of aforementioned prospective biomarkers. Knockout experiments in animal models would serve well to this end. Relatedly, some of the proteins identified, ApoE in particular, may have relevance beyond simple differences in expression levels, and may have allelic significance. Genetic characterisation of these proteins would therefore also be of

interest. With respect to the modelling of neurology via routine blood markers, as with any statistical model, external validation is needed to properly support the findings. To this end, access to data from another spinal injury centre is being finalised at time of writing, the findings from which will be sought to be published in 2022.

Secondly, experiments to uncover the scope of functional improvement governed by the liver/metabolic status. Of particular interest with respect to the liver is how important liver status preceding injury relative to post-injury. If most of the impact on functional recovery is determined by pre-injury status then it is less likely to have a significant clinical impact beyond supporting broader public health messaging as to the value of maintaining good health via diet and excise. However, if modulating metabolic health post-injury has a larger impact on function outcomes than previously appreciated, there is great scope for improving outcomes in a low-risk and low-cost manner. Experiments of modulating diet, including use of pre- and pro-biotics would be of particular interest and human trials could be conducted in this space at relatively low-cost and risk. Whist there are currently several clinical trials underway related to diet in SCI, most of these focus on obesity or nutritional deficiencies, rather than investigating diet as a potential regenerative strategy.[744]

5.7 Concluding comment

A growing body of evidence, including the work conducted here, implicates the liver and the complement cascade as being of particular importance in determining heterogeneous outcomes of SCI. This is likely scope for improvement of function outcomes through modulation of these pathways. However, a great deal of further research is needed to reveal the precise biochemistry at play, and how they might be targeted as part of a regenerative strategy.

Appendix A

Supplementary data

A.1 Session Information

	-
platform	aarch64-apple-darwin20
arch	aarch64
os	darwin20
system	aarch64, darwin20
status	
major	4
minor	1.3
year	2022
month	03
day	10
svn rev	81868
language	R
version.string	R version 4.1.3 (2022-03-10)
nickname	One Push-Up

Table A.1: 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
RColorBrewer	1.1.3	2022-04-03
ggVennDiagram	1.2.0	2021-10-19
BiocManager	1.30.16	2021-06-15
glmnet	4.1.3	2021-11-01
Matrix	1.4.0	2021-12-08
rlang	1.0.2	2022-03-04
DiagrammeR	1.0.9	2022-03-04
lubridate	1.8.0	2021-10-03
lime	0.5.2	2021-02-24
mice	3.14.0	2021-11-23
readxl	1.4.0	2022-03-28
bookdown	0.25	2022-03-16
huxtable	5.4.0	2021-05-14
knitr	1.38	2022-03-25
rmarkdown	2.13	2022-03-09
caret	6.0.91	2022-03-11
zoo	1.8.9	2021-03-06
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
thesisdown	0.2.0.9000	2022-04-06
remotes	2.4.2	2021-11-30

A.2 Model coefficients

Table A.2: Linear regression model coefficients with elastic net penalisation for perliminary paper cohort.

Variable	coefficients

Discharge total motor	
(Intercept)	148.7857
Age at injury	1.3335
Initial sensor prick	7.8484
Initial motor	23.3331
Initial sensor total	17.2439
Discharge sensor touch	
(Intercept)	78.3065
Admission AIS grade	1.1696
Admission AIS grade	1.3169
Central cord syndrome	1.4873
Initial sensor touch	19.1824
Discharge sensor prick	
(Intercept)	75.1475
Admission AIS grade	0.2496
Initial sensor touch	7.5697
Initial sensor prick	6.2208
Initial sensor total	7.5470
Discharge AIS motor	
(Intercept)	60.0656
Comorbidities	2.3476
Previous ailments	0.8146
Diabetes (either type)	0.3047
Age at injury	2.3568
Admission AIS grade	1.1362
Admission AIS grade	4.6308

Central cord syndrome	1.5975
Initial sensor touch	4.8084
Initial motor	11.5082
Initial sensor total	6.2058
Time from injury to 12-month neurology	4.6400
Phosphate (mmol/L)	1.0182
Magnesium (mmol/L)	0.3072
Discharge total sensory	
(Intercept)	152.6500
Comorbidities	0.9377
Admission AIS grade	0.8483
Initial sensor touch	15.8772
Initial sensor prick	9.1066
Initial sensor total	16.4096
Month-12 total motor	
(Intercept)	154.7609
Age at injury	1.1149
Admission AIS grade	1.8241
Central cord syndrome	5.3870
Initial total motor	2.2743
Initial sensor touch	11.2706
Initial sensor prick	12.1746
Initial motor	10.3513
Initial sensor total	11.8338
Time from injury to 12-month neurology	4.8506
Month-12 sensor touch	

(Intercept)	75.4464
Comorbidities	0.1377
Admission AIS grade	1.2667
Central cord syndrome	0.0248
Initial sensor touch	18.6094
Urea (mmol/L)	0.2457
Month-12 sensor prick	
(Intercept)	74.7273
Comorbidities	0.0031
Admission AIS grade	1.7354
Central cord syndrome	0.4710
Initial sensor touch	18.3270
Month-12 AIS motor	
(Intercept)	66.1053
Comorbidities	0.7853
Diabetes (either type)	0.4561
Medications	0.7261
Age at injury	2.3692
Admission AIS grade	2.6408
Admission AIS grade	1.1615
Admission AIS grade	0.2192
Lumbar injury	0.5967
Central cord syndrome	2.9550
TetPara2	0.2152
Initial total motor	2.3796
Initial sensor touch	3.9991
Initial sensor prick	3.7589

Initial motor	3.0354
Initial sensor total	3.8996
Time from injury to 12-month neurology	2.0866
Red blood count (10*12/L)	0.0172
Haematocrit (L/L)	0.7445
Mean Cell Volume (fL)	0.2150
Prothrombin Time (s)	0.5558
International Normalized Ratio	0.4419
Month-12 total sensory	
(Intercept)	149.5893
Admission AIS grade	8.0131
Initial sensor touch	35.6752

Table A.3: Final elastic net model parameters for preliminary data. Alpha is a value between 0 and 1, where 0 is pure ridge regression, 1 is pure LASSO and values between are a mixture of both. Lambda is the shrinkage factor applied to model coefficients

Model target	alpha	lambda
at3mMotor	1.0000	10.641
at3mTouch	1.0000	2.702
at3mPain	0.7375	4.080
at3mASIAMotor	1.0000	1.325
at3mASIASensory	0.8875	6.788
at1yMotor	0.3625	15.470
at1yTouch	1.0000	3.818
at1yPain	1.0000	3.835
at1yASIAMotor	0.1375	12.259

at1yASIASensory	0.9250	9.277

The subsequent coefficients are from the follow-up study.

Table A.4: Linear regression model coefficients with elastic net penalisation

Variable	coefficients
Discharge motor	
(Intercept)	14.3837
Haemoglobin (g/L)	0.5603
Mean Cell Hb (pg)	0.3425
Mean Cell Volume (fL)	0.2967
Monocytes (10*9/L)	0.7138
Admission AIS grade C	8.1192
Admission AIS grade D	7.2730
Alcohol Drinking status	0.6651
Initial motor	0.6812
Initial sensor prick	0.0847
Initial sensor touch	0.0076
Initial scim	0.0483
Discharge sensor prick	
(Intercept)	16.6749
Creatinine (umol/L)	0.2147
Haemoglobin (g/L)	0.9800
Monocytes (10*9/L)	0.9356
Total Bilirubin (umol/L)	0.9606
Type 2 diabetes	0.1297

Admission AIS grade C	5.9749
Admission AIS grade D	1.5147
Initial motor	0.1653
Initial sensor prick	0.5645
Initial sensor touch	0.1297
Discharge sensor touch	
(Intercept)	21.2664
Creatinine (umol/L)	1.0464
Haematocrit (L/L)	1.5520
Mean Cell Volume (fL)	0.2252
Monocytes (10*9/L)	1.1150
Total Bilirubin (umol/L)	0.7580
Type 2 diabetes	1.9635
Admission AIS grade B	2.8478
Admission AIS grade C	10.3250
Admission AIS grade D	5.2434
Lumbar injury	0.4891
Alcohol Drinking status	0.0460
Initial motor	0.0640
Initial sensor prick	0.0308
Initial sensor touch	0.6542
Discharge SCIM	
(Intercept)	24.1514
Alanine Transaminase (u/L)	0.7447
Albumin (g/L)	0.0002
Alkaline Phosphatase (u/L)	0.7343
Creatinine (umol/L)	1.4794

Gamma GT (u/L)	0.1805
Mean Cell Volume (fL)	0.9019
Monocytes (10*9/L)	0.0579
Platelets (10*9/L)	0.2511
Total Protein (g/L)	0.7742
White blood count (10*9/L)	0.6978
Type 1 diabetes	4.8809
Neuro level T	0.9719
SexMale	1.9700
Alcohol Drinking status	1.8840
Fracture	0.6968
Surgery	1.2685
Initial motor	0.2630
Initial sensor prick	0.0829
Initial scim	0.5765
Month-12 motor	
(Intercept)	20.4833
Creatinine (umol/L)	0.0160
C-Reactive Protein (mg/L)	0.8684
Haematocrit (L/L)	0.0032
Haemoglobin (g/L)	1.6359
Mean Cell Hb (pg)	0.0101
Mean Cell Volume (fL)	0.4416
Monocytes (10*9/L)	0.8522
Potassium (mmol/L)	0.4038
Total Bilirubin (umol/L)	0.3863
Type 1 diabetes	3.3723

Admission AIS grade B	0.2769
Admission AIS grade C	9.0082
Admission AIS grade D	9.3897
Smoking yes	0.6993
Alcohol Drinking status	1.7475
Initial motor	0.5762
Initial sensor prick	0.1257
Initial scim	0.0270
Month-12 sensor prick	
(Intercept)	14.3668
Haematocrit (L/L)	0.4243
Haemoglobin (g/L)	0.7408
Mean Cell Hb (pg)	0.2106
Monocytes (10*9/L)	0.9859
Total Bilirubin (umol/L)	1.1180
Admission AIS grade C	6.0284
Admission AIS grade D	1.4502
Lumbar injury	1.4482
Age at injury (Median years)	0.0503
Initial motor	0.1932
Initial sensor prick	0.4227
Initial sensor touch	0.2290
Month-12 sensor touch	
(Intercept)	15.4688
Haematocrit (L/L)	0.7836
Haemoglobin (g/L)	0.7718
Mean Cell Hb (pg)	0.0059

Mean Cell Volume (fL)	0.0712
Monocytes (10*9/L)	1.1749
Total Bilirubin (umol/L)	0.6719
Urea (mmol/L)	0.1630
Type 2 diabetes	1.8745
Admission AIS grade C	8.8281
Admission AIS grade D	2.4381
Age at injury (Median years)	0.0246
Alcohol Drinking status	0.6486
Initial motor	0.0838
Initial sensor prick	0.0851
Initial sensor touch	0.6322
Month-12 SCIM	
(Intercept)	23.9075
Alanine Transaminase (u/L)	0.1683
Mean Cell Volume (fL)	0.1370
SexMale	1.4721
Initial motor	0.2209
Initial sensor prick	0.0910
Initial scim	0.5909

Table A.5: Final elastic net model parameters. Alpha is a value between 0 and 1, where 0 is pure ridge regression, 1 is pure LASSO and values between are a mixture of both. Lambda is the shrinkage factor applied to model coefficients

Model target	alpha	lambda
Discharge motor	1.0	0.6789

Discharge sensor prick	1.0	0.7379
Discharge sensor touch	0.6	0.7180
Discharge SCIM	0.6	0.6318
Month-12 motor	1.0	0.6361
Month-12 sensor prick	0.4	1.6684
Month-12 sensor touch	1.0	0.7224
Month-12 SCIM	1.0	1.4694

Table A.6: Linear regression model coefficients without elastic net penalisation

Variable	Estimate	Std. Error	t value	Pr(>|t|)
Discharge motor				
(Intercept)	19.6211	5.0156	3.912	0.0001
Admission AIS grade C	12.4549	2.5435	4.897	0.0000
Admission AIS grade D	11.2485	3.4104	3.298	0.0011
Initial motor	0.7052	0.0635	11.103	0.0000
Discharge sensor prick				
(Intercept)	14.8141	5.2033	2.847	0.0047
Total Bilirubin (umol/L)	1.4040	0.5962	2.355	0.0192
Admission AIS grade C	9.9100	2.6387	3.756	0.0002
Initial motor	0.2102	0.0659	3.190	0.0016
Initial sensor prick	0.5555	0.0611	9.091	0.0000
Discharge sensor touch				
(Intercept)	22.4386	4.5386	4.944	0.0000
Creatinine (umol/L)	1.8437	0.9352	1.972	0.0496
Admission AIS grade B	6.9628	2.4569	2.834	0.0049

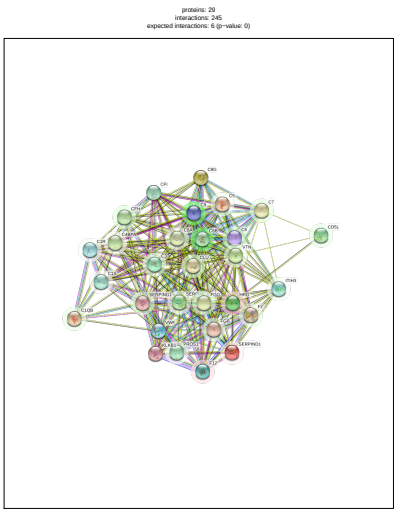
Admission AIS grade C	14.4758	2.3016	6.289	0.0000
Admission AIS grade D	9.9643	3.0860	3.229	0.0014
Initial sensor touch	0.6640	0.0537	12.364	0.0000
Discharge SCIM				
(Intercept)	24.1869	5.7906	4.177	0.0000
Creatinine (umol/L)	2.9559	1.1932	2.477	0.0138
Urea (mmol/L)	-2.7153	1.2468	-2.178	0.0302
Type 2 diabetes	-6.6866	2.8519	-2.345	0.0197
Admission AIS grade B	-7.2569	3.1347	-2.315	0.0213
Admission AIS grade D	-10.5479	3.9374	-2.679	0.0078
Age at injury (Median years)	-0.2094	0.0617	-3.397	0.0008
Time to first blood test (Days)	-0.1088	0.0393	-2.768	0.0060
Smoking unknown	-5.1847	2.3579	-2.199	0.0287
Initial motor	0.3017	0.0733	4.114	0.0001
Initial scim	0.5909	0.0605	9.770	0.0000
Month-12 motor				
(Intercept)	25.6322	6.1014	4.201	0.0000
Admission AIS grade C	14.6021	3.0942	4.719	0.0000
Admission AIS grade D	15.5894	4.1487	3.758	0.0002
Initial motor	0.5712	0.0773	7.394	0.0000
Month-12 sensor prick				
(Intercept)	13.1846	5.5887	2.359	0.0190
Total Bilirubin (umol/L)	1.5684	0.6403	2.449	0.0149
Admission AIS grade C	11.3218	2.8342	3.995	0.0001
Admission AIS grade D	7.7599	3.8001	2.042	0.0420
Fracture	-4.3905	2.1918	-2.003	0.0461

Initial motor	0.2088	0.0708	2.950	0.0034
Initial sensor prick	0.4343	0.0656	6.617	0.0000
Initial sensor touch	0.1734	0.0661	2.623	0.0092
Month-12 sensor touch				
(Intercept)	17.6031	5.3289	3.303	0.0011
Total Bilirubin (umol/L)	1.3712	0.6106	2.246	0.0255
Admission AIS grade C	13.7938	2.7024	5.104	0.0000
Admission AIS grade D	8.1825	3.6234	2.258	0.0247
Drinker status	3.5715	1.6582	2.154	0.0321
Initial sensor touch	0.6177	0.0631	9.796	0.0000
Month-12 SCIM				
(Intercept)	20.9250	6.4651	3.237	0.0013
SexMale	4.7296	2.1960	2.154	0.0321
Age at injury (Median years)	-0.2280	0.0688	-3.312	0.0010
Time to first blood test (Days)	-0.1031	0.0439	-2.349	0.0195
Initial motor	0.2753	0.0819	3.362	0.0009
Initial scim	0.5940	0.0675	8.797	0.0000

A.3 Proteomic plots

A.3.1 iTRAQ

StringDB plots



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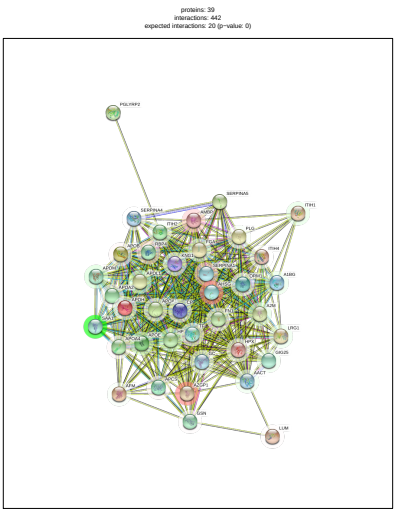

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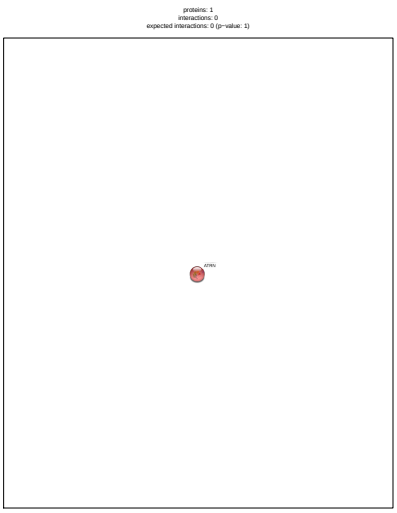
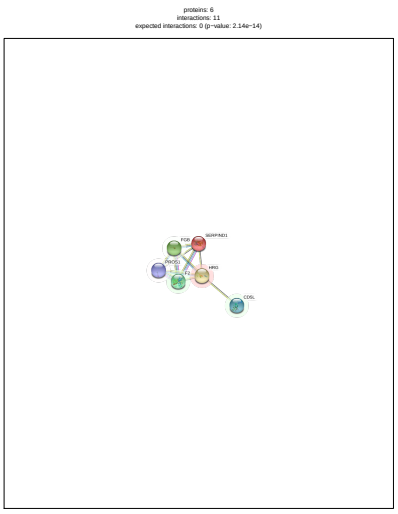


(c) Cluster 3

Figure A.1: Clustered interaction network of differentially abundant proteins from 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 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



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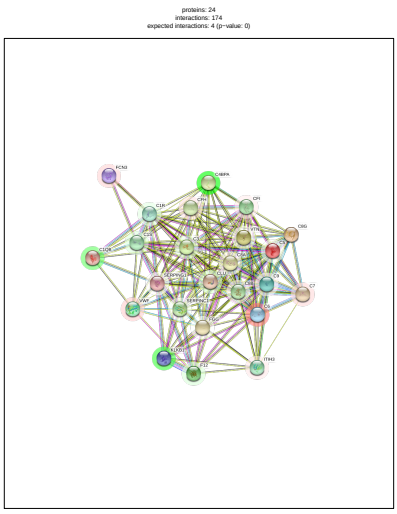
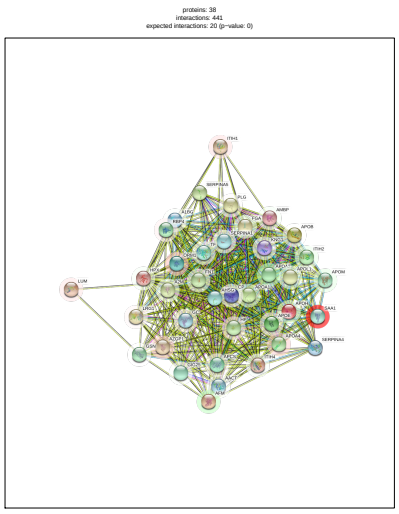
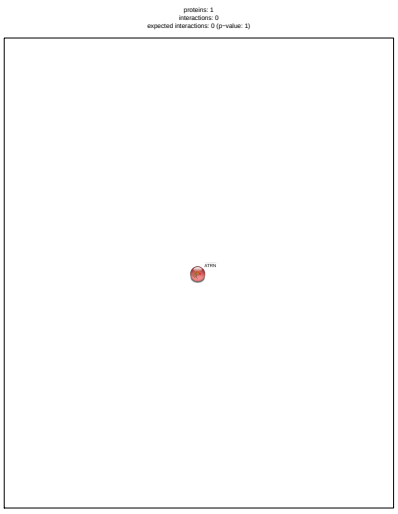


(b) Cluster 2



(c) Cluster 3



(d) Cluster 4

Figure A.2: Clustered 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

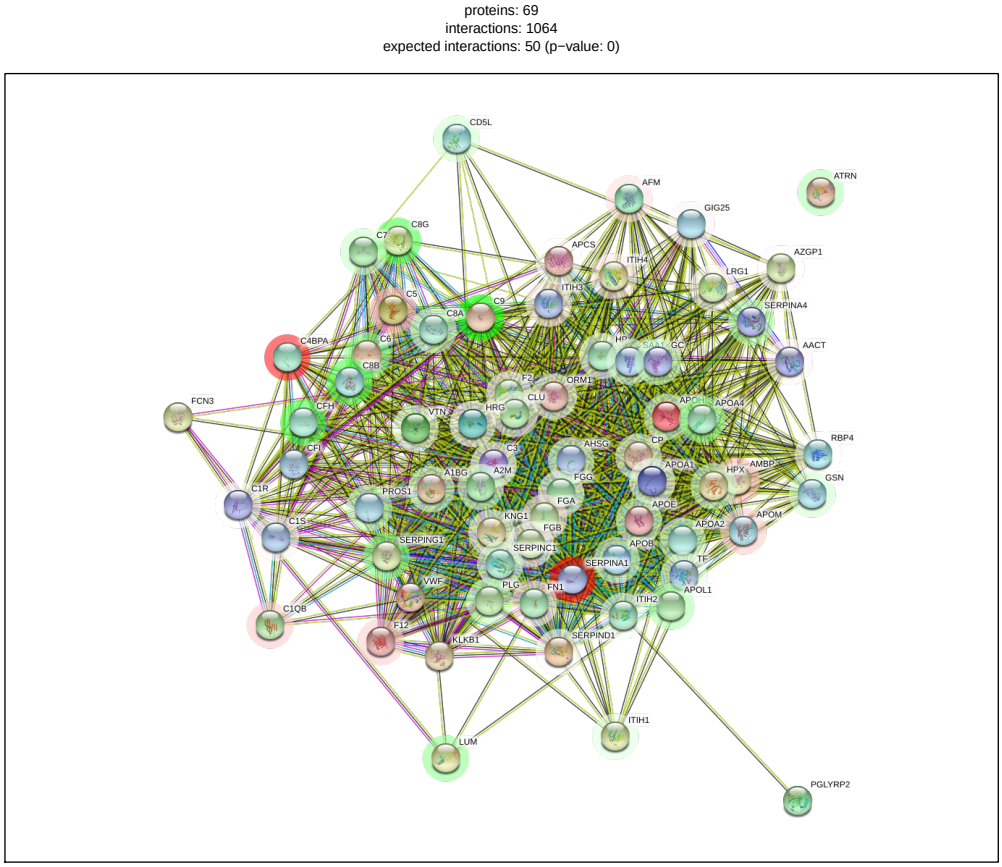
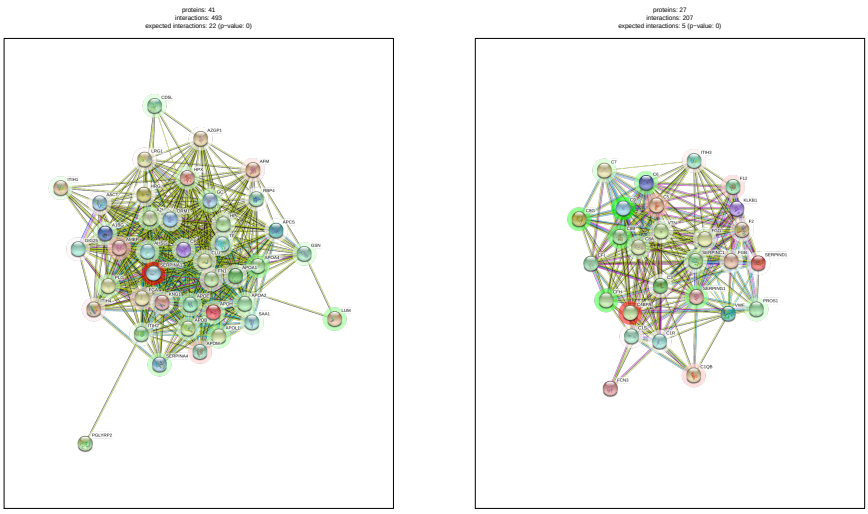
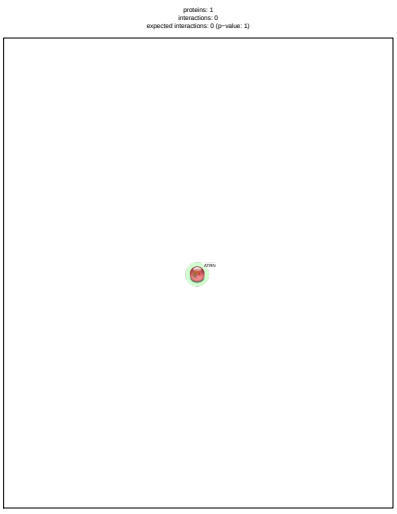


Figure A.3: Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance.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



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.4: Clustered 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

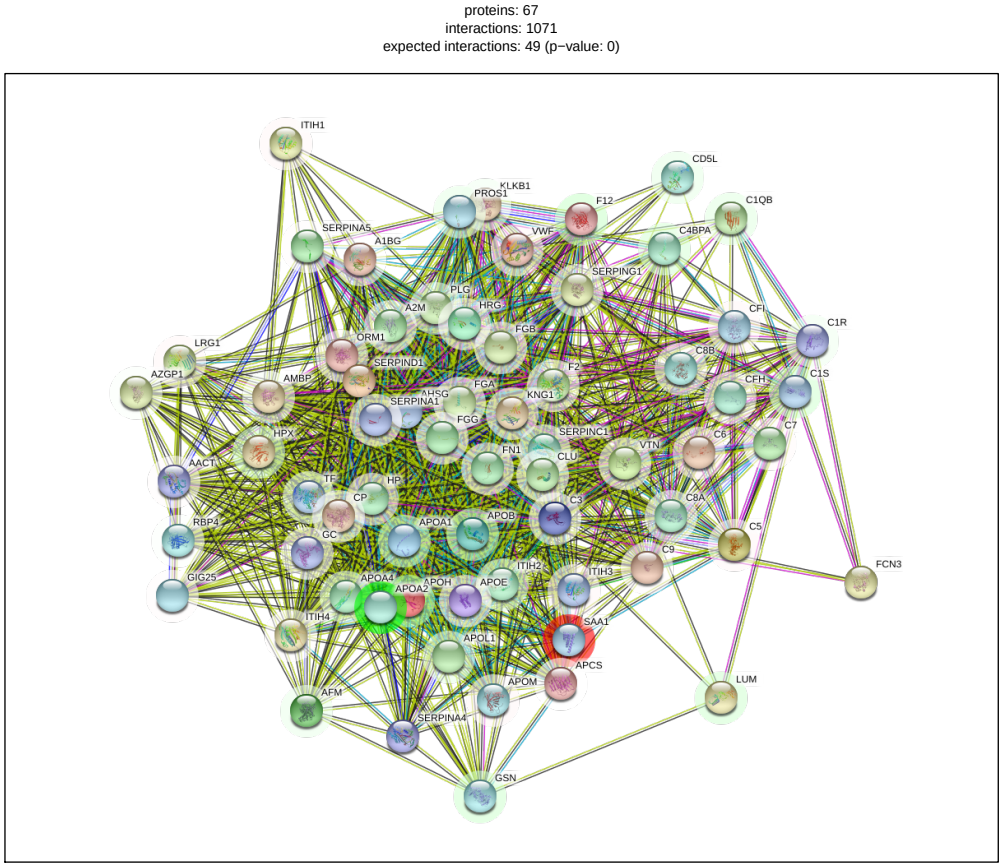
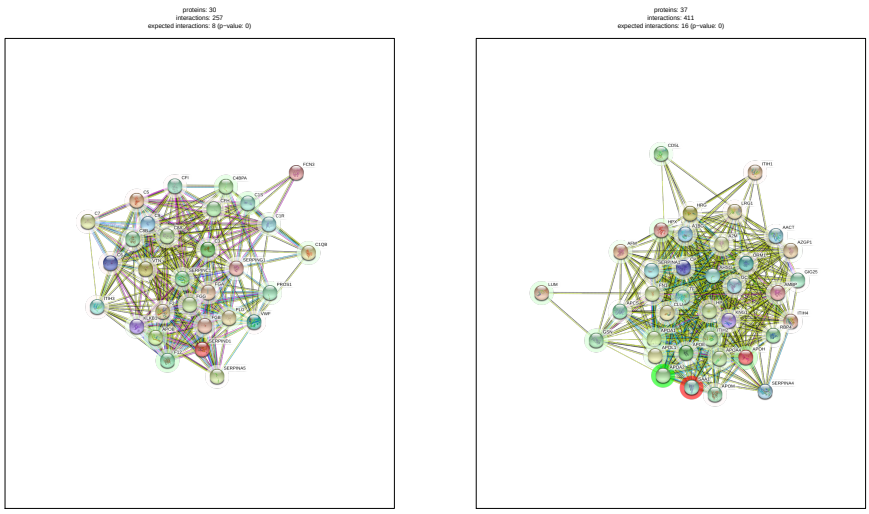


Figure A.5: Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .



