A comprehensive proteomic and bioinformatic analysis of human spinal cord injury plasma identifies proteins associated with the complement cascade and liver function as potential prognostic indicators of neurological outcome

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Abstract

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

Blood samples were taken, following informed consent, from ASIA impairment scale (AIS) grade C "Improvers" (those who experienced an AIS grade improvement) and "Non-Improvers" (No AIS change), and AIS grade A and D at <2 weeks ("Acute") and approx. 3 months ("Sub-acute") post-injury. The total protein concentration from each sample was extracted, with pooled samples being labelled and non-pooled samples treated with ProteoMiner™ beads. Samples were then analysed using two 4-plex isobaric tag for relative and absolute quantification (iTRAQ) analyses and a label-free experiment for comparison, before quantifying with mass spectrometry. Data are available via ProteomeXchange with identifiers PXD035025 and PXD035072 for the iTRAQ and label-free experiments respectively.

Proteomic datasets were analysed using OpenMS (version 2.6.0). R (version 4.1.4) and in particular, the R packages MSstats (version 4.0.1) and pathview (version 1.32.0) were used

for downstream analysis. Proteins of interest identified from this analysis were further validated by enzyme-linked immunosorbent assay (ELISA).

The data demonstrated proteomic differences between the cohorts, with the results from the iTRAQ approach supporting those of the label-free analysis. A total of 79 and 87 differentially abundant proteins across AIS and longitudinal groups were identified from the iTRAQ and label-free analyses, respectively. Alpha-2-macroglobulin (A2M), retinol binding protein 4 (RBP4), serum amyloid A1 (SAA1), Peroxiredoxin 2, Apolipoprotein A1 (ApoA1) and several immunoglobulins were identified as biologically relevant and differentially abundant, with potential as individual candidate prognostic biomarkers of neurological outcome. Bioinformatics analyses revealed that the majority of differentially abundant proteins were components of the complement cascade and most interacted directly with the liver.

Many of the proteins of interest identified using proteomics were detected only in a single group and therefore have potential as a binary (present or absent) biomarkers, RBP4 and PRX-2 in particular. Additional investigations into the chronology of these proteins, and their levels in other tissues (cerebrospinal fluid in particular) are needed to better understand the underlying pathophysiology, including any potentially modifiable targets. Pathway analysis highlighted the complement cascade as being significant across groups of differential functional recovery.

Keywords: Spinal cord injury, Biomarker, Proteomics, Complement cascade

Introduction

Spinal cord injury (SCI) is the transient or permanent loss of normal spinal sensory, motor or autonomic function, and is a major cause of disability. Globally, SCI affects around 500,000 people each year and is most commonly the result of road traffic accidents or falls.[1] Patients typically require extensive medical, rehabilitative and social care at high financial cost to healthcare providers. The lifetime cost of care in the UK is estimated to be £1.12 million (mean value) per SCI, with the total cost of SCI in the UK to the NHS being £1.43 billion in 2016.[2] Individuals with SCI show markedly higher rates of mental illness relative to the general population.[3] Complications arising post-SCI can be long-lasting and often include pain, spasticity and cardiovascular disease, where the systemic inflammatory response that follows SCI can frequently result in organ complications, particularly in the liver and kidneys.[4–6]

The recovery of neurological function post-SCI is highly variable, requiring any clinical trials to have an impractically large sample size to prove efficacy, hence the translation of novel efficacious therapies is challenging and expensive.[7] Being able to more accurately predict patient outcomes would aid clinical decisions and facilitate future clinical trials. Therefore, novel prognostic biomarkers that allow for stratification of injury severity and capacity for neurological recovery would be of high value to the field.

