VALIDATION AND IDENTIFICATION OF SERUM BIOMARKERS TO PREDICT NEUROLOGICAL RECOVERY AFTER ACUTE SPINAL CORD INJURY

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

Validation and Identification of Serum Biomarkers to Predict Neurological Recovery after Acute Spinal Cord Injury

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Abstract

Neurological recovery after acute spinal cord injury is highly variable and therefore difficult to predict. Besides initial injury characteristics, there remains a need for potential objective serum biomarkers that can predict neurological recovery. The main objective of this thesis was to identify such biomarkers.

In chapter 2, I aimed to validate serum albumin as a valid biomarker for long-term neurological recovery after acute spinal cord injury. I performed unbiased recursive partitioning (URP) to examine the relationship between neurological outcomes and serum albumin concentration from the Spinal Cord Injury Rehabilitation study. Results showed that serum albumin could be used as a crude prognostic biomarker, particularly in cases where examination for injury characteristics is not complete.

In chapter 3, I aimed to identify novel serum biomarkers that can predict long-term neurological recovery after acute spinal cord injury. I performed URP and Factor Analysis to investigate the relationship between neurological recovery and all baseline (i.e., up to 72 hours after injury) serum biomarkers from the Sygen clinical trial. I found that blood factor (including red blood cells, hematocrit, and hemoglobin) is significantly associated with neurological outcomes. However, similarly to results in chapter II, these blood factor markers can only serve as crude prognostic biomarkers, in cases where individuals have incomplete neurological examination.

Taken together, these data demonstrate that serum biomarkers, including albumin, red blood cells, hematocrit, and hemoglobin, can predict neurological recovery after acute spinal cord injury. While further research is needed, these biomarkers can be useful for individuals who have incomplete injury characteristics examinations.
Lay Summary

Acute spinal cord injury is a life-debilitating trauma altering motor, sensory, and autonomic functions. It is then reasonable for individuals with acute spinal cord injury to question if they are going to recover. Hence, for clinicians and scientists alike, predicting their recovery is always prioritized. However, predicting neurological recovery after acute spinal cord injury is difficult. Objective predictors, such as biomarkers, are yet to be identified. This thesis then examines different biomarkers that can predict neurological recovery. We have identified serum albumin and blood markers, such as red blood cells, hematocrit, and hemoglobin, are potential objective biomarkers. Even though these biomarkers are just crude estimate for neurological recovery post-injury, they hold promising potentials from clinical perspectives.
Preface

I was the lead investigator of my projects. I was responsible for data entry, data cleaning, data analyses, interpretation of data, and drafting the manuscripts. Dr. Jan Schwabb and Dr. Lukas Grassner were responsible for data interpretation and revising the manuscripts for intellectual contents. Dr. Fred Geisler and Dr. Gale Whitneck were responsible for the primary data collection, data interpretation, and revising the manuscripts for intellectual contents. Dr. Catherine Jutzeler was responsible for study funding, interpretation of data, and revising the manuscript. Dr. John Kramer was the supervisory author on the projects and was involved in concept formation and thesis revision. The data analyzed in this these have also been presented for conference poster. Both analyses in chapter 2 and 3 are currently being prepared for publication.
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List of Abbreviations

ADDEP ............ Archive of Data on Disability to Enable Policy and research
AIS ................. ASIA Impairment Scale
ALS ................ Amyotrophic lateral sclerosis
ASIA ............... American Spinal Injury Association
CFA ................ Confirmatory factor analysis
CSF ................ Cerebrospinal Fluid
EFA ................ Exploratory factor analysis
FDA ................ United States Food and Drug Administration
FIM ................ Functional Independence Measure
GFAP .............. Glial fibrillary acidic protein
GM-1 ............... Monosialotetrahexosylganglioside ganglioside
IL-1β .............. Interleukin-1β
IL-6 ............... Interleukin-6
ISNCSCI .......... International Standards for Neurological Classification of Spinal Cord Injury
LEMS ............. Lower extremity motor scores
MCP-1 ............. Monocyte Chemoattractant Protein-1
NASCIS .......... National Acute Spinal Cord Injury Study
SCIRehab .......... Spinal Cord Injury Rehabilitation
TBI .................. Traumatic brain injury
TNF-α .............. Tumor necrosis factor-α
URP-CTREE .... Unbiased recursive partitioning conditional inference tree
URP ............... Unbiased recursive partitioning
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Thank you for the Wings for Life Research Foundation for the grant to make this project possible. I hope the work described here would contribute to the quest for the “cure” of acute spinal cord injury.
Dedication

Luận án này dành cho ba Nghĩa, má Thom, và bé Gấu. Không ai ngờ con sẽ có thể làm được từ mọi người. Cảm ơn đã luôn tin tưởng ở Đệ.
Chapter 1. General Introduction

1.1 Epidemiology/Etiology

Acute spinal cord injury involves primary and secondary mechanisms. The primary mechanism refers to the initial physical injury (e.g., vehicle accident), whereas the secondary mechanism consists of biochemical and cellular processes initiated by the primary mechanism\(^1\). Acute spinal cord injury occurs worldwide up to 57 cases per million annually, with motor vehicle accidents, falls, violence, and sports accounting for more than 50\% of the etiologies\(^1,2\). Recreational and sports-related injuries occur more often in younger populations (e.g., 20 to 50 years of age), whereas falls are more common in the older cohorts\(^1\). Although acute spinal cord injury has relatively low incidence compared to other traumas (e.g., stroke)\(^1,3\), it results in lifelong health-related consequences that demand intense care from health care professionals.

The majority of individuals with acute spinal cord injury experienced secondary complications necessitating hospitalization (e.g., urinary tract infection, pain, pressure sores, and depression)\(^4,5\). In addition to the psychological and physical trauma of acute spinal cord injury, the monetary costs to the individuals and society are substantial. In Canada, direct costs (i.e., expenditure by the health care system, and/or by the caregivers) were estimated to be up to 1.57 billion Canadian dollars; whereas indirect costs, which refers to the lost potential output due to reduction/elimination of work, are expected to be roughly 1.1 billion Canadian dollars\(^6\). Major direct costs contributors include hospitalizations, health care practitioners visits, equipment, home modification, and attendant care\(^6\). Thus, the physical, psychological, and economical aspects of acute spinal cord injury are severely devastating.
1.2 Translational Barriers in Acute Spinal Cord Injury

More than five decades of research, advances in medical, surgical, and rehabilitative care have been made for individuals with acute spinal cord injury\(^7,8\). However, despite years of global research efforts, clinically validated novel therapeutic interventions still remain elusive. That is to say that there are currently no pharmacological therapies that improve neurological function, beyond the spontaneous recovery\(^9,10\). Most preclinical research on new treatments is conducted on rodent models of acute spinal cord injury. This builds a foundation of acute spinal cord injury pathophysiology knowledge, assuming biological aspects between animal and human models are comparable\(^11\). However, plethora of positive therapeutic interventions in animal models failed to demonstrate the same clinical efficacy in human trials\(^12–16\). This suggests that important biological differences might exist between human and animal models. Indeed, the translational gap from animal models to human trials was apparent in previous clinical trials.

One example was the Sygen (GM-1 monosialotetrahexosylganglioside ganglioside) clinical trial, the multicenter for a novel acute spinal cord injury treatment\(^17\). Previously, GM-1 has been reported to have acute neuroprotective and long-term regenerative effects in multiple animal experimental models\(^12,18–20\). However, the Sygen trial, which randomized 797 individuals with acute spinal cord injury into placebo, or GM-1 treated groups, failed to demonstrate any significant differences between these two groups. The unsuccessful efficacy of GM-1 treatment in human clinical trials indicates a translational barrier between human and animal biology.

Another example involved three large scale double-blinded multicenter trials reported as the National Acute Spinal Cord Injury Study (NASCIS) I, II, and III, which investigated the effects of methylprednisolone. In animal experimental models, methylprednisolone had beneficial effects on functional recovery\(^15,16\). The subsequent NASCIS trials supported this notion\(^21–23\).
Specifically, they reported that individuals received early treatments of methylprednisolone (within 8 hours post-injury) showed 5-point improvement in motor scores. However, their analysis remained to be controversial due to differences in baseline characteristics between experimental and control groups. Indeed, subsequent analyses found no significant differences in neurological recovery between placebo and methylprednisolone-administered groups. Despite methylprednisolone beneficial effects on animal models, research has identified its’ risks in humans including immunosuppression, gastrointestinal ulcers, and pulmonary insufficiency. Even though the NASCIS studies have embedded the administration of methylprednisolone into the standard of clinical practice for acute spinal cord injury, the controversy and criticism surrounding its effects led to its discontinuation in some centers. This again reinforces the translational difficulty between animal and human conditions.