(a) Cluster 1 (b) Cluster 2

Figure A.6: Clustered 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-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .

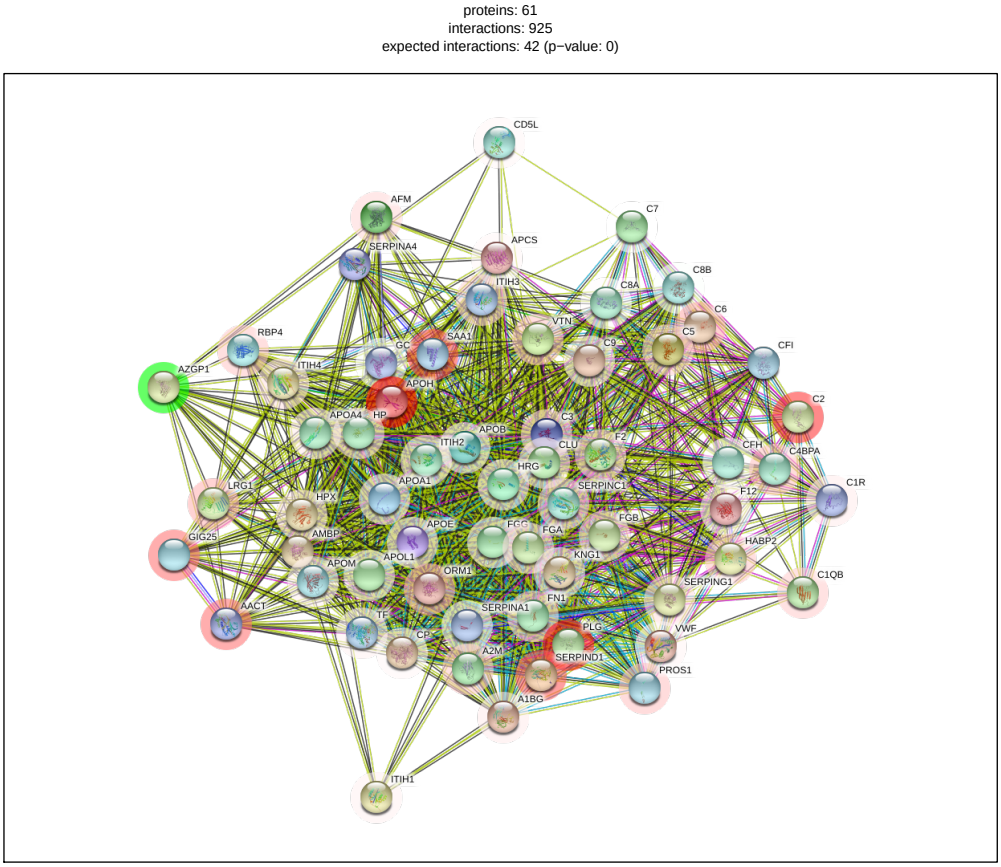
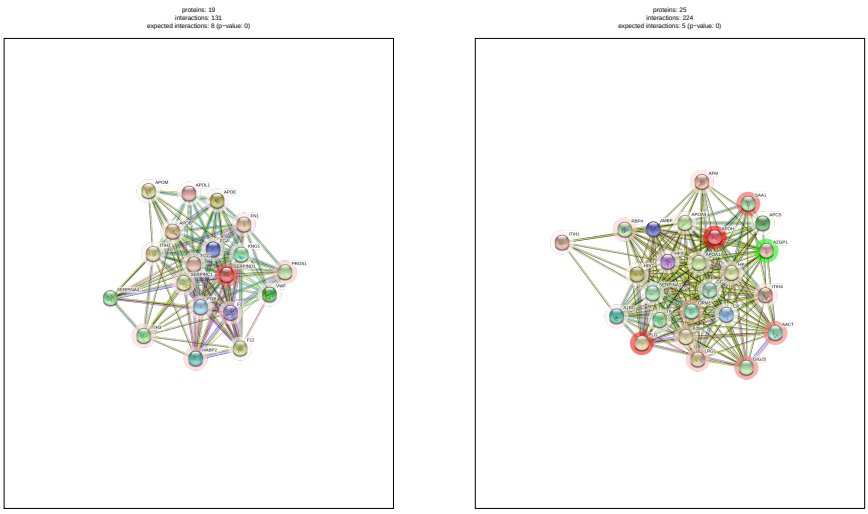
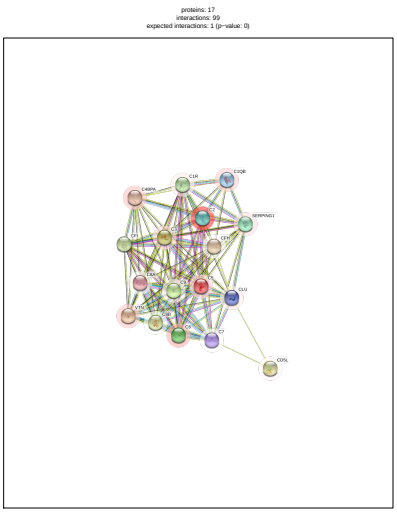


Figure A.7: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.8: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

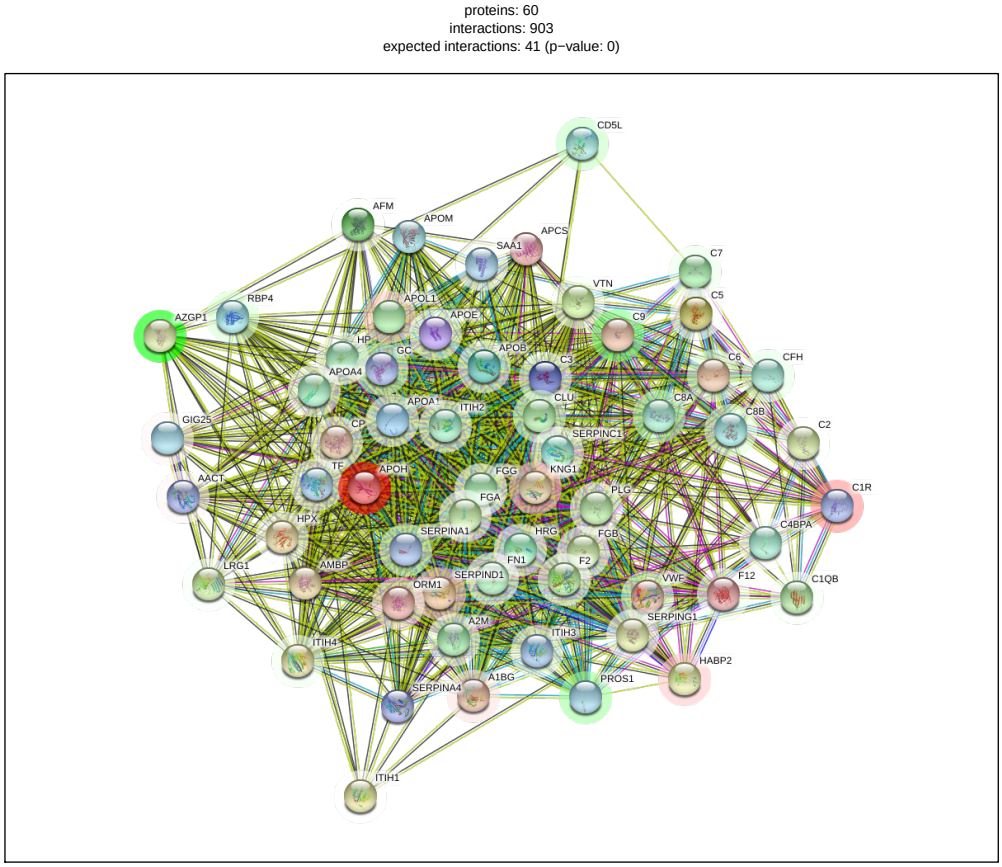
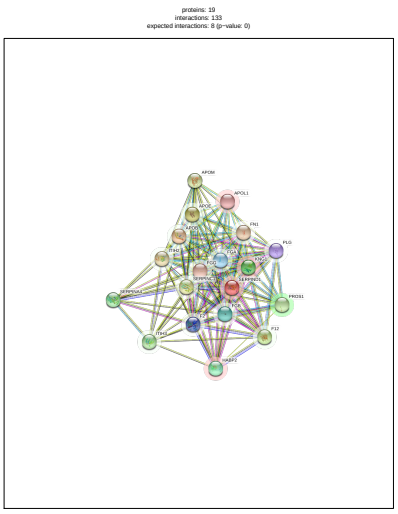


Figure A.9: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



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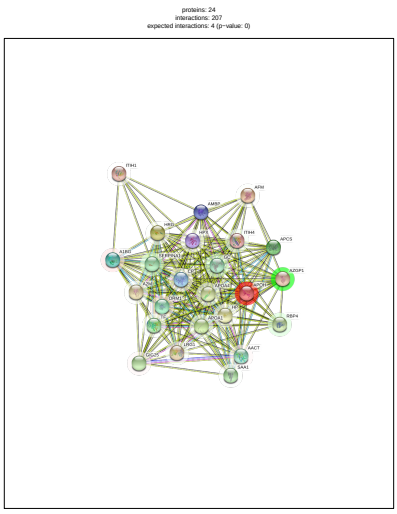
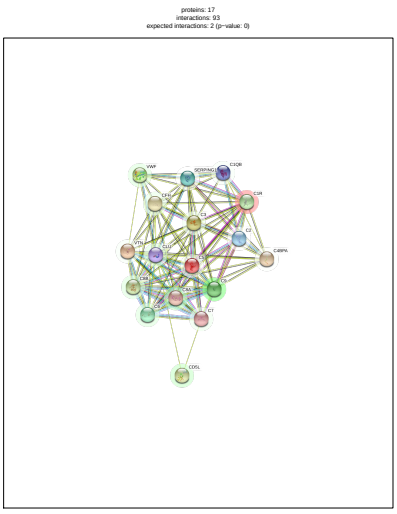


(b) Cluster 2



(c) Cluster 3

Figure A.10: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

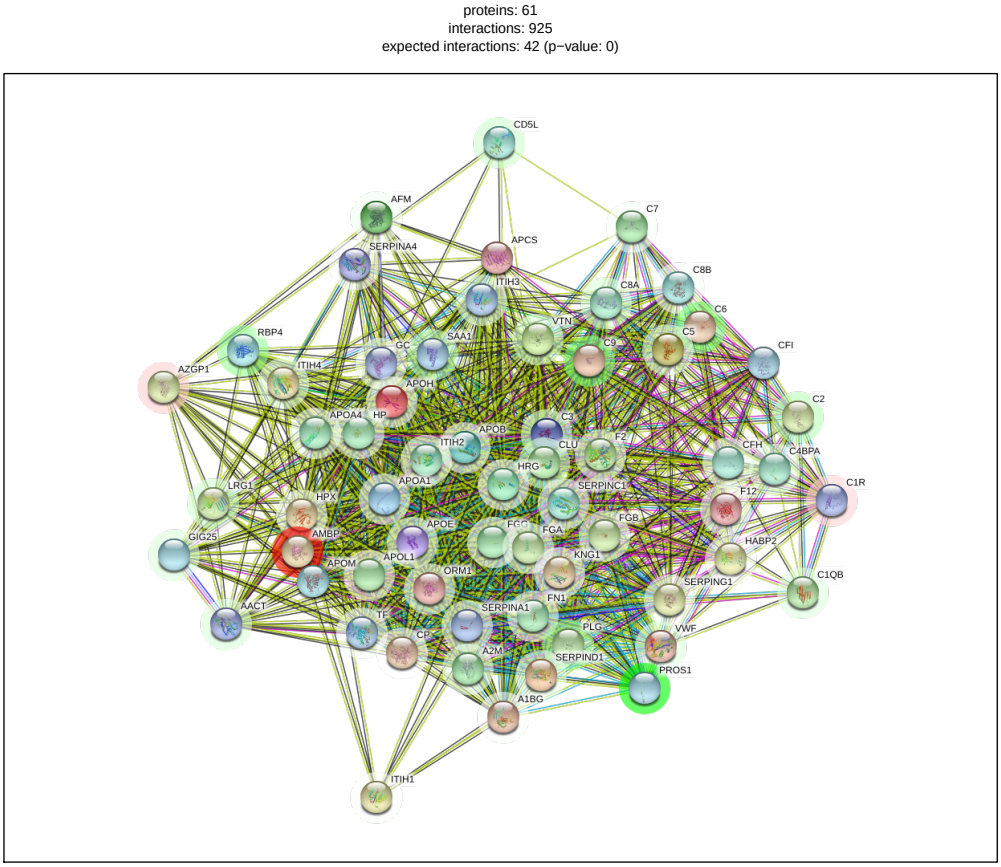
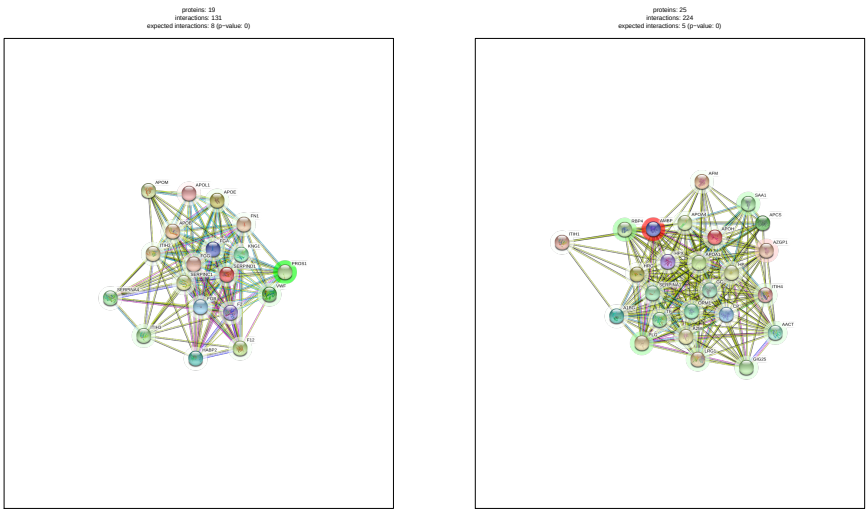
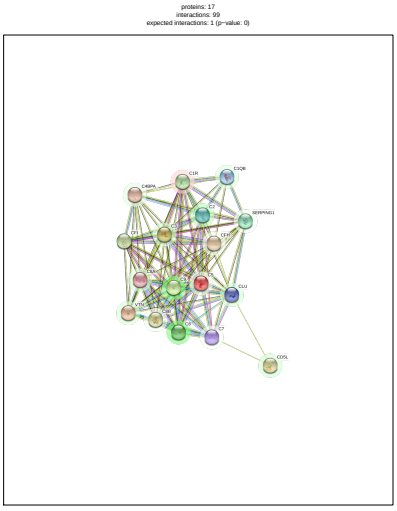


Figure A.11: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.12: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

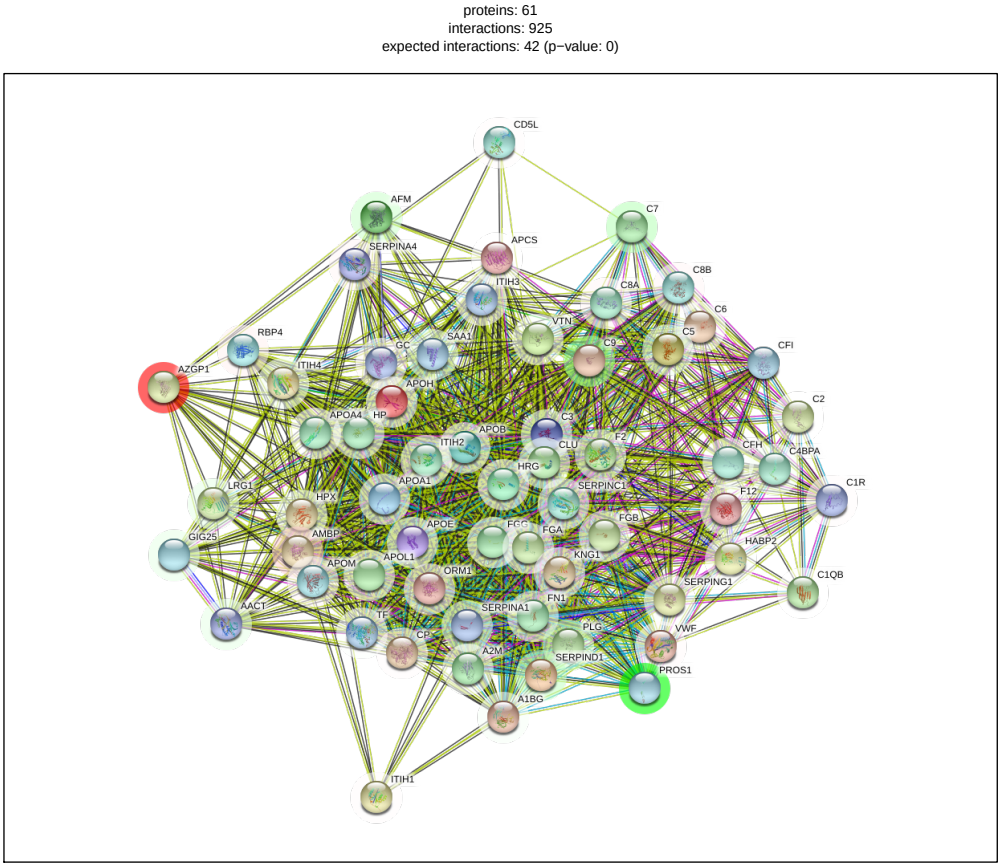
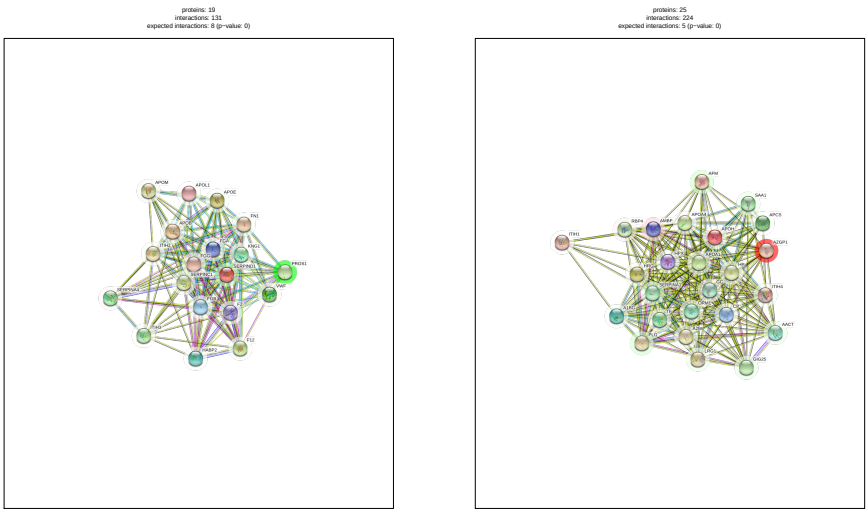
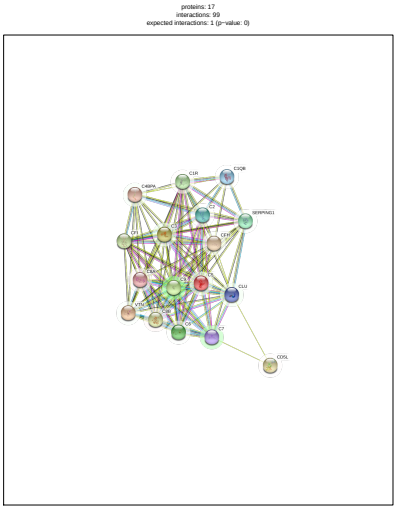


Figure A.13: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.14: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

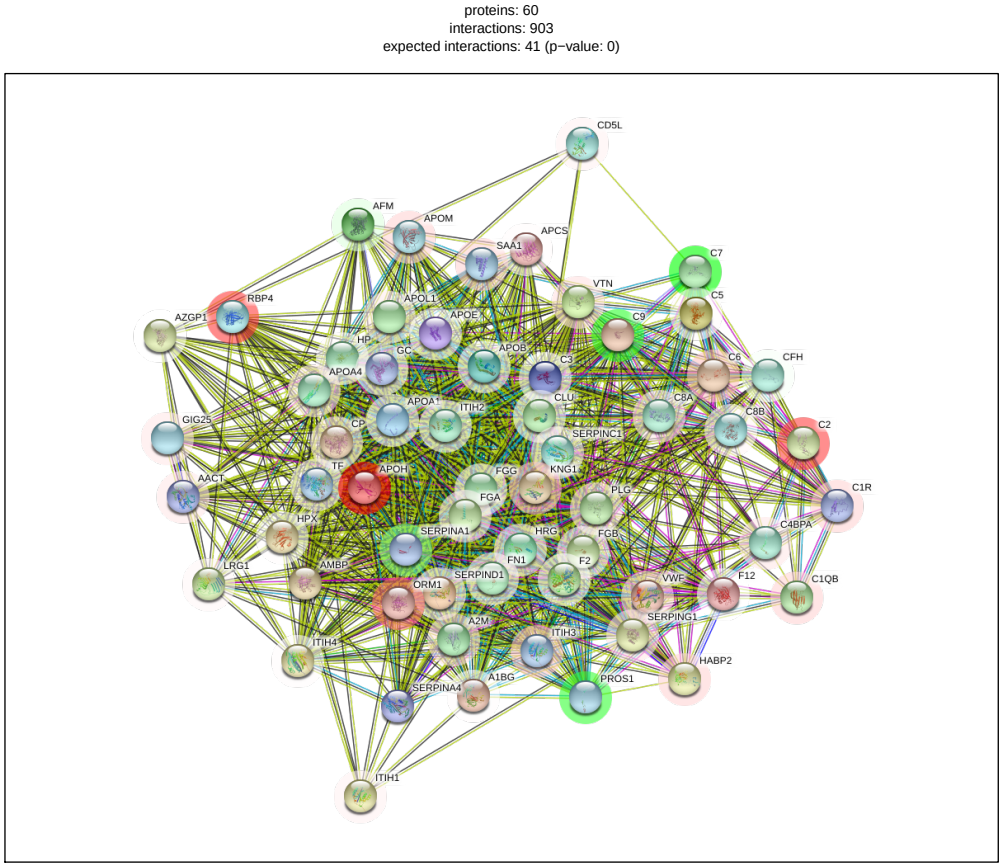
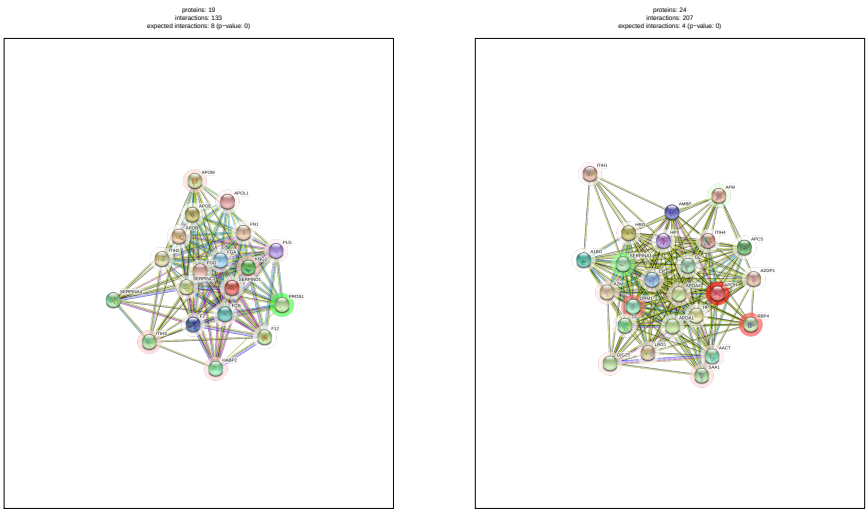
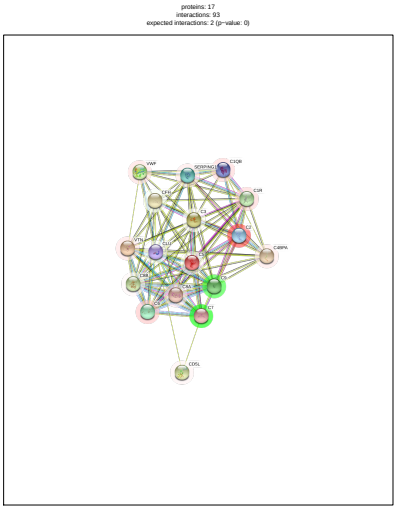


