MICRORNA BIOMARKERS FOR ACUTE TRAUMATIC SPINAL CORD INJURY

by

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Abstract

Spinal cord injury is a devastating condition with variability in injury mechanisms and neurologic recovery. Spinal cord impairment is measured and classified by a widely accepted standard neurologic examination, however this examination is extremely challenging to conduct due to the fact that patients are often sedated, unconscious, or have multiple injuries. The lack of objective diagnostic or prognostic tools is a barrier for clinical trials. Biological markers (biomarkers) are promising as they represent an unbiased approach to classify injury severity and predict neurologic outcome.

MicroRNAs are attractive biomarker candidates in neurological disorders due to their stability in biological fluids, conservation between humans and model mammals, and tissue specificity. These features of microRNAs motivated my research to identify the changes in expression of microRNAs following different injury severities in human patients with spinal cord injury, as well as in a large animal model of spinal cord injury using pigs.

In Chapter 1, I provide background on the diagnosis and prognosis of spinal cord injury and discuss the current status of biomarkers for spinal cord injury.

In Chapter 2, I provide the historical context for the use of animal models for studying spinal cord injury and review the current status of such animal models and injury paradigms in spinal cord injury research.

In Chapter 3, I used a porcine model of thoracic spinal cord injury to study the effects of injury severity on microRNA expression. I identified a set of microRNAs that are diagnostic for injury severity and prognostic for behavioural and histological outcome.

In Chapter 4, I identified changes in microRNA expression following acute spinal cord injury in a cohort of 44 human patients. I identified a set of microRNAs that are diagnostic for baseline injury severity and prognostic for neurologic outcome.

These data describe the alterations in the microRNA profiles following acute spinal cord injury and identify a common set of microRNAs that can be used as diagnostic and prognostic tools. Furthermore, the data obtained and analyzed in pigs and humans with spinal cord injury provides a reference dataset for future work as well as for correlative pig-human investigations.

Lay Summary

Identifying biological markers of severity in patients with spinal cord injury would improve the accuracy of our current assessment practices and allow more patients to participate in clinical trials. MicroRNAs are small, stable RNA molecules that regulate nearly all cellular processes and are thought to be released from injured tissues, representing promising targets as markers for spinal cord injury severity. Using a porcine model of spinal cord injury and next-generation sequencing for molecular analyses, I have identified a microRNA signature in the blood related to spinal cord injury severity. In a parallel study, I have identified microRNAs in the cerebrospinal fluid and blood of human patients with traumatic spinal cord injury. By identifying common microRNA signatures that relate to injury severity in both humans and pigs, we can provide an objective outcome measure to better test therapies in a preclinical, large animal model, prior to large-scale clinical trials in humans.

Preface

The majority of the scientific work presented in this dissertation has been peer-reviewed and accepted by internationally recognized scientific journals.

A version of Chapter 1 has been published as: **Tigchelaar S,** Kwon B. (2017). Serum and cerebrospinal fluid biomarkers to predict functional recovery after spinal cord injury. AOSpine Master Series, Volume 7, Chapter 3. DOI: https://doi.org/10.1055/b-0036-137992.

In Chapter 1, I provide the background on the diagnosis and prognosis of spinal cord injury and discuss the current status of biomarkers for spinal cord injury. I provide a review of microRNA, their biogenesis and function, and the current evidence of microRNAs as biomarkers for central nervous system injuries. Brian Kwon initiated the review, provided project support, and edited the manuscript.

A version of Chapter 2 has been published as: **Tigchelaar S,** Kwon B. (2017). Translation: relevance of animal models in spinal cord injury. Neurological Aspects of Spinal Cord Injury, Volume 1. DOI: https://doi.org/10.1007/978-3-319-46293-6_25.

In Chapter 2, I provide the historical context for the use of animal models for studying spinal cord injury and review the current status of such animal models and injury paradigms in spinal cord injury research. Chapters 1 and 2 serve to provide the appropriate background and reasoning for the investigation of microRNA changes in spinal cord injury, as well as in a large animal pig model

of spinal cord injury. Brian Kwon initiated the review, provided project support, and edited the manuscript.

The work presented in Chapter 3 has been published as: **Tigchelaar S**, Streijger F, Sinha S, Flibotte S, Manouchehri N, So K, Shortt K, Okon E, Rizzuto M, Malenica I, Courtright A, Eisen A, Van Keuren-Jensen K, Nislow C, Kwon BK. (2017). Serum microRNAs reflect injury severity in a large animal model of thoracic spinal cord injury. Scientific Reports 7(1), 1376.

In Chapter 3, I conducted or supervised all of the animal work, including the sample collection, night animal monitoring, behavioural assessments, histological assessments, RNA purification, library preparation, and bioinformatic pipeline development. I oversaw experimental design, performed the bioinformatic analysis, outcome assessment data analysis and wrote the manuscript. Femke Streijger provided project supervision and performed animal surgeries. Sunita Sinha provided project supervision and performed next-generation sequencing (NGS). Stephane Flibotte helped develop the bioinformatic pipeline and provided bioinformatic support. Neda Manouchehri performed animal surgeries and helped perform weekly behavioural analysis. Kitty So and Katelyn Shortt helped with surgeries, sample collection, and weekly behavioural analysis. Elena Okon and Michael Rizzuto helped perform histological analyses. Ivana Malenica helped with bioinformatic pipeline development and data analysis. Amanda Courtright provided microRNA-sequencing training and reaction optimization support. Andrew Eisen, Kendall Van Keuren-Jensen, Corey Nislow and Brian Kwon initiated the study, provided project support, edited the manuscript and advised on experimental design.

The work presented in Chapter 4 has been published as: **Tigchelaar S**, Gupta R, Shannon CP, Streijger F, Sinha S, Flibotte S, Rizzuto MA, Street J, Paquette S, Ailon T, Charest-Morin R, Dea N, Fisher C, Dvorak MF, Dhall S, Mac-Thiong JM, Parent S, Bailey C, Christie S, Van Keuren-Jensen K, Nislow C, Kwon BK. (2019). MicroRNA biomarkers in cerebrospinal fluid and serum reflect injury severity in human acute traumatic spinal cord injury. Journal of Neurotrauma.

In Chapter 4, I conducted or supervised all the experimental analysis of clinical samples including RNA purification, and microRNA sequencing. I performed the bioinformatic analysis, data analysis, wrote the manuscript, and oversaw experimental design. Rishab Gupta helped perform microRNA library preparation and qRT-PCR. Casey Shannon helped perform machine learning analyses of sequencing data. Femke Streijger and Sunita Sinha provided project supervision. Stephane Flibotte provided statistical analysis and bioinformatic support. Michael Rizzuto helped perform RNA purification and data analysis. John Street, Scott Paquette, Tamir Ailon, Raphaele Charest-Morin, Nicolas Dea, Charles Fisher, Marcel Dvorak, Sanjay Dhall, Jean-Marc Mac-Thiong, Stefan Parent, Christopher Bailey, Sean Christie and Brian Kwon performed surgeries and collected clinical samples. Kendall Van Keuren-Jensen, Corey Nislow, and Brian Kwon initiated the study, provided project support, edited the manuscript and advised on experimental design.

Taken together, these data demonstrate a microRNA signature related to acute traumatic spinal cord injury severity within the cerebrospinal fluid and serum and that there is a shared set of microRNAs that are related to injury severity in both a porcine model of traumatic thoracic spinal cord injury and in human patients with acute traumatic spinal cord injury. These microRNAs

represent promising and powerful diagnostic and prognostic markers of spinal cord injury severity status.

All animal experiments were conducted in accordance with the University of British Columbia Animal Care Committee (A16-0311). All procedures strictly adhere to the guidelines issued by the Canadian Council for Animal Care. All clinical data was collected and analyzed in accordance with the University of British Columbia Clinical Research Ethics Board (H10-01091).

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List of Abbreviations

AGO – Argonaute

AIS – ASIA impairment scale

ANOVA – Analysis of variance

ASIA – American spinal injury association

AUC – Area under the curve

AUROC – Area under the receiver operator curve

BBB – Basso, Beattie, and Bresnahan locomotor scale

BCL-2 – B-cell lymphoma 2

BSL – Baseline

CAP-miRSeq – Comprehensive analysis pipeline for microRNA sequencing data

CCL2 – Chemokine C-C motif ligand 2

CCS – Central cord syndrome

CNS – Central nervous system

CSF – Cerebrospinal fluid

CT – Computed tomography

CTRL - Control

DE – Differential expression

DGCR8 – DiGeorge Syndrome Critical Region-8

DPI – Days post injury

EC – Eriochrome cyanine

ESCID – Electromagnetic spinal cord injury device

EV – Extracellular vesicle

FDR – False discovery rate

GCS – Glasgow coma scale

GFAP – Glial fibrillary acidic protein

HIF-1α – Hypoxia-inducible factor 1-alpha

HPI – Hours post injury

IF – Intermediate filament

IH – Infinite horizon

IL – Interleukin

ISCNSCI – International standards for neurological classification of spinal cord injury

L2FC – Log_2 fold change

LEMS – Lower extremity motor score

MAP-2 – Microtubule associated protein 2

MASCIS – Multicenter animal spinal cord injury study

MCP – Monocyte chemoattractant protein

MeCP2 – Methyl CpG-binding protein 2

miRISC – MicroRNA-induced silencing complex

MMP-9 – Matrix metallopeptidase 9

MS – Motor score

Nf - Neurofilament

NfH – Neurofilament heavy polypeptide

NfL – Neurofilament light polypeptide

NfM – Neurofilament medium polypeptide

NGS – Next-generation sequencing

NINDS – National institute of neurological disorders and stroke

NOX4 – NADPH oxidase 4

NOx – Nitric oxide

NSE – Neuron specific enolase

PCR – Polymerase chain reaction

Pre-miRNA – Precursor microRNA

Pri-miRNA – Primary microRNA

PTIBS – Porcine thoracic injury behaviour scale

RhoA – Ras homolog gene family, member A

RNA – Ribonucleic acid

ROC – Receiver operator characteristic

RT – Reverse transcription

S100β – S100 calcium-binding protein beta

SBDP – Spectrin breakdown products

SC – Spinal cord

SCI – Spinal cord injury

SEM – Standard error of the mean

sPLS-DA – Sparse partial least-squares discriminant analysis

TAAA – Thoracoabdominal aortic aneurysm surgery

TBI – Traumatic brain injury

TNF – Tumor necrosis factor

UCH-L1 – Ubiquitin carboxy-terminal hydrolase-L1

UEMS – Upper extremity motor score

UTR – Untranslated region

VEGF – Vascular endothelial growth factor

XIAP – X-linked inhibitor of apoptosis protein

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I have had the great honour and privilege of spending the last 10 years being surrounded by the incredible minds and selfless individuals who are relentless in their advocacy for our community at the International Collaboration on Repair Discoveries (ICORD). I have never known how to appropriately thank the countless mentors I've had during that time who stuck their necks out for me, believed in me, pushed and stretched me, and encouraged me throughout the long haul of my career. I have been incredibly fortunate to have been supervised and mentored by Dr. Brian Kwon, who took a risk on an inexperienced kid when I joined his team and who always provided an environment open to learning and growth as a trainee, and as a scientist. Over the last decade, Dr. Kwon has been a pinnacle of inspiration, and has helped guide me along a path that I had never dared imagine. Our biggest limitations are what we **believe** is possible of ourselves; Dr. Kwon never ceased to show us that our capacity stretches far beyond what we believe of ourselves. I hope to never forget that lesson and it is one that I *continue* to learn every day.

I will relentlessly strive to represent the Kwon lab and ICORD and I hope my path leads me back one day. I owe every individual in our team a lifetime of thanks for the lessons they have taught me in teamwork, communication, perseverance, and dedication. As curious and passionate scientists, they have been a beacon of positivity and role models in scientific trailblazing and they too, have taught me what hard work really means – my project would not be possible without the incredible support they provided. In particular, I am eternally indebted to Jae Lee for taking me under his wing, and for setting a standard for the role of a 'trainee' that will never be matched.

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Finally, it has certainly been a massive undertaking for my community to support me in this endeavour. This has been a team sport, and my PhD will be as much mine, as it is my family's. Thank you Joy, Paul, and Michelle for being my test audience and sitting through hours of my presentations. Thank you for reminding me to relax when I didn't know how, and to get back to work when I got too comfortable. I am grateful for your faithful (and financial) support. I'm sure it looked like this would never be over, but we made it.

While my PhD is coming to an end, my research has not. I hope to continue fighting for the improvement of quality of life for our patients and to advocate for individuals who need our advocacy.

Chapter 1: General introduction

1.1 Introduction overview

In the last four decades, improvements in the medical, surgical, and rehabilitative care have extended the lifespan and increased the quality of life for individuals with spinal cord injury (SCI) – once considered an imminently fatal condition. Numerous therapeutic interventions have shown promise in animal models of SCI, and a handful of these have emerged from the laboratory to be tested in the clinical setting in human SCI. Unfortunately, none have succeeded in demonstrating convincing neurologic benefit in large-scale clinical trials. With limited treatment options for acute SCI patients, there is clearly an urgent need not only for the preclinical scientific development of novel therapeutic interventions, but also the subsequent clinical validation of these treatments in human clinical trials. Unfortunately, to date there have been few clinical trials due to the limited diagnostic and prognostic tools for injury severity classification and neurologic outcome prediction. For these reasons, objective, biological markers are needed to improve our ability to diagnose injury severity and predict outcome.

This thesis focuses on the discovery of a set of biological markers that are associated with injury severity following acute traumatic SCI. Biological markers of SCI that objectively stratify the severity of cord damage could greatly expand the size and scope of clinical trials and enable the testing of novel therapies for acute SCI. Here, we focused on the discovery of a particular biomolecule type, called microRNA. MicroRNAs are short, ~22 nucleotide long non-coding RNAs (whose biological roles include post-transcriptional regulation of gene expression) that have emerged as attractive biomarker candidates owing to their stability in biological fluids, their

conservation between humans and model mammals, and their tissue specificity[1-4]. A number of microRNAs have been implicated in SCI and diverse neurological processes[5-13]. To date, there does not exist a cross-species biological marker for SCI severity, and there have been no studies investigating the changes in microRNA expression in human patients following acute traumatic SCI. Here, I focus on identifying common, cross-species microRNAs that relate to injury severity in a large animal, porcine model of acute traumatic thoracic SCI, as well as in human patients with acute traumatic SCI.

In Chapter 1, I review the current status of SCI diagnostics and the landscape of SCI biomarkers research as it stands today. In Chapter 2, I review the injury models and paradigms available for SCI research and provide justification for using our large animal model of contusive SCI for this investigation of SCI-related biomarkers. In Chapter 3, I describe our preclinical investigation of biomarkers for SCI using a large animal, porcine model of SCI. I begin by developing an injury severity paradigm in a porcine model of acute traumatic thoracic SCI in which cerebrospinal fluid (CSF) and serum samples can be collected for biomarker analysis. I develop an experimental protocol for microRNA analysis using next-generation sequencing, along with a bioinformatic pipeline for data analytics. Using these tools, I profile the microRNA changes within the CSF and serum over 5 days following traumatic, thoracic, contusive SCI in pigs. In Chapter 4, I use the experimental protocol and bioinformatic infrastructure developed in Chapter 3 to profile the microRNA changes within the CSF and serum over 5 days following acute traumatic SCI in human patients.

1.2 Classification of spinal cord injury severity

A major impediment to the clinical testing and validation of novel SCI treatments is our reliance upon the manual *functional* neurologic assessment, performed according to the International Standards for Neurological Classification of SCI (ISNCSCI). In short, the ISNCSCI examination requires the injured individual to demonstrate motor strength in the upper and lower extremities, voluntary anal contraction, and to report their perception of pinprick and light touch throughout the body, including perianal sensation and deep anal pressure (Figure 1.1). The ISNCSCI is an excellent, widely used clinical tool, which standardizes the methodology with which neurologic impairment after SCI can be measured. However, experienced clinicians recognize that in the acute SCI setting (notably, a key time for when patients are considered for recruitment into clinical trials of neurorestorative treatments), it is often impossible to conduct this assessment validly, particularly in those who have multiple injuries, brain trauma, or who are intoxicated or sedated pharmacologically. In a study evaluating factors that influence the reliability of the initial ISNCSCI examination in acute SCI, Burns and colleagues reported that almost two thirds of patients had issues such as mechanical ventilation, intoxication, and/or head injuries, that would likely affect examination reliability[14]. In a review of over 400 acute SCI patients admitted to our institution over a 4-year span, Lee and colleagues reported that concomitant injuries and co-morbidities would have made obtaining a valid ISNCSCI examination impossible in at least 30% of the patients, thus automatically excluding them from recruitment into an acute clinical trial[15]. This inability to accurately establish a functional baseline injury severity severely limits the pool of "recruitable" patients for such studies and is a major impediment to the execution of clinical trials in acute SCI.

An additional challenge for clinical trials is that even when an ISNCSCI examination can be performed and a "baseline" ASIA Impairment Scale (AIS) grade assigned to an acute SCI patient, there is considerable variability in their subsequent spontaneous neurologic recovery. This makes it necessary to recruit large numbers of patients in order to have sufficient statistical power to detect even a modest (yet meaningful) improvement in neurologic function[16]. The imprecision with which the functional ASIA grading predicts eventual neurologic outcome forces investigators to spend years enrolling large numbers of patients to achieve adequate statistical power. The challenge this imposes is illustrated by the clinical evaluation of the drug, Sygen (GM-1 Ganglioside), for which a 760-patient Phase 3 randomized clinical trial took 28 neurotrauma institutions approximately 5 years to complete recruitment[17].

Figure 1.1 International standards for neurological classification of spinal cord injury scoring sheet.