In addition to innate biological differences between human and animal models, the methods used in animal models require further refinement before moving forward into humans trials. Specifically, clinically relevant experimental models induce acute spinal cord injury from squeezed/compressed, sharply cut (transection), and/or contusion methods. However, in humans, it is reasonable to assume that the acute spinal cord injury resulting from a young male driver vehicle accident would be much different from that of an elderly woman falling down the stairs. Hence, narrowly controlled experimental paradigms in animal models are less likely to reflect this variable reality in human acute spinal cord injury. In regards to acute neuroprotective therapies, the therapeutic windows between animal and human conditions remain the one of most puzzling issues for clinicians and scientists. Predicting humans therapeutic window based on a therapy shown to be neuroprotective on rats 1h post-injury would be highly spectaculative. The difficulty to clinically validate these therapeutic treatments is a frustrating barrier for acute spinal
cord injury community. Considering the lack of clinically relevant treatments during acute spinal cord injury phase, neurological examination tools that can help predict the neurological recovery in individuals with acute spinal cord injury are highly valued.

1.3 Neurological Examinations

The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) examination is an excellent method of neurological assessment. To date, the American Spinal Injury Association (ASIA) Impairment Scale (AIS) is the best standard for stratifying injury severity and predicting outcomes\textsuperscript{28}. Briefly, AIS grades are the clinical examination conducted to classify the severity of acute spinal cord injury. The importance of injury severity in the prediction of outcomes has been extensively investigated\textsuperscript{29,30}. Individuals with severe injury recovered to a lesser degree compared to individuals with mild/moderate injury. The AIS grades requires individuals with acute spinal cord injury to demonstrate their residual arms and legs strength (i.e., key muscles from C5 to T1 and from L2 to S1) and their sensation (i.e., using pin prick and light touch test) throughout their body, including peri-anal region. The degrees of injury severity include AIS A (complete injury - no sensory or motor function in fourth and fifth sacral), AIS B (sensory incomplete - sensory but not motor function is preserved), AIS C (motor incomplete - motor function is preserved with muscle grade less than 3), and AIS D (motor incomplete - motor function is preserved with muscle grade of 3 or greater)\textsuperscript{31}. The motor and sensory components of AIS grades are also tested for their reliability\textsuperscript{32,33}. Both motor and sensory examinations are reported to be reliable when conducted by trained examiners. The intraclass correlation coefficients for both sensory and motor components of AIS grades are within 0.98 to 0.99 range\textsuperscript{32}. However, AIS grades have some major limitations that could delay the progress of clinical trials.
First, due to the self-report nature of ISNCSCI examinations (i.e., individuals demonstrate their strength in key muscles groups in arms and legs, and report their sensation to light touch and pin prick tests), they are difficult to conduct in acute spinal cord injury settings if individuals are intoxicated, sedated, and/or too severely injured to make proper assessment\textsuperscript{34}. Second, even with same baseline AIS grades, the extent of which individuals recover is highly variable. For instance, individuals with AIS B have 15% to 40% rate of conversion to AIS C, and almost 40% rate of conversion to AIS D\textsuperscript{35}. The assessment of AIS grade conversion depends on the experience of examiners, patients self-reports, measurement procedures, and other comorbidities such as brain injury\textsuperscript{10}. This imprecision to predict neurological recovery from AIS grades in turn demands large number of individuals for adequate statistical power. It has been estimated that over 300 acute spinal cord injury individuals would be needed to detect a 5 point difference in motor scores\textsuperscript{35}. Taken altogether, the lack of objective baseline assessment represents an enormous problem for clinical validation of novel therapies. Clearly, a new objective assessment is urgently needed.

\subsection*{1.4 Biomarkers}

Biomarkers, by definition, are objective and quantifiable characteristics that can reflect individuals’ biological processes to represent their health and wellbeing\textsuperscript{36}. Based on their function, biomarkers can be classified into three categories: diagnostic, predictive, and prognostic (\textbf{Figure 1.1}). Diagnostic biomarkers are used to detect diseases and classify individuals into clinically relevant groups (i.e., diseased versus not diseased)\textsuperscript{37}. Predictive biomarkers aim to forecast a given treatment response\textsuperscript{37,38}. Prognostic biomarkers provide insights into the natural progression of a disease\textsuperscript{37}. As diagnostic biomarkers are used to detect disease onset, they are not relevant to diseases caused by physical trauma such as acute spinal
cord injury (e.g., vehicle accident). Due to the lack of approved treatments for neurological recovery after acute spinal cord injury, predictive biomarkers are also not relevant. For these reasons, this thesis focused on prognostic biomarkers.

1.4.1 Cerebrospinal Fluid Biomarkers

Acute spinal cord injury triggers axonal degeneration, and releases proteins and metabolites into the cerebrospinal fluid (CSF)\(^1\). Due to its proximity to the spinal cord and involvement in the central nervous system, utilizing CSF as biomarker is advantageous. CSF biomarkers can be measured in two ways: they can directly identify structural damage using specific markers of tissue damage, and they can measure aspects of cellular inflammatory cascades involved in the secondary phase. Several inflammatory (i.e., interleukins) and structural CSF proteins (i.e., tau) have been identified to predict neurological outcomes after acute spinal cord injury\(^39-42\).

1.4.1.1 Inflammatory CSF biomarkers

Inflammation plays a critical role in secondary injury mechanisms after acute spinal cord injury. As a result, mediating inflammation is a popular therapeutic target after acute spinal cord injury, as shown in several studies of pharmacologic agents\(^43\), growth factors\(^44\), and cell transplants\(^45\). Animal studies, in which the severity of injury can be controlled, have confirmed a few inflammatory CSF biomarkers. Yang and colleagues performed rodent model of thoracic injuries induced by weight drop from 3cm (mild injury) or 12cm (severe injury). They reported significant increase in concentrations of pro-inflammatory cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)), interleukin-6 (IL-6), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) up to 6 hours post-injury in the severe-injury cohort\(^39\). Chodobski and colleagues found that levels of TNF-\(\alpha\) level were elevated in the acute spinal cord injury cohort compared to sham group as early as 1 hour post-injury\(^42\). In
line with these findings, Lin and colleagues recently found that adenoviral aimed to decrease IL-1β concentration is associated with better recovery of locomotor function, less neuronal loss, and downregulation of TNF-α level\(^{46}\).

Studies of inflammatory cytokines on human acute spinal cord injury also support the concept of injury severity influencing the inflammation process. Kwon and colleagues conducted a study in which CSF samples were collected over a period of 72 hours post-injury in 27 patients with severities ranging from AIS A to AIS C\(^{47}\). A severity-dependent differences across multiple inflammatory cytokines concentrations were reported. Of particular note, IL-6, IL-8, and MCP-1 (Monocyte Chemoattractant Protein-1) concentrations were significantly higher in AIS A patients than in AIS B and C patients as early as 24 hours post-injury. More recently, Kwon and colleagues conducted a similar study in 50 acute spinal cord injury individuals\(^{48}\). In addition to investigating if CSF biomarkers reflected the injury severity at baseline, AIS grade conversion (i.e., change in injury severity from one time-point to another) was examined. Comparable to previous studies, the level of IL-6 was significantly elevated in AIS A patients 24 hours post-injury. Moreover, the concentration of inflammatory CSF biomarkers (i.e., IL-6, IL-8, and MCP-1) were significantly lower for those who had improved over the first 6 months compared to those who did not. Taken altogether, these studies support the notion that the expression of specific inflammatory cytokines can reflect the severity and recovery potential of acute spinal cord injury. Thus, these may inform future therapeutic treatments to reduce secondary damage and to improve outcomes after acute spinal cord injury.

1.4.1.2 Structural CSF biomarkers

Structural CSF biomarkers reflecting injury in neural tissue can be useful for clinical trials. Previously, these structural CSF biomarkers have been investigated extensively in
traumatic brain injury (TBI). Higher tau concentration (a microtubule associated protein) 2 days post-injury has been found to be correlated with poorer outcomes in TBI\(^40\). Other structural biomarkers, including S100\(\beta\) (calcium binding proteins in astroglial and Schwann cells) and glial fibrillary acidic protein (GFAP, released from injured glial cells and axons), have also been investigated extensively. Hayakata and colleagues reported that concentrations of S100\(\beta\) peaked 6 hours after severe TBI, and that there was a positive correlation between S100\(\beta\) concentration and intracranial pressure\(^41\). Similar to S100\(\beta\), severe TBI also elevates the concentration of GFAP within 24 hours of injury\(^49\). Thus, these structural CSF biomarkers have the potential to assess the severity and outcomes of traumatic brain injury.

Unsurprisingly, structural CSF biomarkers can also be used to evaluate the severity of acute spinal cord injury. In one study, high CSF concentration of GFAP 1 day post-injury was correlated with the severity of paralysis\(^50\). Several other studies have also support this finding. Kwon and colleagues reported the severity-dependent of these structural CSF biomarkers at 24 hours post-injury\(^47\). Specifically, Tau, S100\(\beta\), and GFAP all have significantly higher concentration in AIS A patients than those in AIS B and C patients. In a subsequent study, the same results were replicated, and CSF biomarkers were able to predict the AIS improvement at 6 months post-injury\(^48\). These findings support that these structural CSF biomarkers can measure for injury severity and predict outcome.