Biomarkers studies in SCI often investigate protein changes in cerebral spinal fluid (CSF) as the closer proximity of this medium is thought to be more reflective of the parenchymal injury.[8,9] Whilst this makes CSF potentially more informative for elucidating the pathology of SCI, the repeated use of CSF for routine analysis presents challenges in clinical care due

to the risk and expense associated with the invasiveness of the collection procedure. In contrast, systemic biomarkers measurable in the blood represent a source of information that can be accessed and interpreted both a lower cost and risk. Studies of traumatic brain injury have demonstrated that protein markers identified in CSF are also detectable in both plasma and serum.[10] More recently, circulating white blood cell populations have also been identified as potential SCI injury biomarkers, with a 2021 study showing that elevated levels of neutrophils were associated with no AIS grade conversion, while conversely an increase in lymphocytes during the first week post-SCI were associated with an AIS grade improvement.[11]

A number of individual proteins have been shown to be altered in the bloods post-SCI, including multiple interleukins (IL), tumour necrosis factor alpha (TNF-α) and C-reactive protein (CRP).[12–14]

Further, changes in inflammatory marker levels detected in acute SCI patients were found to be mirrored in donor-matched blood and CSF, albeit at lower absolute concentrations systemically.[15]

Previously, we have shown that routinely collected blood measures associated with liver function and inflammation added prognostic value to AIS motor and sensor outcomes at discharge and 12-months post-injury.[16,17] The current study uses an unbiased shotgun proteomic approach to investigate differentially expressed proteins in SCI patients, coupled with bioinformatics pathway and network analyses.

Methods and Materials

Patients

Blood samples were taken from SCI patients who had provided informed consent and in accordance to ethical provided by the National Research Ethics Service (NRES) Committee North West Liverpool East (11/NW/0876). “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 (Tables 1 & S1). Due to low recruitment numbers, AIS B patients were excluded from this study.

Plasma collection and storage

Plasma samples were collected within 2 weeks of injury (acute) and at approximately 3 months post-injury (subacute). Upon collection in EDTA (ethylenediaminetetraacetic acid) coated tubes samples were processed within 20 minutes, centrifuged at 600g for 15 minutes, to pellet erythrocytes and the resultant plasma fraction was aspirated and divided into aliquots for long-term storage in -80°C briefly and liquid nitrogen in the longer term.

Rationale for Proteomics Analytical Approaches

Two independent proteomic techniques were used to assess protein presence and abundance across the biological groupings. Profiling the proteome of plasma samples is technically difficult with high abundance proteins often limiting the capacity to detect protein differences of lower abundance proteins.[18] Therefore, an initial analysis was

performed to determine whether protein differences could be detected between AIS improvers and non-improvers using iTRAQ proteomics. This method relies on the chemical labelling of peptides from different biological groups, such that small amounts of sample can be pooled and run through the mass spectrometer as a single sample, significantly reducing the required instrument running time and hence cost.[18] iTRAQ analysis could be used to demonstrate that protein differences were detectable between the biological groups, using only minimal amounts of important clinical samples and via an inexpensive and quick method.

Subsequently, a further analysis was conducted in which protein abundance was normalised using ProteoMinerTM technology to help account for the high dynamic range of proteins within plasma and to support detection of low abundance proteins.[19] To allow better assessment of biological variability between these samples, label-free quantitation proteomics was applied with samples being analysed individually. This requires significantly longer amounts of mass-spectrometry instrument time, increasing the costs and volume of biological sample which is used (for the ProteoMinerTM normalisation).

Sample preparation and analysis using iTRAQ proteomics

Thawed plasma samples ($2\mu l$) each were diluted with distilled water ($98\mu l$). Total protein was quantified using a Pierce™ $660nm$ Protein Assay (Thermo Fisher Scientific, Hemel Hempstead, UK).[20]

A total of $100mg$ 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.