Figure A.15: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.16: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

Heatmaps

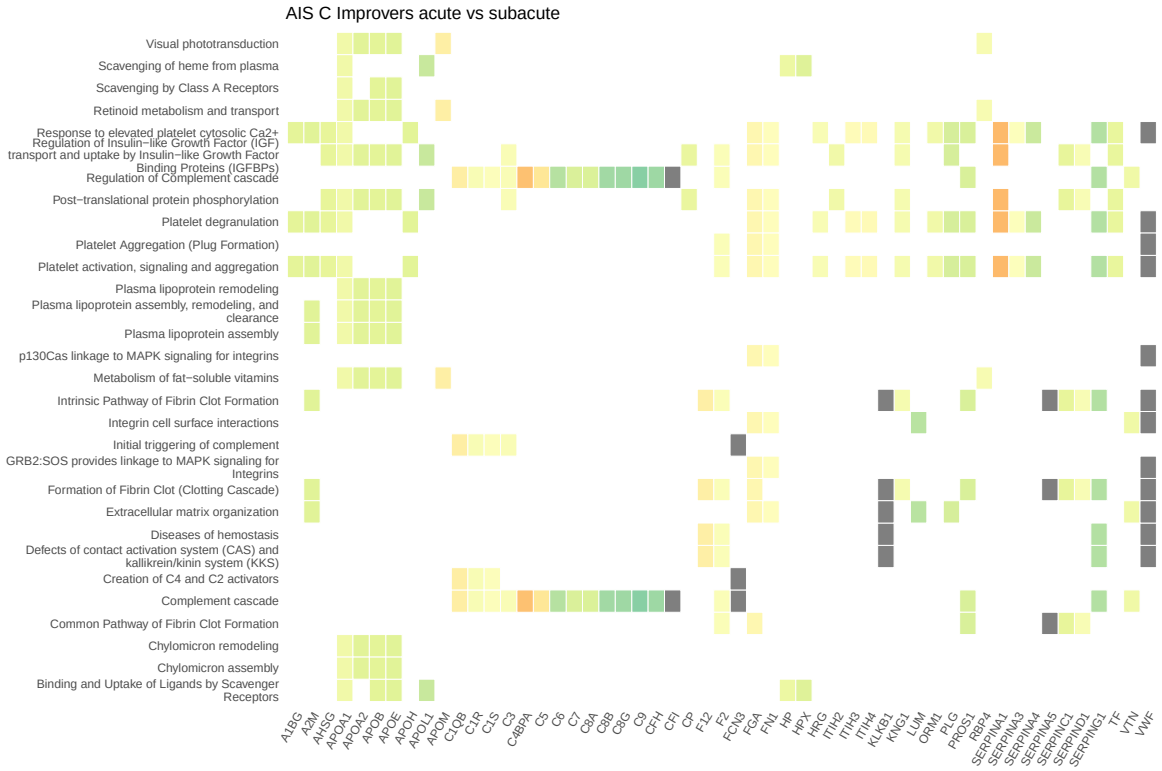


Figure A.17: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

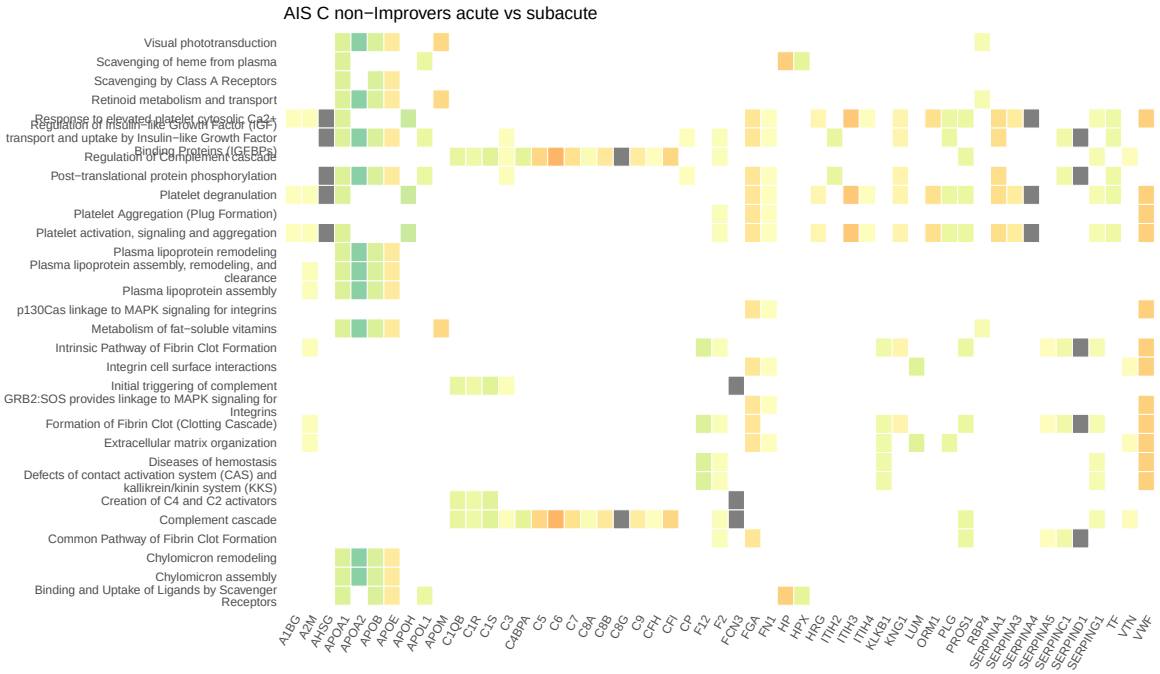


Figure A.18: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

Figure A.19: 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.

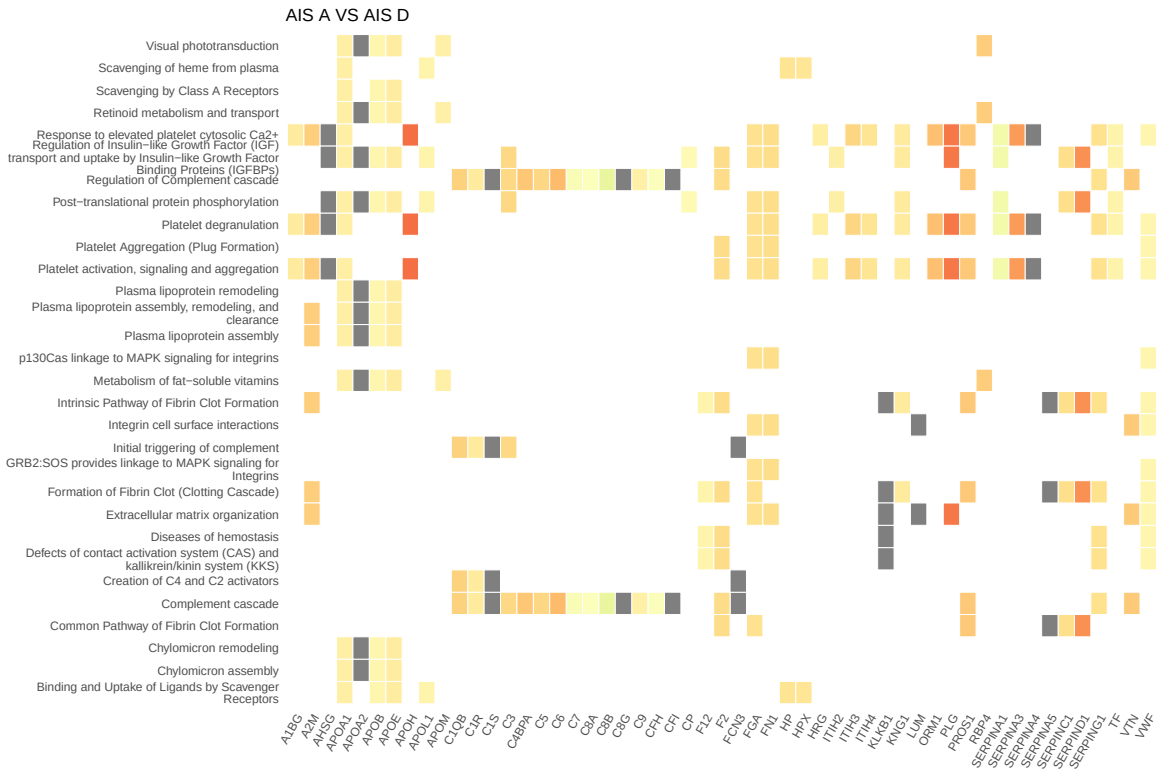


Figure A.20: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

Figure A.21: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

Figure A.22: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

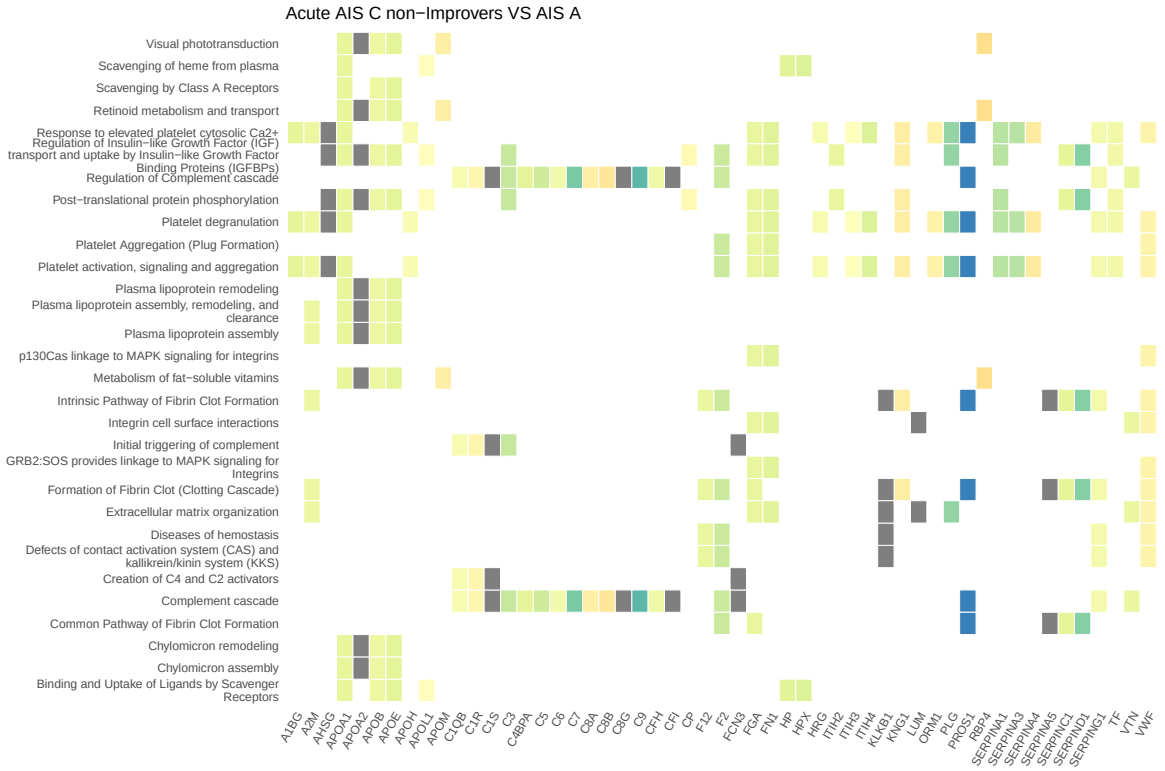


Figure A.23: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

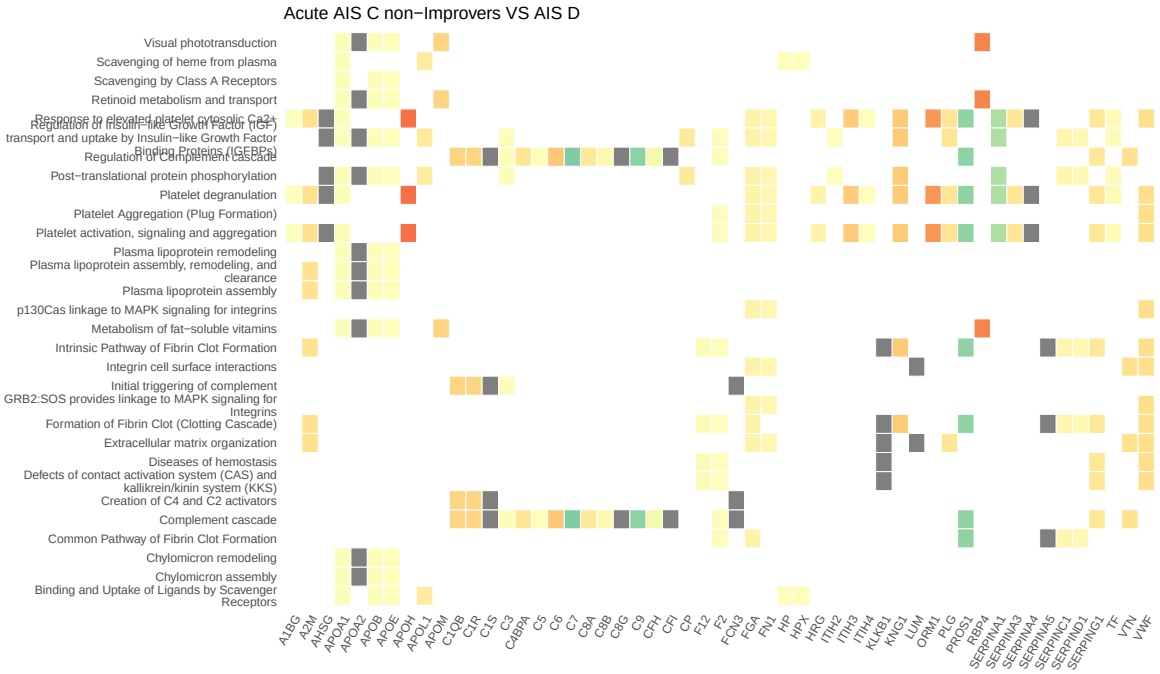


Figure A.24: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

Cnetplots

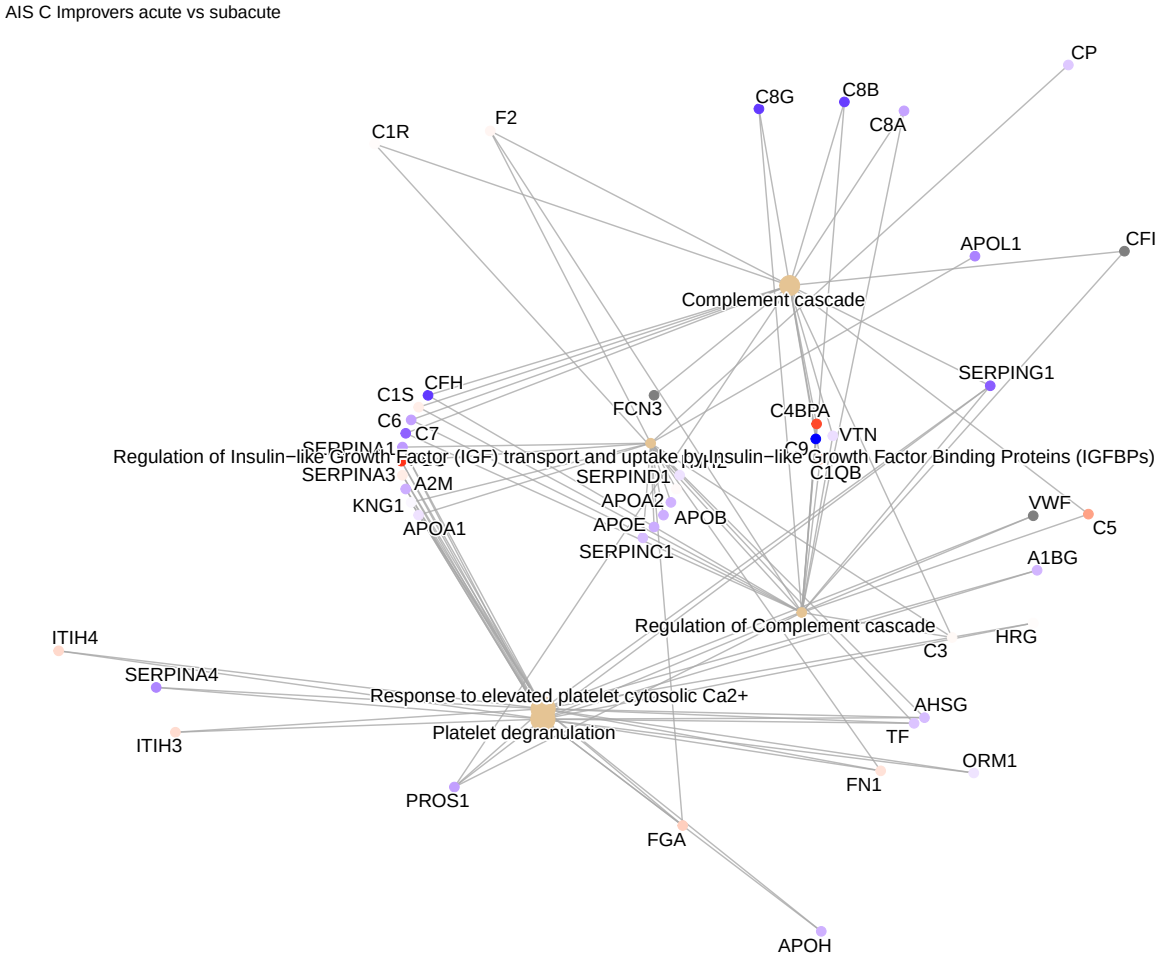


Figure A.25: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

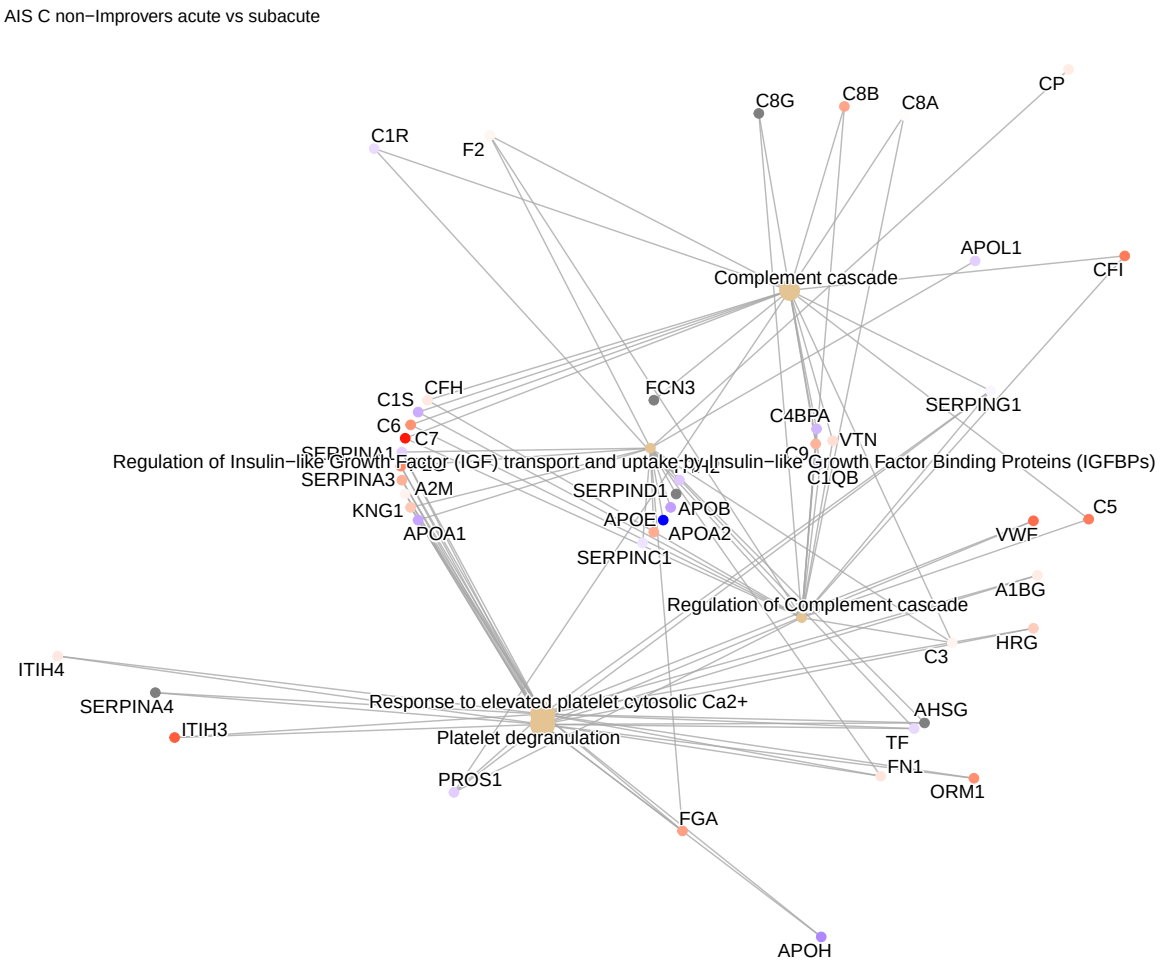


Figure A.26: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

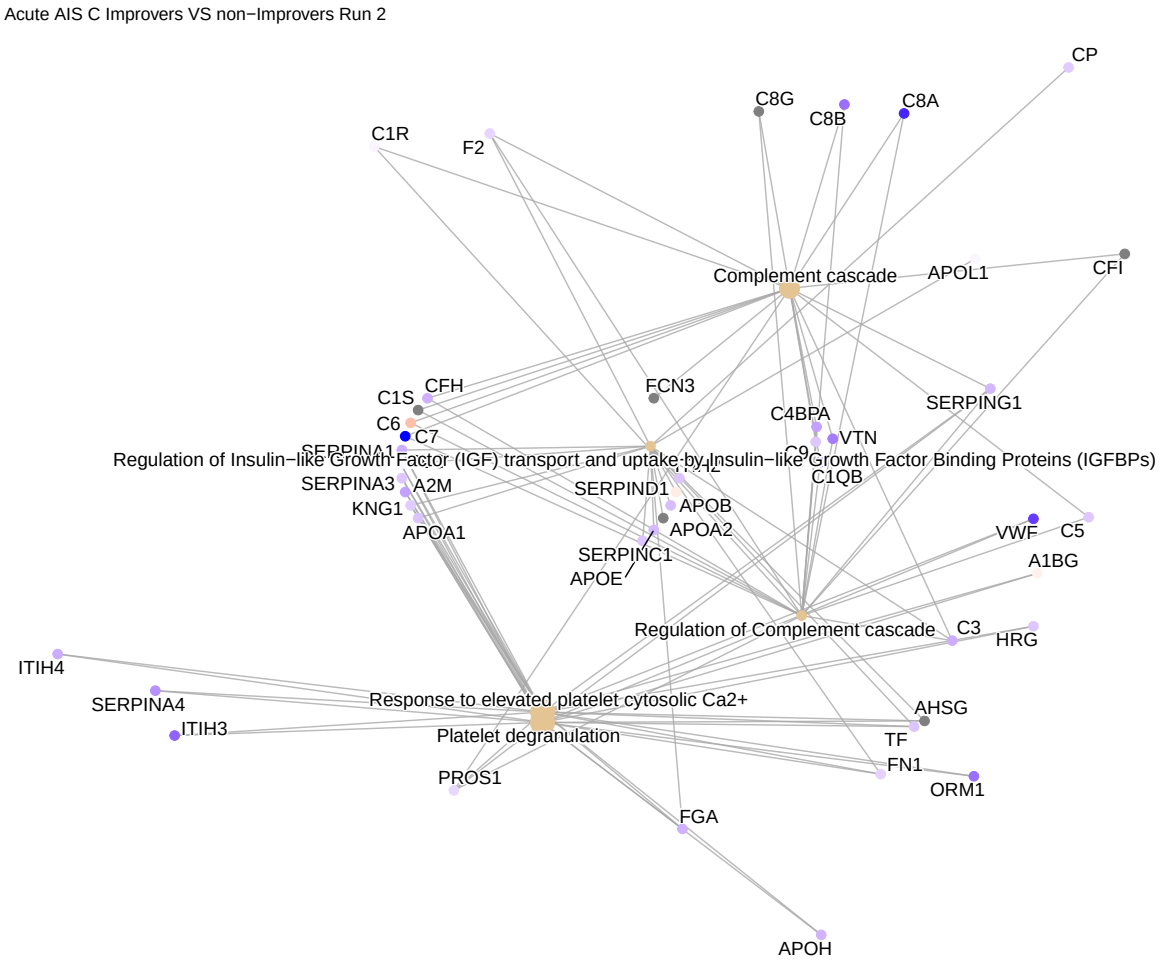


Figure A.27: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and those who did not from the second 4-plex iTRAQ experiment.