1.4.1.3 CSF biomarkers limitations

Despite some potential therapeutic benefits of CSF biomarkers, the act of acquiring them (i.e., lumbar puncture) can sometimes be a major obstacle. This is because lumbar puncture has been associated with several side effects, including reduced CSF pressure, hearing loss, postural puncture headache, cranial nerves and vestibulocochlear dysfunction\(^51\). Risks factors for these
complications (i.e., lesser body mass index, age, sex, etc.) need to be taken into account prior to lumbar puncture\textsuperscript{52}. Unsurprisingly, lumbar puncture can induce anxiety and fear into patients\textsuperscript{52}. In addition, acute spinal cord injury clinical trial requires multiple centers collaboration; hence, limited numbers of labs that can perform lumbar puncture make this requirement unfeasible.

1.4.2 Hematological Biomarkers

Researchers have also looked into major structural proteins in bloodstream as potential biomarkers. Indeed, serum neurofilament light chain was reported to reflect acute spinal cord injury severity and outcome\textsuperscript{53}. Lower serum neurofilament light chain levels were associated with better motor outcome, and serum neurofilament light chain levels were higher in motor-complete individuals versus motor-incomplete individuals\textsuperscript{53}. Other structural proteins, such as S100$\beta$, were also analyzed in serum. Ma and colleagues measured serum S100$\beta$ between acute spinal cord injury rats and control\textsuperscript{54}. Within 72 hours post-injury, serum S100$\beta$ concentration in acute spinal cord injury rats had significantly increased than that of the control animals. This suggests that serum S100$\beta$ might be used as an early biomarker to detect neural damage in acute spinal cord injury. In humans, serum S100$\beta$ was also a good candidate biomarker after acute spinal cord injury\textsuperscript{47}.

However, analyzing structural proteins in serum has unique challenges. A comparison between CSF and serum proteins concentrations indicated that CSF concentrations were more potent than serum counterparts\textsuperscript{47,55}. This suggests CSF biomarkers hold more potentials than that of serum counterparts. Furthermore, due to short half-life, some of these serum proteins require to be assessed in very acute settings (<24h), which might not be possible (e.g., polytrauma)\textsuperscript{56}. Most of these serum protein biomarkers are also not widely available in standard clinical chemistry lab. They are regarded as “specialty analysis”, and only available in designated
laboratories. Taken together, these limit the accessibility to clinical uses and collaboration of clinical trials.

Given the limitations of these inflammatory and structural proteins in CSF and serum, collection and analysis of peripheral blood (i.e., blood draw procedure) represents a less risky, more cost-effective, and clinically preferable approach. In fact, several other trauma studies have employed peripheral blood biomarkers (e.g., albumin). In a population-based study assessing the relationship between serum biomarkers and amyotrophic lateral sclerosis (ALS) outcomes, serum albumin and creatinine were significantly associated with ALS outcomes in both sexes. Specifically, serum albumin and creatinine concentrations were lower in individuals with ALS compared to control individuals. In another study of 750 individuals for acute ischemic stroke, higher serum albumin levels were associated with decreased risk of poor outcomes. They reported that high-dose (up to 2.5g/kg) of serum albumin administered up to 4 hours after stroke significantly improved neurological outcomes. In TBI, serum albumin was also found to be a significant prognostic biomarker for outcome. After adjusting for sex, age, and other clinical factors, serum albumin still remained a significant predictor for TBI outcome. In a randomized clinical trial, Bernard and colleagues reported that each serum albumin increase by 1g/L may have potential benefits on neurologic outcomes after TBI. Overall, these findings build a strong foundation that peripheral blood biomarkers are valid in prognosis in various neurological diseases.

Surprisingly, analysis of peripheral blood biomarkers for acute spinal cord injury is still in its infancy. Up to date, only one study examined the utility of serum biomarker, namely serum albumin, in predicting long-term outcome after acute spinal cord injury. The lack of diversity of prognostic serum biomarkers in acute spinal cord injury fuels this thesis. Here, my thesis has
two aims. First, I aimed to validate serum albumin. Second, I aimed to identify new potential biomarkers.

1.5 Aims and Hypotheses

1.5.1 Aim 1

To validate if serum albumin is associated with neurological outcomes after acute spinal cord injury in a contemporary cohort.

*Based on previous preliminary observations, I hypothesized that low serum albumin concentration is correlated with poor neurological recovery (i.e., changes in motor scores and marked recovery) after acute spinal cord injury.*

1.5.2 Aim 2

To determine the combination of hematological and serum biomarkers (measured at the earliest timepoint) that best predict neurological recovery after acute spinal cord injury.

*In chapter 3, I hypothesize that a multitude of serum biomarkers at baseline admission is positively correlated with neurological recovery (i.e., lower extremity motor scores) after acute spinal cord injury.*

Thus, my thesis conceptual overview is encapsulated in **Figure 1.2**.
Figure 1.1: Illustration of clinical application of biomarkers.

Figure 1.2: Thesis Conceptual Overview
Chapter 2. Validation Study

2.1 Introduction

Albumin is the most abundant protein in blood plasma, primarily served as blood transporter and involved in maintenance of normal plasma oncotic pressure\textsuperscript{62}. Hypoalbuminemia (i.e., concentration of blood albumin that is abnormally low) is attributed to a number of factors, including reduced synthesis associated with liver dysfunction, increased catabolism in the wake of infection, and altered distribution by an increase in transcapillary escape rates\textsuperscript{63}. Decades of medical observation have demonstrated a relationship with hypoalbuminemia and increased risk of morbidity and mortality\textsuperscript{64–67}. More recently, studies have identified hypoalbuminemia as a risk factor associated with faster disease progression and worse outcomes in ALS, Guillain-Barré syndrome, and stroke\textsuperscript{58,59,68}.

Given the extent of trauma associated with acute spinal cord injury, need for surgical interventions, and high incidence of infections, hypoalbuminemia is unsurprisingly common in the acute phases of injury\textsuperscript{64,66}. In addition to increasing the risk of mortality\textsuperscript{63,65}, lower concentrations of serum albumin early after injury (≤1 month) are also associated with more severe acute spinal cord injury and poor neurological recovery\textsuperscript{61}. This was demonstrated in a secondary analysis that utilized data from a completed phase III clinical trial testing the efficacy GM-1 gangliosides in acute spinal cord injury\textsuperscript{69}. In principle, the association between serum albumin and neurological outcomes supports a potential application as an objective biomarker of injury severity.

The overall goal of the current analysis was to validate the relationship between serum albumin and neurological outcomes after acute, traumatic spinal cord injury. A specific aim was to utilize contemporary data, reflecting modern acute spinal cord injury management practices.
Based on our previous findings, we hypothesized that serum albumin would be related to neurological recovery after acute spinal cord injury. To do so, we performed a secondary analysis of open source data from the Spinal Cord Injury Rehabilitation (SCIRehab) study (2007-2010).

2.2 Methods

2.2.1 Study Design and Data Source

The primary data used for this study was accessed from the SCIRehab study through Archive of Data on Disability to Enable Policy and research (ADDEP)\(^7^0\). Design, recruitment, inclusion criteria, and enrollment details have been previously described in detail\(^7^0\). Briefly, the SCIRehab study enrolled individuals aged ≥12 years with acute spinal cord injury rehabilitating at six participating centers in the United States from 2007 to 2009. Participant demographics and injury characteristics were extracted from the participant medical record (part of the National Institute on Disability and Rehabilitation Research Spinal Cord Injury Model Systems Form I).

2.2.2 Inclusion Criteria

For the purpose of our analyses, we included individuals with cervical and thoracic injuries only to remain consistent with the previous study\(^6^1\). Additional inclusion criteria were applied for prediction of 1-year post-injury outcomes, such that individuals who were non-ambulatory at admission to rehabilitation were included, and completed assessment of albumin and neurological outcomes (i.e., lower extremity motor scores and AIS grades).

2.2.3 Outcomes (Dependent Variable)

As a measure of residual function caudal to both thoracic and cervical lesions, lower extremity motor scores (LEMS) and AIS grades were examined as dependent variables at admission to rehabilitation. In brief, key muscles in LEMS were examined according to the
ISNSCI\textsuperscript{28}, with a maximum scores of 25 points for each left and right sides (for a maximum total scores of 50). AIS grades are an aggregate of sensory and motor function, ranging from AIS A (most severe) to D (least severe). To further differentiate AIS D injuries, we employed Functional Independence Measure (FIM)\textsuperscript{71} to categorize individuals based on their ambulatory status. Information from the ISNSCI (i.e., LEMS and AIS grades) and FIM were collected as part of the National Institute on Disability and Rehabilitation Spinal Cord Injury Model Systems Form I.

To examine the relationship between serum albumin concentration and neurological recovery, we focused on change of LEMS and marked recovery at 1-year post-injury. Change of LEMS was calculated as LEMS 1-year post-injury minuses LEMS at admission. Marked recovery occurred for an individual converting 2 AIS grades (i.e., AIS A to C, or AIS B to D) or from AIS C/D to walking. Similar to what was done at admission, walking was determined based on 1-year post-injury examination of FIM.