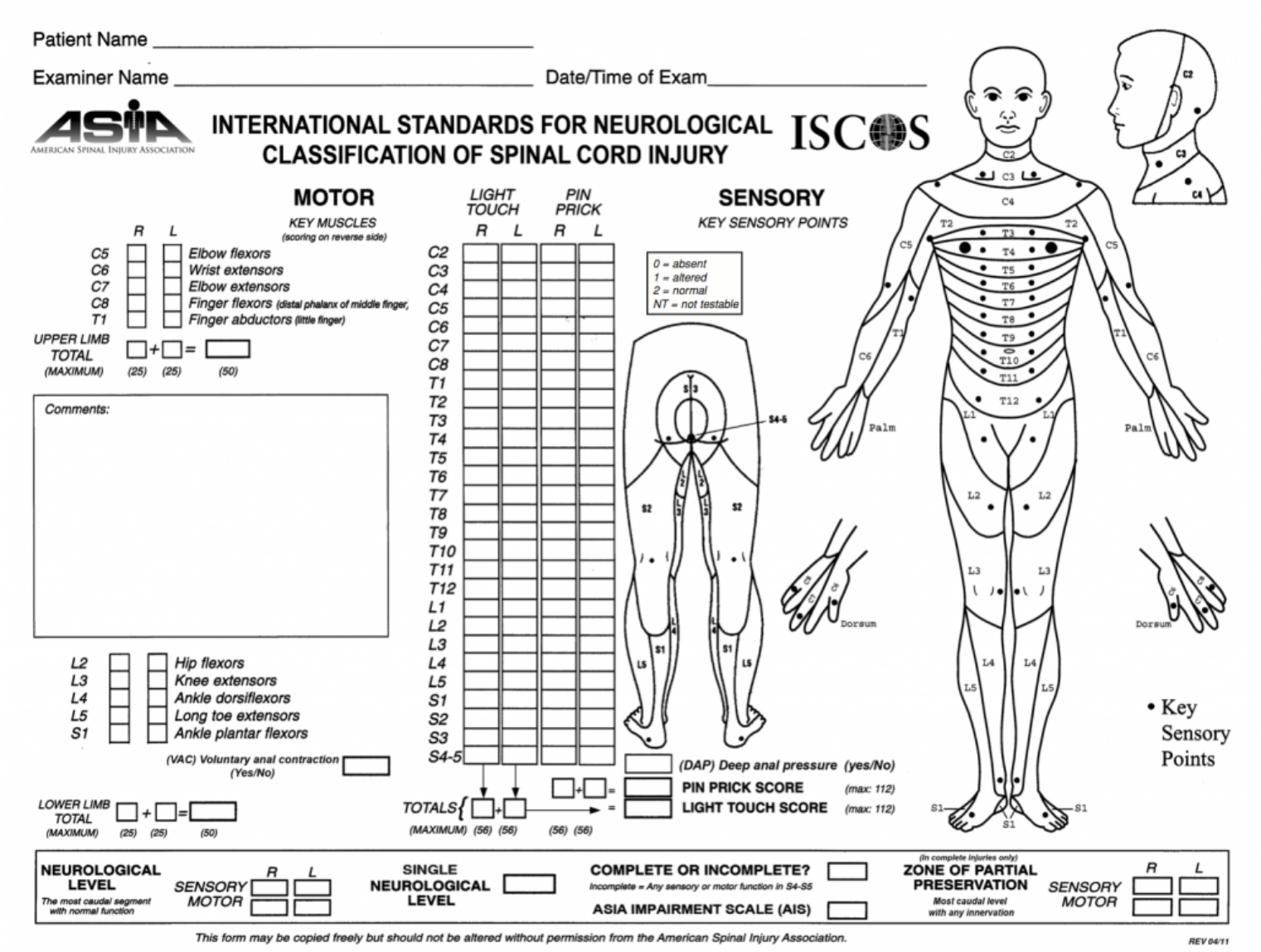


Table 1.1 American spinal injury association international standards impairment scale grades

Grade	Definition
A	**Sensorimotor complete**: No motor or sensory function is preserved in the sacral segments S4-S5
B	**Motor complete**: Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-S5
C	**Incomplete**: Motor function is preserved below the neurological level, and more than half of key muscles below the neurological level have a muscle grade less than 3.
D	**Incomplete**: Motor function is preserved below the neurological level, and at least half of key muscles below the neurological level have a muscle grade of 3 or more.
E	Normal

1.3 Biological markers for spinal cord injury

A biological marker, or 'biomarker' is defined by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[18]. Biomarkers may have a number of uses in SCI. Firstly, biomarkers may reflect the biological extent of damage to the spinal cord, and thus be useful as objective measures of injury severity. A biomarker of injury severity could supplement or - *in theory* - even be used in place of the ISNCSCI assessment of injury severity for the stratification of patients within an acute SCI trial. Secondly, biomarkers may be able to more precisely predict spontaneous neurologic recovery over time. Having a better ability to predict recovery would reduce the numbers of patients that would be needed to sufficiently power clinical trials. An objectively measured biomarker could also be useful as a surrogate biological outcome measure for a novel therapeutic intervention. While the ultimate goal for neuroprotective treatments is to promote

functional recovery, the ability to demonstrate that a treatment is even having the intended biological effect is far from trivial. Due to the high variability in spontaneous recovery, it may be near impossible to determine in a small phase 2 study whether the intervention is actually having any functional effect. Decisions about whether to proceed to large phase 3 trials would be greatly facilitated if there were some objective biological measure of whether the intervention – at the specific dose and timing of administration - was having the expected response in the cord. Biomarkers that provide a measure of such a therapeutic response could be extremely valuable in deciding whether to pursue further clinical evaluation of a drug, and if so, in determining important parameters, such as dose, time window of intervention, and monitoring schedule for a more definitive clinical trial. Additionally, biological surrogate outcome measures that crossed species could be used in both the preclinical development and subsequent clinical evaluation of novel therapeutics.

In order to establish biomarkers that are representative of acute injury within the spinal cord, it is clearly not possible to obtain samples of spinal cord parenchyma from human patients. The "tissue", then, that is in closest proximity to the injured cord is the CSF. Trauma to the spinal cord causes an acute disruption of the spinal cord parenchyma, followed by a secondary axonal degeneration and further degeneration or death of nerve cells either by apoptosis or necrosis; these processes may take days to weeks[19, 20]. Damage to the spinal cord releases proteins, metabolites, and genetic information into the environment, with the closest "environment" being the CSF that surrounds the spinal cord. It is this process that allows for the study of "neurochemical biomarkers" in the CSF[21].

Various investigators have evaluated the CSF in animal models to evaluate the relationship between neurochemical markers within the CSF and the spinal cord parenchyma after SCI. In a rodent model of acute contusive SCI, Wang et al. demonstrated that increases in IL-1β concentrations within the spinal cord tissue over the first 72 hours post injury were paralleled by increased IL-1β concentrations within the CSF[22]. Further, it was shown that this relationship between the cord and surrounding CSF was quite specific, and that systemic (serum) concentrations of IL-1β did not correlate with the concentrations in the injured spinal cord. Harrington et al. demonstrated this phenomenon with tumor necrosis factor-alpha (TNF-α)[23]. Again, the TNF-α concentrations in the cord correlated closely with those measured in the CSF, while serum TNF-α concentrations were much lower and did not correlate with spinal cord concentrations. The lack of correlation between serum and spinal cord levels was possibly due to the fact that concentrations of proteins were orders of magnitude lower and more dilute in serum, compared to CSF{Kwon, 2017 #977}.

Unfortunately, relatively little research has been conducted on CSF biomarkers for acute, traumatic SCI compared to acute traumatic brain injury (TBI). This disparity is related to the accessibility of CSF in SCI patients and the relative ease with which CSF is obtained in TBI patients through extraventricular drains for intracranial pressure monitoring. Such monitoring is possible, but not typically performed in patients with traumatic SCI, making it more challenging to obtain CSF samples at either single, or serial time points[24]. A number of CSF biomarkers have been identified with the potential ability of diagnosing acute traumatic SCI, including proteins that indicate structural damage or neuroinflammation. This chapter will focus on markers that have been described in the CSF and blood after neurological injury.

1.3.1 Classification of injury severity and prediction of recovery with biomarkers

Currently, the validation of biomarkers is tied closely to the evaluation of neurologic function using the ISNCSCI standards; however, it is conceivable that biomarkers could, if validated, by themselves provide accurate information about the extent of neurologic injury in patients who cannot be examined reliably. Because it is well established that the baseline severity of neurologic impairment after SCI is one of the most important predictors of eventual neurologic recovery, it would be expected that biomarkers of injury severity would also be able to predict neurologic recovery. After identifying injury-severity dependent expression of the protein biomarkers IL-6, IL-8, MCP-1, tau, GFAP, and S100β in the CSF of acute SCI patients, Kwon et al. generated a prediction model that used a combination of these markers at a 24-hour post-injury time point to correctly classify baseline AIS grade. Furthermore, these biomarkers were slightly better at predicting segmental motor recovery in cervical SCI than the AIS classification[25].

Kuhle et al. investigated the ability of serum neurofilament light chain (NfL) levels to predict neurological outcome as defined by the mean of the motor and sensory scores at time points 3, 6 and 12 months. NfL levels over the first 7 days post injury were higher in patients with a poor outcome compared to those with a better outcome, with an increasingly stronger correlation over time for NfL measurements after 24 hours[26].

In addition to the value of having a biological measure of injury severity and objective predictor of outcome, it would be extremely valuable to have biomarkers that could be used as biological surrogate outcome measures in acute SCI. These could then be used to monitor outcome and the

effect of interventions. For example, Kwon et al. measured CSF NfH concentrations as an indicator of minocycline treatment[21], having potential neuroprotective properties. Early NfH concentrations (days 1 – 3) were reduced, suggesting a biological effect on secondary injury mechanisms. In a rat model of SCI, NfH was used as a marker for minocycline treatment as well and was found to be reduced in plasma at 3- and 4-days post injury in association with minocycline treatment[27]. In animal models of SCI, IL-1β, MMP-9 and NOx were reduced after minocycline treatment, and in theory, such markers could be evaluated in the CSF or serum of acute SCI patients to document the biological effects of minocycline[28, 29].

Finally, while the measurement of biological markers within the CSF enjoys the advantage of its proximity to the injured spinal cord, it would obviously be of great utility to establish blood-borne biomarkers of injury. The challenge, of course, is in establishing a serum marker that is specific to the injured process within the central nervous system (CNS) and has a sufficient signal-noise ratio. As is seen in (Table 3.2), for studies that have reported both CSF and blood levels of a given marker, the CSF concentrations are typically much higher, although there are certainly candidates that are detectable and therefore appear promising in serum. In TBI, several blood-based biomarkers have been investigated, however, when examination is restricted to only a single molecule associated with damage, such as S100β, any conclusions may lack specificity due to external sources of S100β[30]. Furthermore, systemic inflammatory markers are involved in a vast number of processes and thus, are difficult to individually link to TBI. In the future, a multi-marker approach in characterizing the outcome of acute SCI would be helpful, since the evaluation of multiple markers to establish a "biological signature" could increase diagnostic and prognostic accuracy.

1.3.2 Structural biomarkers of spinal cord injury

Structural proteins that reflect injury to neural tissue are likely to be useful choices for biomarkers of injury severity in SCI. Trauma to the spinal cord causes an acute disruption of the spinal cord parenchyma and results in the release of proteins from the nervous tissue into the CSF. While structural proteins have been studied extensively in TBI and stroke[31, 32], only a handful of structural protein biomarkers have been investigated as biomarkers in acute SCI.

1.3.2.1 Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is an intermediate filament (IF) protein that is expressed in the astroglial cytoskeleton. It is found exclusively in the CNS and is released from injured glial cells. Evidence suggests it may be a useful marker for various types of brain damage, neurodegenerative disorders, stroke, and severe TBI[33].

As a biomarker of acute traumatic SCI, Kwon et al. measured CSF GFAP levels in 27 acute SCI patients and, similar to results obtained with Tau, found that CSF GFAP levels at 24 hours post injury were also dependent upon injury severity and were significantly different between AIS A, B, and C patients[25]. Pouw and colleagues found that the concentration of GFAP in AIS A patients that remained AIS A at follow up six months later was 9.6-fold higher than in AIS A patients who neurologically "converted" to AIS B[34]. More recently, Ahadi et al. investigated serum GFAP levels in 35 patients with SCI[35]. Of these 35 patients, 10 were classified AIS A, 7 were AIS B, 9 were AIS C or D, and 9 had spinal fractures with no evident trauma to the spinal cord. Increased concentrations of GFAP were found in SCI patients compared to control patients at 24, 48, and 72

hours post injury. GFAP levels at 24 hours post injury were significantly higher in the patients classified as AIS A or B compared to those classified as AIS C or D. Additionally, the 24-hour post-injury levels of GFAP were significantly higher in non-survival patients, compared to those that survived.

Aside from traumatic SCI, GFAP has been evaluated as a biomarker in ischemic SCI and in TBI. In a study of 39 patients undergoing elective thoracoabdominal aortic aneurysm surgery (TAAA), GFAP levels in the CSF of patients with and without ischemic complications were compared[36]. The patients with spinal cord ischemia had significantly higher concentrations of GFAP (571-fold), and the authors concluded that GFAP is a very promising marker for identifying patients at risk for post-operative delayed paraplegia after aortic aneurysm surgery. GFAP levels in the blood of patients with moderate-to-severe TBI were increased at admission and 12 hours after admission in patients with unfavorable neurological outcome, as well as in those patients that died [37]. GFAP has been investigated in many different acute neurologic conditions in both human and animal settings and appears to be a promising biomarker of injury severity.

1.3.2.2 Microtubule associated protein-2

Microtubule associated protein-2 (MAP-2) is primarily expressed in the nervous system and is one of the most abundant proteins in the brain[33]. It is important for microtubule stability and neural plasticity, and represents a potentially useful marker of dendritic injury.

MAP-2 has yet to be reported as a biomarker of human SCI, either in the CSF or serum. In a rat model of SCI, the extent and time window of decreases in MAP-2 levels were evaluated within

the spinal cord tissue and it was found that within 1 – 6 hours after SCI, there is a rapid loss of MAP-2 at the injury site[38]. Papa et al. found significant increases in MAP-2 protein within the CSF of patients with severe TBI. In a study of 152 patients with severe TBI, peak MAP-2 levels within the CSF were higher in patients that did not survive, compared to those that did survive[39]. Thus, it would appear that MAP-2 has potential to serve as a structural biomarker for acute neurotrauma, although studies specifically in acute SCI are lacking.

1.3.2.3 Neurofilaments

Neurofilaments (Nf) are a major cytoskeletal component of axons, neuronal soma, and dendrites. The Nf heteropolymer consists of a light chain (NfL), a medium chain (NfM), a heavy chain (NfH) and alpha-internexin polypeptides. The structure of NfM and NfH includes sidearm domains of differing lengths and, following trauma, proteolysis of these domains induces compaction of NFs resulting in impaired transport and accumulation in disconnected axons[40].

As a biomarker of traumatic SCI, neurofilaments have been studied in both the CSF and serum. Pouw and colleagues investigated the CSF-NfH concentrations within 24 hours of injury in 16 patients with traumatic SCI and found elevated levels in motor complete SCI compared to motor incomplete SCI[34]. Additionally, they found that NfH levels differed significantly between AIS B and AIS C patients. Ahadi et al. investigated the phosphorylated form of NfH (p-NfH) in the serum of patients with SCI[35]. Increased concentrations of serum p-NfH were found in SCI patients compared to control patients at 24 and 48 hours post injury and levels were higher in patients classified as AIS A, B or C, compared to those classified as AIS D. Finally, serum p-NfH levels were significantly higher in non-survival patients at 48 hours post injury, compared to patients that

did survive.

Kuhle et al. measured serum NfL concentrations in patients with central cord syndrome (CCS), the most common form of cervical SCI, resulting from trauma and leading to major injury to the central grey matter of the spinal cord[26]. Early serum NfL levels were higher in complete and incomplete SCI patients than in healthy controls or CCS patients. Furthermore, the NFL levels increased over time and remained higher in complete SCI patients compared to incomplete and CCS patients. In 2003, Guez et al. evaluated NfL concentrations in the CSF of a small series of six patients with SCI and reported that increased concentrations of NfL correlated with severity of paralysis [41]. This analysis was based on an evaluation of only three patients in whom CSF samples were collected at an early time point (24 hours post injury), while the rest were collected at three weeks post injury. In 2012, Hayakawa et al. reported in 14 acute cervical SCI patients that serum NfH levels were elevated as early as 12 hours post injury and remained elevated at 21 days post injury. The NfH levels were also statistically different in motor complete patients compared to motor incomplete. Finally, in the single AIS A patient that converted to AIS C within a 5-month period, serum levels of NfH were much lower than the AIS A patients that did not convert[42]. In addition to traumatic SCI, a study investigating NfL changes in the CSF after TAAA surgery found elevated levels in five patients with ischemic SCI compared to patients without SCI[36].

Neurofilaments have also been studied as potential biomarkers in animal models of neurotrauma. Experiments in a rodent model of SCI have reported an upregulation of NfM within spinal cord tissue between 6 and 24 hours post injury[43]. In a rodent SCI model, serum NfH levels increased following injury, showing an initial peak at 16 hours post injury and a second, usually larger peak

at 3 days post injury, returning to baseline levels by 7 days[44]. In a porcine model of nerve root injury, CSF levels of NfL were increased at 1week post injury, and in a separate porcine model of nerve root injury, increased NfL levels in CSF were also found compared to sham animals, 1 week post injury[45, 46].

Finally, neurofilaments have also been reported as biomarkers in TBI. Recently, a study using a porcine model of blast-induced TBI identified significantly increased CSF NfH concentrations at 6 hours post injury compared with pre-injury levels[47]. Consistent with these observations, in a rodent model of TBI, serum NfL levels were increased in a severity dependent fashion, with levels peaking at 24 – 48 hours post injury[48].

1.3.2.4 Neuron specific enolase

Neuron specific enolase (NSE) is one of five isozymes of the glycolytic enzyme enolase. It is localized to the cytoplasm of neurons and is not normally excreted into its environment from intact neurons. However, structural damage of neuronal cells causes leakage of NSE into the extracellular compartment, the CSF, and the bloodstream[49].

NSE has been evaluated as a biomarker of traumatic and ischemic SCI in human and animal systems. Pouw and colleagues measured CSF NSE concentrations in 16 human patients with traumatic SCI. NSE concentrations were significantly correlated with injury severity (motor complete vs motor incomplete)[34]. Ahadi et al. investigated serum NSE levels in 35 patients with SCI [35]. NSE concentrations in the serum are significantly higher at 24 and 48 hours post injury, compared to control patients. There were significantly higher levels of NSE in the serum of patients

classified as AIS C, compared to those classified as AIS D ($p < 0.05$). In a behind-armour blunt trauma model of SCI using pigs, NSE levels were increased in the CSF and serum 3 hours after injury with CSF levels of NSE reaching approximately 3 times that of the serum levels[50]. In a weight-drop contusion rodent model of SCI, serum NSE was significantly higher at 6 hours post injury, compared to control animals[51]. In another rodent SCI study of CSF and serum NSE, levels were significantly higher at 2 hours post injury compared to control, reached peak levels at 6 hours post injury, and correlated with injury severity[52]. Interestingly, the concentrations of serum NSE were very similar to those that were reported in the CSF. A study of ischemic SCI from TAAA repair reported elevated levels of NSE in CSF during aortic cross-clamping and during reperfusion[53].

As a potential biomarker for TBI, NSE was found to be significantly elevated in the serum of patients with severe TBI that did not survive, compared to those who did survive[54].

1.3.2.5 S100β

The S100 proteins are a family of calcium binding proteins that help to regulate intracellular calcium. The S100 proteins are found in astroglial and Schwann cells, in addition to adipocytes, chondrocytes, and melanocytes.

Amongst all neurochemical markers, S100β has been relatively frequently studied as a potential biomarker of traumatic and ischemic SCI in human and animal systems. Kwon et al. measured CSF levels of S100β in acute SCI patients and found that 24-hour post-injury concentrations were elevated in an injury severity-dependent fashion[25]. In a separate study of 16 patients with either

'motor complete' (AIS A, B) or 'motor incomplete' (AIS C, D), the mean CSF S100β concentration in motor complete patients was significantly higher compared with motor incomplete patients[34].

In a rodent compression model of SCI, Ma et al. reported that serum S100β levels rapidly increased following injury such that by 72 hours post injury, they had reached concentrations that were nearly five times that of the control animals[55]. In a rodent model of contusion SCI, significantly increased serum levels of S100β were observed compared to control animals at 6 hours post injury[51]. Zhang et al. documented an increase in both serum and CSF levels of S100β in a behind-armor blunt trauma model of SCI using pigs[50]. Concentrations of S100β in the CSF were reported to be approximately 10 times higher than the S100β concentrations in the serum.

Aside from traumatic SCI, S100β has been evaluated in TBI and ischemia-induced SCI. In patients with severe TBI, CSF levels of S100β were significantly higher in patients with unfavorable outcomes and were correlated with elevated intracranial pressure[56]. In a separate study of pediatric TBI, serum levels of S100β were significantly higher in patients with worse outcomes[54]. In patients undergoing TAAA repair, S100β concentrations in CSF were most elevated five minutes after reperfusion, while there were no changes found in the concentration of S100β in the serum[57, 58]. Winnerkvist et al. evaluated S100β concentrations in the CSF of 39 patients undergoing TAAA surgery and found elevated S100β concentrations in five patients who suffered an ischemic SCI, as compared with the S100β levels in those who did not[36]. Additionally, in individuals with spinal cord compression secondary to epidural abscess or metastatic lesions, elevated S100β levels were associated with a poor motor recovery[59, 60].