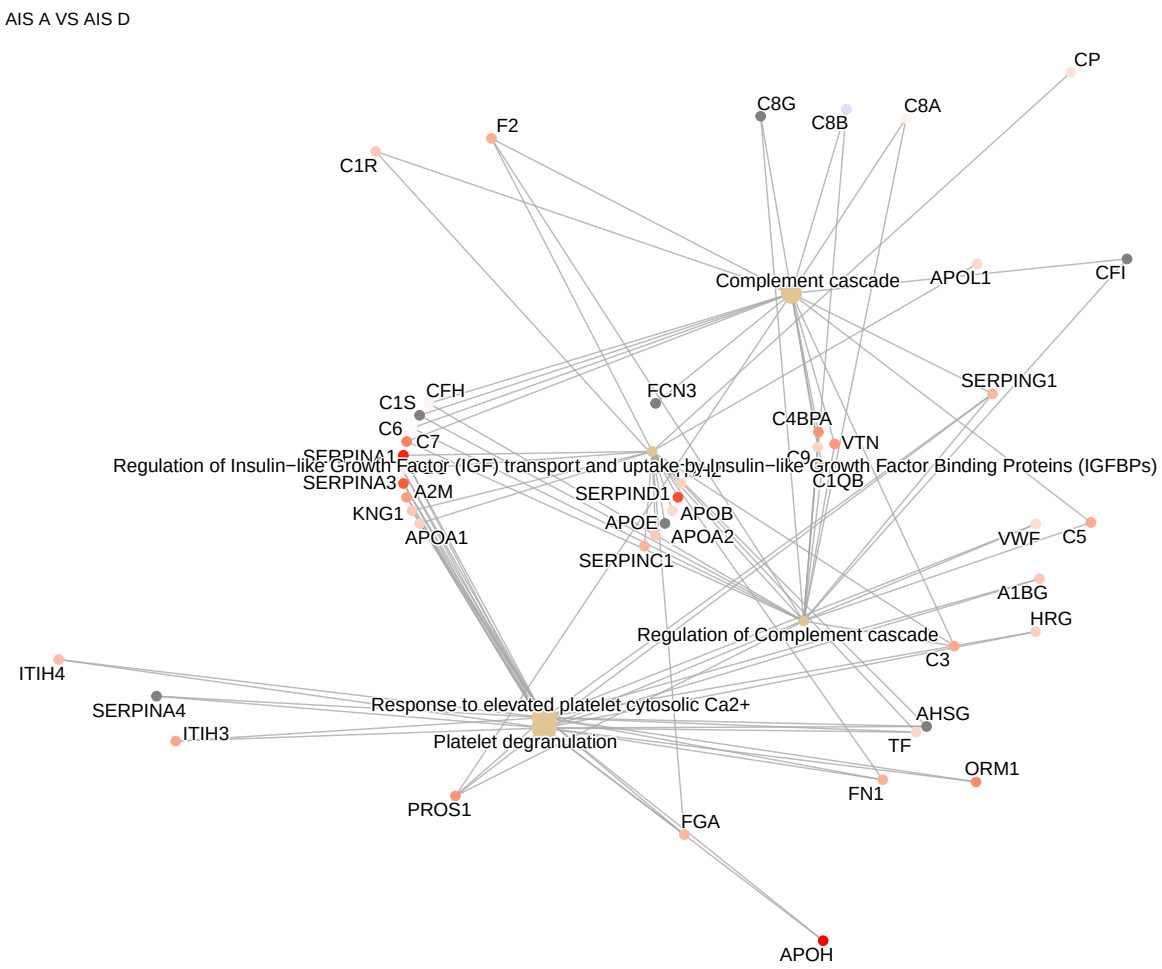


Figure A.28: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

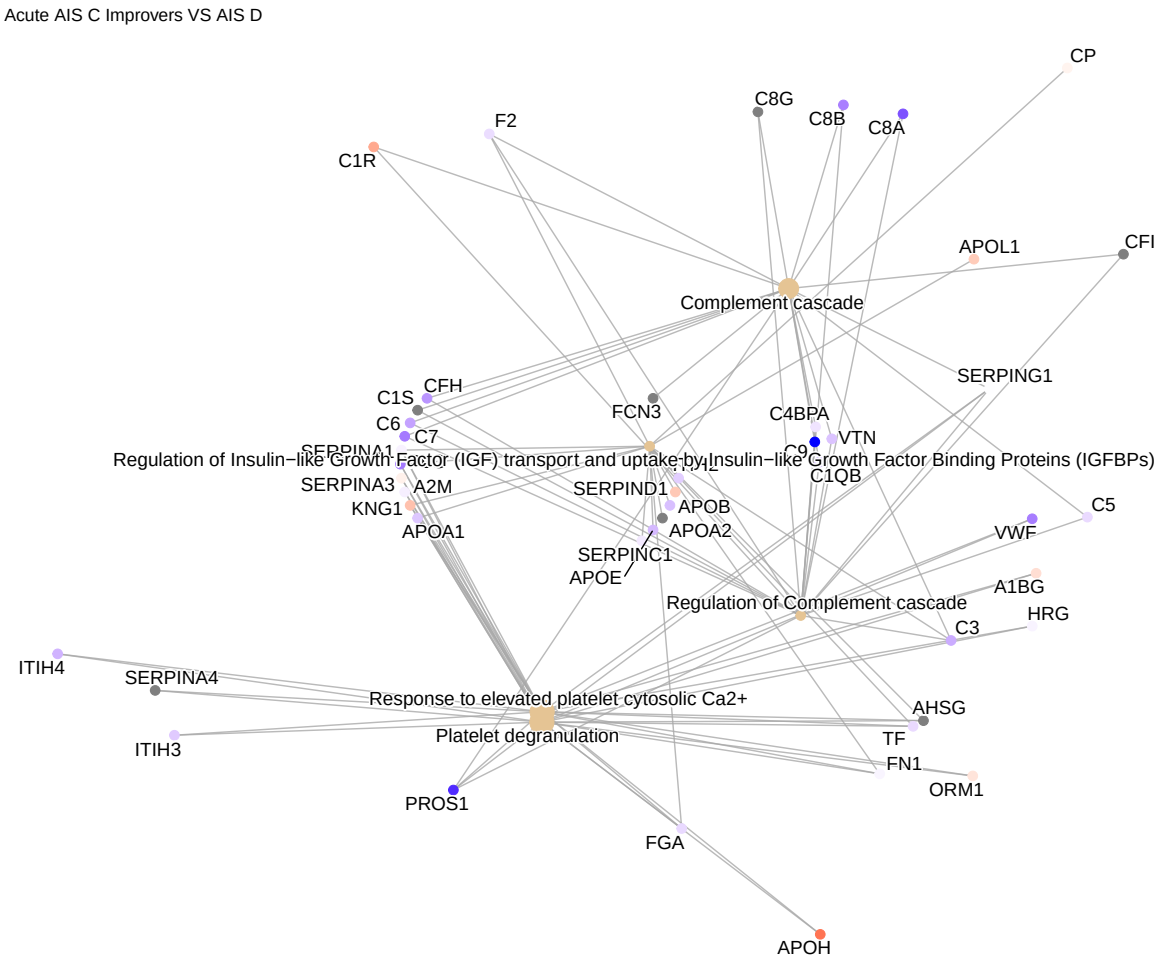


Figure A.29: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

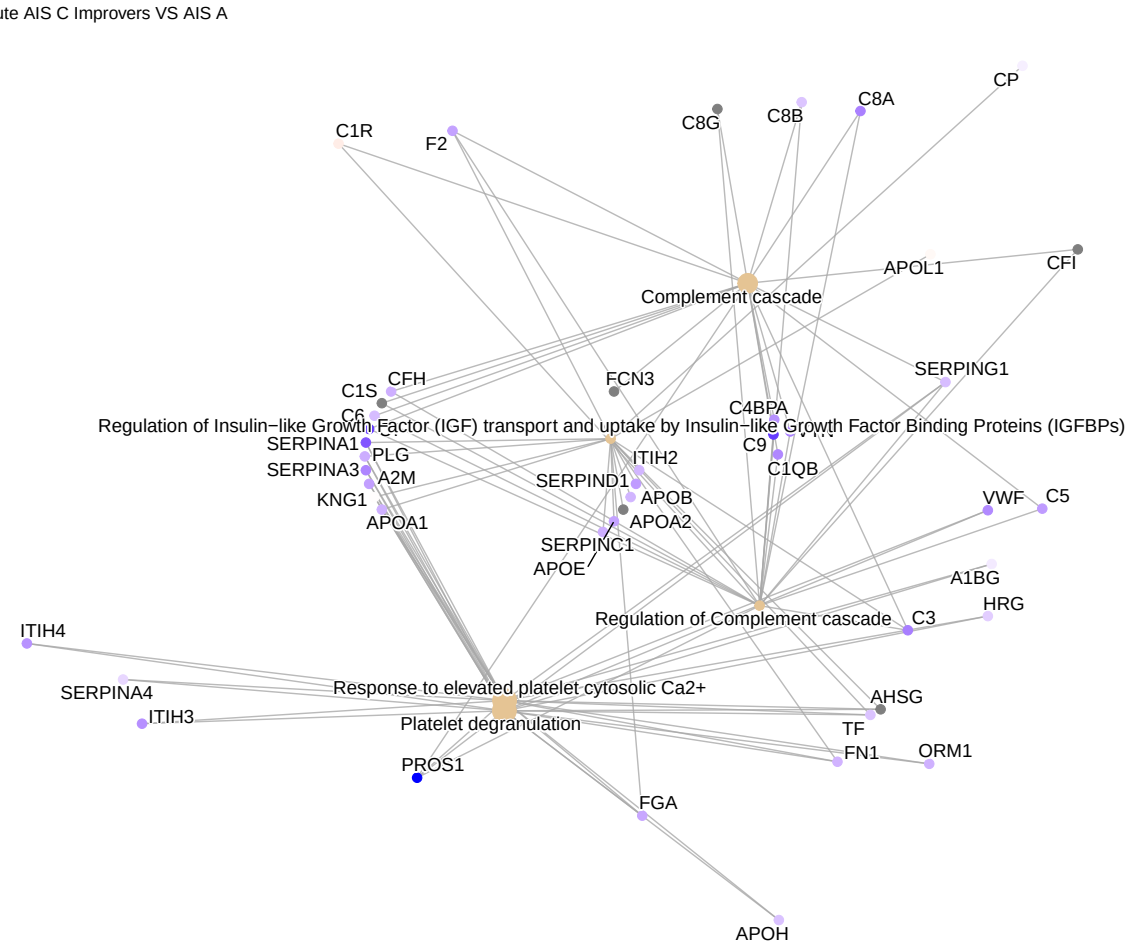


Figure A.30: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

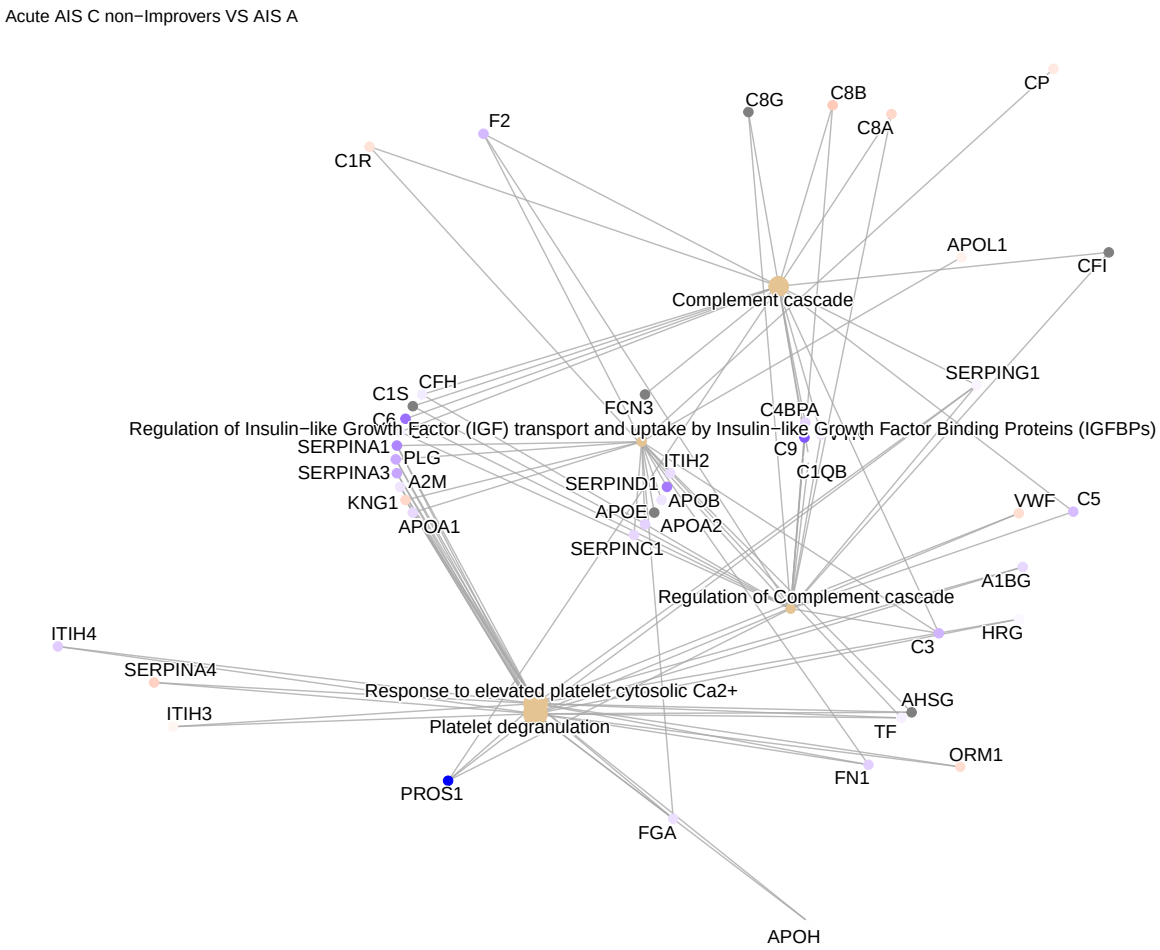


Figure A.31: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

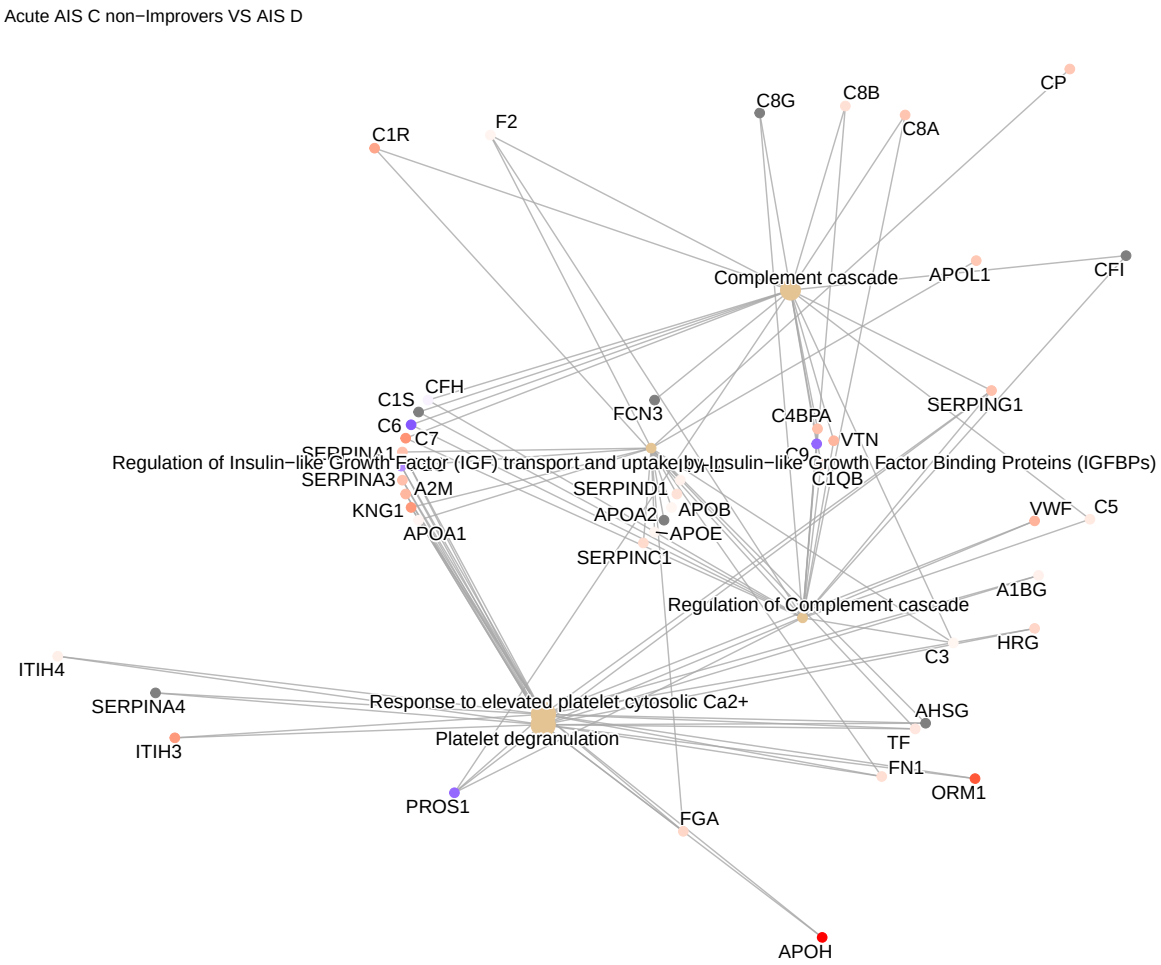


Figure A.32: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

A.3.2 Label-free

Volcano plots

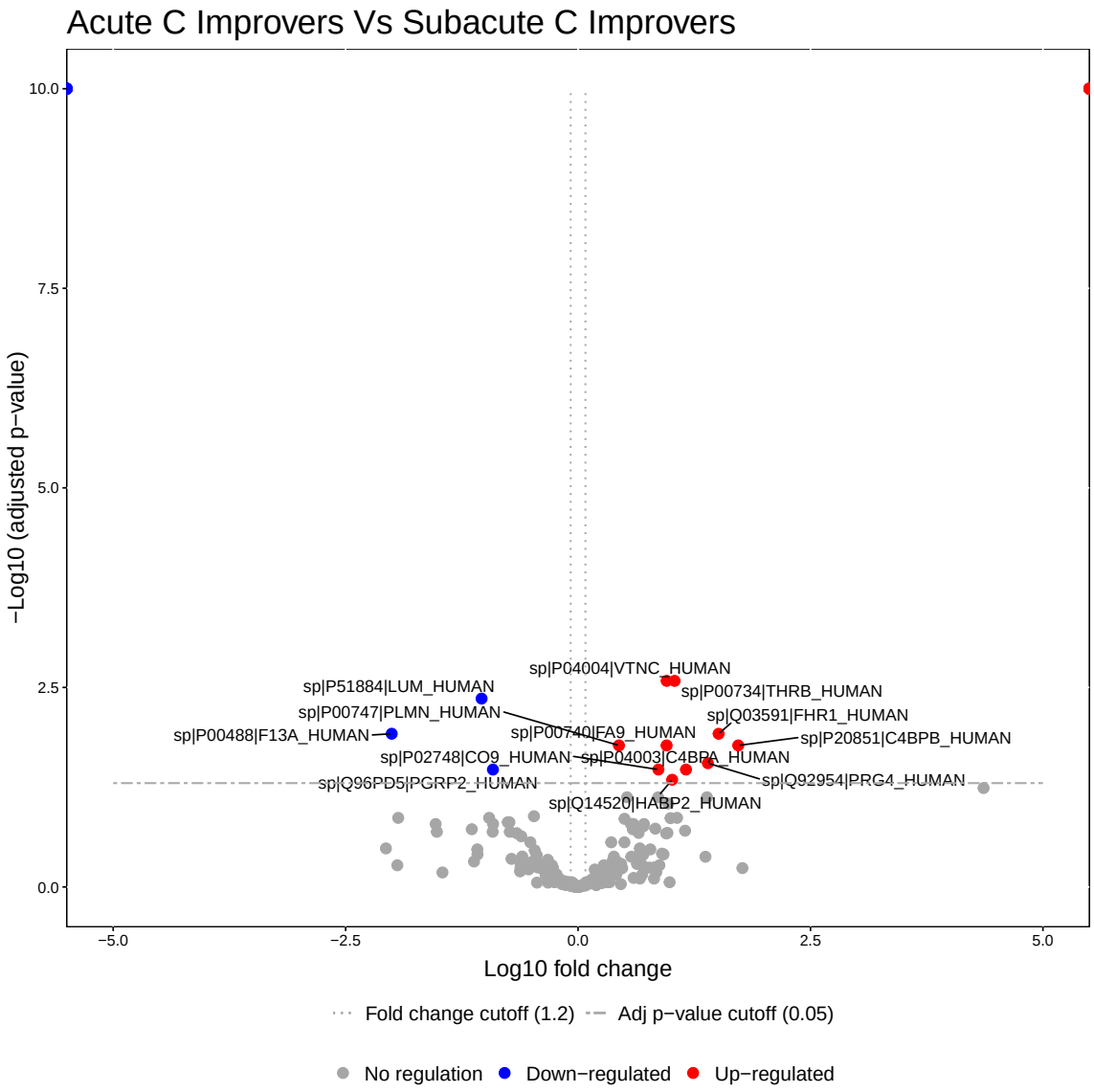


Figure A.33: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

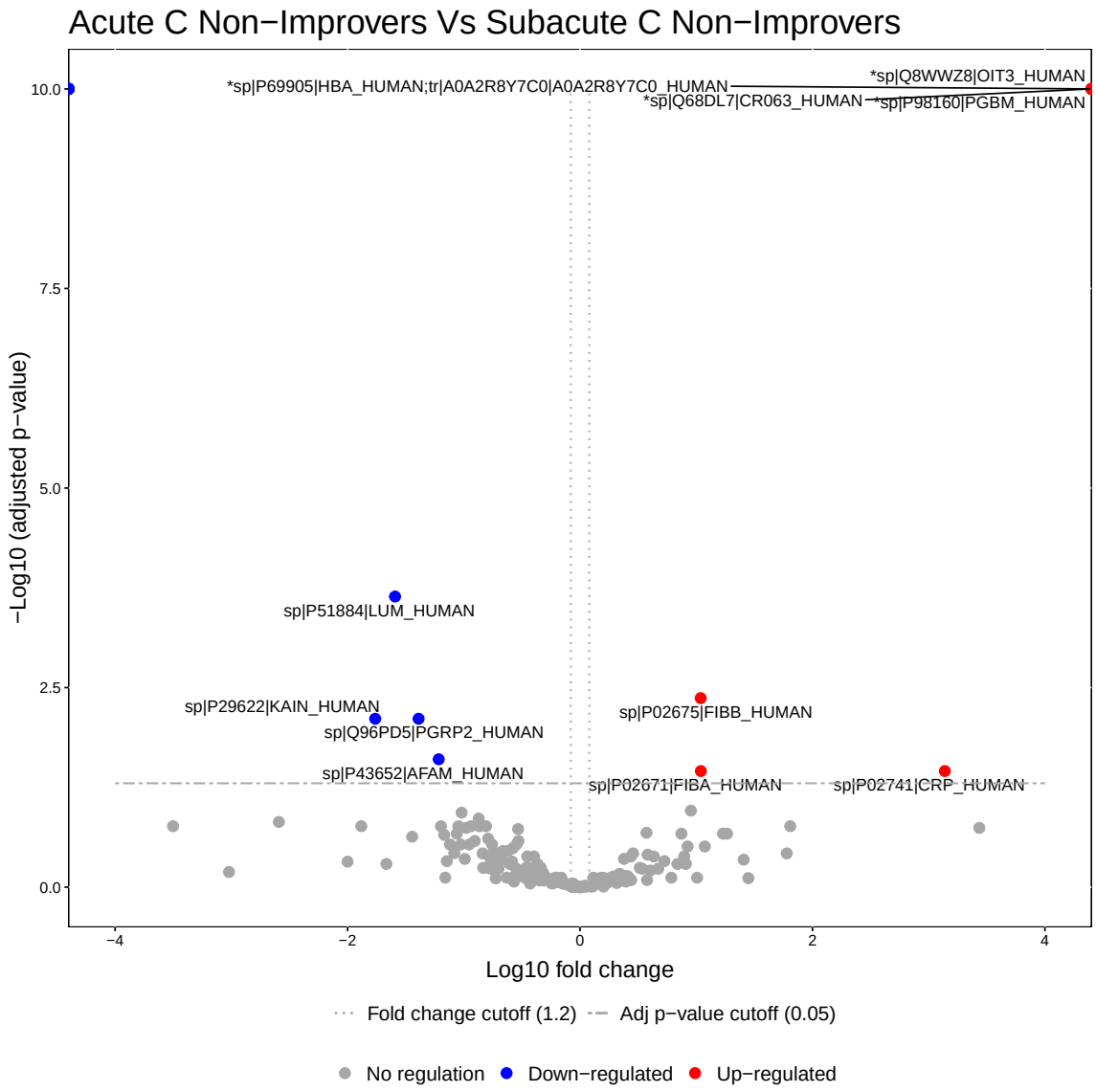


Figure A.34: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

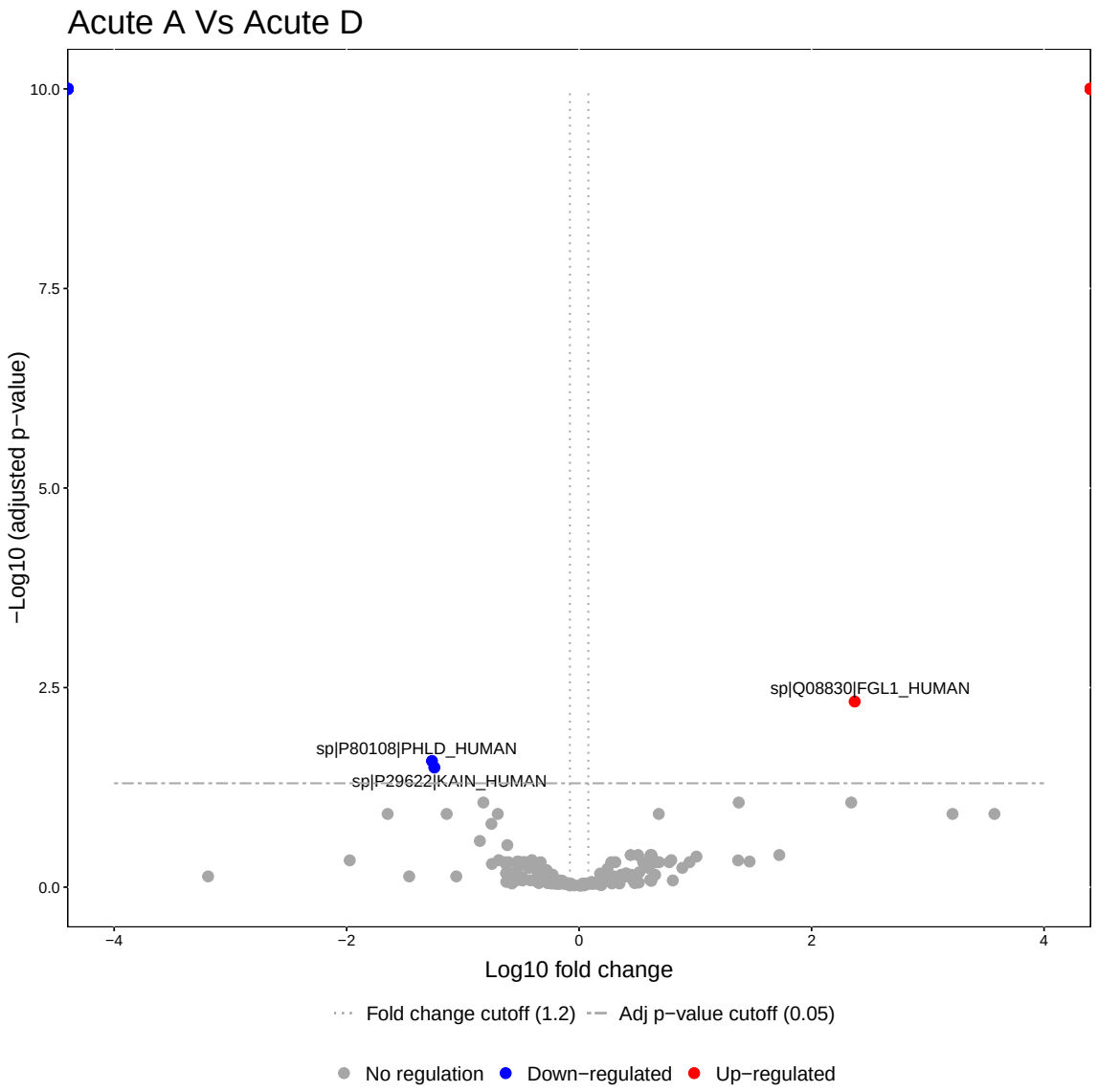


Figure A.35: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A and AIS D patients. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