\subsection*{2.2.4 Predictor (Independent) Variables and Covariates}

The primary independent variable was lowest albumin concentration (CRLOWALBUMIN). In the Spinal Cord Injury Rehabilitation study, albumin concentrations were abstracted from participant medical records. As an aggregate for each participant, mean, highest, and lowest albumin concentrations were determined through rehabilitation (i.e., raw values are not available). Lowest recorded serum albumin concentrations were a priori selected for our analysis. This was done based on our previous study, where the lowest concentration, on average, yielded the highest predictive function of future neurological function\textsuperscript{61}. The average number of elapsed days from injury date to rehabilitation admission was also reported. Potential confounders included LEMS at admission to rehabilitation.
2.2.5 Statistical Analyses

Demographics, injury characteristics, and albumin concentrations were compared between included and excluded cohorts to assess selection bias. Specifically, chi-squares tests were used for categorical variables (i.e., demographics and injury characteristics), while t-tests were used for continuous variables (i.e., albumin concentrations).

2.2.5.1 Unbiased Recursive Partitioning: Conditional Inference Tree

Our primary statistical analysis applied the unbiased recursive partitioning (URP) conditional inference tree (URP-CTREE). In brief, URP is a tree-based regression model using sequential tests of independence between predictors (e.g., albumin concentration, and LEMS admission) and outcomes (e.g., change of LEMS and marked recovery).

Fundamentally, URP follows two steps, which are repeated after each split of the original heterogeneous population. First, the algorithm assesses whether any of the predictors is statistically associated with the outcome by multiple-testing corrected p value (i.e., Bonferroni correction). If the initial null hypothesis between predictors and outcome cannot be rejected (no statistically significant association between predictors and outcome), the algorithm stops producing any split. If the null hypothesis can be rejected (at least one predictor is significantly associated with the outcome), the algorithm splits by selecting the predictor with smallest P value. Second, after the tree has split, the algorithm then calculates another possible split based on the new subset created from previous significant predictor. Each split is evaluated by a two-sample linear statistic (e.g., Fisher’s exact test for nominal responses). If none of the variables included in a model is significantly associated with outcome, no splits are created for the data.
Most importantly, URP provides cut-off values that are valuable application for clinical settings. Thus, URP has been previously applied in a number of acute spinal cord injury studies\textsuperscript{73–75}.

For the association analyses, bivariable URP associations between lowest albumin concentrations and AIS grades, and between lowest albumin concentrations and LEMS at admission were performed. For the prediction analyses, first bivariable URPs were done for lowest albumin concentration and each outcome (i.e., change of LEMS and marked recovery). Multivariable URPs for each outcome were also created adjusting LEMS admission (for change of LEMS outcome) or AIS grades admission (for marked recovery outcome) while holding lowest albumin concentration constant.

2.2.6 Data availability statement

The data utilized in the current study was derived from the ADDEP database, which is a publicly accessible database https://www.icpsr.umich.edu/icpsrweb/ADDEP/studies/36567. Furthermore, we make our methods and the scripts that are required to reproduce the analyses and figures publicly available at https://github.com/AnhKhoaVo/ADDEP. The exact variables (searchable in the ADDEP database) were: REVIEWASIAGRADEADM (i.e., AIS grade at admission), BASAImp (i.e., AIS grade at 1-year), AFLMODRB (i.e., FIM-walking at admission to rehabilitation), and BFIMLMMod (i.e., FIM-walking 1-year post-injury). Our calculated variables included: Marked_Recovery_Annual_2 (i.e., based on REVIEWASIAGRADEADM and BFIMLMMod), LOWER_MS_REHAB (i.e., based on sum of left and right motor scores from L2 to S1 at admission to rehabilitation), LOWER_MS_ANNUAL (i.e., based on sum of left and right motor scores from L2 to S1 at 1 year post-injury), and Change_Scores (i.e., LOWER_MS_ANNUAL – LOWER_MS_REHAB).
2.3 Results

2.3.1. Cohort Summary

We included 430 and 439 individuals in the association analyses of LEMS at admission and AIS grades at admission, respectively (Table 2.1). A total of 117 individuals and 167 individuals were analyzed in the prediction analyses of change of LEMS and marked recovery at 1-year post-injury, respectively (Table 2.2). The average elapsed days from injury date to rehabilitation admission date is 30 days (± 27). Most included and excluded individuals shared similar demographics and injury characteristics.

2.3.2. Association Analysis

The relationship between LEMS and AIS grade and serum albumin concentrations at admission to rehabilitation are shown in Figure 2.1A and 2.1C. At admission, URP of LEMS identified two participant cohorts based on a cut-off serum albumin concentration of 2.6 g/dL (p<0.001) (Figure 2.1B). For URP of AIS grades at admission to rehabilitation, two participant cohorts were also identified, also based on an albumin cut-off concentration of 2.6 g/dL (p<.001) (Figure 2.1D). Both analyses, of LEMS and AIS grade at admission to rehabilitation, support that lower serum albumin concentrations are significantly associated with more severe injuries.

2.3.3. Prediction Analysis

The relationship between serum albumin concentrations and neurological recovery are shown in Figure 2.2. For change in LEMS between rehabilitation admission and 1 year post-injury, URP yielded two participant cohorts based on a serum albumin cut-off of 2.8 g/dL (p<0.001, Figure 2.2A). Adjusting for LEMS at admission, URP again yielded two participant cohorts, based only selected a cut-off value for LEMS (p<0.001, Figure 2.2B). The absence of serum
albumin in the adjusted model suggests that concentrations did not provide any additional prognostic information beyond baseline LEMS.

At a concentration of 3.1 g/dL, serum albumin yielded two terminal nodes for marked recovery (p=0.001, Figure 2.2C). Similar to the URP for change in LEMS, in a model adjusted for AIS grades admission and albumin concentration, three cohorts of individuals were identified (nodes 2, 4, and 5) based on AIS grades admission (p<0.001, Figure 2.2D). The absence of serum albumin in the “tree” suggests that albumin concentration did not perform better than AIS grades at admission.

2.4 Discussion

The goal of this study was to validate the relationship between serum albumin concentrations and neurological outcomes after acute spinal cord injury. In support of our hypothesis, injury severity at admission to rehabilitation was related to serum albumin concentrations. Without adjustment for baseline injury characteristics, serum albumin concentrations were also associated with neurological recovery. However, the relationship with long-term neurological outcome did not persist after baseline adjustment for injury severity. These observations, based on a contemporary collection of data, partially validate our previous findings that serum albumin is associated with the severity of acute spinal cord injury.

In a previous study, serum albumin concentrations measured between 24 hours and one month were associated with both baseline injury severity and long-term neurological outcome. These seminal observations were made using data from a completed Sygen clinical trial, where serum albumin was collected to assess the therapeutic safety of GM-1 gangliosides in acute spinal cord injury. An obvious limitation is that the Sygen trial ran from 1992 to 1997, and as a result, was subject to outdated acute management practices (i.e., administration of
methylprednisolone). As a result, methylprednisolone could affect blood chemistry values\(^\text{77}\). To address this concern, we performed an analysis on a contemporary data source, where serum albumin concentrations were abstracted from medical records as part of a large observational study\(^\text{70}\).

Consistent with previous studies\(^\text{61,66}\), lower serum albumin at admission to rehabilitation was associated with more severe acute spinal cord injury. This was evidenced for both AIS grade and LEMS, based on a cut-off serum albumin concentration identified by URP. By nearly all standards, albumin concentration of 2.6 g/dL up to 45 days post injury represents a state of hypoalbuminemia\(^\text{61}\). Mechanistically, this may reflect a number of factors, including a higher degree of trauma and rates of complications (e.g., infections) in the initial days to weeks after acute spinal cord injury among individuals with more severe injuries\(^\text{78}\).

Beyond an association at admission to rehabilitation, we aimed to determine if serum albumin concentrations could improve the prediction of neurological recovery, to a similar degree or beyond that already attributable to baseline injury characteristics. A significant relationship was evidenced for changes in LEMS and marked recovery. Specifically, serum albumin concentrations > 3.1 and 2.8 g/dL (as identified by the URP) were associated with double the proportion of individuals achieving marked recovery and with greater recovery of LEMS, respectively. However, serum albumin concentration did not improve the prediction of neurological recovery from that which is possible based on baseline measures derived from the ISNCSCI (see Figure 2.2B and D). The exclusion from the “tree” ultimately suggests that serum albumin concentration is only useful for cases where neurological details at admission (i.e., LEMS or AIS grade at baseline) are not available. Since serum albumin concentration failed to improve the prediction of neurological recovery beyond the existing ISNCSCI measures, the use
of albumin in acute spinal cord injury clinical trials, as a biomarker to enhance patient stratification, may be limited.

As a point of interest, both the current analysis and previous study evaluated the impact of albumin on neurological outcomes using data collected for alternative purposes. Specifically, the Sygen trial was planned to evaluate the efficacy of GM-1 gangliosides on sensorimotor recovery from acute spinal cord injury\(^7^6\), and the Spinal Cord Rehabilitation study was designed to identify which rehabilitation interventions are strongly associated with positive outcomes\(^7^0\). The investigators of the latter have made their data entirely open, which is accessible through the ADDEP [https://www.icpsr.umich.edu/icpsrweb/ADDEP/studies/36567]. The advantages of secondary analyses of existing data are reduction in time and costs associated with data collection. In addition, secondary analyses of existing data have demonstrated disease modifying effects of various events and interventions associated with acute spinal cord injury, including the impact of infections\(^7^9\) and acute pain medications\(^7^3,8^0,8^1\).