1.3.2.6 Spectrin breakdown products

Spectrin breakdown products (SBDP) are generated by the calpain-mediated degradation of sub-membrane cytoskeletal proteins, principally spectrins. The generation of calpain-cleaved degradation products of spectrins has repeatedly been used as a biomarker of various brain pathologies, including those induced by trauma[61].

Recently, Yokobori et al. reported increased levels of SBDP in the CSF and serum of one patient with traumatic SCI, compared to control patients with either hydrocephaly or unruptured aneurysms[33]. In a weight drop model of rodent SCI, Yokobori et al. have reported increases in SBDP120 in the spinal cord tissue 6 hours after injury, and SBDP150/145 in the CSF 4 hours after injury. In another rodent SCI study, Schumacher et al. reported increased levels of SBDP in spinal cord tissue as early as 15 minutes post injury, with levels peaking at 2 hours post injury[62].

In a study of human TBI patients, SBDP levels were measured in serially collected CSF samples taken every 6 hours for 7 days post injury[63]. SBDP concentrations were increased in patients with TBI compared to controls at every time point examined, and poor survival was associated with higher SBDP levels.

1.3.2.7 Tau

Tau is an intracellular protein that is highly enriched in neurons. It is a highly soluble microtubule binding phosphoprotein and assembles into stable axonal microtubule bundles. Upon injury, activated calpain depolymerizes microtubules. Hyperphosphorylated tau (leading to

neurofibrillary degeneration) aggregates into filamentous inclusions, termed neurofibrillary tangles that are a strong indication of axonal injury[64]. Tau from damaged microtubules is then released into the CSF and to some extent also into the systemic circulation.

Tau within the CSF has been evaluated as a biomarker of traumatic and ischemic SCI. In a study of traumatic SCI conducted by Kwon et al, CSF samples were collected from 27 human patients with complete or incomplete SCI (AIS A, B, C)[25]. Intrathecal catheters were inserted for CSF drainage and samples were collected over 72 hours. In this study, tau concentrations in CSF at 24 hours post injury were found to be dependent upon injury severity. In fact, in a more recent study, tau concentrations within 24 hours post injury were found to be 2.5-fold higher in AIS A patients who remained AIS A at follow up six months later, compared to AIS patients who neurologically "converted" to an AIS B[34]. In an investigation of 51 dogs with traumatic SCI caused by thoracolumbar or cervical intervertebral disc herniation, CSF tau levels were found to be significantly higher in dogs with lower extremity paralysis as compared to healthy dogs[65]. Furthermore, dogs that improved by one neurological grade within one week had significantly lower tau compared to dogs that needed more time to recover or did not recover.

In the setting of ischemic SCI secondary to TAAA repair, Shiiya and colleagues reported that elevated CSF levels of tau were associated with neurologic impairment[66].

Tau has also been investigated as a biomarker for TBI. The presence of tau in the CSF is a highly sensitive indicator of axonal injury in patients with diffuse axonal brain injury where its levels were shown to increase from 500- to 1000- fold at 1 hour post injury, to 40,000-fold at 24 hours

post injury and return to normal over the course of a few days[67, 68]. Shaw and colleagues evaluated the presence or absence of tau in the serum of patients with closed head injuries[69]. Patients with tau present in their serum were more likely to have an intracranial injury compared to patients with an isolated skull fracture or no CT abnormality, and were more likely to have a poor outcome, defined as either dying or requiring nursing home placement at hospital discharge. Increases in serum tau were found in a controlled cortical impact model of rat TBI[70]. In this study, serum tau at 6 hours post injury was nearly 2.5 times higher in injured animals.

In summary, tau within CSF has shown promise as a biomarker of injury severity in human and animal traumatic SCI, ischemic SCI, and in TBI.

1.3.2.8 UCH-L1

Ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) is highly abundant in the neuronal soma and is involved in the addition or removal of ubiquitin from proteins that are destined for metabolism[33].

Using a weight drop model of SCI in rats, Yokobori et al. measured an increase in CSF UCH-L1 as early as 4 hours post injury. In a single human patient with traumatic SCI, Yokobori et al. measured increases in both CSF and serum UCH-L1 at 2 days post injury with CSF concentrations reaching 50 times the serum UCH-L1 levels[33]. Currently, these preliminary studies are the only existing studies that have investigated the potential of UCH-L1 as a biomarker for acute SCI.

Mondello et al. investigated the temporal profile over seven days of UCH-L1 within the CSF and serum of 95 patients with severe TBI[71]. They found serum and CSF levels of UCH-L1 to be

increased in patients with severe TBI, with CSF levels reaching approximately 30 times that of the serum levels. The CSF and serum concentrations of UCH-L1 distinguished severe TBI survivors from non-survivors, with non-survivors having significantly higher and more persistent levels of serum and CSF UCH-L1.

1.3.3 Neuroinflammatory markers of spinal cord injury

Inflammation is thought to play a central role in the pathophysiology of secondary injury following acute SCI, and inflammatory mediators such as interleukins and other cytokines may be useful as biomarkers. Cytokines are the central mediators of cell activation and recruitment and are upregulated at the sites of traumatic injury by resident tissue cells, activated resident and recruited leukocytes, cytokine-activated endothelial cells, and some neurons. Cytokine expression is upregulated within minutes to hours of injury to the CNS, and increased levels are maintained for several days after initial trauma[72]. The timeline of heightened levels of pro-inflammatory cytokines after SCI has been summarized by Thompson et al.[73].

1.3.3.1 Interleukins

The interleukins are a group of cytokines that are involved in the inflammatory response and can be either pro- or anti-inflammatory, or both, depending upon the temporal pattern of expression. Kwon et al. found a severity dependent expression of IL-6 and IL-8 in the CSF of human patients with complete or incomplete SCI[25]. In a Taiwanese study of 7 patients with acute SCI, an increased concentration of IL-6 and IL-8 was observed in the CSF of patients with complete SCI[74].

Using a rodent weight-drop, contusive model of thoracic, mild and severe SCI, Yang and

colleagues reported that concentrations of the inflammatory proteins, IL-1β, IL-6, and TNF-α mRNA and protein in the spinal cord were significantly increased after a severe, but not mild injury[75].

In addition to traumatic SCI, the study of non-traumatic SCI is relevant to the discussion of inflammatory biomarkers. In the CSF of patients with SCI secondary to transverse myelitis, Kaplin et al. demonstrated a 262-fold increase in CSF IL-6 concentrations when compared to control patients[76]. They also found a strong correlation between IL-6 concentrations and the clinical severity of paralysis. In a study of patients undergoing TAAA repair, Kunihara et al. found that the occurrence of ischemic paralysis was associated with increased concentrations of IL-8 in the CSF[77]. In patients with severe TBI, CSF levels of IL-1β were significantly higher in patients with unfavorable outcomes[56] and Kushi et al. have found increases in CSF and blood concentrations of IL-6 and IL-8 following severe head injury[78].

1.3.3.2 Monocyte Chemoattractant Protein-1

The monocyte chemoattractant protein-1 (MCP-1), also known as the chemokine (C-C motif) ligand 2 (CCL2), is a pro-inflammatory cytokine that appears to be associated with a poor outcome in studies of brain injury. Over-expression of MCP-1 increases brain infarct volume, and exacerbates secondary damage after brain injury, and mouse mutants deficient in genes for MCP-1 show decreased inflammatory infiltration and infarct size[72]. MCP-1 is recognized as having an important role in the recruitment of type I monocytes and is the most potent activator of signal transduction pathways that leads to monocyte transmigration[72].

Kwon et al. reported a marked increase in MCP-1 concentrations in the CSF collected from human patients with complete or incomplete SCI and upregulation was positively correlated with injury severity[25]. In a Taiwanese study of 7 patients, an increased concentration MCP-1 was observed in the CSF of patients with complete SCI[74].

In a mouse contusion model of SCI, MCP-1 mRNA increased in the spinal cord after 5 minutes of injury and remained elevated for 4 days[79]. In a rodent model of SCI in which the cord was subjected to impactor forces of 100, 150, or 200 kilodynes, there was an increased expression of MCP-1 in the 150 and 200 kilodyne injuries as compared to the mild 100 kilodyne injury, and MCP-1 levels correlated with subsequent neuropathic pain[80].

In addition to SCI, MCP-1 levels in the blood of patients with moderate-to-severe TBI were increased at admission and 12 hours after admission in patients with unfavorable neurological outcome, as well as in those patients that died[37].

1.3.3.3 Tumor Necrosis Factor

Tumor Necrosis Factor (TNF) is a pro-inflammatory cytokine mainly expressed by microglia that plays an important role in the control of cell proliferation, differentiation, and apoptosis[81]. TNF recruits macrophages following nerve injury and modulates the expression of cell adhesion molecules, which are required for the migration of leukocytes to sites of injury[82].

Despite likely playing an important role in secondary injury, TNF-α has not yet been reported in the CSF or serum of traumatic SCI patients as a potential biomarker. Yang et al. reported elevated

TNF-α mRNA and protein in the spinal cord after severe injury but not mild injury[75].

Following human TBI, increases in TNF-α levels have been reported in both the CSF and serum. Hayakata et al.[56] examined CSF from 23 patients with severe TBI and found an increase in CSF TNF-α concentrations at 24 hours post injury. The serum concentrations of TNF-α were over 10 times lower than in CSF and did not significantly change over time. Stein and colleagues analyzed CSF and serum from 24 patients with severe TBI and reported that increased serum, and not CSF concentrations of TNF-α moderately correlate with increases in intracranial hypertension and cerebral hypoperfusion[83].

Table 1.2 Summary of studies describing potential biomarkers of spinal cord injury

Biomarker	Description	Evidence as a biomarker in Human SCI	Evidence as a biomarker in Animal SCI Models	Evidence as a biomarker in TBI
SBDP	Generated by the calpain-mediated degradation of sub-membrane cytoskeletal proteins, namely spectrins.	Increased CSF and serum levels in one patient with traumatic SCI[33].	Increased levels of SBDP120 in spinal cord tissue at 6 hpi and increases in CSF SBDP150 at 4 hpi in a rodent SCI model[33]. Increased in spinal cord tissue as early as 15 minutes post-injury, peaking at 2 hours post-injury[62].	Increased CSF levels in patients with TBI compared to controls. Levels were significantly higher in non-survival patients[63].
GFAP	Intermediate filament protein that is expressed in the astroglial skeleton. Released from injured glial cells.	Increased serum levels in SCI patients compared to controls[35]. Severity dependent expression in CSF from patients with acute SCI[34]. Severity dependent expression in CSF from patients with acute SCI [25, 84].		Increased levels in peripheral blood correlated with unfavorable outcome in patients with moderate-to-severe TBI[37].
MAP-2	Dendritic-specific protein important for microtubule stability and neural plasticity.		Rapid loss of MAP-2 at the injury site in a rodent SCI model[38].	Increased levels in the CSF of patients with severe TBI[39].
NfH	Heavy chain polypeptide of neurofilament heteropolymer that forms a major cytoskeletal component of axons.	Increased serum levels in SCI patients compared to controls[35]. Severity dependent expression in CSF from patients with acute SCI[34]. Severity dependent expression in serum from patients with acute SCI[42].	Increased serum levels in a rodent model of SCI[44].	Increased CSF levels in pigs after blast-induced traumatic brain injury[47].
NfL	Light chain polypeptide of neurofilament heteropolymer that forms a major cytoskeletal component of axons.	Severity dependent expression in serum from patients with acute SCI[26].	Increased levels in CSF using a nerve root injury model in pigs[45].	Severity dependent expression in serum using a rat model of TBI[48].

Biomarker	Description	Evidence as a biomarker in Human SCI	Evidence as a biomarker in Animal SCI Models	Evidence as a biomarker in TBI
		Severity dependent expression in CSF (3 patients) from patients with acute SCI[41].		
NfM	Medium chain polypeptide of neurofilament heteropolymer that forms a major cytoskeletal component of axons.		Increased NfM in spinal cord tissue between 6 and 24 hours post injury in a rodent model of SCI[43].	
NSE	Isozyme of the glycolytic enzyme enolase. It is localized to the cytoplasm of neurons and usually only elevated following cell injury.	Increased serum levels in SCI patients compared to controls[35].	Increased CSF and serum levels in a pig behind-armor blunt-trauma model of SCI[50].	Increased serum levels in non-survival group of patients with severe TBI, compared to survival group[54].
		Severity dependent expression in CSF from patients with acute SCI[34].	Severity dependent levels in serum and CSF in a rodent model of SCI[52].	Severity dependent expression in CSF from children with brain injury. Lower NSE Levels correlated with better outcome[85].
			Increased serum levels in a weight drop contusion model of SCI using rats[51].	
S100-β	Calcium binding protein found in astroglial and schwann cells.	Severity dependent expression in CSF from patients with acute SCI[34].	Increased CSF and serum levels in a pig behind-armor blunt-trauma model of SCI[50].	Increased CSF levels in patients with unfavorable outcomes. Levels correlated with intracranial pressure[56].
			Increased serum and CSF levels at 6 hours post injury in a rodent model of SCI[51].	
		Severity dependent expression in CSF from patients with acute SCI[25, 84].		Increased serum levels in non-survival group of patients with severe TBI, compared to survival group[54].
			Increased serum levels in a rodent model of SCI[55].	

Biomarker	Description	Evidence as a biomarker in Human SCI	Evidence as a biomarker in Animal SCI Models	Evidence as a biomarker in TBI
Tau	Microtubule-binding phosphoprotein highly enriched in axons. Released from damaged microtubules due to activation of calpain depolymerization upon injury.	Severity dependent expression in CSF from patients with acute SCI[34].	Severity dependent expression in CSF in a canine model of SCI secondary to intervertebral disc herniation[65].	Increased CSF levels of tau in patients with hydrocephalus[67].
		Severity dependent expression in CSF from patients with acute SCI[25, 84].		Elevated CSF levels in patients with TBI correlated with clinical outcome[68].
UCH-L1	Highly abundant in the neuronal soma and is involved in the addition or removal of ubiquitin from proteins that are destined for metabolism	Increased levels in CSF and serum of one patient with traumatic SCI[33].	Increased levels in CSF at 4 hours post-injury in a rodent model of SCI[33].	Increased serum and CSF levels in patients with severe TBI. UCH-L1 levels were significantly higher and more persistent in non-survivors compared to survivors[71].
Interleukins	A group of cytokines that are involved in the inflammatory process.	Severity dependent expression of IL-6 and IL-8 in CSF from patients with acute SCI[25, 84].	Increased expression of IL-1b, and IL-6 in severe, but not mild injury in a rodent model of SCI[75].	Increases in IL-6 and IL-8 in the CSF and peripheral blood after severe head trauma[78].
		Increased levels of IL-6 and IL-8 in the CSF of patients with complete SCI[74].		Severity dependent expression of IL-6 in the CSF of children with severe head trauma[86].
MCP-1	A pro-inflammatory chemokine, involved in the recruitment of type I monocytes and is the most potent activator of signal transduction pathways that lead to monocyte transmigration.	Severity dependent expression in CSF from patients with acute SCI[25, 84].	Severity dependent expression in spinal cord in a rodent model of SCI. Levels correlated with neuropathic pain[80].	Increased levels of MCP-1 in peripheral blood correlated with unfavorable outcome in patients with moderate-to-severe TBI[37].
		Increased levels in the CSF of patients with complete SCI[74].	Increases in MCP-1 in the spinal cord tissue within 5 minutes of injury, remaining elevated for 4 days[79].	
TNF	Pro-inflammatory cytokine mainly expressed by microglia. Plays an important role in the	Early elevations in TNF-R1 within CSF of patients with acute SCI[21].	Increased levels in spinal cord tissue after severe, but not mild injury in a rodent model of SCI[75].	Increased levels in CSF at 24 hours post injury in patients with brain injury[56].

Biomarker	Description	Evidence as a biomarker in Human SCI	Evidence as a biomarker in Animal SCI Models	Evidence as a biomarker in TBI
	control of cell proliferation, differentiation, and apoptosis			

1.3.4 MicroRNA

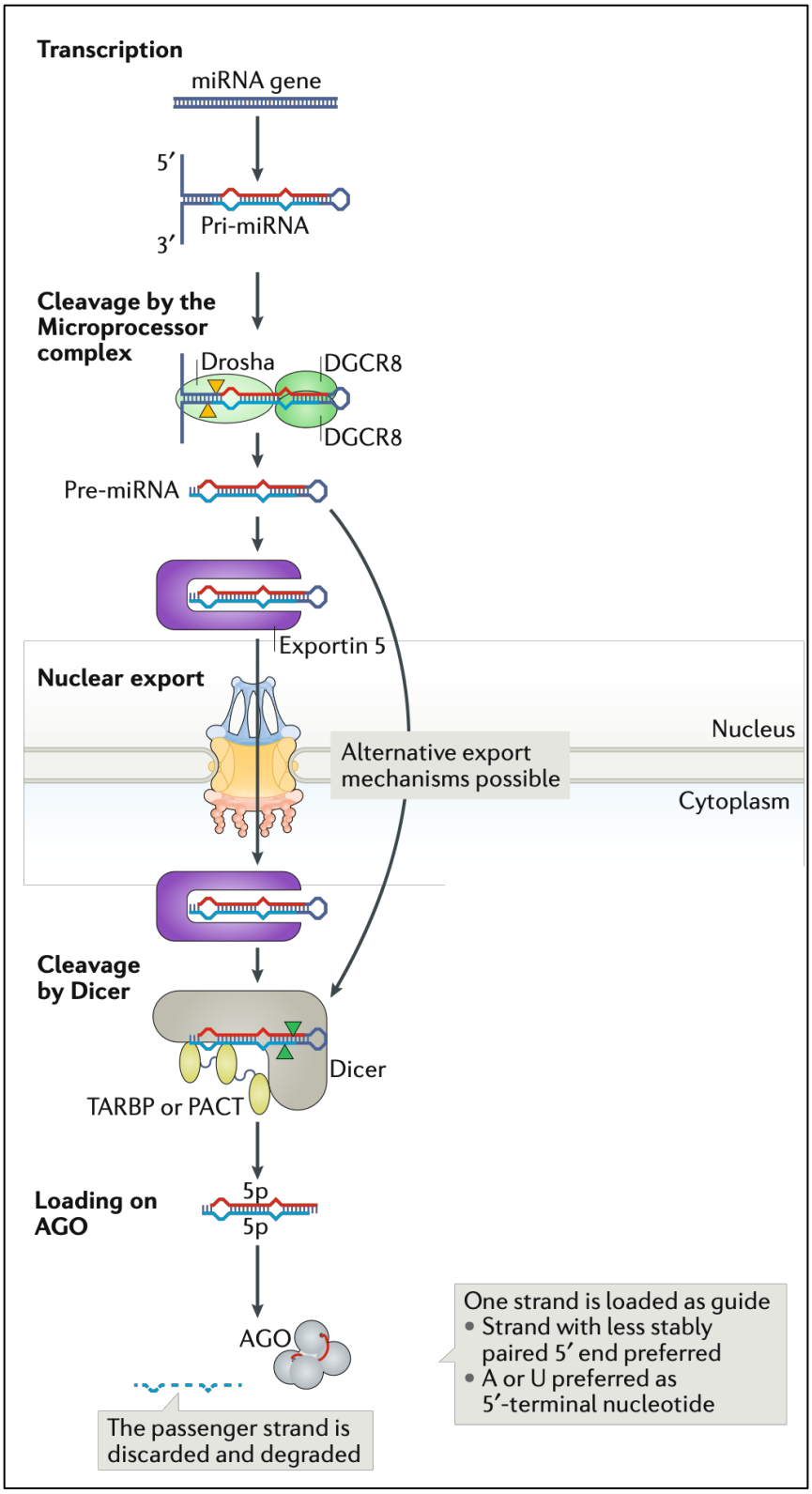
Since the serendipitous discovery of microRNAs in 1993 by Victor Ambrose[87] and Gary Ruvkun[88], microRNAs have emerged as key negative regulators of biological processes. These small RNA molecules are approximately 22 nucleotides in length and form complex networks that regulate nearly all developmental and pathological processes. There are now over 2,000 microRNAs that have reportedly been discovered in humans, and it is believed that they collectively regulate one third of the genes in the human genome[89]. Dysregulation in microRNA expression is increasingly found to be associated with disease, and the detection of dysregulated microRNAs has been of growing interest in developing biomarkers for disease.