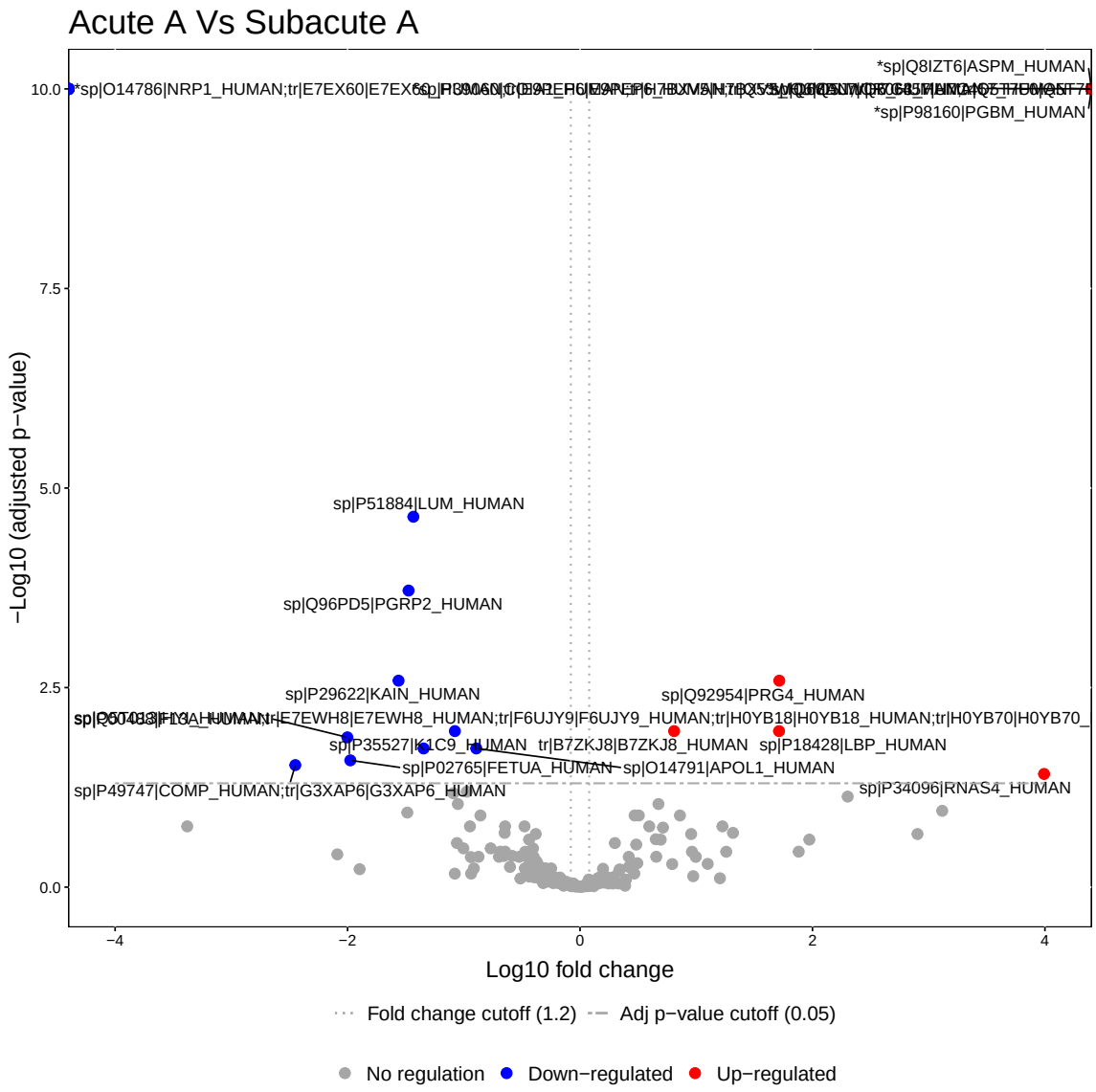


Figure A.36: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS A patients. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

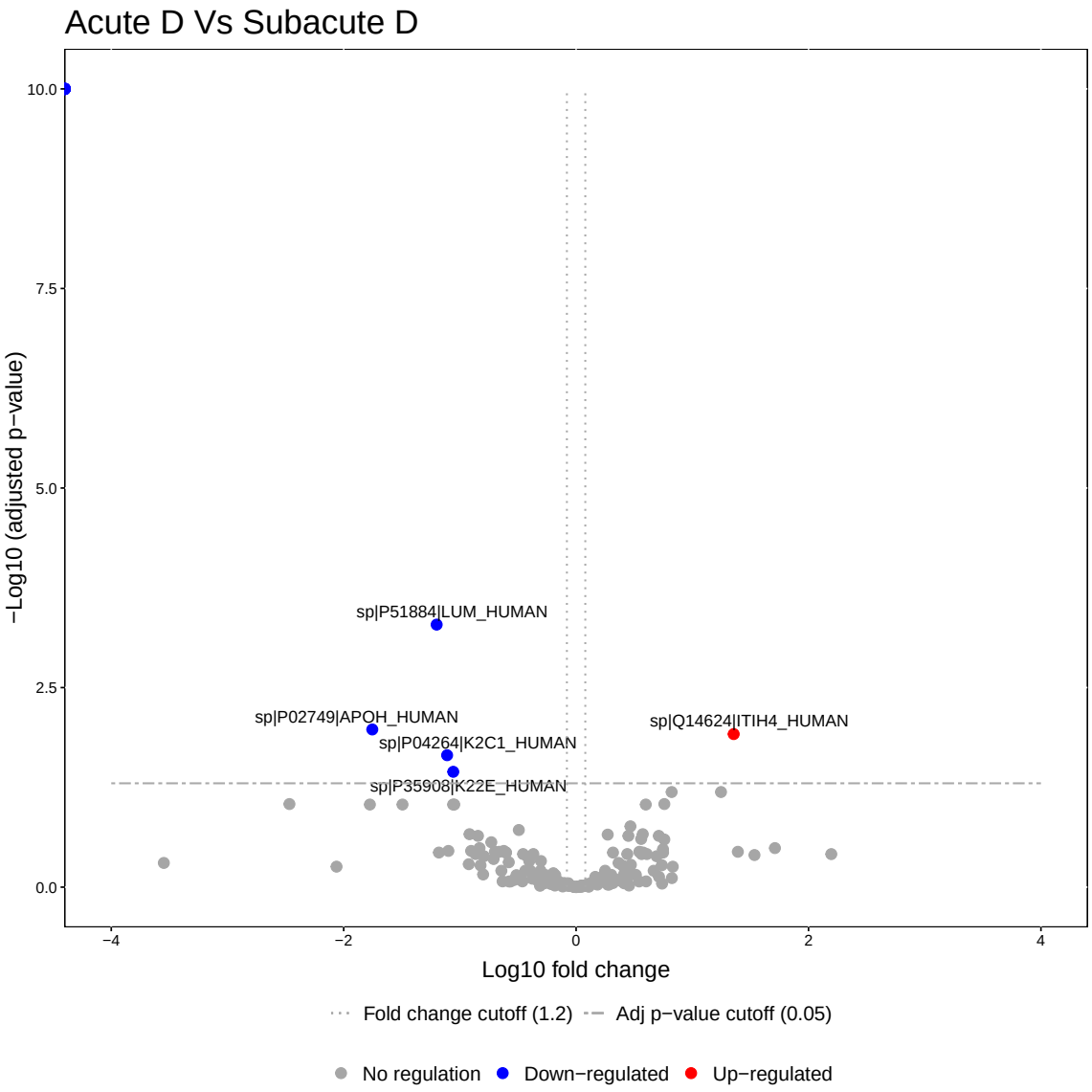


Figure A.37: Volcano plot of $\log_10$ fold change and $\log_10$ adjusted p-value for plasma proteins from 2-weeks and 3-months post-injury from AIS D patients. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

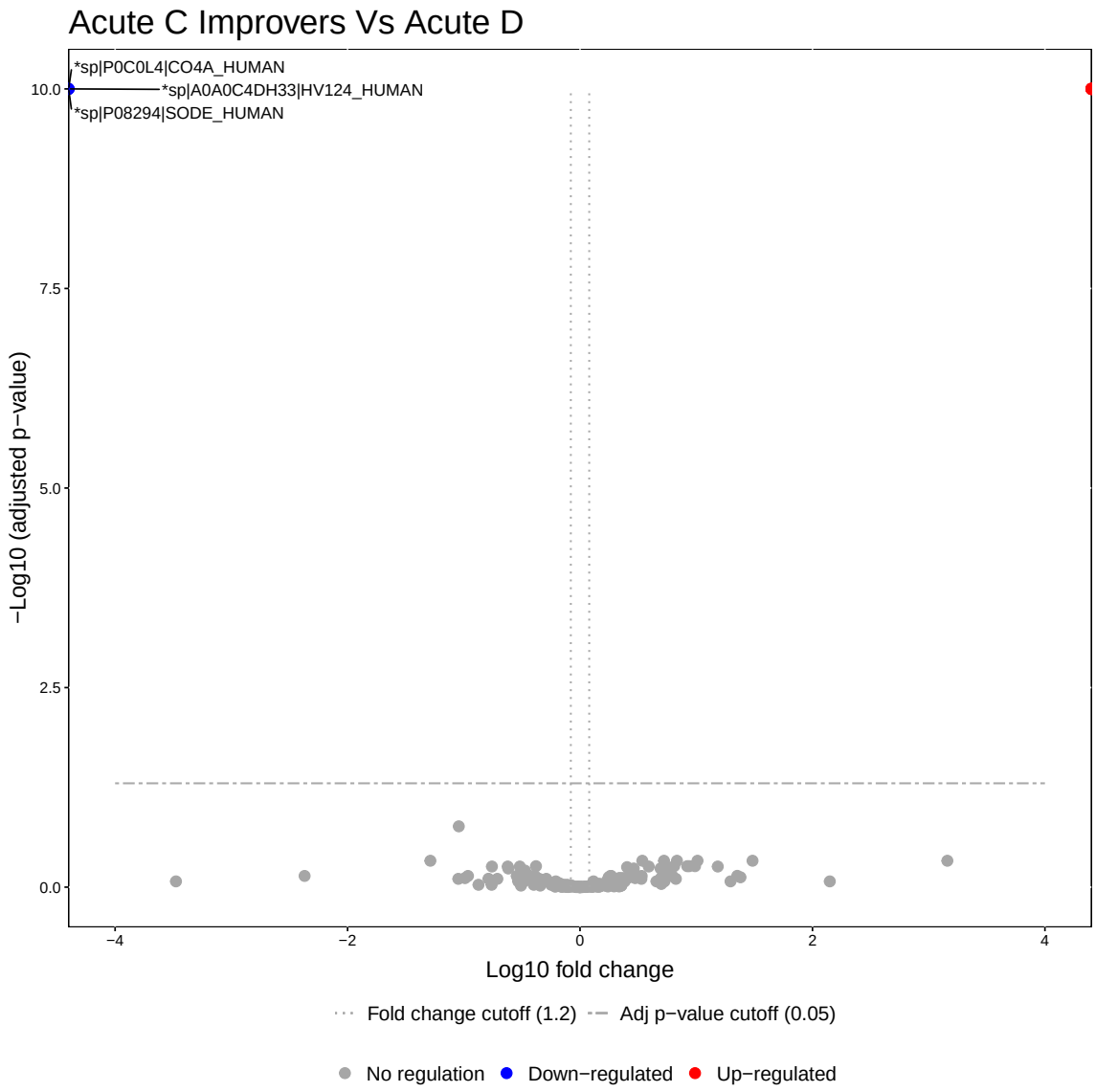


Figure A.38: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who experienced an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

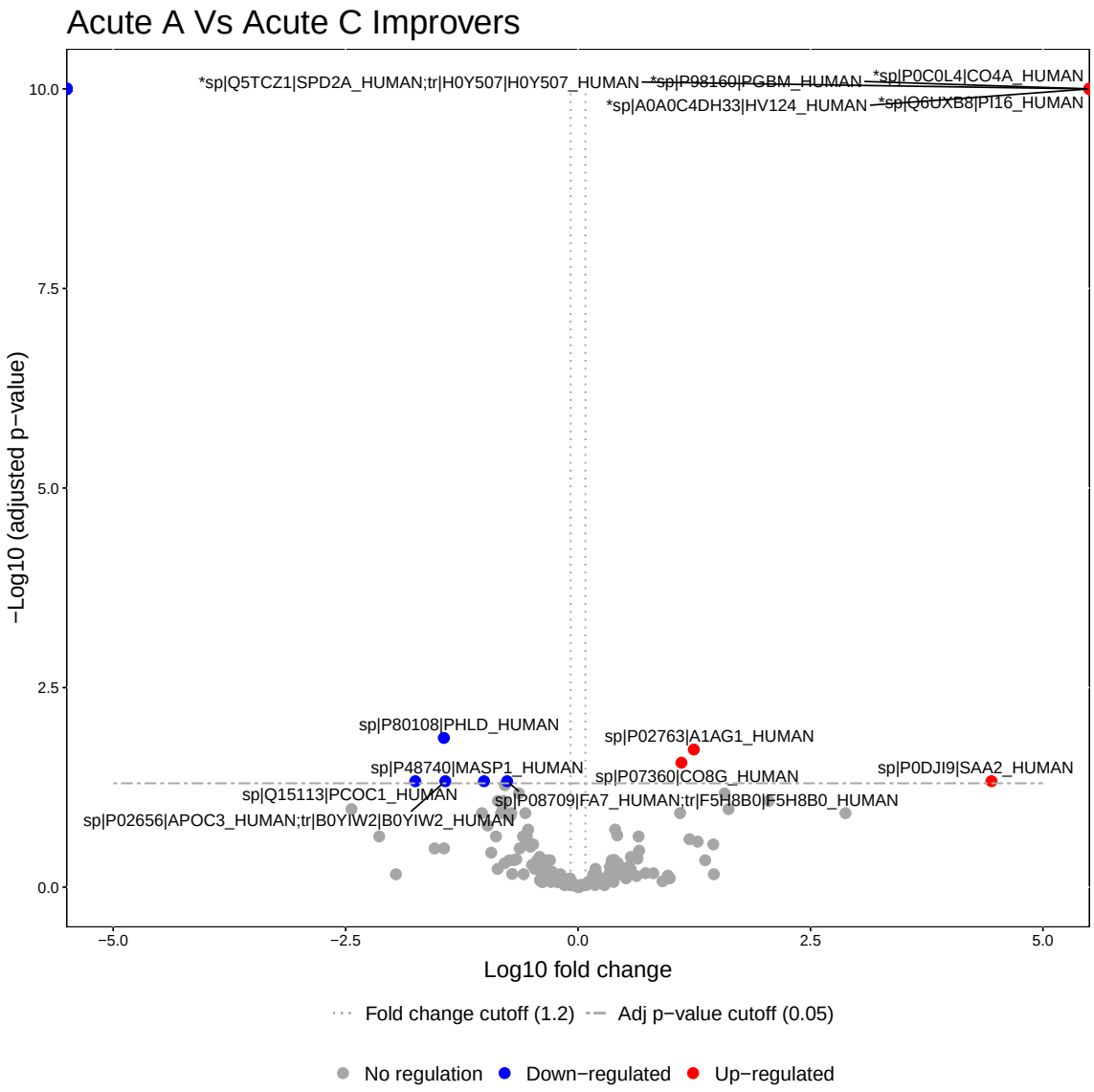


Figure A.39: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who experienced an AIS grade conversion. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

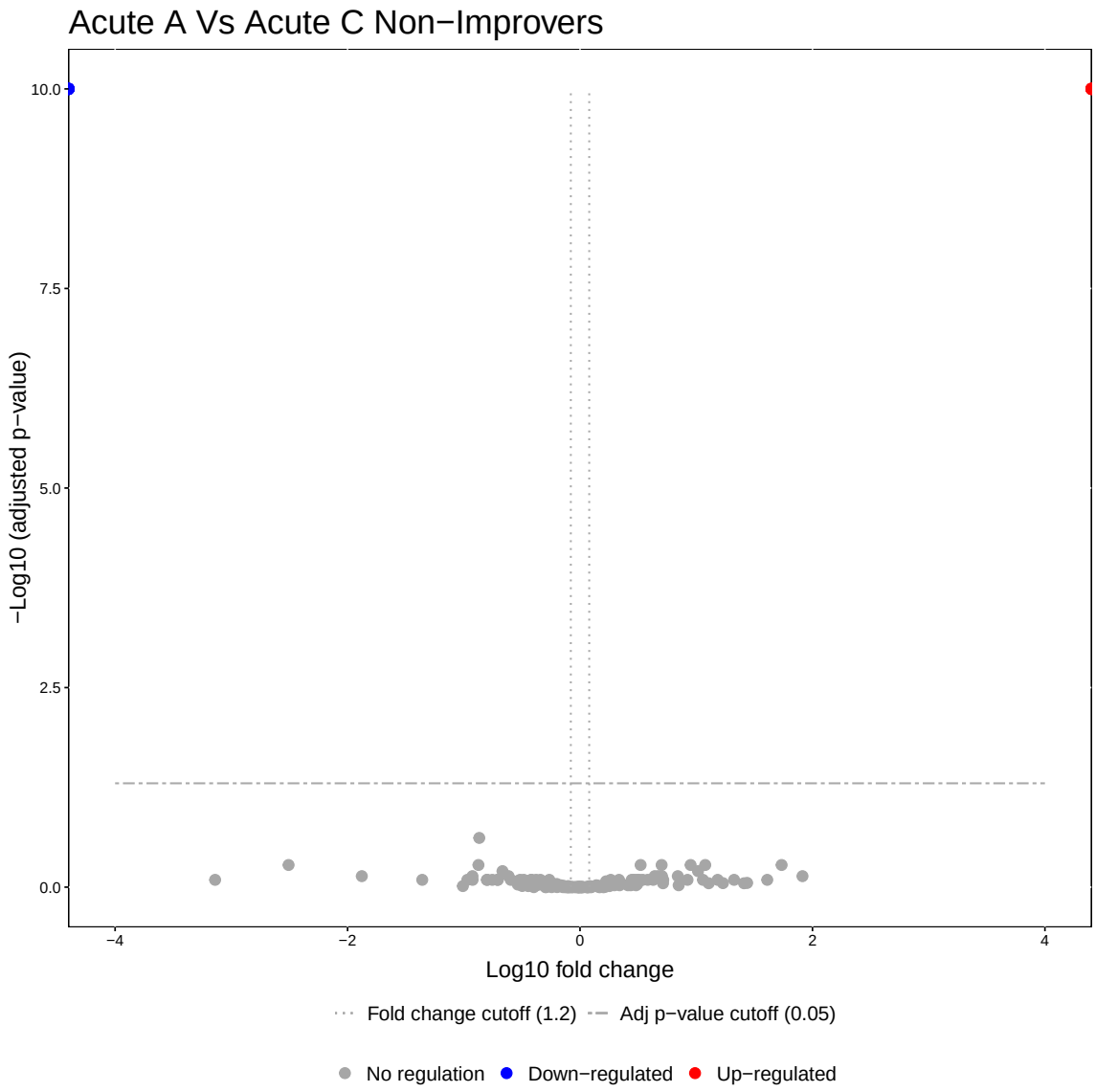


Figure A.40: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS A patients and AIS C patients who did not experience an AIS grade conversion. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

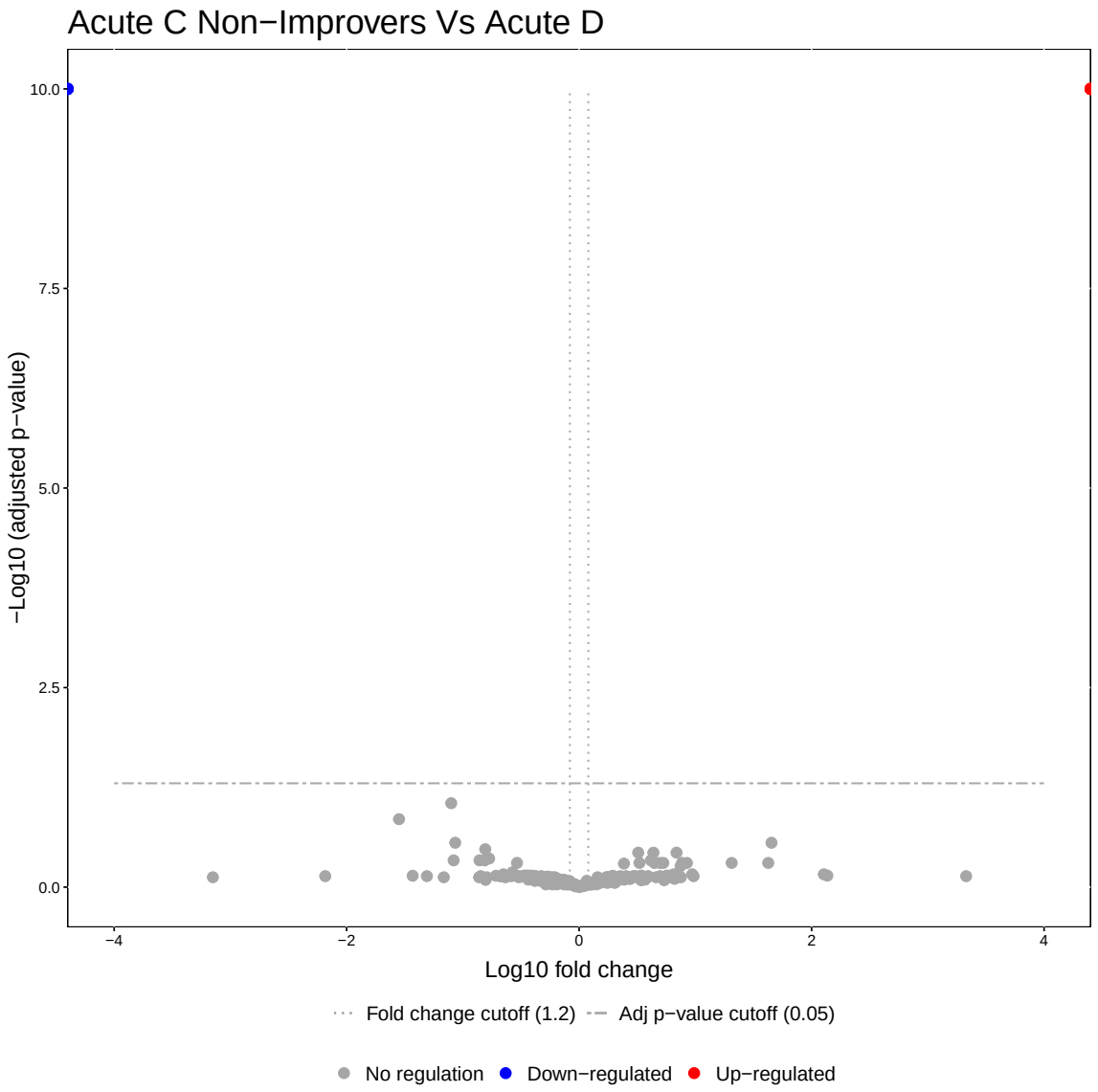


Figure A.41: Volcano plot of $\log_1 0$ fold change and $\log_1 0$ adjusted p-value for plasma proteins from 2-weeks post-injury between AIS C patients who did not experience an AIS grade conversion and AIS D patients. Proteins with a fold changes beyond ± 1.2 and an adjusted p-value less than 0.05 are labelled.