We employed a machine learning approach, URP\(^7^2\), over traditional linear regression for several reasons. Recent studies in individuals with acute spinal cord injury have demonstrated URP as a robust tool to predict neurological recovery based on initial injury characteristics\(^7^4\). From a technical perspective, URP also manages various types of independent and dependent variables (e.g., neurological recovery as a continuous or dichotomous outcome), and does not rely on assumptions of data normal distribution. In terms of application, URP is advantaged over linear and logistical regression for its’ discrete cut-off values on which clinical decision making relies. Overall, URP demonstrated that serum albumin concentrations were related to neurological outcomes after acute spinal cord injury, pointing to the strength of the association.
Differences between this and the previous study could be attributable to differences in design. First, in the Spinal Cord Injury Rehabilitation Study, serum albumin was only recorded in a subset of individuals, where monitoring serum albumin was deemed clinically important. In comparison, blood chemistry was routinely collected in all individuals meeting the inclusion criteria of the Sygen trial. Second, serum albumin abstracted from medical records were reduced to a single concentration, which was then utilized in our URP analysis. In comparison, serum albumin concentrations in the Sygen trial were evaluated at specific time-points after injury (e.g., 1-month). Thirdly, given the differences in study design (i.e., observational study versus prospective clinical trial), the Spinal Cord Injury Rehabilitation Study incurred a higher dropout at 1-year post injury in terms of neurological outcomes than the Sygen trial, potentially leading to various other types of selection bias (e.g., sampling and attrition). Finally, the statistical approach varied between studies. Our analysis of the Spinal Cord Injury Rehabilitation Study utilized a machine learning approach, URP\textsuperscript{72}, whereas the previous study applied more conventional linear and logistical regressions.

In summary, we have validated that albumin concentration is associated with baseline characteristics and long-term neurological recovery after acute spinal cord injury. At admission to rehabilitation, serum albumin concentrations provide a crude estimate of injury severity and future neurological recovery. The potential application of serum albumin to the clinic warrants further prospective study.
Figure 2.1: (A) Scatter plot between albumin concentration and Lower Extremity Motor Scores (LEMS) at rehab admission. (B) Unadjusted unbiased recursive partitioning (URP) between LEMS at admission and albumin concentrations. Albumin concentration was a significant variable at a cutoff value of 2.6[g/dL]. Cohort with albumin >2.6[g/dL] has the most favourable outcome, but cohort with albumin ≤2.6[g/dL] has the least favourable outcome. (C) Boxplot between albumin concentration and AIS grades at admission (i.e., AIS A, B, C, D, and D&Walk). Albumin concentration in AIS C and D&Walk is significantly different from albumin concentration in AIS A and AIS B. (D) URP between albumin concentration and AIS grades at admission. Albumin concentration was a significant variable with cutoff values of 3.2[g/dL] and 2.6[g/dL]. For cohort of albumin ≤2.6[g/dL], the outcome was the least favourable. In cohort of albumin >2.6[g/dL], the outcome was the second least favourable. However, in cohort of albumin >3.2[g/dL], then
the outcome was the most favourable.

**Figure 2.2:** (A) The bivariable URP model between lowest albumin concentration and change of lower extremity motor scores revealed albumin to be significant (p<.001). In the albumin ≤2.8[g/dL] cohort (N = 66), the outcome (change of LEMS) was the least favourable (median = 0, and mean = 2.36); however, in the albumin >2.8[g/dL] cohort (N=51), change of LEMS was the most favourable (median = 2, and mean = 9.35). (B) In the multivariable URP adjusting for both lowest albumin concentration and LEMS at admission.
admission, only LEMS at admission showed up suggesting that lowest albumin concentration was not predictive of outcome. (C) Albumin concentration was significant (p = .001) in splitting the original sample size into two cohorts: in albumin ≤3.1[g/dL] cohort (N =129), the outcome is the least favourable (less than 20% achieved marked recovery); whereas in albumin >3.1[g/dL] cohort (N = 38), the outcome is the most favourable (almost 50% achieved marked recovery). (D) A multivariable URP model for marked recovery with AIS grades as predictor and serum albumin was also created.

Patients with more severe injuries (AIS A and B) had roughly 10% chance of achieving marked recovery, but patients with less severe injuries (AIS C and D) had up to almost 80% chance of achieving marked recovery.

### Table 2.1: Summary of Individuals Characteristics and Albumin Concentration at Admission to Rehabilitation.

<table>
<thead>
<tr>
<th></th>
<th>Lower Extremity Motor Scores (Admission to Rehabilitation)</th>
<th>AIS grades (Admission to Rehabilitation)</th>
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<tr>
<td></td>
<td>Included</td>
<td>Excluded</td>
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<tr>
<td>Total</td>
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<td>Sex, n</td>
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<tr>
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<tr>
<td>Male</td>
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<td>5</td>
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<tr>
<td>Age [yrs], n</td>
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<tr>
<td>&lt;50</td>
<td>312</td>
<td>5</td>
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<tr>
<td>≥50</td>
<td>118</td>
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<td>AIS grades at admission, n</td>
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</tr>
<tr>
<td>A</td>
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<td>1</td>
</tr>
<tr>
<td>B</td>
<td>73</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
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</tr>
<tr>
<td>D</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>D&amp;Walk</td>
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<td>3</td>
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<tr>
<td>Neurological Levels, n</td>
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<td>7</td>
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<tr>
<td>Thoracic</td>
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<td>3</td>
</tr>
<tr>
<td>Albumin [g/dL]</td>
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### Table 2.2: Summary of Individuals Characteristics, Albumin Concentrations, and Days Elapsed at 1-year post-injury.

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<th>Pairwise comparison</th>
<th>Included</th>
<th>Excluded</th>
<th>Pairwise comparison</th>
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<tr>
<td><strong>Total</strong></td>
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<td>265</td>
<td></td>
<td>167</td>
<td>215</td>
<td></td>
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<tr>
<td><strong>Sex, n</strong></td>
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<tr>
<td>Male</td>
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<tr>
<td><strong>Age [yrs], n</strong></td>
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<td>&lt;50</td>
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<td>.854</td>
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<td><strong>AIS grades at admission, n</strong></td>
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<td></td>
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<td>&lt;.001</td>
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<td>D</td>
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<td></td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Neurological Levels, n</strong></td>
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<td>.41</td>
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<td>.957</td>
<td>111</td>
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<td>95</td>
<td></td>
<td>56</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td><strong>Albumin [g/dL]</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>265</td>
<td>.916</td>
<td>167</td>
<td>215</td>
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<tr>
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<td>2.69 ± 0.86</td>
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<td>2.78 ± 0.96</td>
<td>2.62 ± 0.57</td>
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</tr>
<tr>
<td><strong>Number of days between injury date and date of albumin recorded</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45 ± 36</td>
</tr>
<tr>
<td>mean ± SD</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>Number of days between injury date and POC admission date</strong></td>
<td></td>
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<td>30 ± 27</td>
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</table>
Chapter 3. Identifying New Biomarkers

3.1 Introduction

Previously, Tong and colleagues have reported that low serum albumin concentration (i.e., hypoalbuminemia) is associated with poor neurological recovery after acute spinal cord injury\textsuperscript{61}. In chapter 2, we have partially confirmed these results. This begs another question if other conventional serum biomarkers or combination of serum biomarkers measured at baseline (i.e., 24-72 hours post-injury) can predict neurological recovery after acute spinal cord injury.

In order to address the role of other conventional serum biomarkers in the prognosis of neurological outcomes after acute spinal cord injury, we applied machine learning to existing data (i.e., Factor Analysis and Unbiased Recursive Partitioning). While regression techniques are technically simple to perform and straightforward to interpret, they have several notable disadvantages, including the need for defined statistical interactions and multi-collinearity (i.e., very strong correlations between predictors in a multivariable model). The latter is of particular concern as it is well established that there are strong correlations between biomarkers derived from bodily fluid or tissues\textsuperscript{82}.

Advanced multivariable techniques, flexible in their application (e.g., independent of data distribution), are used to explore complex interactions between biomarkers and generate profiles useful in prediction across different subgroup of individuals. To this end, we applied a multivariate statistical method to determine baseline serum biomarkers that predict neurological recovery after acute spinal cord injury. We hypothesized that combinations of conventional serum biomarkers at baseline admission are positively correlated with neurological recovery (i.e., lower extremity motor scores) after acute spinal cord injury.
3.2 Methods

3.2.1 Study design and data source

This was a secondary analysis of a completed United States Food and Drug Administration-sponsored (FDA) clinical trial. Specifically, we analyzed data gathered from a prospective phase III, placebo controlled, multi-centre Sygen study testing the efficacy of GM-1 ganglioside in acute, traumatic spinal cord injury\textsuperscript{76}. Details about study design, recruitment, and enrollment have been previously published\textsuperscript{69}. Briefly, individuals were required to have at least one lower extremity motor with a substantial motor deficit. Individuals with spinal cord transection or penetration were excluded, as were individuals with a cauda equina, branchia, lumbrosacral plexus, or peripheral nerve injury. Multiple trauma cases were included if they were not too severe to prevent neurological evaluation. The Sygen trial, which ran from 1992 to 1998, failed to demonstrate the efficacy of GM-1 treatment over placebo. Subsequent analyses of the data have been performed to characterize the trajectory and amount of spontaneous recovery from acute spinal cord injury\textsuperscript{10,35}.