The pooled plasma samples were precipitated by incubation of the sample in six times the volume of chilled acetone for 1 hour at -20°C. The samples were then centrifuged at 6,000G for 10 minutes at 4°C, and re-suspended in 200μl of triethylammonium bicarbonate buffer. Sequencing Grade Modified Trypsin (10μg/85μg of protein; Promega, Madison, WI, USA) was then added to the samples for overnight digestion at 37°C. Peptides 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. Two individual iTRAQ experiments were set up, the first to assess acute and sub-acute improvers or non-improvers and the second to assess acute improvers and non-improvers to AIS grade A and D patients. 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.

iTRAQ mass spectrometry analysis

The samples were analysed at the BSRC St. Andrews University Mass Spectrometry and Proteomics Facility. 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.[21] Each fraction (10μl) was then analysed by nanoflow LC-ESI-MSMS, as described previously.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035025 and 10.6019/PXD035025.[22]

Sample preparation and analysis using label-free proteomics

Thus, protein abundance was quantified for each sample, whereupon mean protein abundance across experimental groups was calculated to assess protein changes.

To reduce the dynamic range of proteins, ProteoMiner™ beads (BioRad, Hemel Hempstead, UK) were used.[19] 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, and processed as described previously.[20,23]

Label free mass spectrometry analysis

Tryptic peptides 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.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035072 and 10.6019/PXD035072.[22]

iTRAQ OpenMS analysis

TripleTOF 5600 tandem mass spectrometer output files produced in the ABSciex proprietary `.wiff` file format were converted to an open file format, `.mzML` for analysis with OpenMS (version 2.6.0). The docker image of ProteoWizard version 3.0.20287 was used for conversion, and peak picking was applied on conversion[24]. OpenMS version 2.6.0 was used for further analysis.[25] 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).[26]

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 preparation 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`.

Label free OpenMS analysis

For quantification, the raw spectra files were analysed via OpenMS (version 2.6.0) command line tools, with the workflow from the prior section (Section 3.6) 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.[25,27] Unless otherwise stated, default arguments were used throughout.

The decoy database generated in the prior section (iTRAQ OpenMS analysis) was also re-used. The CometAdapter was used to run the search.[28] 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. 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.[29]

Network and pathway analysis

The Bioconductor package ReactomePA, which employs the open-source, open access, manually curated and peer-reviewed pathway database Reactome was used for network analysis.[30,31]

Enzyme-linked immunosorbent assays

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

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 as prognostic biomarkers. 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 performed using the statistical programming language R version 4.3.1 (2023-06-16). Pairwise t tests with bonferroni adjusted P-values with the R `rstatix` package were used to assess differential abundance.

Results

Plasma from American Spinal Injury Association (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 abundance in AIS grade A (n=10) and grade D (n=11) patients was also examined.

In the interest of brevity, only the plots of acute and subactue AIS C improvers VS non-improvers are included here, please see the supplemental data for the other comparisons (section Section 10).

Differential protein abundances

AIS C improvers had 18 more abundant proteins and 49 less abundant proteins at the acute phase relative to non-improvers. Similarly, at the subacute phase, AIS C improvers had 34 more abundant proteins and 34 less abundant proteins relative to non-improvers. The AIS A group had 56 more abundant and 9 less abundant proteins respectively relative to non-improvers. Acutely, AIS C improvers relative to AIS A and D had 21 and 53 more abundant and 46 and 12 less abundant proteins.

Please see the Tables S2, S3 & S4 for a full list of protein fold change changes.

Heatmaps

The majority of the pathways associated with the proteins identified by these iTRAQ experiments are related to the complement cascade and platelet activity (Figures 1, 2). There are also several pathways implicated in metabolic processes, particularly with apolipoproteins and retinoids.

Similarly to the iTRAQ data, many of the Reactome pathways are associated with the complement cascade and platelets activation (Figures 3, 4).

Network analysis of Differentially Abundant Proteins between AIS C improvers and non-improvers

(Figures 5, 6). Similar to the heatmaps, network plots highlighted that the majority of proteins changes were associated with the complement cascade and pathways linked to platelet activity (Figures 5, 6).

Similarly to the heatmaps and the iTRAQ data, network plots derived using the label-free proteomic data highlighted that the majority of differential proteins were associated with the complement cascade and pathways linked to platelets (Figures 7, 8).