1.3.4.1 MicroRNA biogenesis and regulation

MicroRNAs are single-stranded RNA that exhibit tissue-specific expression patterns[90]. MicroRNA biogenesis is summarized in Figure 1.2. Briefly, microRNAs are derived from hairpin precursor microRNAs (pre-miRNA). RNA polymerase II or III first transcribes a molecule in the nucleus of the cell, producing a large primary transcript, called a pri-microRNA[91]. The pri-microRNA is then cleaved by a nuclear RNase III enzyme complex of Drosha[92] and DiGeorge Syndrome Critical Region-8 (DGCR8)[93] releasing a hairpin molecule of ~70 nucleotides in length, called the pre-microRNA. The pre-microRNA hairpin end has a 2-nucleotide overhang at the 3' end, a 5' phosphate, and a 3' hydroxyl, which are typical of RNase III products[94]. The resulting overhang is recognized by exportin-5, a Ran-GTP-dependent molecule that translocates the pre-microRNA molecule into the cytoplasm, where it is processed into a ~22 nucleotide microRNA duplex by the cytoplasmic RNase III enzyme Dicer[95, 96]. Dicer recognizes the 5' phosphate group, the 3'

overhang, and the loop structure, and cleaves the pre-microRNA, resulting in a mature microRNA duplex. Only one of the duplex's strands ('guide' strand) are incorporated into the argonaute complex (AGO), whereas the other ('passenger' strand) is discarded[97]. The preference for incorporating one strand and not the other onto the AGO protein is given to the strand with the least stable 5' end[98]. AGOs constitute a large family of proteins that use single-stranded small nucleic acids as guides to complementary sequences in RNA or DNA targeted for silencing[99]. The AGO protein, bound to a microRNA forms the complex that targets mRNAs, called the microRNA-induced silencing complex (miRISC), which promotes degradation or repression of translation in targeted mRNAs[100]. A single microRNA can silence hundreds of target genes and multiple microRNAs can regulate the same gene[101]. Once bound to AGO, microRNAs are thought to be relatively stable, but turnover rates are dictated by multiple factors and can range from minutes to days[102]. Turnover rates can depend on tissue context, with faster turnover rates having been measured in neuronal microRNAs compared with other tissues[103].

Figure 1.2 MicroRNA biogenesis and incorporation into the argonaute protein. *Adapted from Gebert et al*[104]



1.3.4.2 Extracellular microRNA

Besides those microRNA that are present within cells, substantial amounts of microRNA have been detected outside of cells as well, and in nearly all known biofluids including blood, plasma, serum, milk, tears, saliva, urine, cerebrospinal fluid, and semen[2, 105, 106]. Due to their association with carrier proteins, these extracellular microRNAs were found to be extremely stable: resistant to high doses of RNAses, freeze-thaw cycles, and pH fluctuations, and concentrations of microRNAs did not significantly change with prolonged incubation of blood plasma at room temperature[2, 107]. A fraction of these circulating microRNAs were found to be localized in extracellular vesicles (EVs)[107, 108]. Nearly all cell types form and excrete several types of EVs including microvesicles and exosomes. Microvesicles are formed after outward budding of the plasma membrane and are between 100 – 1000 nm in size[109], while exosomes are released after a fraction of multivesicular bodies are fused with the plasma membrane and are much smaller in size (40 – 100 nm)[110]. MicroRNAs are also found in apoptotic bodies, which are approximately 1 – 4 uM in size[111]. Besides EVs and apoptotic bodies, a large fraction of microRNAs are solely free-floating complexes with AGO proteins[108].

The biological significance of secreted microRNAs still remains unclear[112-114]. Cells may excrete microRNAs to get rid of unwanted or 'trash' RNAs[115]. For example, after reducing target mRNA expression, unused microRNAs can become redundant and disposed of in exosomes[116]. Alternatively, the finding of sequence-specific microRNA sorting into EVs[117] suggests a system for cell-to-cell communication via circulating microRNAs. To date, a variety of studies have revealed that microRNA can be transferred between different cell types through EVs and regulate target genes in the recipient cells[118]. It remains unclear whether only a small fraction of circulating

microRNAs travel within exosomes (~10% or less in plasma)[107, 114] or whether exosomal microRNAs make up the majority of circulating microRNAs (83-99% in serum)[119]. While there is evidence for the biological function of exosomal microRNAs[120], it is not currently known if free-floating microRNAs, not packaged into exosomes, have a biological role[114], and if there are mechanisms for AGO-bound microRNAs to penetrate through the membrane of mammalian cells[113]. While there is evidence for both scenarios, the theory that circulating microRNAs play a significant role in translational regulation and cell-to-cell communication, as well as that circulating microRNAs are products of cellular debris and cell death and play little role in such regulation, are questions that extend beyond the goals of this thesis. While each are important questions to address, the impacts on both the experimental design and data interpretation in this thesis are minimal as we can remain relatively agnostic about the role of these molecules, so long as they are able to act as powerful diagnostic and prognostic markers of CNS damage – specifically, as markers of SCI severity. Half of the identified tissue-specific microRNAs are CNS-specific, promoting homeostatic functions on CNS gene expression[121]. With regards to choice of biofluids for the identification of microRNA biomarkers that reflect SCI injury, CSF is advantageous due to its proximity to the injured spinal cord, and may reflect a more representative signature of the pathological processes within the cord. However, the collection of CSF is invasive and a more convenient biofluid would be ideal. Blood serum is a less invasively collected biofluid, more readily available, and may contain CNS-derived microRNA that are informative of the status of the injured spinal cord, however it also contains microRNA signals from all tissues of the body. Circulating microRNAs, whether packaged in EVs or free-floating in the CSF and serum, represent indicators of disease-relevant information, and are attractive candidates for monitoring injury

status after SCI. Profiling cell-free microRNA represent promising targets due to the potential reduction in noise from blood cell and immune-cell derived microRNAs.

1.3.4.3 MicroRNAs in the central nervous system

MicroRNAs have emerged as important molecules that control a diverse range of biological processes, and are predicted to control expression of nearly 30% of all protein coding genes[122]. MicroRNAs are necessary for development: in mice, for example, global deletion of microRNAs through the deletion of the gene that encodes DICER, leads to arrested development at embryonic day 7.5[123]. MicroRNAs are also necessary for development of the CNS, with targeted ablation of the DICER gene in nervous system tissue yielding a smaller cortex[124], and deletion of AGO proteins (and thus microRNA function) preventing neural tube closure[125]. Many microRNAs have been identified in the CNS[126-129], with several microRNAs showing tissue specific expression[130]. Using microarray, real-time PCR, and in-situ hybridization, Bak et al. found 44 microRNAs that showed more than threefold enrichment in the brain or spinal cord and Liu et al. found that nearly 80% of detected microRNAs were expressed in the adult rat spinal cord[126, 131]. MicroRNAs also show cell-specific expression in the CNS, with specific microRNAs being expressed in neurons[130, 132], astrocytes[133, 134], and oligodendrocytes[135]. The high abundance and tissue specificity of microRNAs in CNS tissue make them promising candidates for diagnostic biomarkers in CNS injuries and disease.

Injury to the CNS causes dramatic changes to the cellular environment, which arise as a result of dysregulation in signalling pathways and structural proteins. Since microRNAs have recently emerged as key regulators of a wide array of molecular networks, identifying their specific

modulation following CNS damage could help provide insight into the injury response. MicroRNA expression studies have shown altered microRNA levels in brain, spinal cord, blood, CSF, and/or saliva, following injuries to the CNS[1, 8, 136-138].

1.3.4.4 MicroRNAs in spinal cord injury

SCI develops as a result of a primary injury, followed by a secondary injury[139]. The primary SCI is a result of the physical forces of the initial traumatic event and is often the most important factor in determining injury severity[140]. The primary injury results in shearing of cell membranes, severed axons, ruptured blood vessels, and disruption of the blood-spinal cord barrier[141-143]. Following the primary injury, a cascade of injury processes and inflammation contribute to the secondary injury, including extensive temporal changes in gene expression[140, 144, 145]. Given the specificity of expression of microRNAs within the spinal cord, they represent promising molecules as indicators of injury severity and progression: first, as biomarkers of acute cellular destruction of the primary injury, and second, as indicators of the progression of the secondary injury, as regulators of the cell death and inflammation processes that occur in the days to weeks post injury.

While there have been relatively few studies investigating microRNA changes following traumatic SCI, the effort to describe such changes is increasing, with most studies being restricted to animal models, as outlined in Table 1.3[11, 13, 131, 134, 146-150]. In a contusion SCI model using rats, Liu et al.[131] profiled microRNA changes using microarray analysis and showed that expression of 60 detected microRNAs were altered in the spinal cord tissue following SCI. Of those 60, 30 were upregulated, 16 were downregulated and 14 were upregulated at 4 hours post injury and downregulated at 1 – 7 days post injury. A study by Yunta et al.[10] identified nearly 350 microRNAs with significant

changes in the spinal cord tissue of rats following contusive SCI, with 200 microRNAs being downregulated at 7 days post injury. Using a moderate contusion SCI in rats, Hu et al.[151] identified 9 upregulated microRNAs and 5 downregulated microRNAs at 24 hours post injury in the spinal cord. MicroRNA expression is also dependent on the duration of injury compression[152], with greater dysregulation in the spinal cord of rats that had 1 minute of spinal cord compression versus those that had 1 second of compression. To date, the study described in chapter 4 of this thesis is the only study to have profiled the temporal changes in microRNA expression in human patients with acute traumatic SCI.

1.3.4.5 MicroRNAs in traumatic brain injury

Like SCI, the heterogeneity of injury in TBI cases makes an accurate assessment of the severity of trauma and prediction of patient outcome, challenging. Injuries are classified as mild, moderate, and severe according to the Glasgow Coma Scale (GCS) score, where this scale might underestimate mild TBI cases[153]. Due to the challenge in accurately diagnosing injury severity in TBI, mild and moderate TBI detection is one of the most difficult clinical diagnoses. Furthermore, like microRNAs expressed within the spinal cord, those expressed within the brain show region-specific expression[126], and represent promising molecules as biomarkers for diagnosing injury severity in TBI patients. To date, 6 studies investigating microRNA changes in human TBI patients have been conducted, with a combination of male and female patients, and a wide range of ages[8, 154-158]. Additionally, 15 studies have been conducted in animal models, mostly focusing on male mice and rats with a variety of injury modalities[157, 159-172].

1.3.4.6 MicroRNAs as biomarkers for CNS injury

MicroRNAs represent promising molecules to address two primary goals: developing diagnostic and prognostic tools for CNS injury, and for creating novel therapeutics. A number of studies have identified specific changes in microRNA expression that may function as biomarkers for injury to the CNS. In a mouse model of contusive SCI, Hachisuka et al.[146] showed that serum miR-9 levels increased at 3 hours post injury and proposed miR-9 as a promising candidate marker for SCI. MiR-9 is preferentially expressed in the CNS with an important role in oligodendrocyte differentiation and myelin maintenance[135]. Increases in miR-9 might then be associated with the release of microRNAs from the destruction of myelin in the spinal cord. MiR-10b is enriched in the spinal cord tissue[173] and may represent an important marker of destruction to the spinal cord parenchyma. In patients with glioblastoma, miR-10b is significantly increased and is a promising CSF biomarker[174]. Interestingly, in glioblastoma patients, miR-10b was present in the CSF samples, even when CSF cytology analysis produced negative results, suggesting that miR-10b may serve as a highly sensitive CSF marker. MiR-21 has been repeatedly found to increase following SCI[10, 131, 134], and was found to be upregulated in neurons following cerebral ischemia[175]. MiR-21 likely plays a role in astrogliosis[134] and targets Faslg, a TNF-α family member and an important cell death-inducing ligand[175], and may be important not only as a biomarker but also as a therapeutic target for SCI. MiR-23b has been shown to decrease following SCI, while the levels of its target, NOX4, a reactive oxygen species, increased. Further, Im et al.[176] showed that application of synthetic miR-23b reduced NOX4 levels, and improved neuropathic pain outcomes following SCI. MiR-122 levels are an early marker of cerebrovascular disease, and application of miR-122 improved survival of rat endothelial cerebral cells[177]. MiR-124-3p is reported to be one of the most abundant microRNAs found in neurons, with expression levels nearly 100 times those

in other tissues[178]. Louw and colleagues have proposed the delivery of exogenous miR-124 as a potential therapeutic intervention for the treatment of SCI[179]. MiR-125b has been shown to promote regeneration in a rat model of SCI, in a process involving the direct downstream regulation of Sema4D, a transmembrane axonal repulsion cue[180]. MiR-128 is preferentially expressed in mature neurons[135], and has been shown to be upregulated following contusive SCI in rats, at 4 hours post injury[131]. Yu et al.[181] found that miR-133 is involved in functional recovery post SCI in adult zebrafish, suggesting roles in targeting RhoA, enhancing regrowth of the corticospinal tract, and promoting neuroprotection. MiR-150-5p was upregulated following oxidative stress in rat neuronal spinal cord cells[182]. MiR-181 is highly expressed in the adult CNS[183] suggesting it may be a promising biomarker for CNS damage and cell death. Overexpression of miR-181 leads to increased levels of the anti-inflammatory cytokine, IL-10, through action on its target, MeCP2, the methyl CpG-binding protein 2, and XIAP, an X-linked inhibitor of apoptosis mRNA[183]. MiR-195-5p has been shown to decrease following SCI in a rat model of SCI[184]. Downregulation of miR-195 corresponded to an increase in BCL-2, VEGF, and HIF-1α, suggesting it plays a role in regulating hypoxia-related apoptosis. Interestingly, Tao et al.[184] showed reduced functional recovery, post-SCI, following application of synthetic miR-195 into the rat spinal cord. MiR-204 levels increased in plasma after spinal cord ischemia-reperfusion injury in human patients, where mir-204 targets the apoptotic regulator, BCL-2[185], and regulates cell death in neuronal cells. Further, Gao et al.[186] found that miR-204 negatively regulates neuritin-1 and activates caspase-3, enhancing the sensitivity of Schwann cells to oxidative stress and apoptosis. MiR-219a-5p has been found to increase following mild and severe SCI in a mouse model of contusive SCI, with levels reaching their maximum at 12 hours post injury[146]. MiR-219 directly represses the expression of oligodendrocyte progenitor cell proliferation promoters,

indicating a critical role of miR-219 in coupling differentiation to proliferation arrest in the oligodendrocyte lineage[187]. Finally, Boon et al.[188] showed that levels of miR-208 and miR-499 progressively declined following SCI in skeletal muscle and are thought to regulate skeletal muscle mass. Presence of these microRNAs in serum may indicate muscle atrophy as a result of SCI.

Each of the microRNAs described above have been found to change in human CSF or serum following acute traumatic SCI or in the serum of a porcine model of contusive SCI and often in both humans and pigs, as highlighted in Table 1.4 and as described in chapters 3 and 4. The diversity of these microRNA changes encourage our efforts to develop them as biomarkers for SCI.

Table 1.3 Review of studies investigating microRNA changes in spinal cord injury.

Species	Tissue	MicroRNA detection method	Injury model	Level of injury	Time points	MicroRNA changes
Zebrafish[181]	SC	RT-PCR	Transection SCI	4 mm caudal to brain	- 6 hpi - 1 dpi - 7 dpi	MiR-133b upregulated at 1 dpi and 7 dpi
Mouse[146]	Serum, SC	Microarray	Contusive SCI	T10	- 3 hpi - 12 hpi - 1 dpi - 3 dpi - 7 dpi	29 microRNAs showed severity-dependent expression with higher levels in severe vs mild vs SHAM. MiR-9, miR-219, miR-384 levels peaked between 3-12 hpi, returning to baseline by 7 dpi
Mouse[147]	SC	Microarray	Transection SCI	T11	- 1 dpi - 2 dpi - 3 dpi - 7 dpi	265 microRNAs differentially expressed (specifics not shown or discussed)
Mouse[149]	SC	Microarray	Compressive SCI	T11-12	- 12 hpi	12 hpi: 5 up, 5 down
Rat[189]	SC	Microarray	Ischemic-reperfusion SCI	Lumbo-sacral segments	- 2 dpi	2 dpi: 38 up, 10 down
Rat[151]	SC	Micorarray	Contusive SCI	T10	- 1 dpi - 3 dpi	1 dpi: 9 up, 5 down 3 dpi: 3 up, 5 down
Rat[13]	SC	Microarray	Contusive SCI	T12-13	- 4 dpi - 14 dpi	Between 4-14 dpi 4 up, 32 down
Rat[131]	SC	Microarray	Contusive SCI	T10	- 4 hpi - 1 dpi - 7 dpi	4 hpi: 23 up, 18 down 1 dpi: 27 up, 30 down 7 dpi: 30 up, 30 down
Rat[10]	SC	Microarray	Contusive SCI	T8	- 1 dpi - 3 dpi - 7 dpi	1 dpi: No changes 3 dpi: 5 up, 46 down 7 dpi: 11 up, 192 down

Species	Tissue	MicroRNA detection method	Injury model	Level of injury	Time points	MicroRNA changes			
Pig[4]	CSF, serum	Small RNA-Seq	Contusive SCI	T10	- 1 dpi - 3 dpi - 5 dpi	Severe	Moderate	Mild	SHAM
						1 dpi: 5 up, 0 down 3 dpi: No changes 5 dpi: 2 up, 0 down	1 dpi: 5 up, 0 down 3 dpi: No changes 5 dpi: 2 up, 0 down	1 dpi: 5 up, 0 down 3 dpi: No changes 5 dpi: 2 up, 0 down	1 dpi: 5 up, 0 down 3 dpi: No changes 5 dpi: 2 up, 0 down
Human[190]	CSF, serum	RNA-Seq, RT-PCR	Acute traumatic SCI	Variable	- 1 dpi - 2 dpi - 3 dpi - 4 dpi - 5 dpi	AIS A	AIS B	AIS C	
						1 dpi: 84 up, 26 down 2 dpi: 75 up, 19 down 3 dpi: 73 up, 19 down 4 dpi: 78 up, 25 down 5 dpi: 69 up, 18 down	1 dpi: 51 up, 9 down 2 dpi: 59 up, 12 down 3 dpi: 30 up, 8 down 4 dpi: 22 up, 9 down 5 dpi: 22 up, 10 down	1 dpi: 39 up, 6 down 2 dpi: 52 up, 16 down 3 dpi: 26 up, 8 down 4 dpi: 15 up, 14 down 5 dpi: 17 up, 8 down	

Table 1.4 Review of central nervous system microRNA changes.