3.2.2 Outcomes (Dependent) Variables

The primary outcomes were LEMS at 52 weeks post-injury. Key muscles were examined were examined according to the ISNCSCI\textsuperscript{28,31,33}, with a maximum score of 25 points on each left and right sides (for a maximum total score of 50). The strength of each muscle function is measured on a 6-point scale ranging from 0 (total paralysis) to 5 (active moment, full range of motion against gravity and sufficient resistance). These scores represent neurological function caudal to thoracic and cervical injuries.
3.2.3 Predictor (Independent) Variables and Covariates

As an FDA requirement, detailed information regarding routine blood chemistry (a total of 42 biomarkers) was collected at admission to the trauma-center (hereinafter referred to as baseline), 1, 2, 4, 8, and 52 weeks post-injury. This analysis focused on the baseline blood chemistry. Lower extremity motor scores and four S4-S5 sensory scores (i.e., pin prick and light touch from left and right sides) at baseline was then adjusted for subsequent analyses. S4-S5 sensory scores were coded as binary variables (intact/impaired or absent).

3.2.4 Statistical Analyses

3.2.4.1 Data Reduction Techniques

To examine the relationship between various predictor variables and outcomes, we utilized data reduction techniques, Factor Analysis consisting of Exploratory Factor Analysis (EFA) and Confirmatory Factor Analysis (CFA). EFA is to explore the relationship in our set of observed variables, and CFA is to verify the validity of factors models. Particularly, factor analysis combines highly-correlated variables (i.e., multi-collinear) into several clusters to reduce the dataset into a more manageable size while retaining the original information as much as possible. In other words, EFA grouped multi-collinear variables into same factors, then CFA was used to compose values for each factor. Normalized Z-scores were used to reduce the large variance of the variables. As a result, the values of each factor became unit-less. EFA with varimax rotation and maximum likelihood as factor method were chosen to handle missing data and Heywood case. The relationship between each variable and their corresponding factor, known as factor loadings, and can also be thought as Pearson correlation coefficient, was set to be over 0.5. Factors were retained in the EFA based on their scree plot and eigen values (>=1). CFA was then performed to obtain the values for each factor.
3.2.4.2 *Unbiased Recursive Partitioning: Conditional Inference Tree*

The URP is a tree-based model using sequential tests of independence between predictors and outcomes\(^2\). Briefly, URP is used to divide an original heterogeneous sample into homogenous subgroups dependent on the outcomes of interest. Details about URP’s functions have been previously described in chapter 2.

3.2.4.3 *Analyses*

Of the 797 individuals in the Sygen trial, we included individuals who had measurements of lower extremity motor scores at 52 weeks post-injury. For demographic and injury characteristics, t-test was used for continuous variables and chi-square test was used for categorical variables (or their nonparametric counterparts).

The statistical analyses were performed as follows: first, multivariable-URP were employed for all factors and outcome. After identifying the significant factors from the previous analysis, multivariable-URP were then created to adjust for LEMS baseline and sensory scores. Because of the unit-less values of factors created by Factor Analysis, subsequent multivariable-URP was then constructed using the biomarkers (that were represented by the factors included in the initial model). \( P < .05 \) was regarded as statistically significant. Bonferroni correction was included for multiple comparisons. All analyses were carried out using R. R package “lavaan” was used for factor analysis, and package “party” was used for URP.
3.3 Results

3.3.1 Cohort Summary

We included 634 individuals in the analysis. Individuals demographics and injury characteristics (included and excluded cohorts from our analysis) are shown in Table 3.1. Significant differences were evident between those included and excluded from our analysis. Specifically, those excluded were more likely to have cervical injuries and to be older than the included cohorts (Table 3.1).

3.3.2 Factor Analysis

Out of 42 blood biomarkers, 75%-missing-values markers, unit-less, and urine biomarkers were excluded in our factor analysis. Each biomarker was highly correlated to their corresponding factors (factors loadings over 0.8). Six distinct factors were generated from our factor analysis, according to their Eigen’s values. Factor analysis classified biomarkers with high collinearity into several factors, each represented a specific biological process; thus, we named those factors according to their biological processes (Figure 3.1A). The values of each factor were then obtained using CFA. Since these biomarkers were normalized by using z scores, these factors values were unit-less.

3.3.3 Prediction of LEMS at 52 weeks post-injury

The relationship between all factors and LEMS at 52 weeks post-injury is shown in Figure 3.1B. The presence of only factor 1 (blood factor) indicates a significant predictor. This URP identified two cohorts based on a cut-off factor concentration of 0.426 (p < .001). Adjusting for LEMS baseline, URP yielded two individuals cohorts, based on cut-off values of both LEMS baseline and blood factor. Specifically, for cohorts with LEMS baseline equals to 0, blood factor can further split them into two cohorts (p = .009) (Figure 3.2A). Replacing the blood factor with
their corresponding biomarkers (i.e., red blood cells, hemoglobin, and hematocrit) reveals similar tree, based on a blood cells cut-off of 4.1 mil/mcl (Figure 3.2B).

Similar to URP for LEMS baseline, in a model adjusted for sensory scores and blood factor, blood factor was significant yielding two cohorts (p = .001) for individuals with absent sensory functions (i.e., pin prick and light touch) (Figure 3.3A). For the subsequent URP tree, we replaced the blood factor with the equivalent biomarkers. This URP tree revealed that hematocrit of 38% would be the appropriate cut-off value for individuals with absent pin prick and light touch (Figure 3.3B).

However, after adjusting for both sensory scores and LEMS baseline, blood factor was not selected in the URP (Figure 3.4). The absence of blood factors suggests that blood biomarkers were not as good as baseline injury characteristics at predicting motor recovery 52 weeks post-injury.

3.4 Discussion

The goal of this analysis is to examine the relationship between composites of blood biomarkers at baseline (within 72 hours of injury) and neurological recovery after acute spinal cord injury. Without adjustment for both motor and sensory baseline injury characteristics, our findings indicate that blood factor, specifically red blood cells at 4.1 mil/mcl and hematocrit at 38%, assists the prognosis and stratification of neurological outcomes 52 weeks post acute spinal cord injury. However, this relationship failed to surpass baseline injury characteristics after the adjustment.

Our analyses indicated that blood factors (red blood cell, hemoglobin, and hematocrit) are associated with individuals’ neurological outcome after acute spinal cord injury. Since the biomarkers comprising of blood factor are representative of anemia markers, our blood factor
can also be used reflect anemia. Indeed, in able-bodied population, anemia is calculated based on normal reference range for red blood cell (within 4.5 – 5.9 mil/mcl), hemoglobin (between 14 – 17.5 g/dl), and hematocrit (from 40% to 50%)\textsuperscript{88}. In acute spinal cord injury population, little is known for the reference range of anemia. Here, with the help of URP, we provide evidence of blood factors values in acute spinal cord injury population to assist prediction and stratification of injury. In particular, with LEMS baseline equals 0 (synonymous to severe injury), having anemia (i.e., red blood cell below 4.1 mil/mcl and hematocrit below 38%) would worsen neurological recovery.

When considering either baseline lower extremity motor scores or baseline S4S5 sensory functions, blood factors offered potential stratification for individuals with severe injuries (i.e. lower extremity motor scores baseline is 0). However, these blood factors did not improve prediction of neurological recovery beyond the combination of both baseline injury characteristics. The absence of blood factors in the URP tree suggests that these markers are most effective in cases where complete baseline neurological details (i.e., lower extremity motor scores and sensory functions) are not available (see Figure 3.2B and 3.3B). For instance, if individuals with acute spinal cord injury also have a broken leg, the examiners would be able to get sensory information but not motor testing.

Mechanisms underlying the relationship between anemia (e.g., low blood factor in our cohort) and limited neurological recovery is poorly understood, but we speculate that anemia was due to blood loss at the time of admission. It was previously reported that blood loss caused by bony injury, gastrointestinal infection, and surgery, was a major contributor to anemia\textsuperscript{89}. If overlooked during therapeutic window of opportunity (i.e., 1 to 4 weeks post-injury), anemia due to blood loss can cause systemic hypotension\textsuperscript{90}. This then triggers cascades of deleterious effects.
on health of individuals with acute spinal cord injury, such as reduced cognitive performance\textsuperscript{91}, fatigue\textsuperscript{92}, autonomic dysreflexia\textsuperscript{93}, and atherosclerosis\textsuperscript{93}. In addition, anemia has also been found to signal many chronic inflammatory processes and additional complications, including massive bleeding in acute spinal cord injury\textsuperscript{89,94}. As a result, anemia due to blood loss at baseline admission would potentially lead to poor neurological recovery. To avoid deteriorating the injury outcomes, early treatment to prevent hypotension/blood loss is paramount.