StringDB plots

(a) Cluster 1 (b) Cluster 2

Figure A.42: Clustered interaction network of differentially abundant proteins from 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 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

(a) Cluster 1 (b) Cluster 2

Figure A.43: Clustered 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

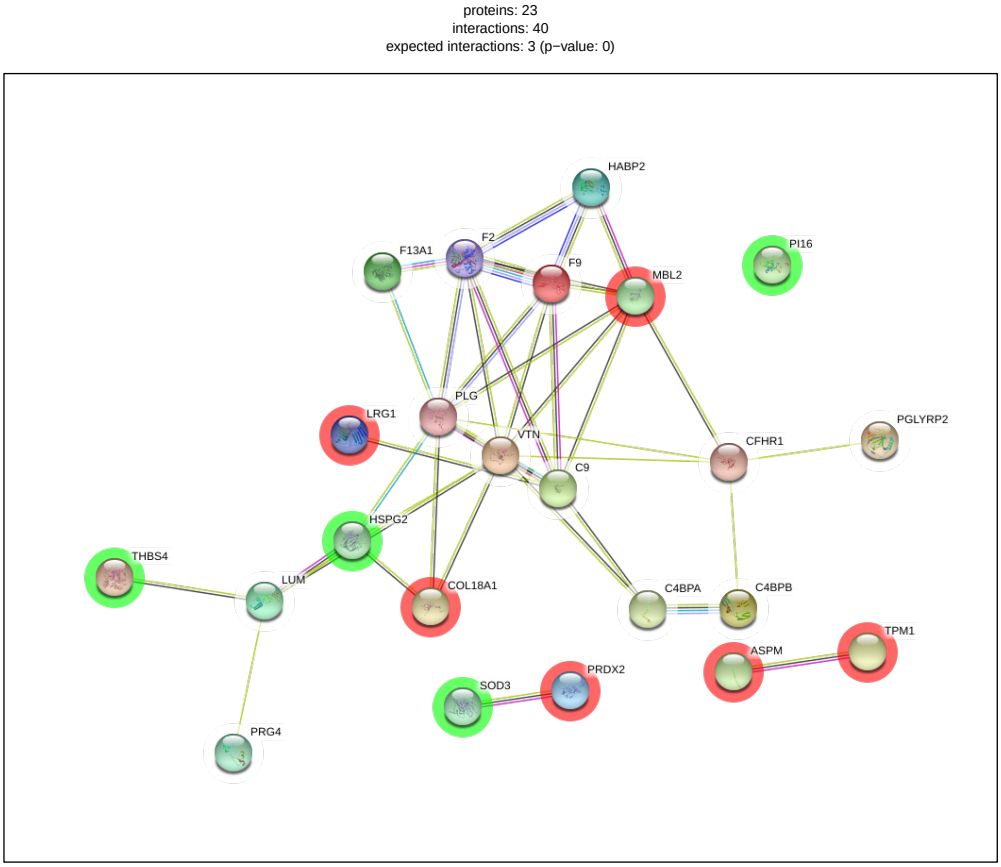
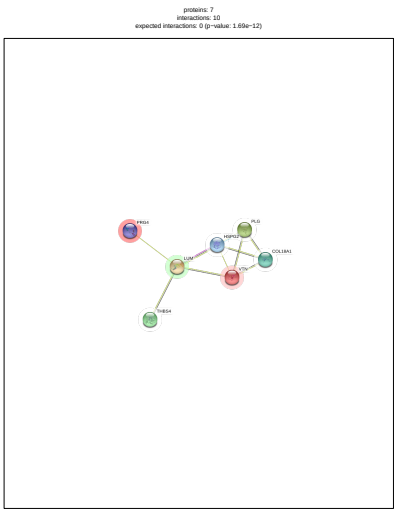
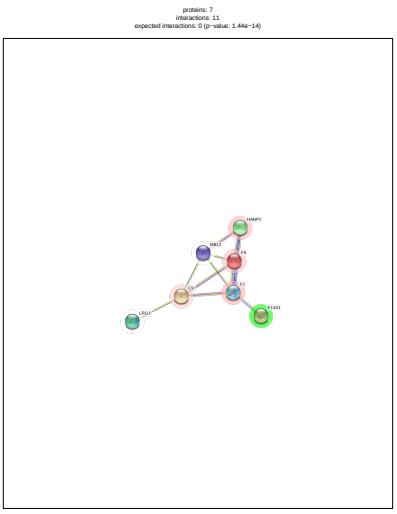


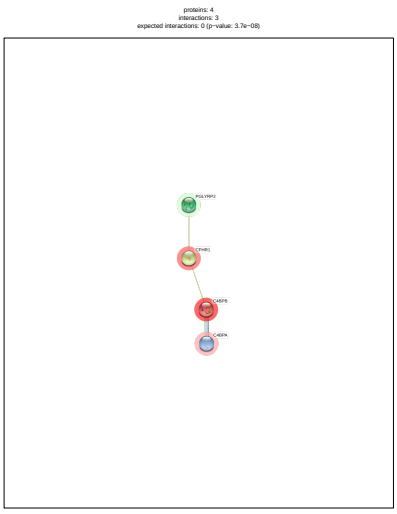
Figure A.44: Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who experienced an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .



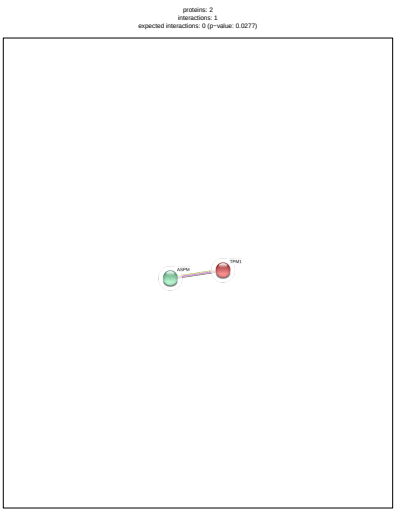
(a) Cluster



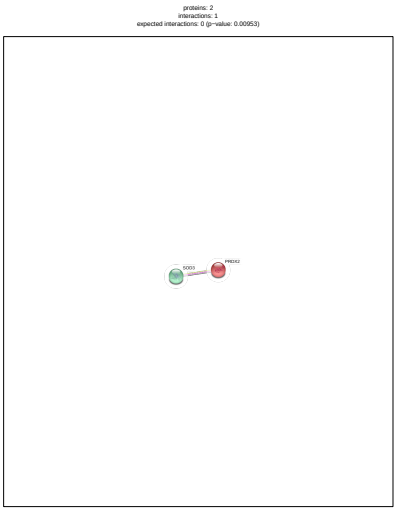
(b) Cluster 2



(c) Cluster 3



(d) Cluster 4



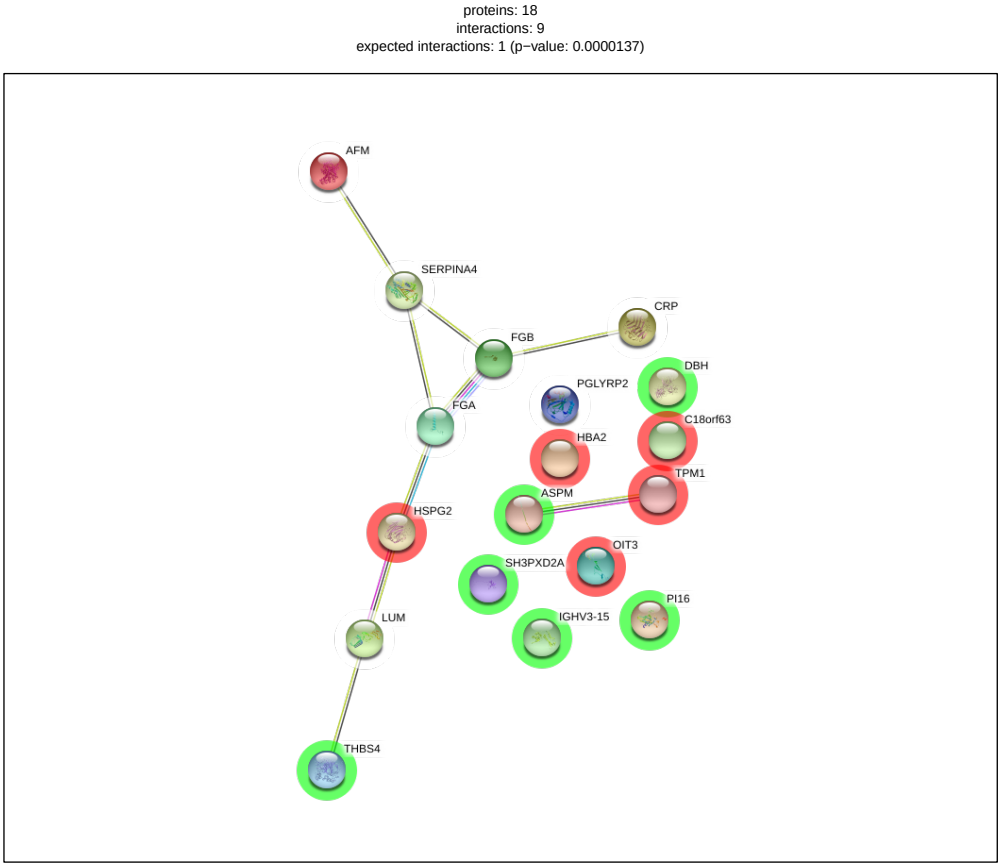
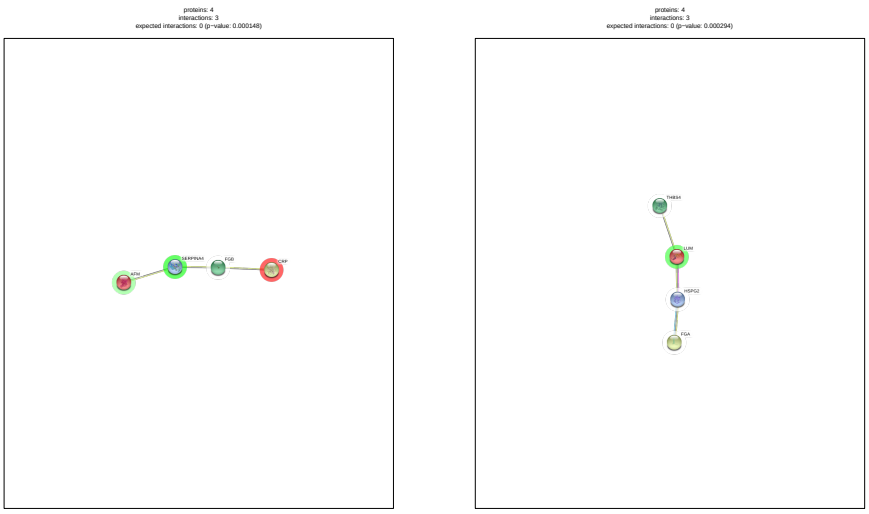
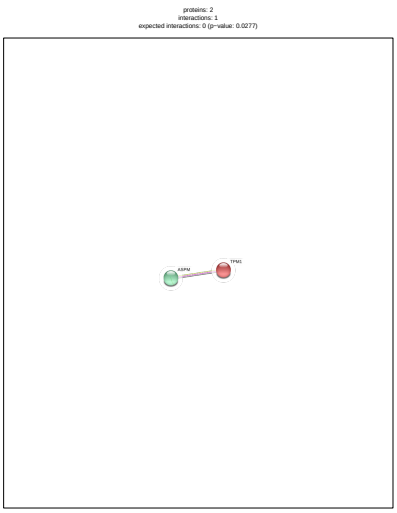


Figure A.46: Interaction network of differentially abundant proteins from plasma 2-weeks and 3-months post-injury for AIS C patients who did not experience an AIS grade conversion. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined . Predicted interactions from: gene co-occurence ; gene fusions ; gene neighbourhood . Others are from gene co-expression ; text-mining and protein homology .



(a) Cluster 1

(b) Cluster 2



(c) Cluster 3

Figure A.47: Clustered 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

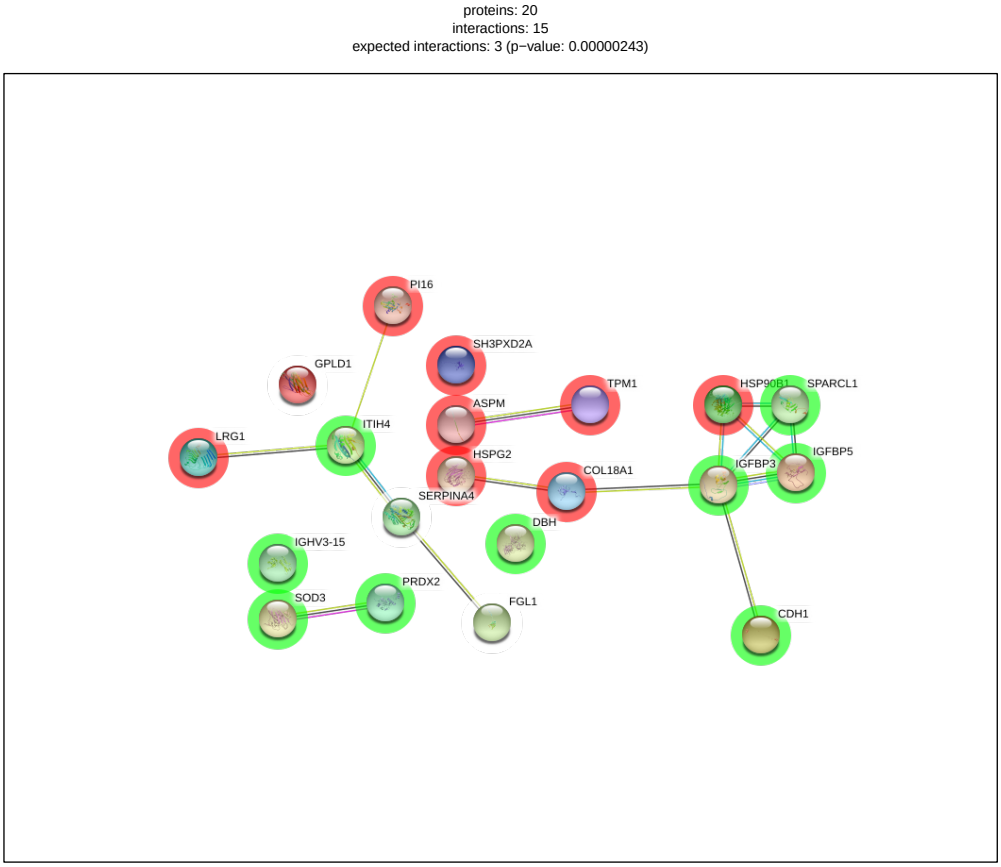
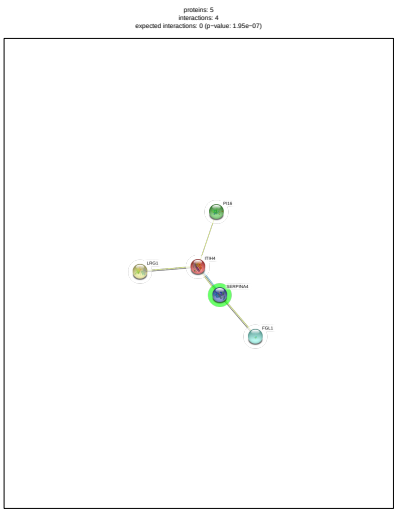
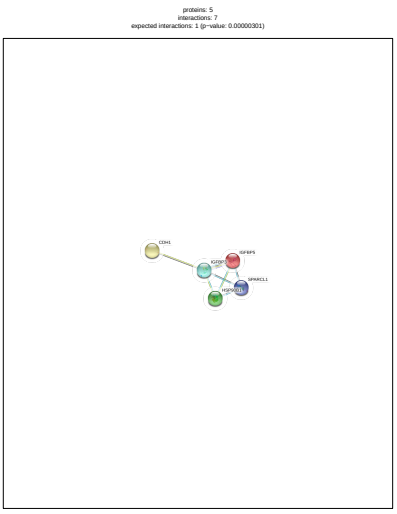


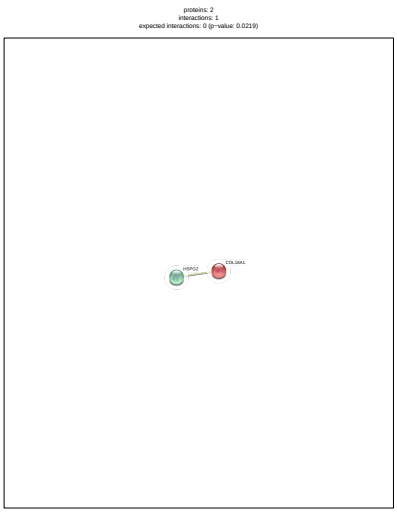
Figure A.48: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS A and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



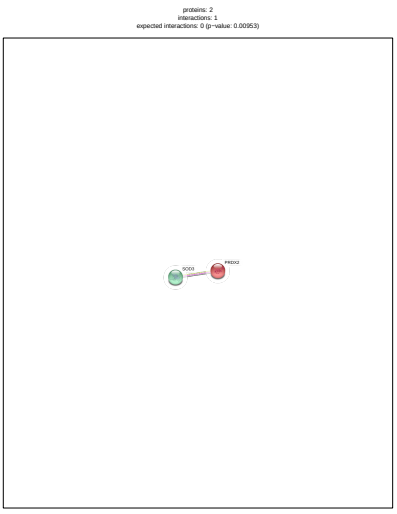
(a) Cluster 1



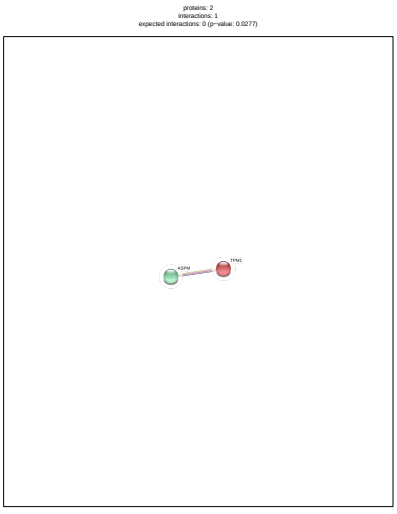
(b) Cluster 2



(c) Cluster 3



(d) Cluster 4



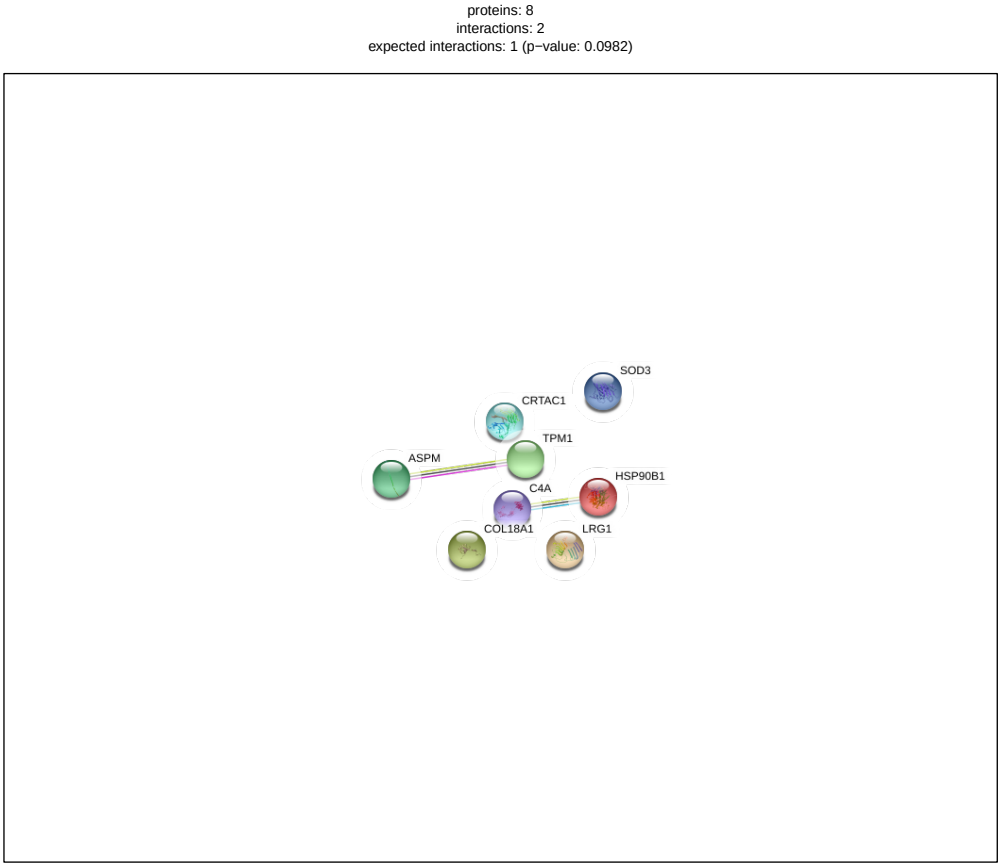


Figure A.50: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

(a) Cluster 1 (b) Cluster 2

Figure A.51: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

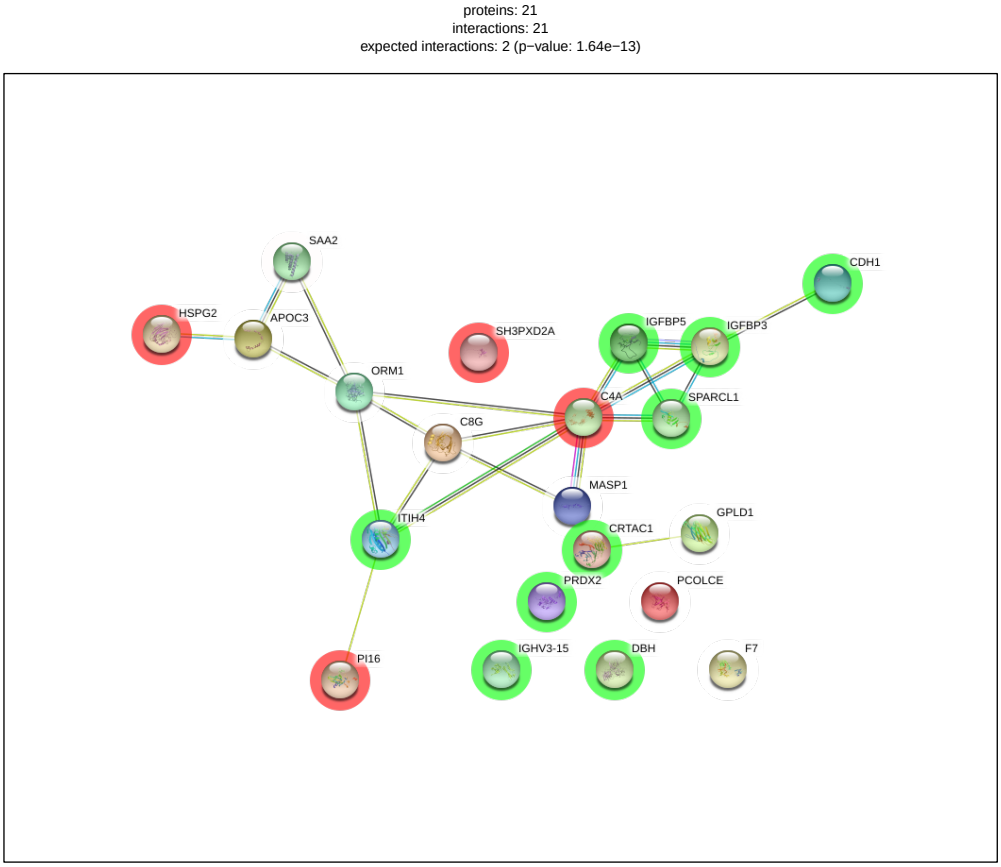
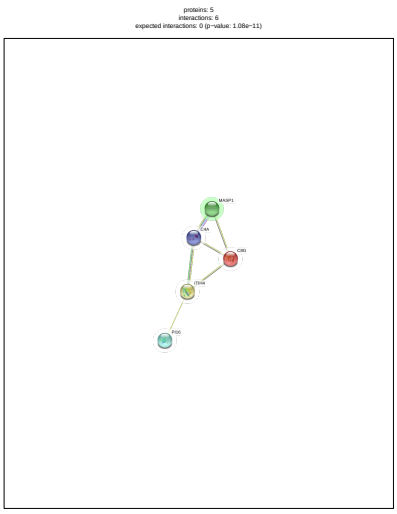
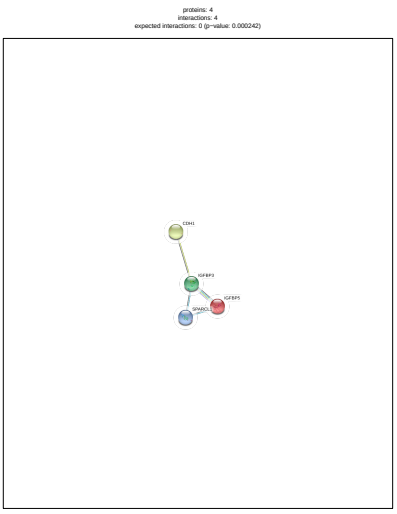


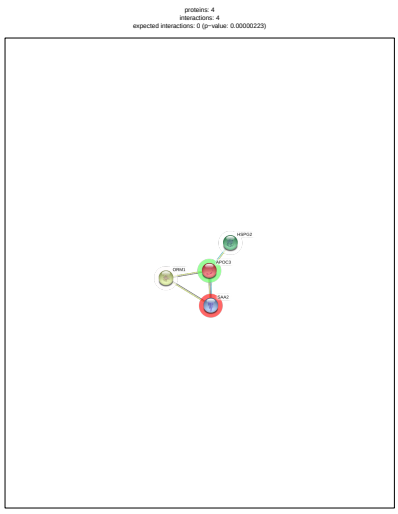
Figure A.52: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C improvers and A. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