MicroRNA	Injury Model	Tissue	Observation	Role	Biomarker	Pig SCI	Human SCI
miR-9[146]	Mouse Contusive SCI	Serum	Upregulated	Oligodendrocyte differentiation, myelin maintenance	Biomarker of myelin damage	No change in serum	Upregulated in CSF
miR-10b[173, 174]	Human patients with glioblastoma	CSF	Upregulated	Enriched in brain and spinal cord tissue; present in glioblastoma patients, even when CSF cytology tests were negative. Not present in healthy controls	Biomarker of neuronal damage, death	Upregulated in serum	Upregulated in CSF
miR-21[10, 131, 134, 175]	Mouse Contusive SCI	SC	Upregulated	Enriched in brain and spinal cord tissue, miR-21 targets Faslg, a TNF-α family member, regulated cell death. Regulates astrogliosis	Biomarker of neuronal damage, astrogliosis	Upregulated	Upregulated in CSF
	Rat Contusive SCI	SC	Upregulated				
	Rat Contusive SCI	SC	Upregulated				
	Rat Cerebral Ischemia (MCAo)	Cortex	Upregulated				
miR-23b[176]	Mouse neuropathic pain	SC	Downregulated	Targets NOX4, a reactive oxygen species. Application of miR-23b reduces neuropathic pain.	Biomarker of inflammatory processes	Upregulated in serum	Downregulated in CSF
miR-122[177]	Stroke prone, hypertensive rats	Brain	Downregulated	Regulates oxidative stress, inflammation, endothelial dysfunction, and apoptosis. Application of miR-122 produced a protective effect on survival of cerebral endothelial cells.	Biomarker of cerebrovascular damage	No change in serum	Downregulated in serum of AIS A patients

MicroRNA	Injury Model	Tissue	Observation	Role	Biomarker	Pig SCI	Human SCI
miR-124[178, 179]	Rat transection SCI	SC	Upregulated	One of the most abundant neuronal microRNAs, with expression levels 100x other tissues. Reduces reactive oxygen species, regulates TNF-α, and reduces expression of MHC-II.	Biomarker of inflammatory response, and neuronal damage	No change in serum	Upregulated in CSF
miR-125b[180]	Axolotl SCI (2 mm of SC removed)	SC	Downregulated	Expression is concentrated in radial glial cells. Inhibition of miR-125b inhibited axon regeneration.	Biomarker of astrocytic damage, axonal regeneration, glial scar response	Upregulated in serum	Upregulated in CSF
	Rat transection SCI	SC	Downregulated	Enriched in astrocytes. Application of miR-125b decreased Sema4D levels, with improved behavioural outcomes. GFAP levels were reduced, and scar size was reduced.			
miR-128[131, 191]	Rat contusive SCI	SC	Upregulated	Enriched in mature neurons. Overexpression resulted in reduced concentrations of TNF-α, IL-1β, and IL-6.	Biomarker of neuronal damage, neuropathic pain	Upregulated in serum	Upregulated in CSF
miR-133[181, 192]	Zebrafish transection SCI	SC	Upregulated	Enriched in the spinal cord, targets RhoA, an axonal growth inhibitor. MiR-133 is important for spinal cord regeneration.	Biomarker of spinal cord damage, AND/OR muscle injury, atrophy	Upregulated in serum	Upregulated in serum
	Human ALS patients	Serum	Upregulated	Higher in spinal ALS patients compared to bulbar ALS patients. Likely released into serum due to muscle atrophy.			
miR-150[182, 193]	Rat neuronal spinal cord cells exposed to	Neuronal spinal cord cells	Upregulated	Role in oxidative stress response.	Biomarkers of spinal cord damage and oxidative stress response	Upregulated in serum	Upregulated in serum

MicroRNA	Injury Model	Tissue	Observation	Role	Biomarker	Pig SCI	Human SCI
	reactive oxygen species						
	Rat cardiac ventricular cardiomyocytes exposed to reactive oxygen species	Ventricular cardiomyocytes	Upregulated	Overexpression of miR-150 exacerbated cardiac myocyte apoptosis. MiR-150 may target c-myb, a regulator of vascular smooth muscle cells.	Biomarker of cardiac myocyte injury, vascular smooth muscle injury		
miR-181[131, 183]	Rat contusive SCI	SC	Upregulated early, downregulated late	May target TNF-α, an inflammatory mediator.	Biomarker of astrocyte injury, inflammation	Upregulated in serum	Upregulated in serum
	Mouse primary astrocyte cultures	Primary astrocytes	Upregulated in astrocytes compared to neurons	Enriched in cultured astrocytes compared to other tissues. Overexpression resulted in increased cell death. Knockdown caused increased levels of TNF-α, IL-6 and IL-1β			
miR-195-5p[184]	Rat contusive SCI	SC	Downregulated	Upregulation of MiR-195 decreased functional recovery of animals. Downregulation corresponded to increases in BCL-2, VEGF, and HIF-1α.	Biomarker of hypoxia-related apoptosis	No change in serum	Downregulated in CSF
miR-204[185, 186, 189]	Rat ischemia-reperfusion SCI	SC	Upregulated	MiR-204 targets BCL-2, and Nrn1, increasing Schwann cells to oxidative stress and inducing apoptosis.	Biomarker of neuronal apoptosis	Upregulated in serum	Upregulated in serum
	Human ischemia–reperfusion SCI	Plasma	Upregulated				
miR-219a[187]	Mouse contusive SCI	Plasma	Upregulated	Plays a role in oligodendrocyte differentiation and myelin maintenance. MiR-219 targets PDGFRα, SOX6, FOXJ3, and ZFP238 highlighting its role in coupling differentiation to	Biomarker of myelin injury	No change in serum	Upregulated in CSF

MicroRNA	Injury Model	Tissue	Observation	Role	Biomarker	Pig SCI	Human SCI
				proliferation arrest in the oligodendrocyte lineage			
miR-208, miR-499[188]	Human patients with non-acute SCI	Muscle tissue	Downregulated	MiR-208 and miR-499 are members of the 'myo-miR' family, regulating muscle morphology. Together, they target myostatin, an inhibitor of muscle growth.	Biomarker of muscle injury, atrophy	Upregulated in serum	Upregulated in serum

Chapter 2: Animal models for spinal cord injury

Animal models allow for the controlled and rigorous study of injury responses and recovery. The animal cohorts can be relatively homogenous, the injury characteristics and conditions can be precisely controlled, and – compared to human clinical trials – the experiments are relatively inexpensive. In addition to tightly controlled experimental conditions, animal models offer the obvious advantage of being able to harvest the injured spinal cord itself from the euthanized animal for a myriad of different analyses (e.g. histology, biochemistry, molecular biology). It is important to recognize that the same factors that are often touted as advantages of animal model studies – tightly controlled conditions, homogeneous cohorts, precisely delivered mechanical injuries – also make such experiments quite distinct from clinical reality. In the latter, there is considerable heterogeneity and variability in baseline health, injury biomechanics, and subsequent treatment. This is a typically under-appreciated limitation of experimental paradigms that employ animal models for SCI research.

2.1 Injury models

2.1.1 Contusion

The majority of traumatic SCIs are caused by blunt trauma related to motor vehicle accidents (43%), falls (18.8%), and sporting injuries (11.1%). These involve a sudden contusive force being applied to the spinal cord as the surrounding bony/ligamentous spinal column fails[194]. Penetrating trauma to the spinal cord from gunshot wounds or knife injuries are relatively uncommon in western societies.

The earliest forms of SCI modeling involved a replication of this contusive form of injury. The first documented animal model of SCI was described in 1911 by Alfred Reginald Allen[195]. In this canine SCI model, a 30-gram mass was dropped from an 11 cm height along a rod, onto the dorsal surface of the exposed spinal cord. Generally speaking, the contusion injury models currently utilized are modifications of the original Allen injury model[196]. The first widely used weight drop SCI contusion device in rodents was the New York University Impactor[197]. Injury severity with the NYU impactor is adjusted by altering the height from which a 10-gram rod is dropped onto the exposed thoracic spinal cord: 6.25 mm (mild), 12.5 mm (moderate), 25 mm (severe), and 50 mm (very severe). The rod velocity is monitored to allow for validation and standardization of the injury mechanics. The NYU impactor was used in the "Multicenter Animal Spinal Cord Injury Study" or "MASCIS" and is often referred to now as the NYU-MASCIS or MASCIS Impactor.

The NYU impactor was followed by the development of an electromagnetic impactor device by the Ohio State University (OSU) Spinal Cord Research Center. This device was computer-controlled to precisely deliver a defined displacement to the dorsal thoracic spinal cord surface[198-201]. The OSU electromagnetic spinal cord injury device (ESCID) was originally designed for use with rats, but was also modified to be applicable in mice as well[200]. It has also been used to model cervical SCI[202]. The computer control allows for the adjustment of displacement and velocity and provides feedback on the maximal impact force delivered.

In 2003, Scheff et al. reported on the ability of a new device, the Infinite Horizon (IH) impactor, to produce graded morphological and behavioural changes in a rodent model following contusion injury to the spinal cord at T10[203]. To control the injury severity with the IH impactor, the impact force (measured in in kilodynes) is computer-controlled and adjusted; this is in contrast to the NYU-MASCIS impactor and OSU impactor in which the injury severity is adjusted by height of weight drop (and conversely velocity) or depth of tissue displacement (mm), respectively. The impactor has been modified to induce unilateral cervical spinal cord injuries [204] and can also be modified for use in smaller rodents such as mice by replacing the impacting tip with a smaller version[205]. A technical challenge with the IH impactor is in grasping the spinal column securely with its clamps. Streijger et al. recently reported on a design for a custom clamping system to improve upon this[206]. Like the NYU-MASCIS impactor, these contusive models are able to create graded injuries within the spinal cord and are characterized by central haemorrhagic necrosis, ischemia, and inflammation[207].

2.1.2 Clip compression

In 1978, Rivlin and Tator developed the "clip-compression" model of SCI in rats, in which the spinal cord was compressed dorsoventrally between the arms of a modified aneurysm clip[208]. The clips can be calibrated to exert a specific force to the spinal cord and induce graded injury severities [209]. This model demonstrated the relationship between the severity of neurologic injury and the severity and duration of compression. The Rivlin-Tator aneurysm clip was originally designed for use in the thoracic cord[208]. Recently, Fehlings and colleagues have developed modified protocols for its use at the C6-C7 and C7-T1 spinal levels[210].

The clip-compression model aims to replicate the effect of persistent spinal cord compression that is commonly found in clinical SCI. Compression, however, is typically applied for one minute, which is vastly different from the duration of persistent compression that is witnessed in human SCI. The clip-compression and contusion injuries both impart a blunt force to the spinal cord. They differ in that clip-compression injury lacks the initial velocity of the contusion injury, while contusion injuries do not impart the sustained spinal cord compression observed in human SCI. Even for commercially available contusion impactors, the velocity with which the spinal cord is impacted is considerably lower than what is estimated to occur in human SCI[211, 212].

2.1.3 Balloon induced compression

Tarlov first proposed the balloon-induced compression injury model in 1953[213]. The model was developed due to its simplicity and non-invasive nature. Initially, a small hemi-laminectomy defect was created to access the epidural space, into which a Fogarty catheter was inserted and advanced cranially to one or two higher spinal levels[214]. Alternatively, the catheter may be installed in the lumbosacral spine and advanced through the epidural space to the thoracic spinal cord. Inflating the balloon at the catheter tip with saline imparts the compressive force against the spinal cord[215]. Again, the injury can be graded using varied volumes within the balloon. This method of injury has been used in a variety of large animal species, including canines and monkeys[215, 216].

2.1.4 Transection

While full transections are rare in the clinical setting[217, 218], the transection models offer the advantages of investigating axonal regeneration/plasticity, degeneration, tissue engineering strategies, and cell transplantation[207]. Transections can be either complete or incomplete, with the

latter intended to precisely interrupt specific tracts[219, 220]. For example, a dorsal hemi-section can be used for selective transection of the corticospinal tracts, while a dorsolateral quadrant lesion can be used to transect the rubrospinal tract[207, 218]. The key advantage to transection models (either full or partial) is the clearer interpretation of axonal regeneration studies.

2.1.5 Photochemical

A photochemical based model of SCI was first developed in 1986[221] and has been used as a method of creating a discrete SCI without significant primary trauma[222]. A dye is infused systemically, and then activated within a discrete area of the spinal cord using an argon laser. The resultant photochemical reaction produces single oxygen molecules on the endothelial surface of spinal cord vessels, which triggers an aggressive platelet response, vessel occlusion, and damage to the parenchymal tissue[223]. The model is considered reliable and reproducible and does not require a full laminectomy. While this model is quite distinct from the high-energy injury models that resemble human injury, it can be useful for studying features of the secondary injury phase in the absence of significant mechanical trauma.

2.1.6 Excitotoxic

Injury in the excitotoxic model is induced by an intraspinal or intrathecal injection of excitotoxins such as quisqualic acid, an AMPA-metabotropic receptor agonist[224]. These excitotoxins are particularly useful for generating rodent models of neuropathic pain with thermal hyperalgesia and mechanical allodynia[225, 226].

2.2 Animal species used in modeling SCI

In addition to the variety of injury mechanisms used to induce SCI, there are numerous animal species that are used for SCI research. Rats and mice are by far the most commonly used animals for *in vivo* SCI modeling and experimentation[227, 228]. Other experimental species used for SCI research include gerbils, guinea pigs, hamsters, cats, dogs, pigs, and non-human primates[214, 229-235]. Other models include invertebrates such as eels[218], whose unique regenerative capacities have been investigated in an effort to understand the regenerative processes and how they might be harnessed in humans. While there is a breadth of animal species available for SCI modeling, one should recognize that each has its own advantages and disadvantages, and none necessarily represent the human condition with perfect fidelity.

2.2.1 Mouse

Mouse models of SCI have the significant advantage of enabling the application of sophisticated genetic/molecular techniques to investigate the role of specific genetic factors that may influence biological responses to injury (e.g. cell death or axonal regeneration). The ability to enhance or delete specific genes allows for the study of their roles in gain of function and loss of function experiments. Using mice with a specific gene deletion has become a standard approach in SCI research, and Cre-Lox technology along with increasing numbers of transgenic mice have provided greater spatiotemporal control of the knockout strategy and a better understanding on specific factors affect axonal outgrowth. The small size of mice, however, makes surgical procedures more difficult and prohibits many device implantations[218, 226]. Similar to rats, investigators have modified the traditional thoracic SCI approaches to study cervical SCI in mice[236-238].

2.2.2 Rat

Rat models are the most commonly used for SCI experimentation and most SCI models were initially described and characterized in the rat[201, 203, 239-243]. They are relatively inexpensive, easy to handle and care for, and have long-established injury mechanisms available (e.g. contusion, clip compression, transection). They have well-described neuroanatomy and there exists a wide array of behavioural tools as outcome measures. Locomotor recovery is typically evaluated with the widely used Basso, Beattie and Bresnahan (BBB) locomotor scale, which has the advantage of being relatively inexpensive and comparable across laboratories[244]. More sophisticated systems for testing locomotor recovery have been established and are commercially available (e.g. Catwalk,)[243, 245]. While traditionally the rodent SCI models involved a mid, or low-thoracic injury and an assessment of lower limb recovery, a number of groups have developed methods for injuring the cervical spinal cord to specifically evaluate forelimb responses[246-251].

2.2.3 Cat

The cat (*Felis catus)* has been used extensively in locomotor studies that have contributed to a greater understanding of spinal networks controlling and regulating locomotor activity. The larger size of cats allows for a wider range of surgical implementation and the opportunity to execute detailed kinematic analyses in locomotor and postural studies. Anatomically, the spinal cord of cats is organized similarly to humans - specifically, the main part of the corticospinal tract is located in the dorsolateral spinal cord, while in rodents it is located below the dorsal columns. In the early 20th century, the demonstration that the spinal cord could generate the basic rhythm of locomotion by itself was first shown in an adult cat model of SCI[252]. The cat SCI model has served as the foundation for seminal insights into locomotor training by researchers such as Serge

Rossignol[253] Reggie Edgerton[254], and Susan Harkema[255]. The evidence of an intrinsic spinal locomotor network that can be activated using different types of stimulation was important for the development of rehabilitation strategies for spinal cord injured humans.

2.2.4 Dog

Within the last two decades, rodent models of SCI have largely replaced the use of dogs (*Canis lupus)* in experimental SCI research. However, there has been increasing interest in 'naturally occurring' SCI that occurs in domestic dogs[256]. Dogs naturally suffer SCI as the result of traffic accidents, or – in some species – acute thoracolumbar disc herniations[257]. Because of this, the mechanisms of injury are often similar to human SCI: vertebral fracture-luxation and disc extrusions, which both produce a combination contusion-compression lesion to the ventral aspect of the cord – something that is difficult to model experimentally[256]. The paradigm of a 'naturally occurring' SCI (in contrast to an experimentally induced SCI) lends itself to the conduct of a true "clinical trial" that resembles how human trials are conducted[258]. Essentially, the clinical population of domestic dogs with SCI forms a "surrogate human" population of clinical patients in which to test the efficacy of potential treatments for SCI. Recently, a population of dogs non-experimental (accidental) SCI was used in a prospective randomized clinical trial of an intraspinal transplantation of glial cells derived from the olfactory mucosa[258]. This study demonstrates the potential utility of this naturally occurring SCI in dogs to conduct a clinical trial of a novel therapeutic much in the same way as it would be conducted in humans.

2.2.5 Pig

The pig (*Sus scrofa)* has the spinal cord and CSF dimensions that are more similar than rodents to those of an adult human[231]. The obvious differences in size and anatomy and potentially greater similarities in biological responses to injury between humans and higher order animals make a porcine model of SCI a useful model for the investigation of biological and cellular transplantation studies[231]. The pig also presents an advantage in its large CSF volume and accessibility within the intrathecal space, unlike the rodent where serial sampling of such biofluids is not possible. Additionally, while the costs associated with conducting studies in large animals are certainly higher than those with rodents, they are a fraction of those associated with primate studies. A number of investigators have described the development of porcine models of SCI using a weight drop impactor, computer-controlled contusion/compression devices, or calibrated vascular clips[231, 232, 235]. Using a weight drop contusion device, Lee et al. developed a porcine model of traumatic thoracic SCI in Yucatan miniature pigs where varying degrees of injury severity can be induced by altering the height (5, 10, 20, 30, 40, and 50 cm) from which a 50 gram weight is dropped onto the dorsal aspect of the T10/11 segment[231]. Locomotor recovery was evaluated using their Porcine Thoracic Injury Behaviour Scale (PTIBS), which was sensitive at distinguishing recovery amongst animals of different injury severities[231]. Using a computer-controlled system, Navarro et al. developed a chronic SCI model in adult Gottingen-Minnesota pigs where they showed consistent development of paraplegia following a 2.5 kg compression force, delivered at a velocity of 3cm/sec on the dorsal aspect of the T12 segment[232]. Zurita et al. developed a vascular clip model of SCI in adult minipigs, where paralysis was caused by the epidural application of two vascular clips for 30 minutes[235].

2.2.6 Non-human primates

Non-human primate studies are primarily limited by their specialized facility requirements and high cost related to the intensity of animal care. Some also have ethical objections to their use. Nevertheless, their 'relatedness' to humans has made them an appealing species to preclinically evaluate novel therapies and has motivated researchers to develop injury models in them. In 2012, Nout et al. reported on a lateral hemi-section model of cervical SCI in adult rhesus monkeys[233]. A lateral hemi-section allows for precise control over the lesion location and extent, and it allows for some preservation of function in experimental animals so that the injury morbidity is reduced. A cervical contusion model of SCI in marmosets was developed by Iwanami et al., where a 17-gram weight is dropped from a height of 50 mm onto the exposed dura matter of the C5 segment[230]. One of the main advantages of a cervical SCI model is the similarity in upper extremity function between humans and non-human primates. As regaining hand function is the highest priority for tetraplegic individuals[259], evaluating therapies in a primate cervical SCI model that influences the recovery of hand function is of translational importance[233]. Demonstrating the restoration of hand function in a primate model of cervical SCI is an important consideration for the translation of invasive and inherently risky cell transplantation therapies.