In a clinical context, our findings support the current hemodynamic management practices following acute spinal cord injury. According to the Clinical Practice Guidelines of the Consortium for Spinal Cord Medicine, the primary treatment for hypotension in patients of acute spinal cord injury is fluid resuscitation (medical practice to refill bodily fluid lost)\textsuperscript{95}. This is necessary for acute spinal cord injury patients to maintain tissue perfusion and to resolve neurogenic shock\textsuperscript{96}. Since avoiding hypotension in early treatments is important, studies have reported favourable outcomes after following the clinical protocols of using fluid resuscitation\textsuperscript{97,98}.

We used machine learning approaches, URP and Factor Analysis, over traditional regression techniques. First, factor analysis can address for multicollinearity problems (e.g., highly correlated biomarkers) by data reduction while preserve the integrity of the data\textsuperscript{82,99}. Recently, a preliminary cohort study also used factor analysis to correlate different factors and neurological outcomes after acute spinal cord injury\textsuperscript{100}. Comparable to our analysis, their serum biomarkers were organized in similar fashion. For instance, red blood cells and hemoglobin were sorted in a same factor, and liver enzymes (i.e., ALT and AST) were also grouped in a same factor in their study. The relatively small sample size in their preliminary study (82 individuals) contribute to their major limitation. On the contrary, our analysis drawn on from over 600 individuals and still
arrived at the same conclusion to their analysis. These similarities confirm the validity of our methods. On the other hand, URP manages various types of interactions without relying heavily on parametric assumptions. In addition, URP produces cut-off values, which are crucial for clinicians in acute clinical settings. As a result, we observed that blood factor is associated with neurological outcome after acute spinal cord injury, pointing to the strength of these machine learning. Recently, different trauma studies, including acute spinal cord injury, traumatic brain injury, and acute heart attack studies, have adopted these machine-learning techniques.

However, this analysis is not without limitations. First, the analysis was done on retrospective dataset, which was completed nearly 20 years ago. Thus, the effects of outdated acute care management (i.e., administration of methylprednisolone) on routine blood chemistry could not be excluded. Second, as shown in Figure 3.2 and 3.3, the inclusion of blood factors only came after baseline injury characteristics (i.e., lower extremity motor scores or sensory scores). In other words, knowing individuals’ blood factors values, namely red blood cells count, hematocrit, and hemoglobin, is only useful after their baseline lower extremity motor scores and sensory scores are calculated. As a result, application of blood factor to stratify individuals with acute spinal cord injury may be limited.

In summary, we have identified that blood factor at baseline admission (i.e., up to 72 hours post-injury) are associated with neurological recovery after acute spinal cord injury. This blood factor is helpful when ISNCSCI examinations are incomplete. At baseline, these markers can serve as a tentative estimate for future neurological recovery. The potential usage of these markers in clinical trials remains to be further investigated.
Figure 3.1: (A) Exploratory Factor Analysis (EFA) was performed to obtain 6 latent factors. Each biomarker was highly correlated to their corresponding factors. Confirmatory Factor Analysis (CFA) was then performed to obtain the values of each factor. However, only Factor 1 (named blood factor) was significant in the subsequently analysis. (B) Multivariable unbiased recursive partitioning (URP) between all six factors and lower extremity motor scores at 52 weeks post-injury. Factor 1 (blood factor) was a significant predictor at a cut-off value of 0.426.

Figure 3.2: (A) In the multivariable URP adjusting for blood factor and LEMS baseline, blood factor was a significant variable with a cut-off of 0.919 in subset of individuals with LEMS baseline equals to 0. (B) When replaced the blood factor with its corresponding biomarkers (i.e., red blood cells, hemoglobin, and hematocrit), red blood cells replaced
blood factor in the URP tree. That is, red blood cells were a significant predictor with a cut-off values of 4.1 mil/mcl in subset of individuals with LEMS baseline of 0.

Figure 3.3: (A) In the multivariable URP adjusting for blood factor and S4S5 sensory scores, blood factor was a significant predictor with a cut-off of 0.89, particularly in a subset of sensory-absent individuals. (B) Replacing blood factor with its appropriate biomarkers (i.e., red blood cells, hematocrit, and hemoglobin) yielded similar URP tree. Specifically, hematocrit of 38% would be a significant predictor for outcome for individuals with no sensory functions.
Figure 3.4: In the multivariable URP adjusting for blood factor, baseline LEMS, and sensory scores, blood factor was no longer a significant predictor for outcome. The exclusion from the URP tree indicates that blood factor failed to offer additional information beyond that was offered by injury characteristics.

Table 3.1: Summary of Individuals Characteristics

<table>
<thead>
<tr>
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<th>Lower Extremity Motor Scores 52 weeks post-injury</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Included</td>
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<tr>
<td>Total</td>
<td>634</td>
</tr>
<tr>
<td>Sex, n</td>
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<tr>
<td>Male</td>
<td>509</td>
</tr>
<tr>
<td>Female</td>
<td>125</td>
</tr>
<tr>
<td>Age [years]</td>
<td>mean±SD</td>
</tr>
<tr>
<td>AIS grades at admission, n</td>
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<td>A</td>
<td>411</td>
</tr>
<tr>
<td>B</td>
<td>103</td>
</tr>
<tr>
<td>C</td>
<td>104</td>
</tr>
<tr>
<td>D</td>
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<tr>
<td>Neurological Levels, n</td>
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<td>Cervical</td>
<td>465</td>
</tr>
<tr>
<td>Thoracic</td>
<td>169</td>
</tr>
</tbody>
</table>
Chapter 4. General Discussion

The overall goal of this thesis was to validate and identify the relationship between conventional peripheral serum biomarkers and acute spinal cord injury outcome. To this end, I have demonstrated a correlation between serum biomarkers (e.g., albumin, red blood cells) and injury severity through the use of machine learning. In addition, I combined hypothesis-driven and data-driven approaches to fully investigate the uses of biomarkers. Indeed, serum biomarkers should be employed as a complement to the current motor and sensory assessment for a complete prognostic tool. While further research is required to determine the reliability of these serum biomarkers, this represents a potential application and identification of new treatment. In this chapter, I discuss the significance of biomarkers for future trials, outline the uses of machine learning techniques for future research in the field of acute spinal cord injury, and highlight the data-driven and hypothesis-driven approaches used.

4.1 Biomarkers

There are several potential applications for biomarkers in acute spinal cord injury. The first is to diagnose the presence and extent of damage in acute spinal cord injury. To do this, neuroimaging outcomes have provided unprecedented insights. This is of pivotal importance as an accurate measure of acute spinal cord injury, which is critical for prognosis of long-term outcomes (e.g., walking) in clinical trials. Furthermore, biomarkers that can reflect individuals’ injury severity can help stratify for clinical trials. Biomarkers can also be used to identify alternative measures of new treatment. As reflected in Sygen clinical trial, every biomarker was collected as potential response to the target drug (i.e., GM-1).
Most biomarker studies examining hematological or CSF have chiefly focused on inflammatory or neuronal/glial markers\textsuperscript{47,48,53,106,107}. This represents a logical focus since damage to the spinal cord involves insult to neurons and glia, leading to inflammation. However, from a pragmatic perspective, this approach necessitates expertise and additional cost due to the advanced procedures required to analyze samples. As an alternative, acute spinal cord injury and other neurological conditions facing similar diagnostic and prognostic difficulties have turned to conventional peripheral hematological markers. Indeed, these conventional serum biomarkers are commonly collected during acute care, but rarely considered for prognosing long-term neurological outcomes. In this thesis, I fully explored the prognostic utility of these biomarkers for long-term neurological recovery.

4.2 Objectives

4.2.1 Objective 1

Validation studies are important. Evidence is needed to assure that effects of treatments or observed proportion of events were similar in groups of individuals from other settings. It is necessary to quantify the performance of prognostic model on a new set of individuals (i.e., different location or time) to ensure generality (i.e., external validation) and clinically credible before applying to clinical practice\textsuperscript{108}. Thus, the first objective of this thesis was to validate if serum albumin is associated with neurological outcomes after acute spinal cord injury. To examine this, I performed a secondary analysis on data from Spinal Cord Injury Rehabilitation study (2007-2009) in Chapter 2. By using this recent dataset, I addressed the concerns regarding our previous secondary analysis on data from the Sygen clinical trial (completed in 1998), which was subjected to outdated acute management (e.g., administration of methylprednisolone). In agreement with the previous analysis\textsuperscript{61}, serum albumin concentrations were associated with
severity of acute spinal cord injury. However, serum albumin concentrations were a crude
prognostic biomarker since it did not improve prediction beyond baseline measures of injury
characteristics.

4.2.2 Objective 2

Given the limited number of conventional serum biomarkers applied in acute spinal cord
injury context, further studies are needed to discover new potential biomarkers. The second
objective of this thesis then was to determine the combination of hematological and serum
biomarkers at baseline admission that best predicted neurological recovery after acute spinal cord
injury. Using machine-learning techniques, I identified blood factor (i.e., red blood cells,
hematocrit, and hemoglobin) as being predictive of neurological recovery in chapter 3. Similar to
the results in chapter 2, this blood factor serve as crude prognostic biomarkers in cases where
complete neurological examinations for individuals with acute spinal cord injury were
unavailable (e.g., unconscious).