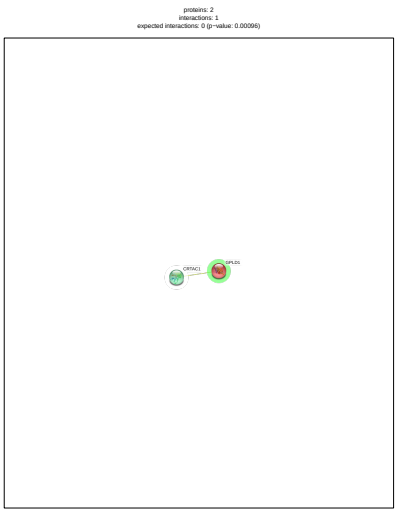
(a) Cluster 1



(b) Cluster 2



(c) Cluster 3



(d) Cluster 4

Figure A.53: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

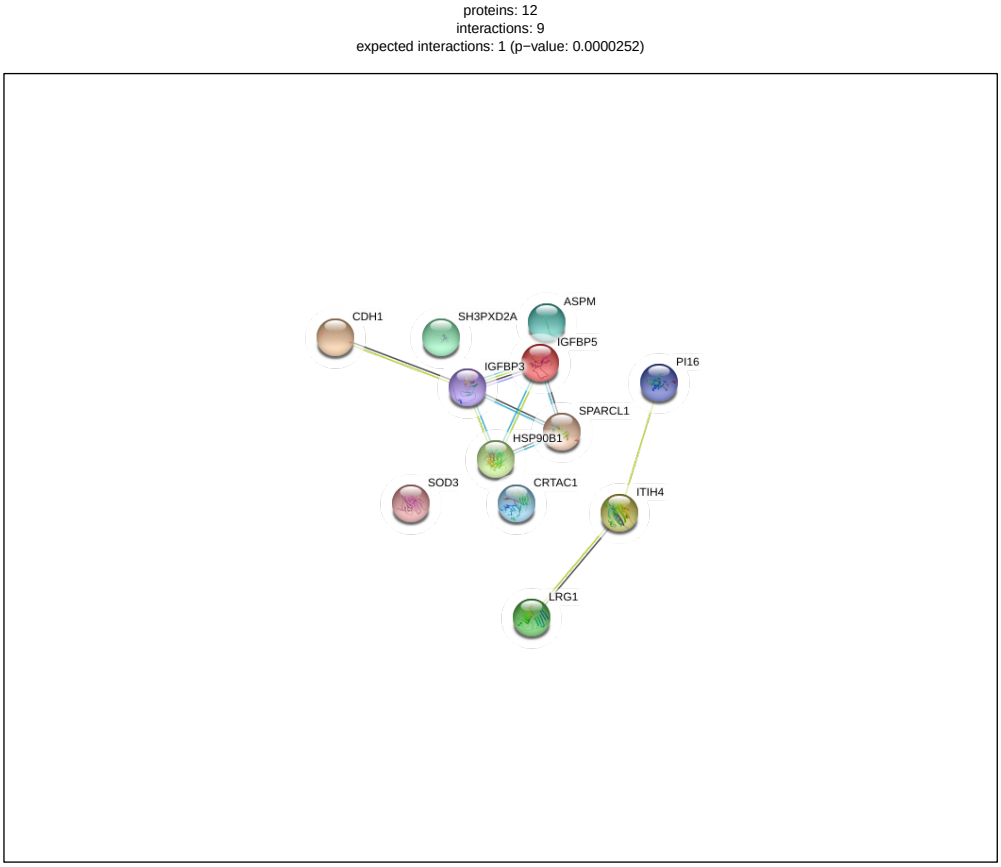
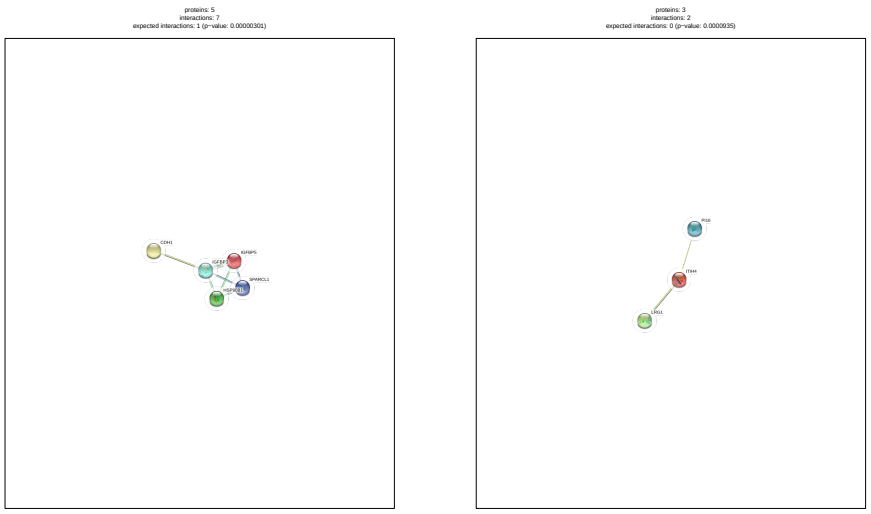


Figure A.54: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and A. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.



(a) Cluster 1 (b) Cluster 2

Figure A.55: Clustered interaction network of differentially abundant proteins from plasma 2-weeks 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-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

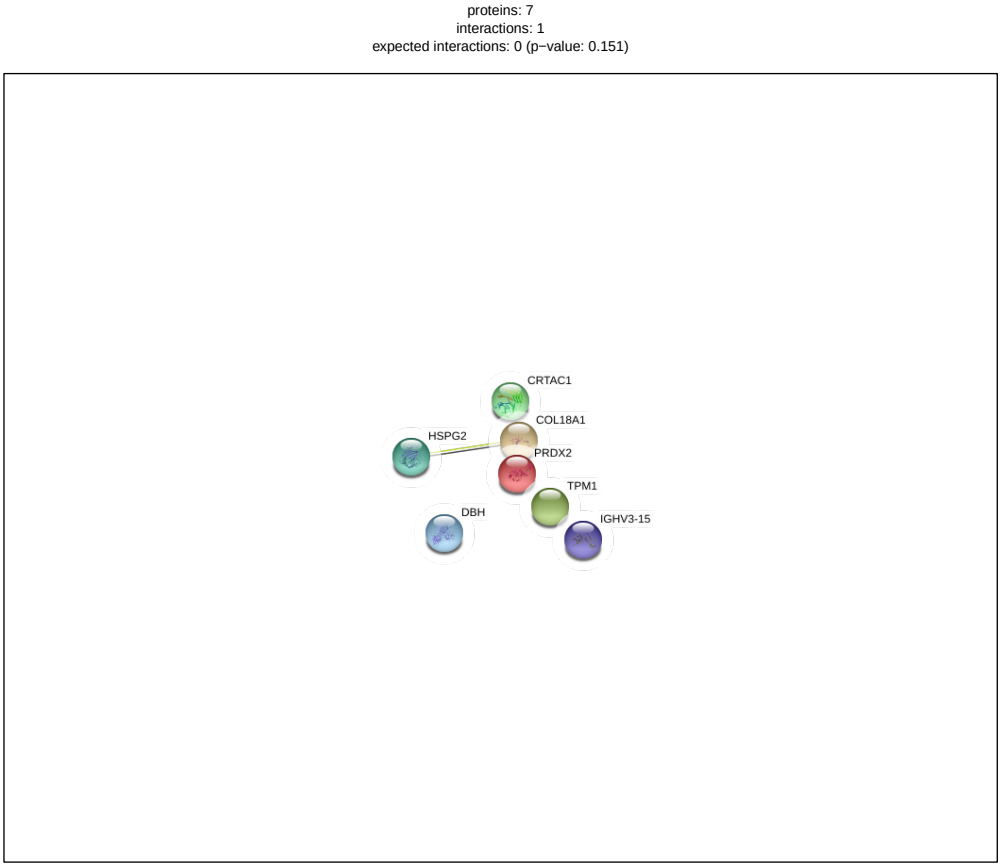


Figure A.56: Interaction network of differentially abundant proteins from plasma 2-week post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

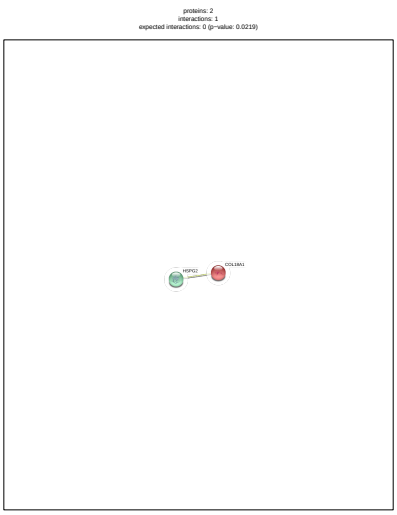


Figure A.57: Clustered interaction network of differentially abundant proteins from plasma 2-weeks post-injury, between AIS C non-improvers and D. The coloured "halo" denotes fold change whereby green indicates that protein is less abundant and red that there is greater abundance. Edges represent protein-protein associations; these are known interactions from: curated databases and those that are experimentally determined. Predicted interactions from: gene co-occurence; gene fusions; gene neighbourhood. Others are from gene co-expression; text-mining and protein homology.

Heatmaps

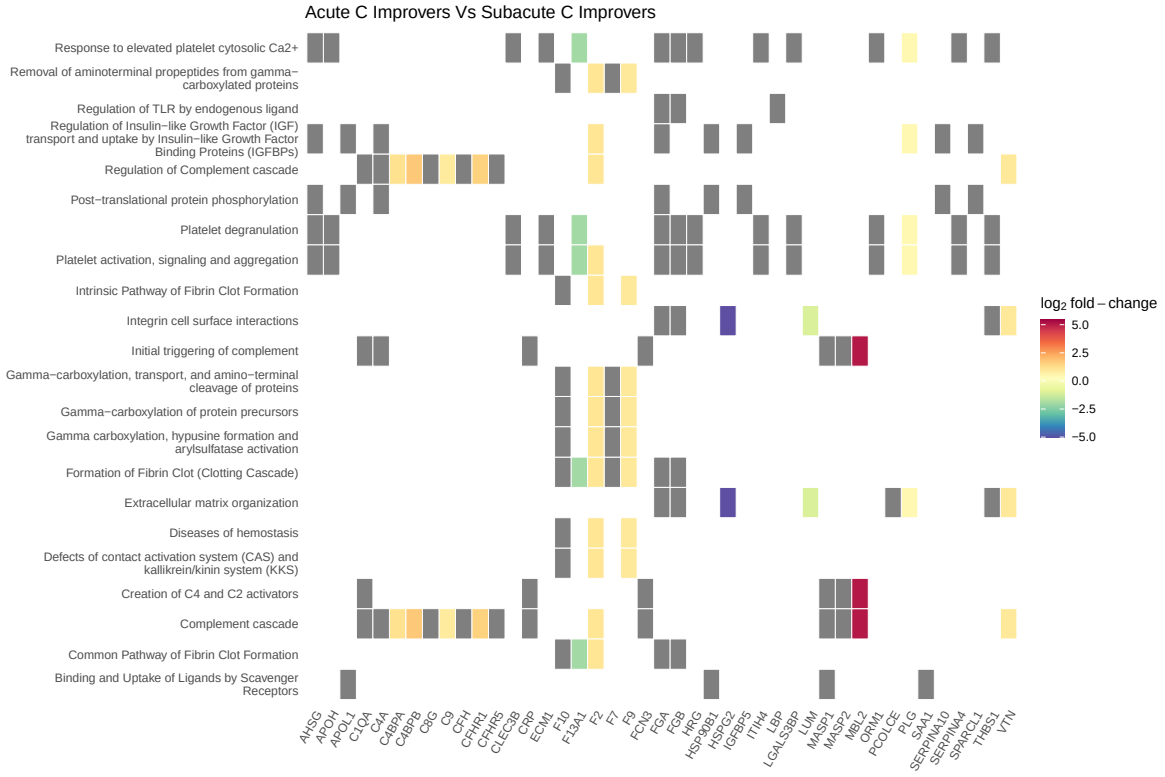


Figure A.58: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury. Grey blocks denote proteins not present in the comparison.

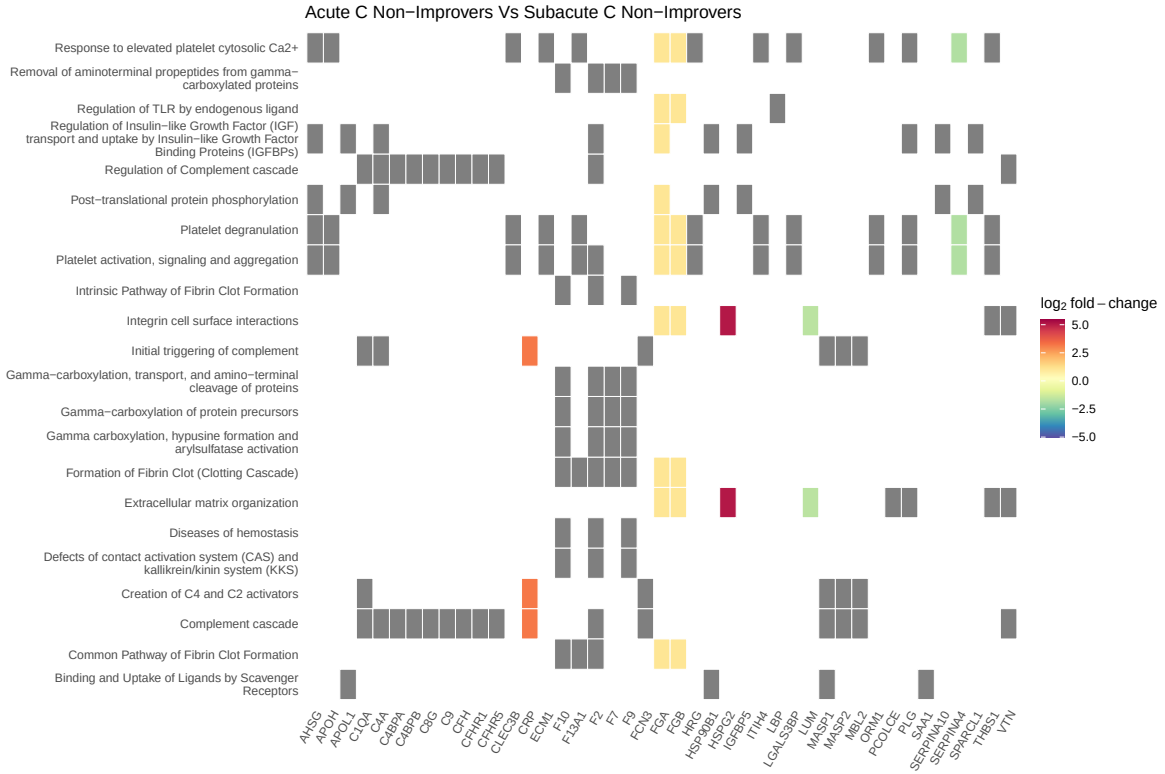


Figure A.59: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury. Grey blocks denote proteins not present in the comparison.

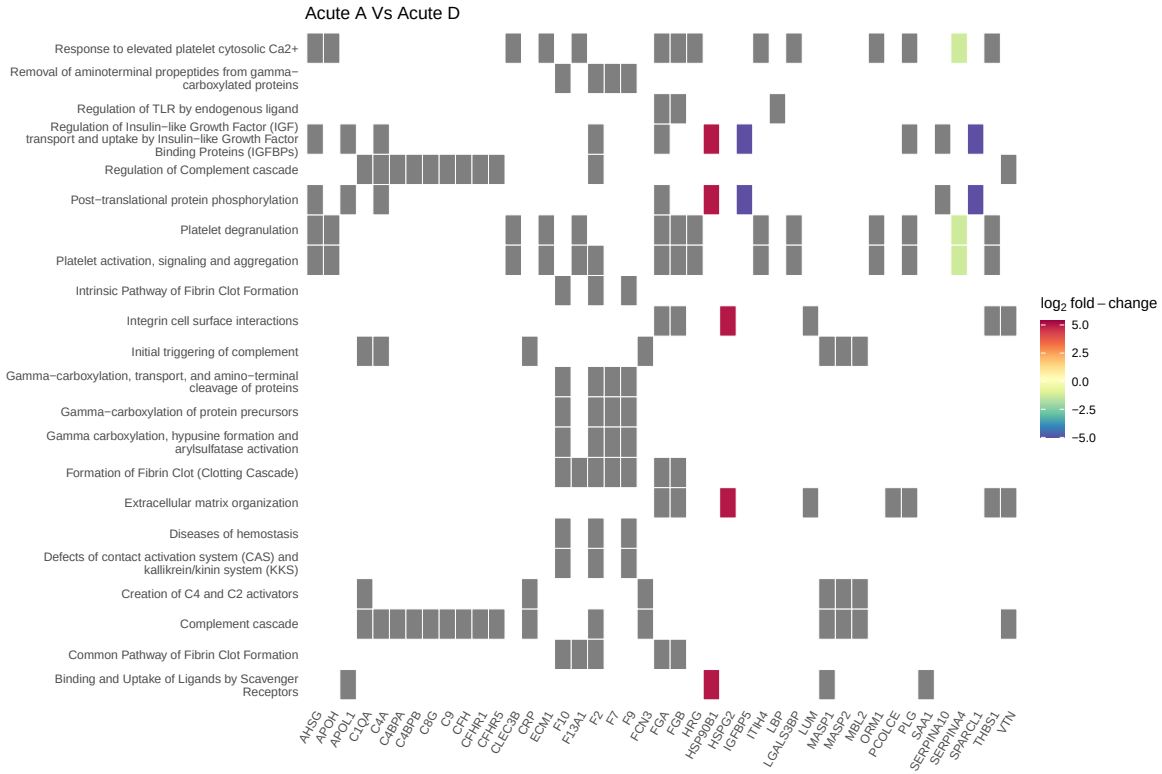


Figure A.60: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients. Grey blocks denote proteins not present in the comparison.

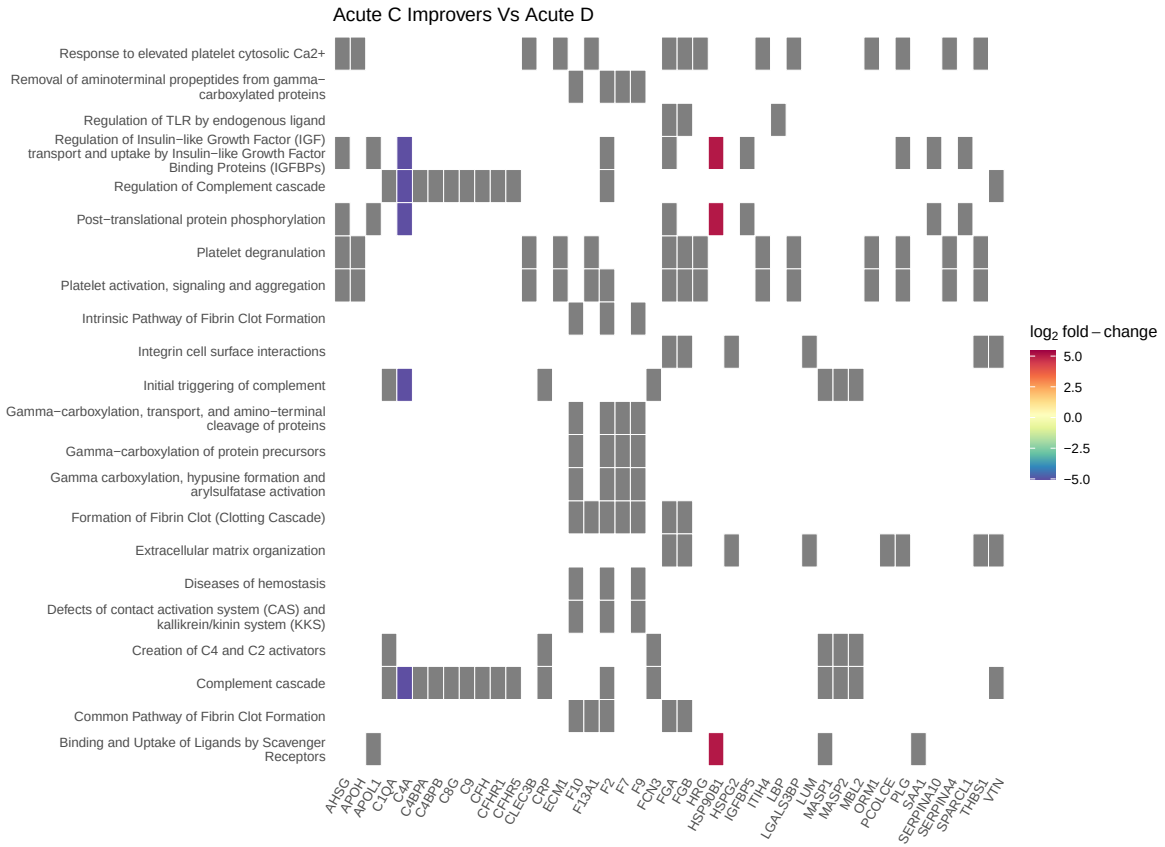


Figure A.61: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients. Grey blocks denote proteins not present in the comparison.

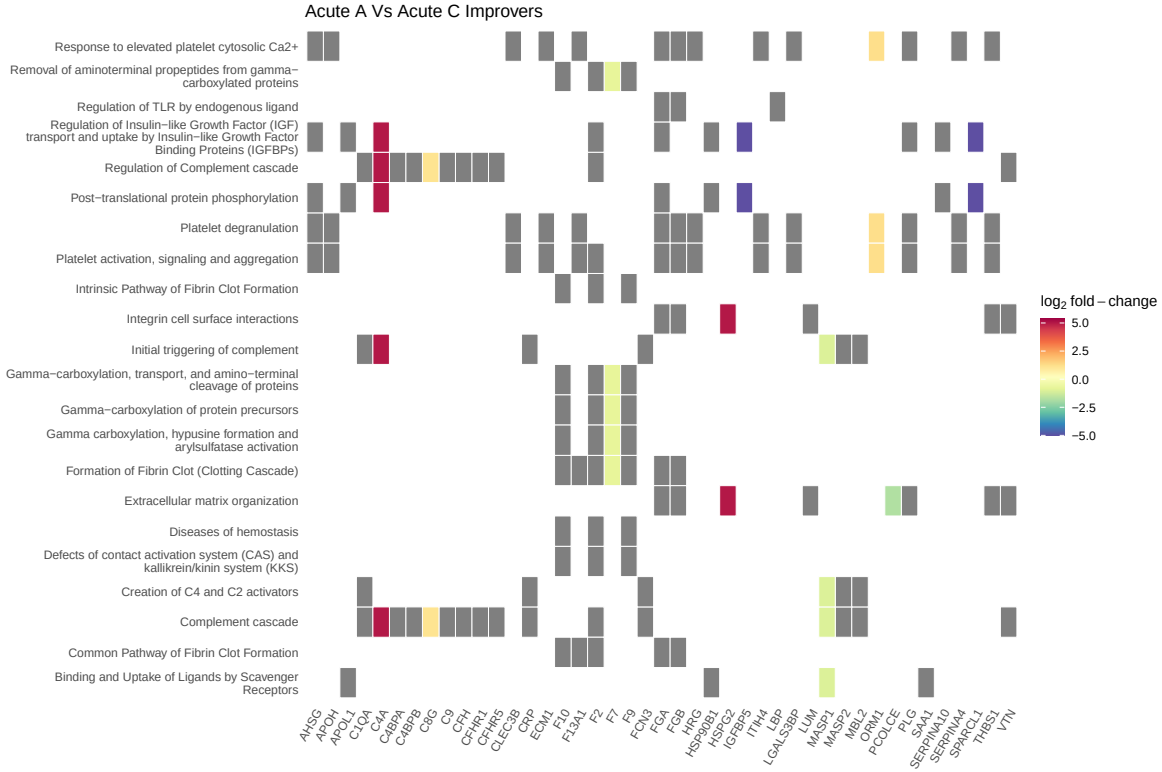


Figure A.62: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.

Figure A.63: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients. Grey blocks denote proteins not present in the comparison.

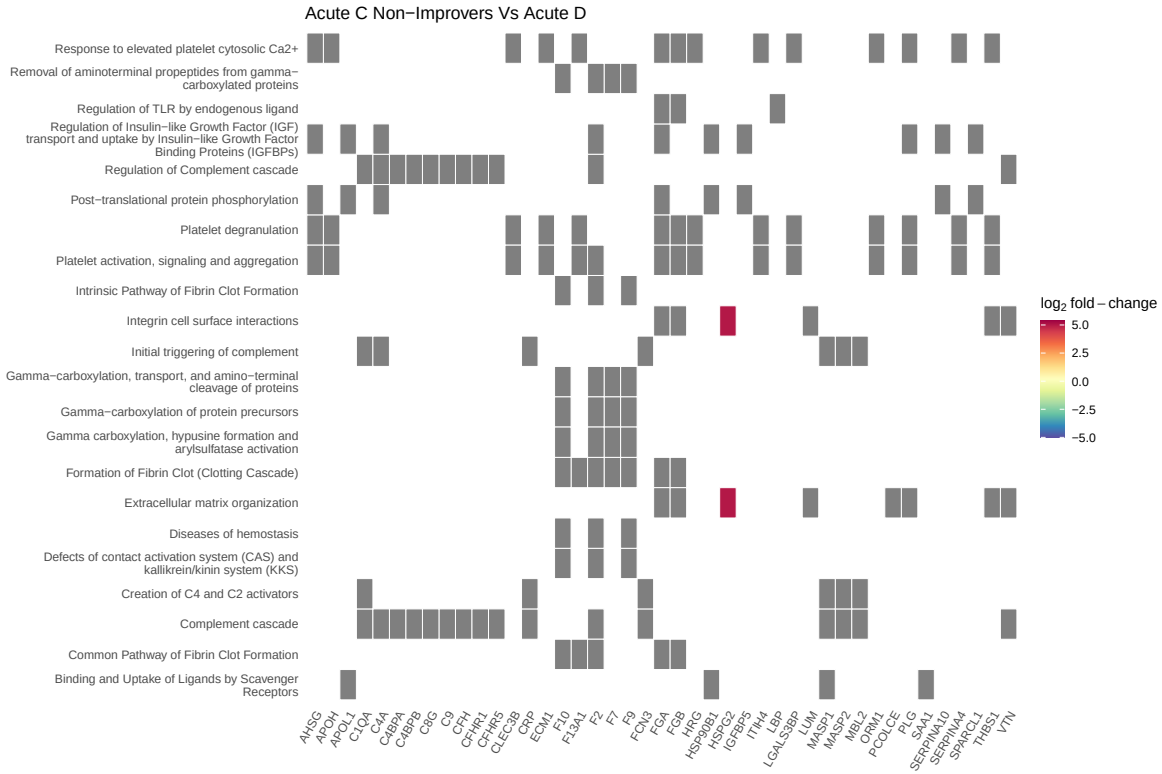


Figure A.64: Heatmap denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients. Grey blocks denote proteins not present in the comparison.