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

Pathway analysis via the `pathview` R package returned the complement and coagulation cascade to be 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 compared with those who did not (Figure 9).

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

Validation of Proteomic Data using ELISA

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

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 11). For ApoA1 plasma abundance was significantly different 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 11). A statistically significant difference was also found between AIS C improvers and non-improvers at 2-weeks post-injury for RBP4 (Figure 11).

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 a modest overlap of 26 proteins found using both techniques.

Discussion

This is the first study, to our knowledge, to investigate the plasma proteome in SCI patients whose AIS scores either improved or did not improve post injury and also to compare these

to AIS grade A and D patients. We have used two proteomic techniques allowing us to profile both high and low abundance proteins, in order to identify protein candidate biomarkers which may have potential to predict neurological improvement within the acute setting. Moreover, this data can better inform us of the biology underlying neurological improvement or stability in a cohort of patients being conservatively managed post SCI.

This discovery phase, explorative study has several limitations, the main being limited sample number. Candidate biomarkers identified in this study would require validation in independent, larger cohorts prior to being able to determine their potential clinical utility. However, this work does provide confidence that there are detectible protein differences between AIS C improvers and non-improvers and that screening for plasma proteins may have potential for patient treatment/ care pathway stratification. Notably, the frequency of patients with polytrauma was high in this study, making it more difficult to determine which plasma protein changes are a direct consequence of the SCI itself and which are indirect effects of the polytrauma. Our analysis indicated that polytrauma was not different between our improver and non-improver cohorts. However, in future larger studies to assess the potential of individual candidate protein markers, multi-regression models would need to be performed, with polytrauma included as a covariate, to confirm the prognostic potential of the protein markers. Regardless, polytrauma is a clinical reality with many road traffic collision (RTC) and fall related SCI patients experiencing other injuries. Moreover, clinical management decisions are even more difficult in the presence of polytrauma and hence, biomarkers that can aid patient stratification in this context are required.[32]

This study has highlighted a number of proteins that may be able to discriminate in the acute phase following injury, between AIS grade C patients who either improve or do not improve by an AIS grade following SCI.

The most promising of these is Retinol Binding Protein 4 (RBP4) which was demonstrated to be increased in improvers compared to non-improvers in the acute phase post injury. Further this change could be confirmed using ELISA, which may provide a more clinically useful means of assessment on a wide scale. RBP4 is synthesised in the liver and binds retinol that is released following vitamin A deficiency.[33] Multiple animal studies have indicated that there may be a role of retinoid signaling in maintaining motor neuron integrity[34-37] and in contribution to the retained plasticity and regenerative potential of the mature spinal cord.[38] The increased levels of RBP4 in the AIS C improvers may relate to an improved capacity for neuronal regeneration/plasticity. However, further studies to determine whether this is due to increased expression or due to higher vitamin A dietary intake would be required.

Alongside RBP4, a number of other protein abundance differences across the different biological comparisons were identified in proteins associated with liver function. Our previous work investigating the potential of routinely measured haematological analytes for predicting neurological outcome in SCI patients also highlighted several proteins that were linked with liver function; thus providing further support to the theory that liver status is relevant to differential functional recover.[16,17] The pathway analysis specifically indicated that the acute phase response (APR) is implicated.

The APR is the bodies first response to infection or injury, including SCI. This systemic response is largely coordinated by factors released from the liver.[4,39-41] This hepatic response is typically transient and quickly fades, but prolonged liver inflammation and pathology has been observed in rodent SCI models.[42,43] 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.[43-47]

The acute (1-7 days) liver response to SCI is well documented; inflammatory cytokines released at the injury site, reach the liver through the bloodstream stimulating the liver to produce acute phase proteins, exacerbating the immune response. [41,48] It has been suggested that liver inflammation and activity of hepatic Kuppfer cells promotes recruitment of leukocytes to the injury site in brain or spinal trauma, potentially enhancing CNS injury.[49,50]