4.3 Clinical Application

Across various conditions, serum albumin concentrations consistently remain a significant
prognostic biomarker. However, why serum albumin concentrations serve prognostic roles in
several neurological conditions is to be further explored. In one theory, serum albumin was
believed to reflect an individual’s overall health conditions. In this way, low albumin
concentrations represent poor health, which then limits the neurological recovery after acute
spinal cord injury. Therefore, it has been suggested that albumin administration would be then
beneficial for long-term outcomes. However, in different conditions, several studies reported
no differences were found between albumin- and saline-administration groups. Another
theory suggests that serum albumin concentrations are surrogate measure of systemic

inflammation instead of underlying cause of poor health. Thus, after acute spinal cord injury, a number of secondary inflammations would be reflected by serum albumin, which would then modulate the outcomes. This relationship between albumin, inflammation, and neurological recovery has been explored in recent study. However, this proposed theory necessitates further investigation.

Although albumin concentrations were limited in their ability to improve the prediction of neurological recovery beyond injury characteristics, they may still prove useful as crude estimates for injury severity. Indeed, serum albumin concentrations, routinely collected during acute phase, are feasible and convenient biomarkers to help stratify individuals with acute spinal cord injury. Although injury characteristics remain the strongest predictors for neurological recovery, albumin concentrations are still suitable to cases where the neurological exam is not available, incomplete, or unreliable (e.g., unconscious or unresponsive individuals). Additionally, since albumin is included in standard blood chemistry workup and commonly evaluated by physicians, it can then be complemented the existing neurological exams for acute spinal cord injury.

Serum albumin concentrations was partially validated as a crude estimate for neurological recovery in chapter 2. However, this begs the question if there are any other serum biomarkers an early time-point after acute spinal cord injury to predict neurological recovery. This question was further explored using machine-learning techniques on multiple potential serum biomarkers in chapter 3, which identified blood factor.

This blood factor holds potential clinical values. As early as 72 hours post-injury, cut-off values of this blood factor could provide threshold information for individuals with acute spinal cord injury. In particular, red blood cells below 4.1 mil/mL, and hematocrit less than 38% in
individuals with severe injury (lower extremity motor scores is 0 or having no sensation in S4-S5 region) would indicate anemia, and therefore worsened recovery in individuals with acute spinal cord injury. For instance, for some individuals with acute spinal cord injury may have only had their sensory information assessed (e.g. injuries prevented full motor testing), their hematocrit threshold could provide information about how much likely they are to recover. Hence, from clinical perspectives, conventional serum biomarkers could still provide with an early clinical prognosis (i.e., up to 72 hours post-injury), despite their incomplete ISNCSCI evaluation.

However, the results from chapters 2 and 3 are not meant to compete, but rather complement one another. In the case of incomplete neurological examination, clinicians can use blood factor to roughly estimate recovery of individuals with acute spinal cord injury as early as 72 hours. However, if blood factor is also unavailable, then serum albumin 4 weeks post-injury would also fill the gap. However, future studies should further investigate other potential biomarkers that could improve prediction on their own. Although our results are far from the ultimate goal (e.g., biomarkers would improve prediction beyond injury characteristics), these markers could still provide valuable information for clinicians.

4.4 Machine Learning

In chapter 2 and 3, my analyses utilized unsupervised machine learning (e.g., factor analysis) instead of traditional regression. Multivariable regression models are not well-equipped to handle complex interactions (e.g., multicollinearity)\textsuperscript{99}. In addition, the parametric assumptions of conventional regression (i.e., outliers, linearity, homoscedasticity, and normality) between predictors and outcome in the biological settings are rarely met. Considering the complex nature of acute spinal cord injury, one or more assumptions are not usually satisfied. Furthermore, traditional regression techniques (e.g., linear, logistic regression) do not provide cut-off points,
which would be valuable for patient stratification for potential clinical trials. Factor Analysis and URP were used to address these limitations. Factor Analysis preserves information as well as eliminates the problems of multicollinearity because it groups highly-correlated biomarkers into several factors, each representing a specific biological process\textsuperscript{82,99}. As a result, factors contain prognostic values of their corresponding biomarkers. URP is advantageous because it (1) does not adhere to parametric assumptions, (2) addresses for interactions between predictors, (3) stratifies cohorts into homogenous subgroups, and (4) provides outcome distributions within each subgroup\textsuperscript{72}. These machine learning techniques also have some limitations, including bias in variable selection, and obscure missing data handling\textsuperscript{113}. In addition, whether URP-CTREE overfit is still subject to fierce debates between statisticians, which warrants further exploration\textsuperscript{113}. Despite these limitations, without the help of machine learning, I could have run multiple linear regressions, which would commit type 1 error. Particularly, adjusting for too many variables in one linear regression inflates the chances of committing type 1 error\textsuperscript{114}. For instance, if linear regression performs 20 independent variables, the chance of at least one variable being significant is 64\%. Thus, the Bonferroni adjustment in URP makes it less likely to commit type 1 error. These unique features of Factor Analysis and URP-CTREE are valuable in the stratification and prediction for future clinical trials.

### 4.5 Hypothesis-driven and Data-driven approaches: Strengths and Limitations

In search for potential biomarkers of acute spinal cord injury recovery, many researchers follow a hypothesis-driven approach. This approach necessitates the researchers to propose a hypothesis, after which they can collect and data to test the hypothesis (e.g., albumin was correlated with neurological recovery)\textsuperscript{115}. This approach determines the focus, and direction of research. Specifically, hypothesis makes it clear what the main focus of the study, and what
results are to be accepted or refuted. Additionally, hypothesis guides the investigators decide what type of data to be accepted or ignored. However, such approach is bound within the researchers’ existing knowledge and experience (e.g., the researchers must first predict the hypothesis then collect data pertaining to it), which would substantially impact the reproducibility and translational potential of their findings\textsuperscript{116}. Our hypothesis-driven approach is highlighted in chapter 2. Based on previous research\textsuperscript{61} and literature background, we hypothesized that serum albumin was positively correlated with recovery after acute spinal cord injury.

In healthcare industry, driven by record keeping, compliance requirements, and patients care, massive quantities of data (“big data”) are stored and rapidly digitized\textsuperscript{117}. This recent emergence of “big data” has encouraged the examination of large quantities of data, and rapidly enabled us to take a different approach for analyses\textsuperscript{118,119}. This can support a wide range of medical and healthcare functions. However, due to its sheer volumes and diversity of data types, big data in healthcare are often overwhelming. In fact, big data in healthcare are often so large and complex that it is difficult to manage with traditional software and/or hardware\textsuperscript{120}. With the appropriate tools, big data analytics can take the advantage of the explosion of data to make better informed decision. Using this strategy, we can first generate or collect data and use this resource to answer a broader questions (e.g. identifying biomarkers)\textsuperscript{116}. Specifically, data-driven approach refers to the usage of data analysis, machine learning, and related methods to understand the pattern in data without making any assumption prior. This approach is highlighted in chapter 3. In using this approach, we minimize the effect of experimenter’s bias (i.e. researchers’ influence on the outcomes of their research), and allow the data to reveal the patterns relating to our broad
objectives. The results of chapter 3 identified blood factor (i.e., red blood cells, hemoglobin, and hematocrit) without making any *a priori* assumptions required for a specific hypothesis.

As such, it is important to note that a combined effort between hypothesis-driven and data-driven approaches is necessary to tackle complex disorder such as acute spinal cord injury. Ideally, big data approaches could first be used to generate useful and specific leads for future studies and hypothesis. Then, follow-up studies could apply traditional hypothesis-driven approach to verify these results. Such combination of data-gathering and hypothesis-driven approaches might be a more reliable procedure to understand a complex trauma with a multitude of underlying processes.

The strengths and weaknesses of both methodologies are highlighted above as well as in chapter 2 and 3. A broader strength of this thesis relies on the secondary analyses of the existing data. The advantages of such analyses are less time-consuming and costs associated with data collection. Instead of spending time recruiting individuals and collecting data, I had more time to analyze the given dataset. In addition, secondary analysis of existing data have identified modifying effects of various interventions associated with acute spinal cord injury, including infections\(^79\) and pain medications\(^80,81\). Similarly, by analyzing two existing datasets, I have partially validated and identified potential serum biomarkers for clinical purposes. Conversely, this thesis also presents with some limitations. Since we do not collect data, our analyses and knowledge are limited to the data given. Additionally, original data are often disorganized; hence, data cleanup needs to be done to make them presentable. Some valuable biomarkers can also be missing because they were not measured, reducing the power of our analyses. Indeed, for this thesis, I had to re-organize data and create new variables. Since I did not collect the data, I would not be able to verify if data are missing at random or not.
4.6 Conclusion

In conclusion, my thesis revealed two major findings. First, I found that serum biomarkers at different time-points (i.e., albumin and blood factor) can provide crude estimate of injury severity for individuals with acute spinal cord injury. Second, data-driven approaches can be used to generate hypothesis. Thus, by employing machine learning, I have discovered blood factor markers (i.e., red blood cells, hemoglobin, and hematocrit) are predictive of neurological recovery after acute spinal cord injury. This knowledge can propel further studies investigating this relationship and its potential, and open new doors for ongoing search for biomarkers in acute spinal cord injury.
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