2.3 Limitations of preclinical experimentation using animal models

While much effort has been made to refine various animal models in different injury species, it is worth acknowledging some of the broad limitations of the experimentation paradigms utilized in preclinical studies. As mentioned earlier, human SCI is incredibly heterogeneous in its causes, consequences, and pathology. Conversely, in animal studies, researchers typically endeavour to minimize experimental variability in as many ways as possible. Animals of the same weight, age,

gender, and sometimes, genetic background, are accrued for an experiment, then subjected to a precise biomechanical injury (which imperfectly simulates the high velocity with which human injury occurs), and are subsequently housed in identical post-injury conditions.

Additionally, conditions inherent to the experiment may influence the outcome. Anaesthesia, for example, is essential for nearly all animal experiments but it may by itself have neuroprotective effects. Pentobarbital and isoflurane, commonly used anaesthetics for SCI experiments, have been shown to reduce infarct volume following ischemia[260-262]. The general anaesthetic, ketamine, has been shown to reduce functional and histopathological indices of injury in gerbils subjected to cerebral ischemia[260]. While there is no alternative to appropriately anaesthetizing the animals in experimental injury models, the potential effects of these drugs (albeit distributed equally across an experiment) need to be considered.

It should also be acknowledged that the functional outcomes and behavioural assessments differ from experimental models and the clinical setting. Much effort has gone into the design of assessment scales and procedures that accurately measure functional recovery in animals. Some of the most commonly used tests in SCI research include the BBB scale[263], the Tarlov open field test[213], and the inclined plane test[208]. Both the Tarlov and inclined plane tests assess general locomotor ability and do not reflect specific changes in motor or sensory function[264]. Alternatively, the BBB scale emphasizes hindlimb function, and does not assess other movements, which require coordinated spinal cord activity[265]. In the end, despite the refinements and widespread use of such outcome measures, the reality is that these measures have no direct physiologic correlate in humans. For example, it is impossible to predict how the achievement of "plantar weight supported

stepping" on the BBB scale would translate into human lower extremity function. Researchers are warned to resist the temptation to extrapolate such changes in animal behaviour to human locomotor recovery. For these reasons, it is imperative to develop objective, cross-species biomarkers of SCI severity with which extrapolation from preclinical animal models to humans may be reasonable.

Finally, the SCI field has become increasingly aware of the challenge of demonstrating the robustness of its novel therapies, as manifested by the difficulty in independently reproducing promising experimental findings. This has, to some extent, been recognized for decades in many areas of biomedical research[266-268]. Recently, the biotechnology industry has reported on the difficulty of replicating many promising findings published in peer-reviewed journals[269, 270]. Poor study design and reporting practices in SCI research have likely resulted in biased results, considerable waste, and arguably, a slowing of progress towards developing effective therapeutics[271]. In the SCI field, a formal replication initiative funded by the National Institute of Neurological Disorders and Stroke (NINDS) resulted in the failure of the majority of promising SCI therapies[272]. Factors like litter-to-litter variability can impact behavioural studies significantly[273]. Concerns have also been raised regarding a number of issues in neuroscience publications, including inappropriate statistics[274, 275], and the use of underpowered sample sizes[276]. Recently there has been support for implementing reporting standards for preclinical research that are analogous to the reporting of clinical trials, as the absence of such standards may influence the interpretation of study results[271]. For example, RhoA/Rock inhibitor and stem cell studies for SCI treatment have been shown to describe more favorable outcomes when the articles do not report whether investigators were blinded during behavioural testing[277, 278]. Reproducibility and

standardization efforts such as these will improve the quality of SCI research and help the field interpret the overwhelming body of literature that is emerging on novel therapeutics.

2.4 Summary

Animal models will continue to be a cornerstone of discovery and validation for SCI. The emergence of increasing numbers of potential therapies will demand vigilance at the preclinical level to demonstrate the robustness of effect and put forth the most promising candidates, lest we overwhelm the clinical community with treatments destined to fail. The wide range of animal models and animal species that are now available give the scientific community new opportunities to address important translational questions prior to human testing. The future challenge will be in balancing the urgent needs of the injured community for effective therapies with the caution to put things into clinical trial without 'sufficient' testing. Ongoing dialogue that involves scientists, clinicians, regulatory agencies, funding agencies, and individuals living with SCI is imperative to ensure that this balance is struck in such a way that we move forward efficiently towards the establishment of effective therapies.

Chapter 3: MicroRNA biomarkers in a porcine model of thoracic spinal cord injury

3.1 Introduction

SCI is a devastating condition, often resulting in life-long disability. The patient population with SCI comprises an extremely heterogeneous group with considerable differences in injury mechanism, location, severity, and patient genetics. Each year, over 17,000 individuals in North America and many thousands more around the world suffer an acute traumatic SCI and are left paralyzed[279]. There are nearly 290,000 individuals living with SCI in the United States alone. The estimated lifetime costs for one SCI ranges from $1 – 4 million, depending on the severity of injury. A small number of acute SCI clinical trials have been conducted; yet none have shown convincing efficacy[17, 280-282]. As a consequence, neuro-restorative treatment options are limited for acute SCI patients.

A fundamental limitation of such clinical trials is their singular reliance on standardized measures of neurologic function for patient enrollment, stratification, and treatment assessment. The baseline clinical assessment of SCI and measurement of neurologic impairment can be inaccurate or impossible to conduct due to intoxication, sedation, or concomitant injuries. In a review of over 400 acute SCI patients, we previously showed that concomitant injuries and co-morbidities would have excluded at least 30% of patients from participation into an acute clinical trial[15]. The inability to determine a baseline functional severity of neurologic impairment further limits the pool of "recruitable" patients for acute SCI clinical trials of new therapies and is a major impediment to

the translation of such therapies. In those who can be reliably examined within the first few hours post injury and the baseline level of neurological impairment ascertained, the variability in spontaneous neurologic recovery is high, forcing investigators to enroll large numbers of patients to have sufficient statistical power[16]. Accordingly, new treatments that show promise in experimental models of SCI are extremely difficult to validate in acute SCI patients.

Biological markers of SCI that objectively stratify the severity of cord damage would greatly facilitate clinical trials of novel therapies for acute SCI. Additionally, such biomarkers may be able to predict spontaneous neurologic recovery over time with greater precision, sensitivity and reproducibility than the standard clinical examination, which in turn would reduce the number of patients needed to sufficiently power clinical trials. Biological outcome measures that can be applied between porcine models and human patients could accelerate the preclinical development and subsequent clinical evaluation of novel therapeutics. It is recognized within and outside the SCI field that biomarkers that enhance patient enrolment and provide surrogate outcome measures would increase the pace of clinical trials and improve the chances of successfully demonstrating efficacy, thus moving us more quickly towards effective treatments. For example, it has been estimated that the use of biomarkers in the selection of subjects for early Alzheimer's trials could reduce sample size by 67% and trials costs by 60% as compared to trials depending solely upon clinical measures[283]. For these reasons, there has been considerable research interest in establishing CSF and serum biomarkers after acute SCI, TBI, and other acute and chronic neurologic conditions[1, 25, 26, 32-34, 284-286]. For example, Kwon et al.[284] have shown significant differences between the proteins IL-6, tau, S100β, and GFAP within the CSF of patients with acute SCI. While these proteins (and their levels in SCI) show promise in defining the severity of SCI, the procedure

of acquiring CSF through a lumbar puncture is relatively invasive. A blood-borne biological marker that could characterize injury severity and predict outcome would therefore be desirable.

In this work, we investigated the potential for microRNAs (short, ~22 nucleotide non-coding RNAs that post-transcriptionally regulate gene expression) measured in serum and CSF to serve as biomarkers of injury severity after acute traumatic SCI in a porcine (*Sus scrofa)* model. MicroRNAs have emerged as attractive biomarker candidates due to their stability in fluids, phylogenetic similarities, and tissue specificity[1-3]. Using this large animal model of contusion plus compression SCI, we collected CSF and serum samples from 16 animals over 5 days and performed next-generation sequencing on the microRNA population within these biological fluids to determine if changes in microRNAs can be used as biomarkers of injury severity. We found a severity-dependent increase in the number and magnitude of microRNAs within the serum of pigs, that was most pronounced at 1- and 3-days post injury. The microRNA levels at these time points significantly correlated with injury severity, measured by force of impact, as well as with functional outcome scores at 12 weeks post injury.

3.2 Material and methods

All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia's Animal Care Committee.

3.2.1 Animals and experimental design

Female Yucatan miniature pigs (*Sus scrofa)* (Sinclair Bio-resources, Columbia, MO) weighing 20 – 30 kg were group-housed at a large animal facility for 5 weeks prior to surgery. Animals were

block-randomized into different injury severity groups, or a SHAM group. Animals in the SHAM-operated surgery group (n = 4) received an identical laminectomy surgery as in the injured groups, but no weight drop injury or compression to the spinal cord. 3 different injury severities were induced by dropping a 50-gram weight onto the exposed spinal cord from a height of 40, 20, or 10 cm (n = 4 per group), followed by 5 minutes of compression with a 150-gram weight. Samples of CSF and serum were collected 15 minutes before injury (baseline, BSL) and then again at 1, 3, and 5 days post injury. Total RNA was isolated from samples of CSF and serum, and using next-generation sequencing, we profiled known microRNAs, identified novel microRNAs, and compared the time-course of microRNA profiles between CSF and serum over 5 days, following injury.

3.2.2 Porcine model of spinal cord injury

Surgical procedures for SCI and post-operative care were performed as previously described[231, 287]. Using anatomic landmarks, the T9, T10, and T11 pedicles were cannulated and instrumented with screws (Select™ Multi Axial Screw, Medtronic, Minneapolis, MN). After the T10 laminectomy was performed, the weight drop device was rigidly secured to the pedicle screws and positioned so that the impactor (weight: 50 g) would fall directly on the exposed dura and spinal cord at T10. The tip of the impactor (diameter: 9.53 mm) was instrumented with a load cell (LLB215, Futek Advanced Sensor Technology, Irvine, CA, USA) to record the force at impact. Immediately following the contusion injury, compression was applied by placing a 100-gram mass on top of the impactor (150 grams total) for 5 minutes.

3.2.3 Cerebrospinal fluid collection

The technique for serially collecting CSF samples in our porcine model post injury has been previously described[287]. In summary, CSF collection was achieved using a 19-gauge epidural catheter (Perifix epidural catheter set; Braun Medical Inc., PA) inserted into the intrathecal space with the catheter tip resting approximately 8 cm caudal to the injury site. A total of 1 mL of CSF was collected at baseline, 15 minutes before injury, and at 1, 3, and 5 days post injury. Immediately after collection, CSF samples were centrifuged at 1,000 xg for 10 minutes at room temperature. The CSF supernatant was aliquoted into a 2.0 mL RNAse free centrifuge tube (Eppendorf™, ON), immediately frozen on dry ice, and stored at -80 ºC for RNA isolation.

3.2.4 Serum collection

Blood collection was performed by inserting an 8F Groshong catheter (Bard Access Systems, Inc, Salt Lake City, UT) in the left external jugular vein. This was connected to a low volume titanium subcutaneous access port (X-port, Bard Access Systems. Salt Lake City, UT) housed in the posterior neck region. A sterile 22-gauge Huber needle (Instech Solomon. Plymouth Meeting, PA) was used to access the port for withdrawal of blood. A total of 5 mL of whole blood was collected at BSL, and at 1, 3, and 5 days post injury. To separate the serum portion, blood was allowed to incubate for 25 minutes at room temperature, and then centrifuged at 10,000 xg for 5 minutes. The serum supernatant was aliquoted into a 2.0 mL RNAse free centrifuge tube (Eppendorf™, ON), immediately frozen on dry ice, and stored at -80 ºC for RNA isolation.

3.2.5 Porcine thoracic injury behaviour scale

To assess hindlimb functional recovery, the PTIBS was used, as previously described[231, 287]. Briefly, four weeks prior to injury, animals were trained to walk straight along a rubber mat (4.0 m x 1.2 m) at a constant speed without stopping. Clicker training, along with a food reward, was used for motivation. Baseline behavior was obtained for each animal, one week prior to surgery: five runs were recorded with three high-definition camcorders placed 30 cm above the ground and behind the animals. Functional assessment resumed at one-week post injury and continued weekly for 12 weeks. The functional assessment footage was analyzed by two independent observers that were blinded to the biomechanical severity of SCI that was induced at the time of surgery. These observers were members of the research team, and familiar with the PTIBS scoring system, but were not directly involved in the surgical or post-operative care aspects of the project and thus were not aware of the severity of injury for each animal. The PTIBS scale ranges from no active hindlimb movements (score 1), to normal ambulation (score 10). PTIBS scores of 1 – 3 are characterized by "hindlimb dragging," scores of 4 – 6 reflect varying degrees of "stepping" ability, and scores of 7 – 10 reflect varying degrees of "walking" ability.

3.2.6 Histological outcomes

At the end of the experiment (12 weeks post injury), animals were euthanized, the spinal cord harvested, post-fixed and cryoprotected as described previously[287]. Subsequently, spinal cords were cut into 1 cm blocks centered on the site of injury, frozen on dry ice, and stored at -80 ºC. Cross-sections (20 µm thick) were then cut using a cryostat. Sections were serially mounted onto adjacent silane-coated SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) such that sections on the same slide were obtained from tissue 400 µm apart and stored at -80 °C. For differentiating

grey and white matter, Eriochrome Cyanine R staining (EC) was performed with Neutral Red as a counterstain. EC-stained sections were examined and micrographs (5x objective) were taken of sections at 800 μm intervals throughout the lesion site (Zeiss AxioImager M2 microscope, Carl Zeiss Canada Ltd., Toronto, ON, Canada). The spinal cord outer perimeter, white matter, and gray matter were outlined, and the area of each was calculated using Zen Imaging Software (Carl Zeiss Canada Ltd., Toronto, ON, Canada). The spared white matter was defined as areas that exhibited dense blue staining. Spared gray matter was defined based on the color of the stains and morphology. We define intact gray matter as tissue containing normal gray matter cytoarchitecture with visible neutral red staining present. The percentages of white matter and grey matter were calculated by dividing the spared white or grey matter by the total area of the spinal cord on a given section and the sum of the two, representing "total spared tissue".

3.2.7 RNA isolation

Total RNA was isolated from 1 mL of CSF or serum using the miRVana PARIS kit (ThermoFisher, Cat#: AM1556) according to the manufacturer's instructions, incorporating the modifications of Burgos et al.[288] for maximum yield. Total RNA was re-suspended in 6 μL of water and used for library preparation with Illumina's TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA). The following modifications were introduced to the library preparation to account for the very low amounts of input RNA in all samples: 1. Each reaction used only half of the recommended reagent amounts, and 2. The PCR cycles during library amplification were increased to 15. Libraries were individually barcoded with Illumina-provided index barcodes so that samples could later be demultiplexed. Libraries were pooled and sequenced on the Illumina MiSeq or HiSeq 2500, generating single-end 36 base pair reads.

3.2.8 Post-sequencing analysis pipeline

Sequencing reads were processed using the Mayo Clinic's Comprehensive analysis pipeline for microRNA sequencing data (CAP-miRSeq)[289]. The pipeline is summarized in Figure 3.4A. Read quality was assessed using FastQC[290] before and after trimming adapter sequences and low quality 3′ bases. Adapter sequences were trimmed with Cutadapt[291] and reads shorter than 17 nucleotides were discarded. Trimmed reads were first aligned using Bowtie[292] where the pipeline conducts two alignment processes: one used internally for miRDeep2 to quantify and predict novel microRNAs and another for all RNA quantification and data visualization. The CAP-miRSeq pipeline generates a summary for each sample's alignment statistics and number of microRNAs detected with reports of raw counts for known microRNAs of all samples in matrix format. Samples with a total sum of mapped microRNA read counts less than 50,000 reads per sample were removed from analysis. This 50,000 read threshold was determined based on the Spearman correlations of randomly selected subsets of reads to the total number of reads in a sample (Figure 3.5).

3.2.9 Statistical analysis

Adjustments for differences in sequencing depth were made using the number of aligned reads, and the counts were adjusted to "microRNA reads, per million aligned reads". In other words, the number of microRNA reads proportional to all other small RNAs.

$$Normalized\ MicroRNA\ Reads = \frac{Raw\ MicroRNA\ Reads}{Aligned\ Reads} \times 1{,}000{,}000$$

This normalization accounts for technical differences in library sizes, where a larger library will be associated with higher numbers of microRNA counts, *as well as* aligned read counts. To ensure

this proportion is unchanged due to technical differences, three biologically different samples were sequenced with differing concentrations, on different devices, and different dates. While the raw number of reads and number of aligned reads varied greatly due to different concentrations being loaded, the ratio of raw microRNA reads to aligned reads remained identical. This provided confidence in the ability of this normalization method to account for technical differences in pipetting, library size, or sequencing efficiency.

Since most normalization strategies rely on the assumption that most genes are not differentially expressed[293], we used an alternative method of normalization. The rationale for this normalization was designed to accommodate differences in global levels of microRNA levels[294-297]. This method is well suited for our NGS dataset, which includes reads for the entire set of microRNAs, rRNAs, yRNAs, tRNAs, snoRNAs, and other R-Fam members that are captured during library preparation. Statistically significant microRNAs were determined using multiple t-tests, while correcting for multiple comparisons using the Benjamini-Hochberg method with adjusted p-values lower than 0.05. Differences in total microRNA counts were determined using two-way analysis of variance (ANOVA) after assessing for distribution and variance. MicroRNAs with a statistically significant area under the receiver operator curve (AUROC) were determined after correcting for multiple testing using the Benjamini-Hochberg method with adjusted p-values lower than 0.05.

3.3 Results

3.3.1 Study overview

Female miniature Yucatan pigs (n = 12) received a contusion SCI by dropping a 50 g weight from a height of either 10 cm (mild), 20 cm (moderate), or 40 cm (severe) onto the exposed spinal cord, followed by 5 minutes of compression with an additional 100 gram weight (150 grams total) (summarized in Figure 3.1). An additional group of pigs (n = 4) received all aspects of the surgical procedure (including laminectomy) but without any SCI, thereby serving as "SHAM" or "NON-SCI" controls. Impact forces recorded with a force sensor were consistent within injury height groups and correlated with injury height (Table 3.1). Samples of CSF and serum were collected at baseline, 15 minutes prior to injury, and then at 1, 3, and 5 days post injury (Table 3.1). Total RNA was isolated and microRNA libraries were created for expression profiling using NGS. Animals performed weekly functional recovery tests for 12 weeks following SCI. At 12 weeks post injury, animals were euthanized, and spinal cords were collected for histological analysis of spared tissue.

Table 3.1 Study parameters. The number of animals in each injury group is shown with their respective injury heights and recorded impact forces in Newtons (N), along with the number of samples collected for each sample type (serum or CSF) and time point.

				Number of Samples (n)			
Drop Height (cm)	**n**	**Impact Force (N)**	**Sample Type**	**Baseline**	**1 dpi**	**3 dpi**	**5 dpi**
10	4	19.22 ± 1.70	Serum	4	4	4	4
			CSF	4	4	4	4
20	4	30.04 ± 2.52	Serum	4	4	4	4
			CSF	4	4	4	4
40	4	47.87 ± 6.93	Serum	4	4	4	4
			CSF	4	4	4	4
NA (SHAM)	4	NA	Serum	4	4	4	4
			CSF	4	4	4	4

3.3.2 Behavioural outcomes

To measure the recovery of gross locomotor performance, hindlimb function was assessed using the PTIBS, designed by Lee et al[231]. Scoring was performed pre-operatively and then on a weekly basis post injury (Figure 3.2). Prior to injury, all animals achieved a baseline score of 10, indicative of normal hindlimb function and locomotion. Following SCI, locomotor function was most severely impaired at 1-week post injury, with mean PTIBS scores of 2, 3.2, and 4 for the severe, moderate, and mild SCI groups respectively, and 10 for the Sham group. As expected, lowest PTIBS scores were observed at the greatest injury severity. At all post-injury time points (from week 1 to 12) all injured animals were substantially and significantly impaired compared to baseline values, independent of their injury severity, while the SHAM group had little to no impairment. By 12 weeks post injury, the mean PTIBS scores were 3, 4.25, 6.5, and 10 for the severe, moderate, mild SCI and SHAM groups, respectively. Injury height had a significant effect on PTIBS scores $F(3, 169) = 803.9$, $p < 0.0001$ with significant differences between all groups

from week 6 to 12. This data provides evidence to confirm the generation of four distinct injury-severity groups that correlated with behavioral recovery.