Cnetplots

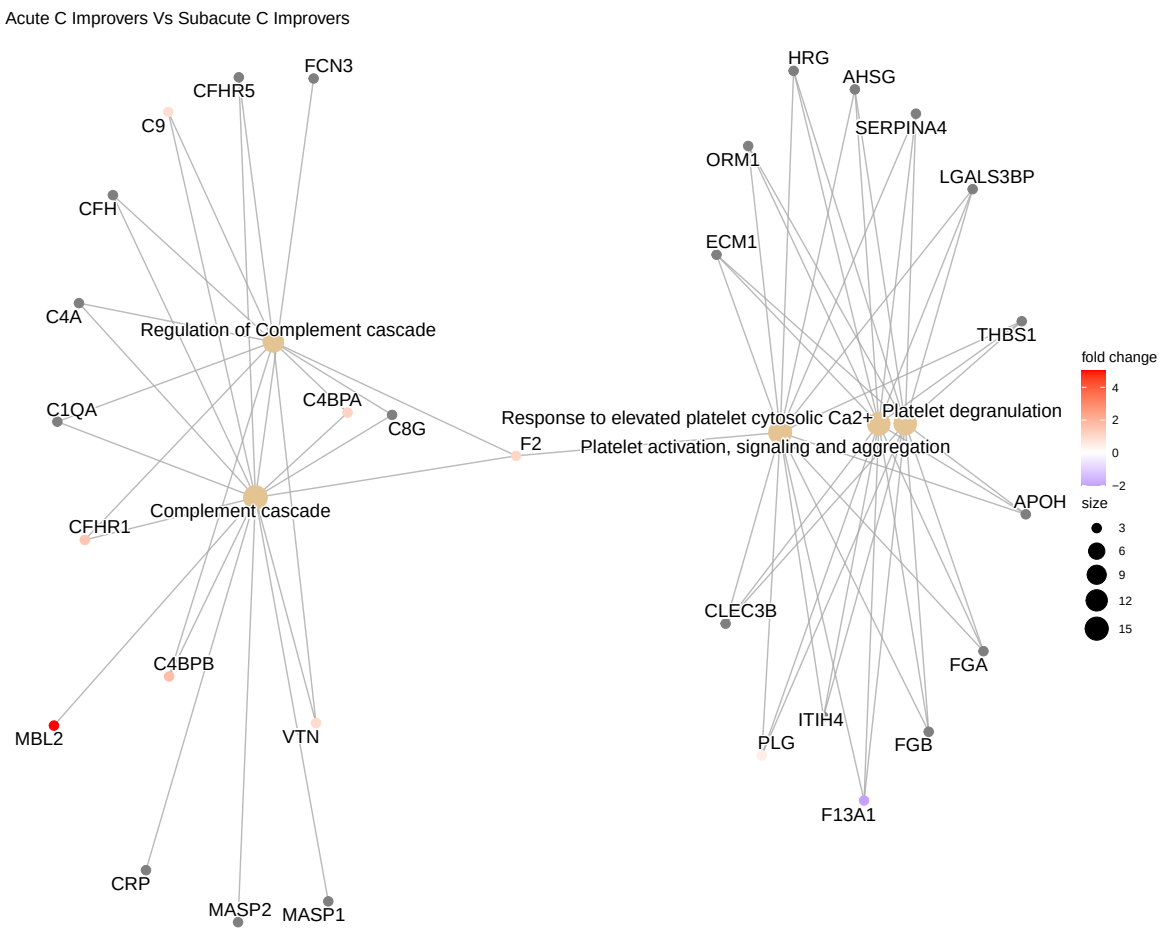


Figure A.65: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement at 2-weeks and 3-months post-injury.

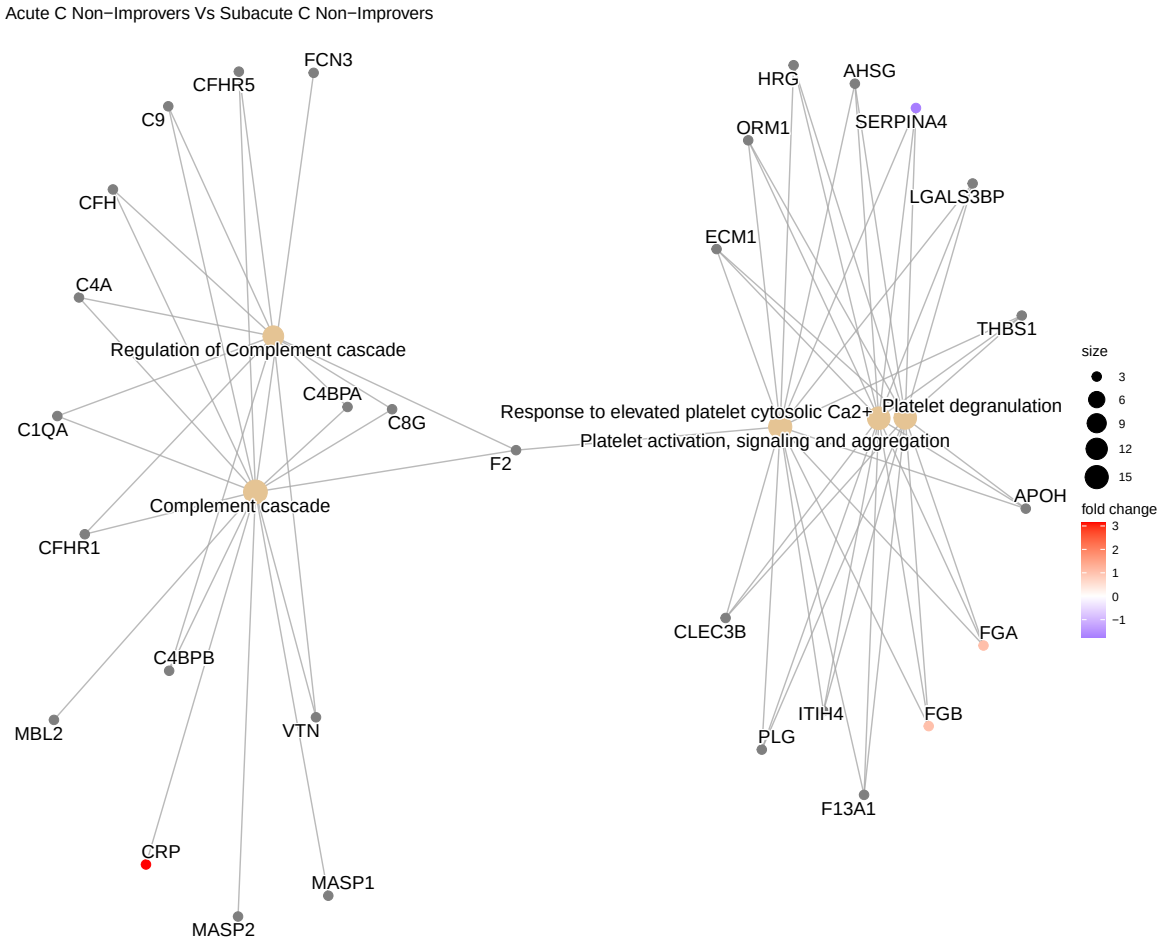


Figure A.66: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks and 3-months post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement at 2-weeks and 3-months post-injury.

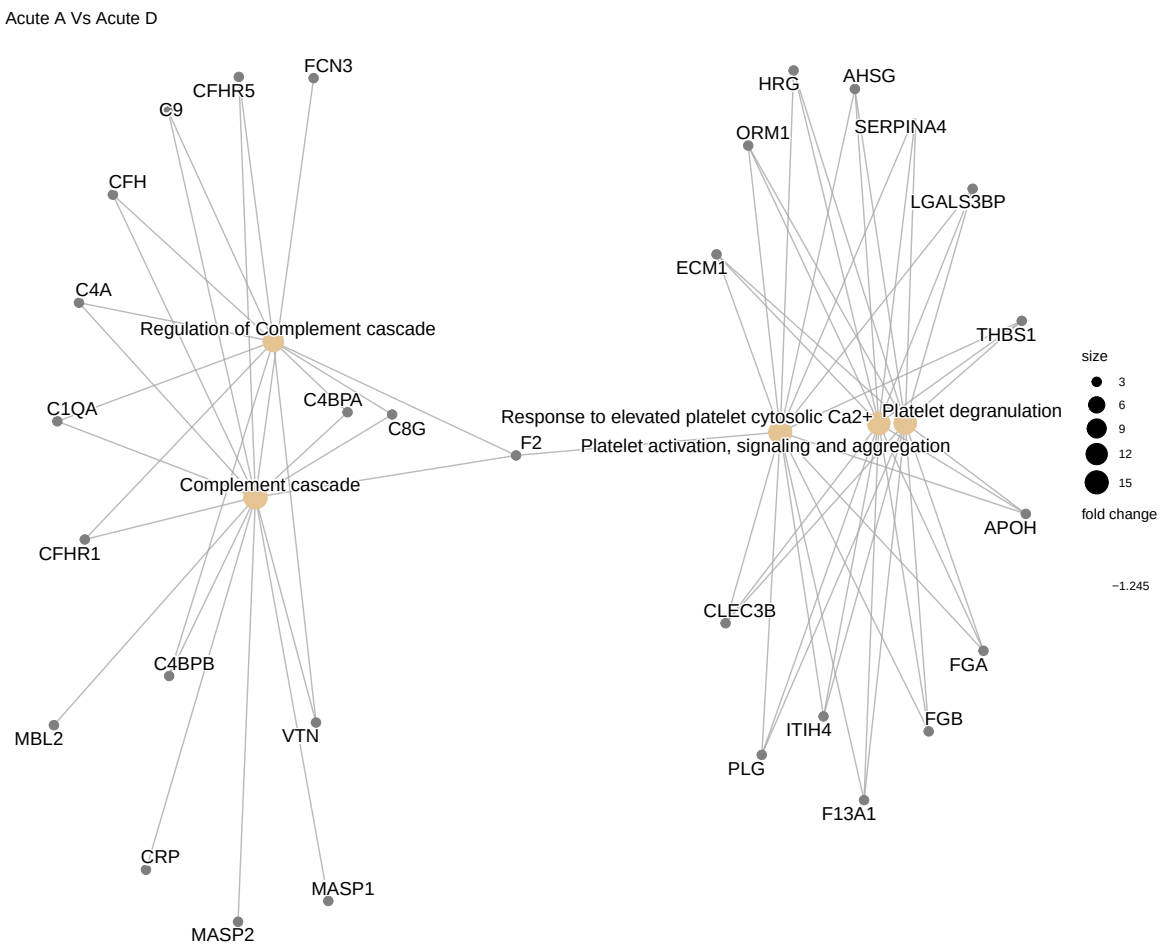


Figure A.67: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS A and AIS D SCI patients.

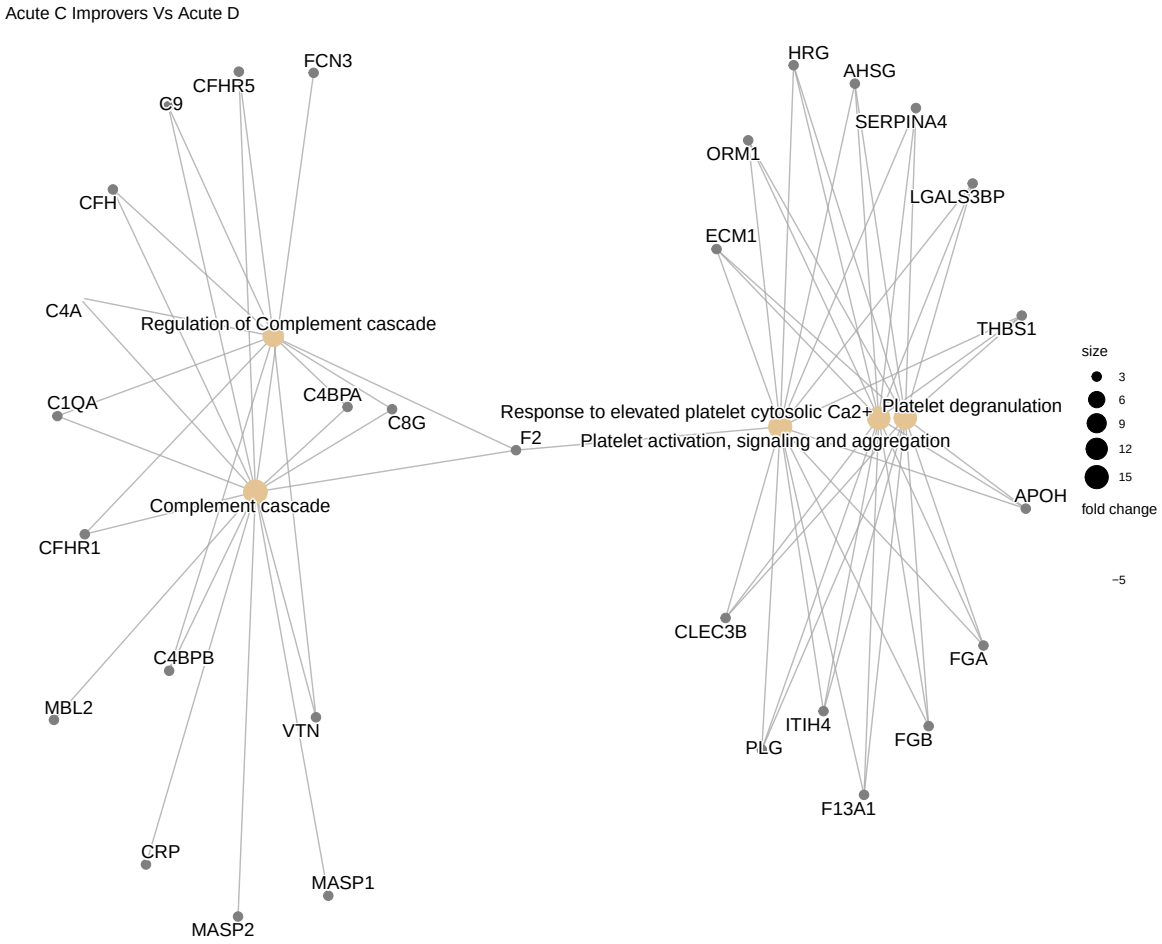


Figure A.68: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS D patients.

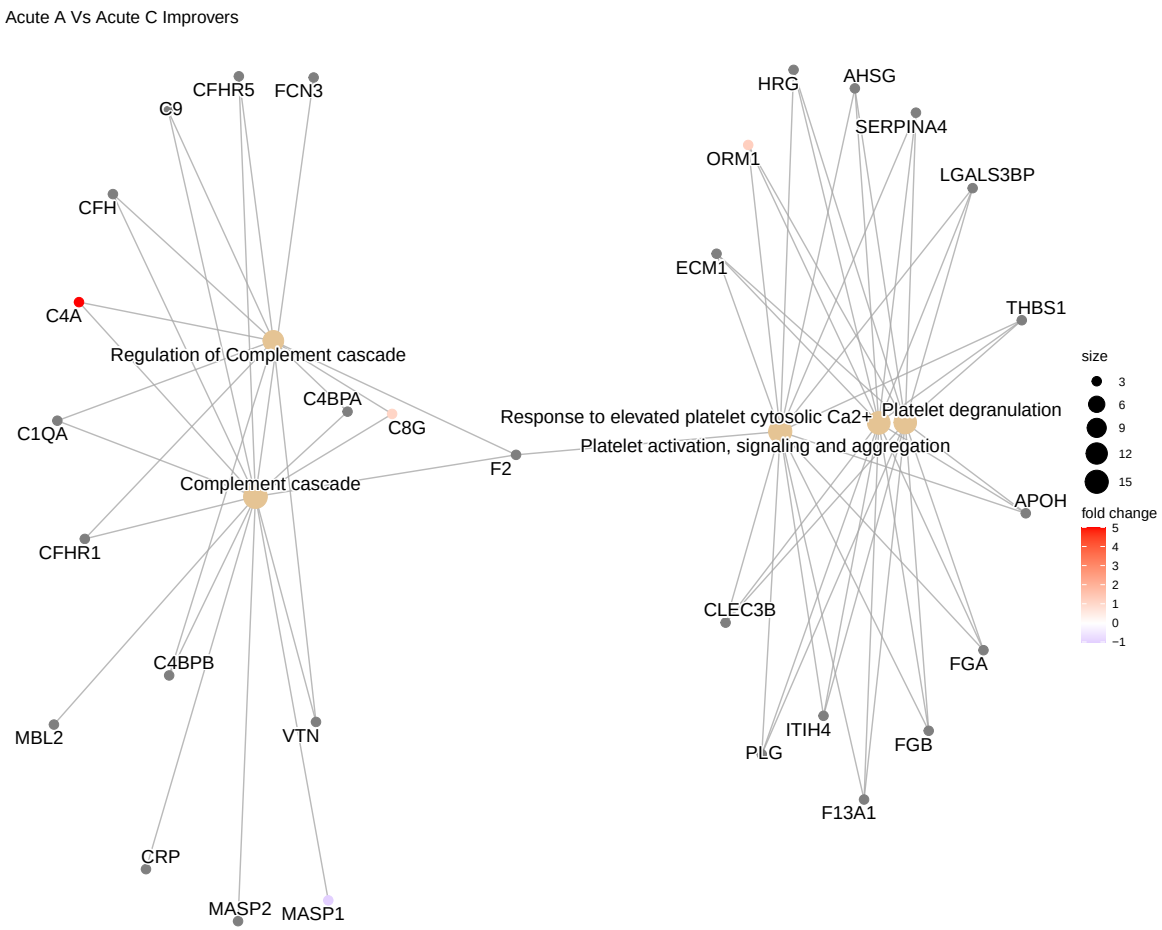


Figure A.69: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who experienced an AIS grade improvement and AIS A patients.

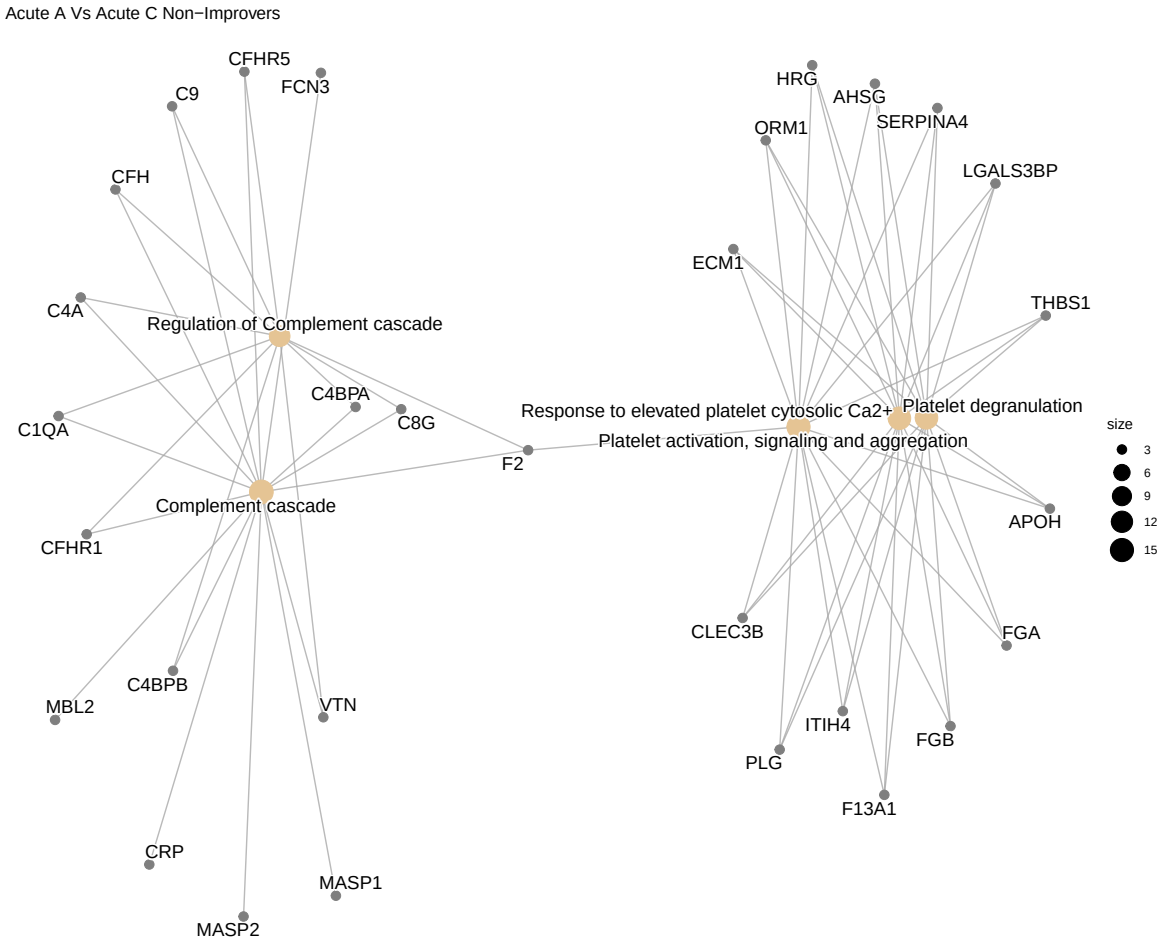


Figure A.70: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS A patients.

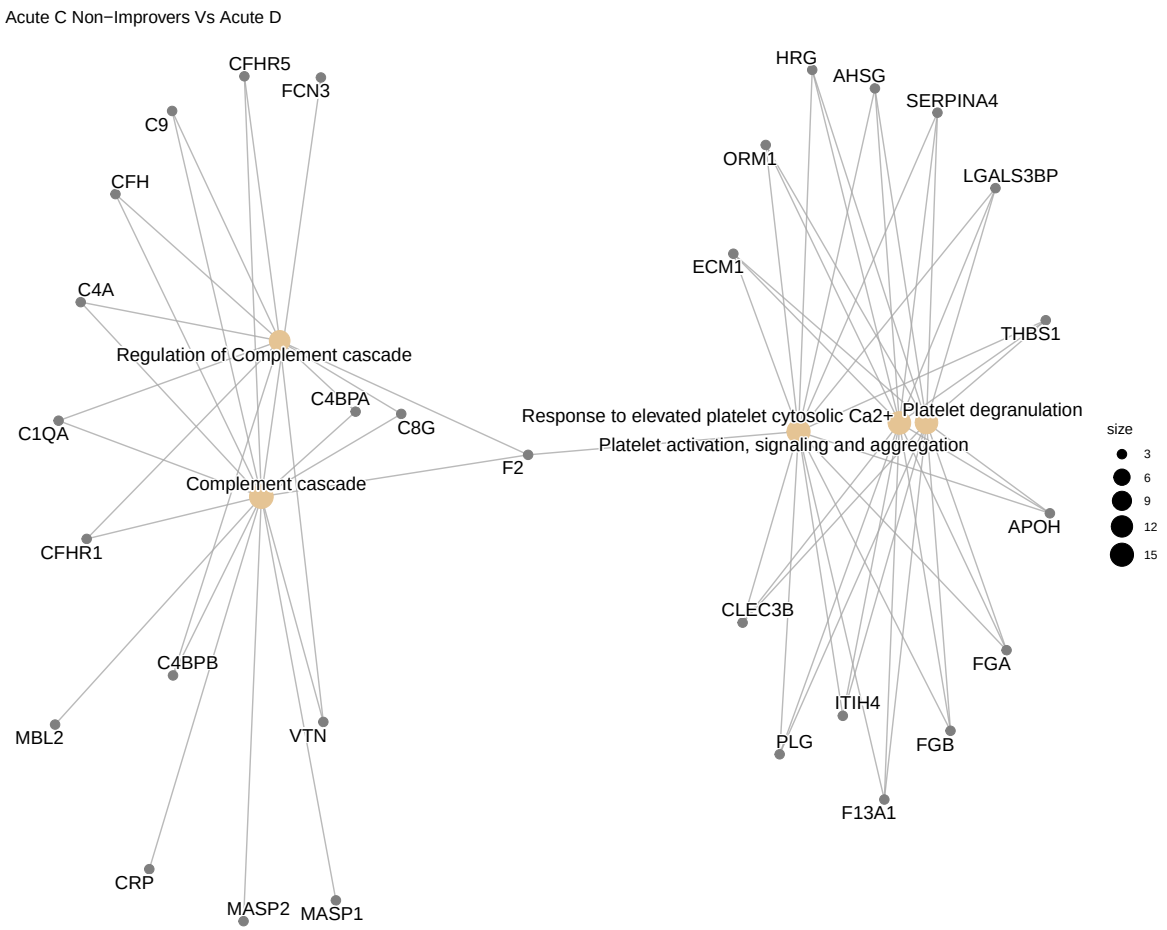


Figure A.71: Network plot denoting the $\log_2$ fold change of proteins in plasma collected 2-weeks post-injury, and the biological pathways these proteins are associated with on Reactome. This compares AIS C SCI patients who did not experience an AIS grade improvement and AIS D patients.

Annex

A.4 Ethical approval letter

NHS
Health Research Authority

NRES Committee North West - Liverpool East
North West REC Centre
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

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31 January 2012

Dr Karina Wright
Research Associate
ISTM, Keele University
ARC/TORCH Building
RJAH Orthopaedic Hospital
SY10 7AG

Dear Dr Wright

Study title:	**Blood sampling for identification of biomarkers to investigate the different pathologies of spinal cord injury.**
REC reference:	**11/NW/0876**
Protocol number:	**None**

Thank you for your letter which was received on 05 January 2012, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
REC application: 93773/271321/1/178		01 December 2011
Protocol	1	28 November 2011
Investigator CV: Karina Therese Wright		
Participant Information Sheet	1.1	03 January 2012
Participant Consent Form	1.1	03 January 2012
Response to Request for Further Information from Dr Karina Wright		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/NW/0876	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp H Penistone

Mrs Jean Harkin
Chair

Email: helen.penistone@northwest.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to:
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