Pathway analysis of both proteomic experiments identified the complement and coagulation cascades as a significant pathway of interest. In the AIS C improvers compared to non-improvers key upstream regulator, C3, was less abundant, thus indicating reduced complement activation. C3 knockout mouse studies have reported improved neurological scores at acute and long-term time points; reduced residual consolidated neurological deficit at 21 days and a three-to-fourfold decrease in neutrophil infiltration, resulting in enhanced regeneration of axons.[51,52] These results imply that the complement cascade is a particularly important component of a differential response to neurological injury which ultimately leads to greater functional recovery. Given the complexity of the complement

cascade and the limited time points in our study, further work is needed to elucidate which facets of the cascade are outcome modifying, and at which stages post-injury.

The small number of statistically significant proteins that were differentially abundant between AIS improvers and non-improvers speaks to the variability of human plasma samples and is likely exacerbated by the window (up to two weeks) for 'acute' sample collection relative to injury in our SCI centre. Thus, a repeat of this experiment with a larger sample size and fixed time points, closer to the time of injury, will likely reveal many more proteins of potential interest. Regardless, this study has highlighted candidate potential prognostic biomarkers of functional recovery. We have also highlighted the complement cascade as being a particularly important pathway in differential recovery for further investigation. Furthermore, a metabolomic analysis of similar samples would greatly compliment this work, particularly with regards to investigating further links to the liver's role in neurological recovery.

Transparency, Rigor and Reproducibility Summary

A total of 73 distinct samples were identified as suitable for this study.[3] These belong to the following groups "Acute D" (n=11), "Subacute D" (n=10), "Actue C non-improvers" (n=7), "Acute C improvers" (n=10), "Subacute C non-improvers" (n=6), "Acute A" (n=11), "Subacute A" (n=9) and "Subacute C improvers" (n=9), all of which were included in the study.[3,4] Sample were analysed in 1 batch for the iTRAQ experiment and in another batch for the label-free.[6] ELISA experiments were run in a third batch, with each batch using separate aliquots taken from liquid nitrogen of the same samples.[6] Plasma samples were

collected within 2 weeks of injury (acute) or at approximately 3 months post-injury (subacute) and were processed to freezing at -80°C within 30 minutes of collection before ultimate transfer to liquid nitrogen storage.[7] Further validation and investigation of the key proteins identified in the study is ongoing.[11] Data are available via ProteomeXchange with identifiers PXD035025 and PXD035072 for the iTRAQ and label-free experiments respectively.[12] All biofluid samples used to conduct the study were obtained by the investigators and 100% of the participants have provided permission for samples to be used for future research without requirement for specific additional informed consent, though limited quantities remain.[14] This paper will be published under a Creative Commons Open Access license, and upon publication will be freely available.[15]

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Author contributions

Gabriel Mateus Bernardo Harrington: writing - original draft (lead); investigation (lead); formal analysis (lead); conceptualisation (equal); software (lead); visualisation (lead). **Paul Cool**: writing - review & editing (equal); formal analysis (supporting); software (supporting); methodology (equal); conceptualisation (supporting); supervision (equal). **Charlotte Hulme**: writing - review & editing (equal); methodology (supporting);

conceptualisation (supporting); supervision (supporting). **Jessica Fisher-Stokes**: writing - review & editing (supporting); formal analysis (supporting); validation (supporting). **Mandy Peffers**: writing - review & editing (supporting); methodology (supporting); conceptualisation (supporting); investigation (supporting). **Wagih El Masri**: writing - review & editing (supporting). **Aheed Osman**: writing - review & editing (supporting); resources (supporting). **Joy Roy Chowdhury**: writing - review & editing (supporting); resources (supporting). **Naveen Kumar**: writing - review & editing (supporting); resources (supporting). **Srinivasa Budithi**: writing - review & editing (supporting); resources (supporting). **Karina Wright**: writing - review & editing (equal); methodology (equal); project administration (lead); conceptualisation (equal); funding acquisition (lead); supervision (equal).

Authors disclosure

The authors have no competing interest to disclose

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