3.3.3 Histological outcomes

To assess the amount of spared (healthy) tissue within the spinal cord, spared gray matter and white matter were quantified by manually tracing the respective cross-sections of spinal cord, 0.8 mm apart, as previously described by Lee et al.[231]. Figure 3.3A shows the total percent spared tissue. Total percent spared tissue was calculated by taking the sum of the spared white and gray matter and dividing that value by the total area of the spinal cord on its respective cross-section. The height of injury had a significant effect on the amount of spared tissue (greater height, less spared tissue), calculated as the area under the curve of the total percent spared tissue over the region from 13.6 mm rostral to 13.6 mm caudal to the site of injury $F(3, 12) = 63.46$, $p < 0.0001$. Cumulative spared tissue was greatest in the SHAM group (3,380 ± 16.0 cumulative %) compared to the mild (2,720 ± 98.0 cumulative %), moderate (1,990 ± 140 cumulative %) and severe (1,650 ± 88.0 cumulative %) SCI groups, respectively ($p = 0.0022$, $p < 0.0001$, $p < 0.0001$) (Figure 3.3B). The cumulative spared tissue was significantly greater in the mild SCI group as compared to the moderate SCI group ($p = 0.0008$) and the severe SCI group ($p < 0.0001$). While the spared tissue was significantly higher between the 3.2 to 11.2 mm sections caudal to the epicenter in the moderate SCI group compared to the severe SCI group, there were no statistical differences between the cumulative areas ($p = 0.12$). This data provides evidence that the four *behaviorally* distinct groups are also reflected by four distinct injury-severity groups with regards to histologically measured tissue damage.

3.3.4 MicroRNA detection in cerebrospinal fluid and serum

MicroRNA libraries were created from total RNA isolated from CSF or serum. RNA concentrations in CSF are reported to be between 15 – 30 ng per mL, while serum has 10 – 60 ng per mL[288]. These relatively low levels of total RNA required the protocol modifications described by Burgos et al.[288] for Illumina's TruSeq Small RNA Library Preparation, which requires 1 ug of total input RNA. This enabled us to generate sequencing libraries for 64 CSF and 64 serum samples. Libraries were sequenced to generate short single-end reads, which were processed using the CAP-miRSeq bioinformatic pipeline (Figure 3.4A).

Trimmed reads were first aligned to the porcine S*us scrofa* (Version 10.2) reference genome[298]. The resulting aligned reads were then mapped to the microRNA database (Version 21), miRBase. The *Sus scrofa* microRNA database from miRBase contains 411 known, mature microRNAs. Most of these (305 microRNAs) are also found in the human microRNA database, which contains over 1,800 mature microRNAs. Aligning our sequencing reads to the human database did not significantly increase the number of detected mature microRNAs, so all further analyses used the *Sus scrofa* database.

Without using any read-count cut-off, we detected a total of 314 previously identified microRNAs that were expressed in one or more time points across all serum samples (Figure 3.4B), which represents over 75% of all known pig microRNAs. Interestingly, there were no microRNAs uniquely detected in CSF. 280 microRNAs were detected in CSF, all of which were also found in serum, while there were an additional 34 microRNAs uniquely detected in serum. Using the confidence cut-offs proposed by Burgos et al.[299] that require a novel microRNA to be present in at

least 30% of samples, our analysis also detected 14 putative novel microRNAs in serum (Figure 3.4B, Table 3.2).

For profiling changes in microRNAs, we only considered samples with a total microRNA read count per sample above 50,000, and an average read count for any individual microRNA of over 5 counts. As shown in Figure 3.5, for samples below this cut-off the Spearman correlation drops below 0.9. 60 serum samples were above this cut-off, unfortunately, only four CSF samples had over 50,000 microRNA reads (Figure 3.4C). Overall, sequence coverage was much lower for CSF samples; the majority of samples had less than 10,000 microRNA reads. This read count was not improved by additional sequencing. The two likely explanations for this result is (1) The abundance of microRNAs in CSF is very low[288], or (2) there may be inhibitors of PCR in the pig CSF samples that interfere with library preparations[300]. While microRNA profiling was not successful in the majority of CSF samples, a correlation analysis was performed between the normalized CSF microRNA counts and serum microRNA counts of all detected genes (Figure 3.6). The profile of CSF microRNAs significantly correlated with the profile of microRNAs within serum ($r = 0.9620$, $p < 0.0001$). In addition, because we found a large overlap between the microRNA content of serum and CSF samples, we proceeded to focus exclusively on serum samples for our further temporal analyses.

Table 3.2 Novel microRNAs. To be listed, the potential microRNA must be present in at least 30% of either Serum or CSF samples, and have more than 5 counts on average across all samples. Column one through three contains the location of the sequence predicted by miRDeep2 for the potential mature microRNAs. Column four is the percentage of serum samples in which the microRNA was detected and column five is the percentage of CSF samples in which the microRNA was detected. Column six represents the total percentage of samples in which the microRNA was detected.

Genomic location						
Chr	**Start**	**Stop**	**Mature Sequence**	**% of serum samples**	**% of CSF samples**	**% of total samples**
chr15	133957559	133957616	gccgacgagccccucgcacaaaccggaccugagcguuuuguucguucggcucgcguga	76.53	50.00	71.31
chr6	870006	870063	cggugcgggucgggggcggggcggacuguccucagugcgccccgggcgggucgcgccg	75.51	50.00	70.49
chrX	39824716	39824776	gccaguuacuuccgcucugauggaucuauuuccauuucuagcggcggcgacucuggacuc	71.43	45.83	66.39
chr12	37079098	37079158	gcucugacuuuauugcacuacuguacuuuacagcuagcagugcaauaguauugucaaagc	71.43	41.67	65.57
chr5	66193719	66193780	ucuuccagcacaguguuggauggucuaauagugaagcuccuaacacugucugguaaagaug	67.35	50.00	63.93
chr13	24885255	24885316	uucaaguaacccaggauaggcugugcaggucccaaggggccuauucuugguuacuugcacg	68.37	41.67	63.11
chr10	6017465	6017551	guccgaugguaguggguuaucagaacuuauuaacguuagugucucuaaaguugguauacaaccccccccacugcuaaauuugacug	64.29	50.00	61.48
chrX	48640827	48640885	uacccauugcauaucggaguugugaauucucaaagcaccuccuaugugcaugguuuac	57.14	33.33	52.46
chr16	51742262	51742327	gcagagucaagccuggguuagcacuuggaugggagaccgucugggcauaccgggugcuguaggcuu	55.10	25.00	49.18
chr7	55687381	55687443	ucugguucugugacccugccuauuugcccuucagccagguaagagcaucugaaccaggggu	46.94	16.67	40.98
chr5	30185306	30185368	gugccuggguccccucccagagguucaaauuuaauugcucugagaugugaccugggcaucc	38.78	37.50	38.52
chr3	42401527	42401607	cugauguggcgguggcaacgugagggcagccagcaccugugaggugguggugcugacguuggcucugcgaggucggcu	39.80	25.00	36.89
chr7	69920245	69920312	cggcggccgggggcgccggaggguccacugagcuuccucccccacccucccgccuccucucgccgcc	37.76	25.00	35.25
chr10	50066936	50066995	ggcgggcccacgggggccccccggugagcccgggccgccgggccccgugggccccccgg	30.61	37.50	31.97
chr12	35722001	35722067	cccuugaugaucguucuccucucucugguugagagaggcagagggagagaacaccgucugaguggu	27.55	33.33	28.69

3.3.5 Identification of serum microRNAs related to spinal cord injury

To determine if microRNA levels were significantly altered following SCI in a severity-dependent fashion, the $\log_2$ fold-change for serum microRNAs was determined, relative to pre-injury baseline levels. MicroRNAs with greater than a $\log_2$ fold change of one, (representing a 2-fold change), and adjusted p-value < 0.05 were identified (Table 3.3). From this analysis, we identified a total of 58 significantly elevated microRNAs in the severe SCI group, 21 in the moderate SCI group, 9 in the mild SCI group, and 7 microRNAs in the SHAM group. In addition, there was one microRNA that was significantly decreased in the moderate and mild SCI groups. The numbers of significantly elevated microRNAs that were common or unique to each group are shown in a Venn diagram in Figure 3.7.

Since all but one significantly altered microRNA increased after injury, we investigated the global change in microRNAs across the top 100 most abundant microRNAs. The aim of this was to determine if, while possibly not significant, all of the expressed microRNAs had increased after injury, or just those that were found to be significant. The top 100 was used as a cut-off due to the fact that below this, microRNAs had fewer than 10 counts. Figure 3.8 shows the profile of the 100 most abundant microRNAs, generated from the average normalized read counts. Interestingly, in the severe and moderate SCI groups, we found that microRNA levels across all 100 genes were elevated in response to injury, compared with baseline (Figure 3.8A, B). In contrast, no increase was seen in the global microRNA levels in the mild SCI or SHAM groups (Figure 3.8C, D).

We explored if the total number of normalized microRNA reads, quantified as a measure of injury severity in each group (Figure 3.9A), could serve as a potential measure of severity. Injury severity

had a significant effect on serum microRNA levels as measured by ANOVA $F(3, 12) = 6.25$, $p = 0.0084$. In the severe SCI group, the total amount of microRNA was significantly elevated compared to baseline ($9.12 \times 10^4 \pm 4.21 \times 10^4$) at 1 ($2.28 \times 10^5 \pm 3.74 \times 10^4$, $p = 0.0004$), 3 ($2.05 \times 10^5 \pm 1.86 \times 10^4$, $p = 0.0031$), and 5 days post injury ($1.77 \times 10^5 \pm 2.51 \times 10^4$, $p = 0.0341$). The moderate SCI group had significantly elevated levels of microRNAs compared to baseline ($6.49 \times 10^4 \pm 1.08 \times 10^4$) at 1 ($1.55 \times 10^5 \pm 3.42 \times 10^4$, $p = 0.024$) and 3 days post injury ($1.52 \times 10^5 \pm 2.01 \times 10^4$, $p = 0.0307$). In contrast, the mild SCI and SHAM groups did not have significantly different levels of microRNAs at any time points after injury ($p > 0.999$ for all). Between groups, the total microRNA level was significantly higher in the severe SCI group ($2.28 \times 10^5 \pm 3.74 \times 10^4$) compared to the mild SCI ($1.01 \times 10^4 \pm 6.27 \times 10^3$, $p = 0.0024$) and SHAM ($1.16 \times 10^5 \pm 2.75 \times 10^4$, $p = 0.0071$) groups at 1-day post injury. At 3 days post injury, the severe SCI ($2.05 \times 10^5 \pm 1.86 \times 10^4$) group had significantly higher total microRNA levels than the mild SCI ($9.03 \times 10^4 \pm 1.92 \times 10^4$, $p = 0.0057$) and SHAM ($8.79 \times 10^4 \pm 4.23 \times 10^3$, $p = 0.0056$) groups. At 5 days post injury, the severe SCI group ($1.77 \times 10^5 \pm 2.51 \times 10^4$) had significantly higher total microRNA levels than the mild SCI group ($7.73 \times 10^4 \pm 3.11 \times 10^3$, $p = 0.024$).

Due to the stability of serum microRNAs, we predicted that the systemic microRNA load would accumulate over-time, and this was confirmed by quantifying the cumulative levels of microRNA over the 5 days post injury (Figure 3.9B). Cumulative microRNA in the severe SCI group ($1.24 \times 10^7 \pm 2.47 \times 10^6$) was significantly higher than the mild SCI ($2.76 \times 10^6 \pm 1.37 \times 10^6$, $p = 0.0074$) and SHAM groups ($2.90 \times 10^6 \pm 1.83 \times 10^6$, $p = 0.0081$) but not the moderate group ($9.01 \times 10^6 \pm 1.83 \times 10^6$, $p = 0.056$). MicroRNA levels in the moderate SCI group ($9.01 \times 10^6 \pm 2.14 \times 10^6$) were significantly higher than the mild SCI and SHAM groups ($p = 0.039$, $p = 0.046$, respectively).

3.3.6 Serum microRNAs correlate with functional outcome measures

In order to determine which time points would most accurately identify injury severity, we investigated the degree to which total microRNA levels at each time point correlate with injury severity outcome measures. Correlations between total microRNA levels, PTIBS scores at 12 weeks post injury, and histological outcome measurements were made. Additionally, the comparison between the correlations of impact force and histological outcome or PTIBS, using the Pearson correlation coefficients, was made. Histological outcome (as measured by total percent spared tissue) and PTIBS scores at 12 weeks post injury, were compared to baseline, 1-, 3-, and 5-day post-injury total microRNA levels.

Expectedly, the amount of microRNA at baseline, before injury, did not correlate with PTIBS scores ($r = 0.043$ $p = 0.88$ respectively) (Figure 3.11A). In contrast, following SCI, total microRNA levels at 1-day post injury significantly correlated with PTIBS scores ($r = -0.56$ $p = 0.023$) (Figure 3.11B), while the total microRNA levels at 3 days post injury correlated strongly with PTIBS scores ($r = -0.79$ $p = 0.0002$) (Figure 3.11C). The total microRNA levels did not reach a significant level of correlation with PTIBS scores at 5 days post injury ($r = -0.46$, $p = 0.075$) (Figure 3.11D).

Prior to injury, microRNA levels at baseline did not correlate with total percent spared tissue ($r = -0.031$ $p = 0.91$ respectively) (Figure 3.12A). Following SCI, total microRNA levels at 1 day post injury significantly correlated with total percent spared tissue ($r = -0.62$ $p = 0.011$) (Figure 3.12B), and at 3 days post injury, total microRNA levels correlated well with total percent spared tissue (r

= -0.79 p = 0.0003) (Figure 3.12C). Finally, at 5 days post injury, the total level of microRNA significantly correlated with total percent spared tissue (r = -0.58, p = 0.018) (Figure 3.12D).

These results suggest that total microRNA at 1- and 3-days post injury is directly related to injury severity and has strong predictive value regarding the cellular integrity of the spinal cord at the site of injury as well as functional recovery observed at 12 weeks post injury. The correlation of microRNA level with outcome measures was reduced by 5 days post injury with regards to both behavioural outcome and histological outcome suggesting that microRNAs are most predictive of outcome early after injury. As expected, the force of injury significantly correlated with PTIBS scores at 12 weeks post injury, and histological outcome ($r = -0.93$ $p < 0.0001$, $r = -0.96$ $p < 0.0001$, respectively) (Figure 3.13A, B).

3.3.7 Diagnostic accuracy of microRNAs for injury severity

To determine the potential of specific microRNAs to diagnose SCI and determine injury severity, receiver operator characteristic (ROC) curves were generated to calculate the area under the area under the curve (AUC). Based on the correlation results (Figure 3.11, Figure 3.12), the levels of each microRNA at 1- and 3-days post injury were used to determine how well they could classify severity of injury. Using the list of dysregulated microRNAs (Table 3.3), the 10 microRNAs that showed the greatest diagnostic accuracy (and smallest p-value) in each pair of comparisons (by AUC) are shown in Figure 3.10. Specifically, in differentiating SCI from SHAM groups, miR-133a-5p[149] (AUC = 0.95, $p < 0.0001$), miR-378[131] (AUC = 0.91, $p < 0.0001$), miR-378b-3p[131] (AUC = 0.90, $p < 0.0001$), miR-365-3p[189] (AUC = 0.89, $p < 0.0001$), miR-133b[150, 181] (AUC = 0.89, $p < 0.0001$), miR-10b[173] (AUC = 0.88, $p < 0.0001$), miR-885-5p (AUC = 0.88, $p < 0.0001$),

miR-130a (AUC = 0.88, p < 0.0001), miR-100[131, 301] (AUC = 0.88, p < 0.0001), and miR-208b[188] (AUC = 0.87, p < 0.0001) showed the highest AUC values, some of which have been shown to have alterations after CNS injuries. In distinguishing severe from mild SCI, miR-423-3p (AUC = 1.00, p = 0.0008), miR-425-5p (AUC = 1, p = 0.0008), miR-486[147] (AUC = 1.00, p = 0.0008), miR-100[131, 301] (AUC = 0.97, p = 0.0016), miR-10b[173] (AUC = 0.94 , p = 0.0033), miR-378[131] (AUC = 0.94, p = 0.0033), miR-204[302] (AUC = 0.92, p = 0.0046), miR-22-5p (AUC = 0.92, p = 0.0046), miR-378b-3p[131] (AUC = 0.92, p =0.0046), and miR-125b[10] (AUC = 0.91, p = 0.0063) showed the highest AUC values some of which have been shown to have alterations after CNS injuries. In distinguishing severe from moderate SCI, miR-130a[303] (AUC = 0.98, p = 0.0011), miR-744[304] (AUC = 0.98, p = 0.0011), miR-425-5p (AUC = 0.97, p = 0.0016), miR-130b (AUC = 0.95, p = 0.0023), miR-423-3p (AUC = 0.95, p = 0.0023), miR-125b[10] (AUC = 0.92, p = 0.0046), miR-152[131] (AUC = 0.92, p = 0.0046), let-7i (AUC = 0.89, p = 0.0087), miR-100[131, 301] (AUC = 0.88, p = 0.011), and miR-30b-5p (AUC = 0.88, p = 0.011) showed the highest AUC values some of which have been shown to have alterations after CNS injuries. Finally, in distinguishing moderate from mild SCI, miR-486[147] (AUC = 0.86, p = 0.012), miR-10b[173] (AUC = 0.85, p = 0.016), miR-100[131, 301] (AUC = 0.82, p = 0.026), miR-301 (AUC = 0.82, p = 0.026), miR-378[131] (AUC = 0.81, p = 0.034), miR-133a-5p[149] (AUC = 0.79, p = 0.043), miR-126-5p (AUC = 0.79, p = 0.043), miR-30b-5p (AUC = 0.79, p = 0.043), and miR-378b-3p[131] (AUC = 0.79, p = 0.043) showed the highest AUC values, some of which have been shown to have alterations after CNS injuries.

Table 3.3 Table of deregulated serum microRNAs. Log_2 fold-change values, relative to baseline after severe (40 cm, red), moderate (20 cm, blue), mild (10 cm, green) injury, or SHAM surgery (non-CNS injury control, grey). Associated p-values are denoted as * ($p < 0.05$), ** ($p < 0.005$), *** ($P < 0.0005$). DPI, days post injury; L2FC, Log_2 fold-change. Only time points with significantly deregulated microRNAs are shown. Total numbers of deregulated microRNAs are shown at bottom.

	Severe SCI (40 cm)						Moderate SCI (20 cm)				Mild SCI (10 cm)		SHAM (SCI Control)			
	1 dpi		3 dpi		5 dpi		1 dpi		3 dpi		1 dpi		1 dpi		5 dpi	
Mature microRNA	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p
let-7a			1.45	*	1.44	*										
let-7d-3p					1.74	*										
let-7d-5p					1.73	*										
let-7e			2.37	**	2.31	*										
let-7f			1.40	*	1.39	*										
let-7g			1.39	*	1.35	*										
let-7i					1.27	*										
miR-1	7.03	**														
miR-100							1.38	**	1.23	**						
miR-10b											1.09	**				
miR-125b	1.29	**														
miR-126-3p			1.96	*	1.90	*										
miR-126-5p									1.26	*						
miR-127	2.70	** *	2.22	**			2.19	** *			1.69	** *	2.76	** *		
miR-128	1.79	**	1.72	**	1.53	*	1.49	**			1.31	**				
miR-1285							0.72	**	0.55	**	0.68	**				
miR-130a	1.58	*	1.54	*	1.55	*	1.33	*	1.40	**						
miR-130b			1.41	*	1.42	*										
miR-133a-3p	1.93	** *	1.53	**			1.89	** *	1.48	**	1.70	** *	2.00	** *		
miR-133a-5p	3.82	** *	3.78	** *	3.00	* *	2.56	** *	2.27	** *						
miR-133b	15.20	** *	11.88	** *			12.29	** *	8.68	** *	12.05	** *	9.33	** *		

	Severe SCI (40 cm)						Moderate SCI (20 cm)				Mild SCI (10 cm)		SHAM (SCI Control)			
	1 dpi		3 dpi		5 dpi		1 dpi		3 dpi		1 dpi		1 dpi		5 dpi	
Mature microRNA	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p
miR-142-3p			1.87	**	1.77	*										
miR-142-5p			1.44	*	1.43	*										
miR-143-3p							1.26	**								
miR-144					2.93	*										
miR-145-3p	1.82	*														
miR-145-5p	1.89	**														
miR-1468									1.31	**					1.39	** *
miR-148b-3p			3.24	*	3.20	*										
miR-149	1.41	**	1.41	**	1.34	* *										
miR-150			1.47	**	1.49	*										
miR-152			1.63	**	1.51	*										
miR-15a	2.10	*	1.96	*	2.01	* *										
miR-204	6.38	**														
miR-208b	5.85	** *	3.33	** *			5.50	** *			4.85	**	7.43	**		
miR-216	3.67	** *														
miR-22-3p	1.18	*	1.16	*			1.21	**								
miR-22-5p	3.26	**	3.20	**	2.57	* *										
miR-221-3p			1.43	** *	1.42	* *										
miR-222			1.67	**	1.73	* *										
miR-23b			3.27	*	3.10	* *										
miR-26a			1.55	*	1.53	*										
miR-27a	1.50	*	1.41	*												
miR-27b-3p	1.29	*					1.20	**	1.11	**						
miR-29c	4.24	*														
miR-301			2.33	**	2.30	* *										
miR-30a-3p									1.33	**						
miR-30a-5p							1.18	*	1.17	**						

	Severe SCI (40 cm)						Moderate SCI (20 cm)				Mild SCI (10 cm)		SHAM (SCI Control)			
	1 dpi		3 dpi		5 dpi		1 dpi		3 dpi		1 dpi		1 dpi		5 dpi	
Mature microRNA	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p	L2FC	p
miR-30b-5p			1.60	*												
miR-365-3p	2.78	*					1.59	*	1.57	*	1.48	** *				
miR-378	1.46	**	1.39	**	1.24	*	1.51	** *	1.40	**	1.32	**	1.37	** *		
miR-378b-3p	2.26	**	2.43	** *	1.96	*	2.43	**	2.38	**					2.26	**
miR-423-3p			1.12	*												
miR-425-3p			2.08	*												
miR-425-5p			1.31	*	1.28	*										
miR-4331	0.79	**	0.78	*	0.63	*										
miR-450b-5p	2.30	** *														
miR-451			1.36	*	1.32	*										
miR-486			1.17	*					1.19	**						
miR-574	1.51	**	1.48	** *	1.46	*										
miR-7134-3p			1.56	*	1.57	*										
miR-7139-5p					1.65	*			1.64	*						
miR-744					1.35	*										
miR-885-5p	8.09	**	6.50	*												
miR-98			1.86	*	1.81	*										
miR-9841-3p			1.78	**	1.73	*										
Total	27		42		37		16		16		9		5		2	

3.4 Discussion

This study represents the first description of the temporal microRNA patterns in serum using a large animal porcine model of SCI in which these molecular assays were corroborated with behavioural and histological analysis. We have identified a set of novel microRNAs in *Sus scrofa*

and our results suggest that the microRNA quantities, as measured using a NGS assay, are proportional to injury severity in the serum of pigs. Analysis of serum microRNAs revealed a global increase in microRNAs with the greatest changes in number and magnitude of microRNAs correlating with injury severity, reflecting a substantial alteration in the post-transcriptional regulatory environment. SHAM laminectomy showed similar profiles to mild injury, which is consistent with the findings of De Biase et al.[305]. Previous studies have highlighted the significant alteration of microRNAs in CNS disorders and have shown the potential of microRNAs to act as markers of neurologic dysfunction[11, 131, 134, 158, 299, 306].

We have shown that microRNA levels at 1- and 3-days post injury significantly correlate with functional and histological outcome measures. Interestingly, microRNA levels at 3 days post injury correlate with PTIBS scores ($r = -0.750$, $p = 0.0008$) and spared tissue ($r = -0.833$, $p < 0.0001$) at 12 weeks post injury nearly as well as the force by which the injury was induced ($r = -0.934$ $p < 0.0001$, $r = -0.959$, $p < 0.0001$, respectively). Our results indicate that animals with higher levels of serum microRNAs at 1- and 3-days post injury had worse functional outcomes 12 weeks post injury, as measured by PTIBS, and had more cellular damage, as measured by spared tissue. These results represent an important finding: that the detection of serum microRNAs at 1- or 3-days post injury is nearly as predictive of 12-week post-injury functional recovery and cellular damage as the force of contusive injury itself, which is the main determinant of injury severity in this type of controlled, preclinical experimental study. The potential for having such an early objective predictor of long-term recovery after acute SCI is very relevant for clinical trials of novel therapies, as the variability in spontaneous recovery based on an early neurologic assessment (when such an assessment can actually be done)) is very high. Our preclinical data suggests that

serum microRNAs have promise as objective biomarkers of acute SCI that can predict long-term outcome.

The mechanisms involved in the pathogenesis of SCI include a primary mechanical injury (impact) and a secondary injury induced by multiple subsequent biological processes, including a local inflammatory response, cytotoxicity, apoptosis, and demyelination[9, 144, 145]. In addition to local inflammation, a systemic inflammatory response, inducing organ damage, has been shown to occur following SCI[307]. Although altered gene expression significantly contributes to the pathogenesis of secondary SCI[144, 145], the regulatory networks that control it are not well understood. An aspect of the complex nature of secondary SCI could be derived from gene regulation by microRNAs[1, 11, 134]. MicroRNAs, as potential indicators of a pathological state, are carried to the periphery and are appealing candidates for monitoring CNS pathophysiology related to SCI.

In order to establish microRNA biomarkers that are representative of acute injury within the spinal cord, the "tissue" that is in closest proximity to the injured cord, and which is obtainable in human patients, is the CSF. Damage to the spinal cord releases proteins, metabolites, and nucleic acids into the CSF[25, 34] and we have previously identified several inflammatory and structural proteins related to injury severity within the CSF of human patients with SCI[21]. While CSF might be the most logical sampling site, logistically the serum samples represent a less invasive biological fluid that is significantly easier to obtain. In this study, we intended on providing strong evidence in support of CSF borne microRNAs that were detectable in serum and showed a severity-dependent pattern of expression. Unfortunately, the profiling of CSF microRNAs proved to be unsuccessful in a majority of pig CSF samples. Therefore, after verifying that the microRNAs seen in CSF are

also observed in serum with significant correlation between their expression levels, we focused on identifying markers of injury severity in the serum.

MicroRNAs have been previously investigated in the setting of SCI in a number of rodent studies. Hachisuka et al.[146] showed that several microRNAs increased following SCI in a severity-dependent fashion in mouse serum. In a microarray study using a contusion model of SCI in rats, the levels of over 35% of the microRNAs expressed in spinal cord tissue were significantly altered within 7 days post injury[131]. A separate study of changes in gene expression within different spinal cord regions showed greater changes in moderate and severe injuries, compared to mild and SHAM injury. As we observed in pig, the expression of some rodent genes was proportional to injury severity[305].

The porcine model is a large animal model of SCI and has several advantages over rodent models of SCI. In addition to the anatomical similarities between pigs and humans, measuring temporal changes in neurochemical markers is possible in pigs because of the ability to serially collect samples over time due to the larger CSF-filled subarachnoid space in pigs compared to rodents. In addition, the microRNA profile of pigs is more conserved with humans compared to rodents[3], with 45% of the known pig microRNAs having human orthologs, compared to only 16% of the known rat microRNAs[308]. Together, these factors make the pig a more relevant model, with regards to clinical translation, for studying the changes in microRNAs following SCI in patients.

The mechanisms that underlie the global, severity-dependent increase in microRNA abundance we observed are not yet clear. To reiterate, all animals in our study (SHAM included) underwent

the same surgical approach, the same extent of laminectomy, and the same sample acquisition protocols – the only difference between groups was the height from which the weight was dropped onto the exposed spinal cord. Therefore, the differences in serum microRNA expression between groups cannot be explained by a difference in *surgical* trauma, since the surgeries themselves were identical, but only by a more severely injured spinal cord. Similarly, the increase seen in the SCI animals versus SHAM control animals cannot be explained by surgical insult because the SHAM animals underwent the identical surgical exposure and laminectomy and only differed by the absence of the contusion injury. The most parsimonious explanation is that the actual traumatic SCI is directly or indirectly driving up the global concentration of microRNAs within serum. In previous studies, a number of structural and inflammatory markers were reported as biomarkers for SCI severity[21, 25, 34, 284], likely arising from acute damage to the spinal cord itself.

In light of these caveats, our observations are consistent with the observed increase in microRNAs reflecting active, biogenic upregulation of microRNAs systemically. While the accumulation of microRNAs we observe could be specific, it may also reflect downstream effects of a systemic inflammatory response that is proportional to the severity of injury. A systemic inflammatory response in relation to SCI has been documented[307]. Additionally, Bao et al.[309] showed evidence for the activation of circulating inflammatory cells after SCI that potentially damage tissues outside the spinal cord. With an increased systemic inflammatory response, and increased cell turnover, there may be elevated levels of free-floating microRNAs in circulation due to cell death, or exosomal microRNA that are contributing to the inflammatory response or programmed cell death.

Interestingly, Yunta et al. have shown a global *downregulation* of microRNAs within spinal cord tissue of rodents following SCI[10]. The authors suggest the changes could be due to the death of specific cell types within the spinal cord. We would hypothesize that the downregulation of microRNAs within spinal cord tissue could contribute to the global upregulation we observe in fluid – the acute damage to the spinal cord causes a massive cell death event, releasing cellular contents into circulation, ie: less microRNAs in tissue, more in circulation. Studies in TBI[310], liver injury[311], and SCI[25, 284] have shown that acute damage to the brain, liver, or spinal cord, resulting in cell death, produces measurable biomarkers in the blood or CSF that can be traced back to the tissue of origin. The fact that the SCI itself, and not the SHAM surgery, inflicts a rise in serum microRNAs in a severity dependent pattern provides a specific, promising biomarker for injury severity.

There are a number of limitations worth discussing with regards to this study. Firstly, global upregulation of microRNAs seems to be a strong indicator of injury severity and correlates well with 12-week post-injury outcome, however the overall cumulative global expression was not found to be statistically different between the mild and sham groups (Figure 3.9). This suggests that mild SCI may be difficult to detect based on overall microRNA expression. While this may be the case, there were 4 microRNAs found to be differentially expressed in the mild SCI group (3 of which were also dysregulated in other SCI groups), but not in the SHAM group (Figure 3.7). These microRNAs may provide the subtle discrimination when the differences between groups are not easily detectable and may indicate the difference between CNS injury and surgical treatment. Secondly, the numbers of replicates in this study are relatively low. Initially, six biological replicates were used in each group (24 animals). Unfortunately, sample collection was a

challenging aspect of this study. We therefore selected animals in which a full set of corresponding CSF and serum at all four time points was present, resulting in four animals per group. One advantage to this porcine model of SCI, however, is the ability to serially collect samples from the same animals – to be able to investigate expression changes, over time, in the same animal. While this study describes four experimental groups, they are not mutually exclusive; each injury severity group is a gradation of the same treatment, and the results show a corresponding gradation of microRNA expression. We felt that the inclusion of four experimental severities and six replicates per group were stronger than, say, two experimental groups (SCI and control, for example) and 12 replicates in each group. This gives us confidence in the statistical power of the study, as well as in the observed effects. Thirdly, the associated costs with surgery, post-surgical care, and 12 weeks of animal housing, in addition to sample preparation costs, were also limiting. With four replicates and four time points across four treatment groups, next-generation sequencing was performed on 128 CSF and serum samples. Finally, while this study lacks the validation of specific microRNAs using PCR-based detected methods, this was a strategic decision to focus on surveying all detectable microRNAs in both CSF and serum. Our ultimate goal is in the development of a clinical and pre-clinical biomarker that can be used in both humans and our porcine model. Validation of microRNAs that might be pig-specific would be premature. Following this study, we are profiling microRNA changes in the CSF and serum collected from human patients with traumatic SCI. While there is a relatively high cost associated with microRNA sequencing, the economic benefit will far outweigh the cost of sample preparation, and the ability to accurately diagnose patients will be invaluable. Once we have identified top microRNA biomarker candidates that exist in *both* human and pig biofluids, we will aim to validate these microRNAs in both species and generate a panel of genes that are highly diagnostic of SCI severity, and predictive of outcome.

3.5 Conclusions

Our study provides a comprehensive description of the changes that occur across microRNAs during the early post-injury phase of acute SCI. The microRNAs detected in porcine serum increased globally in an injury severity dependent manner and provides promising targets for markers of injury severity in SCI. The results from this study will guide the investigation of temporal changes in microRNAs within human samples of CSF and serum, collected from patients with traumatic SCI. With the continuous emergence of new SCI therapies that are seeking validation in clinical trials, the field of SCI is in dire need of new approaches for classifying injury severity and improved methods of predicting outcome. MicroRNA biomarkers are promising solutions to this bottleneck in the pipeline of developing clinically relevant therapies for SCI.

Figure 3.1 Study outline. Miniature Yucatan pigs received either SHAM laminectomy (black), or a contusion SCI using a weight dropped from 40 (red), 20 (blue), or 10 cm (green), followed by 5 minutes of compression. For microRNA expression profiling, CSF and serum samples were collected before injury (baseline) and at 1, 3, and 5 days post injury. Functional recovery was assessed weekly for 12 weeks using PTIBS. At 12 weeks post injury, animals were euthanized, and the spinal cord processed for spared white and gray matter quantification.

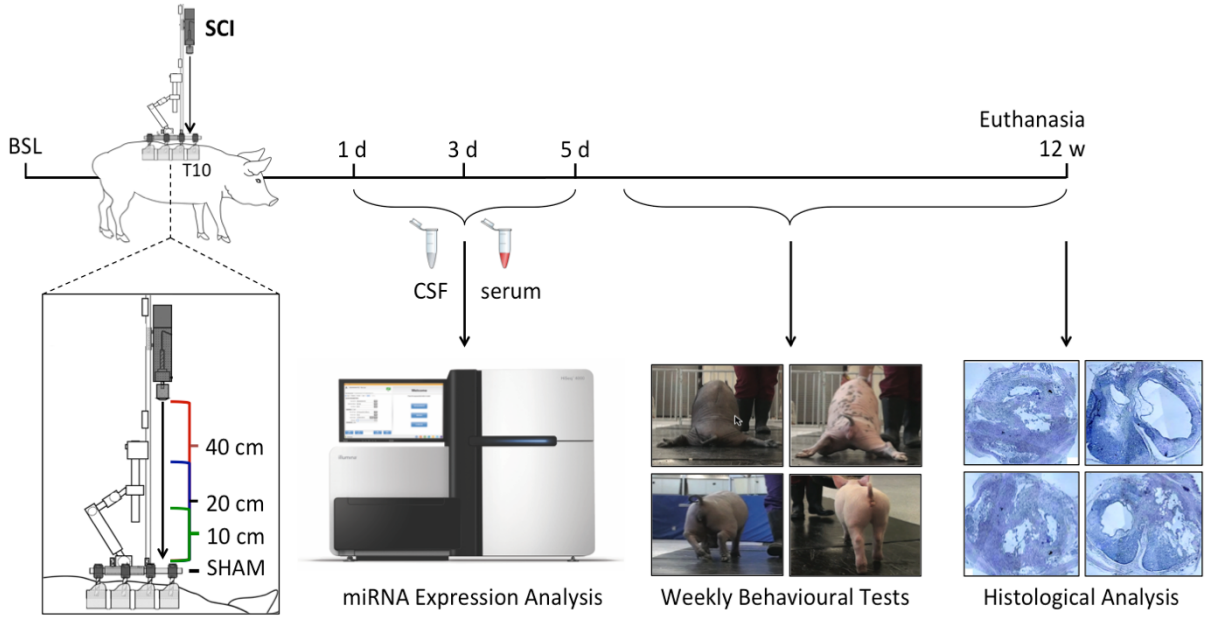


Figure 3.2 Effect of injury severity on locomotor recovery after spinal cord injury. PTIBS scores were measured before injury (baseline) and weekly for 12 weeks post injury for the 40 (red), 20 (blue), 10 cm (green), and SHAM (black) groups. Data points represent mean ± SEM for n = 4 animals per group (see Table 1).

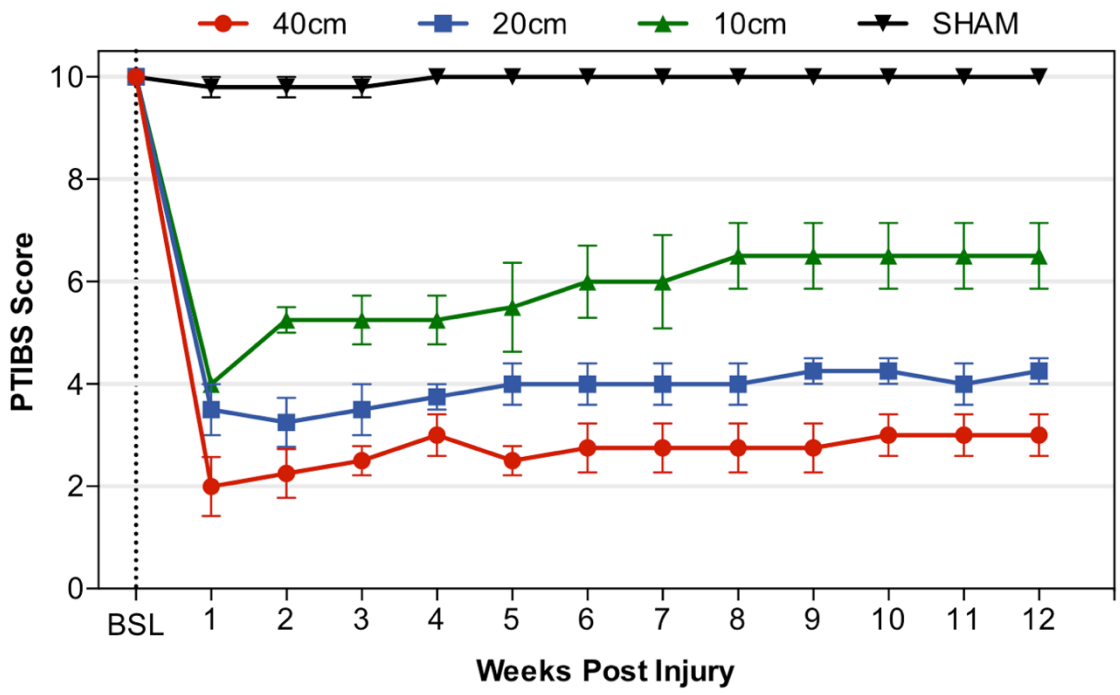


Figure 3.3 Effect of injury severity on tissue sparing 12 weeks after spinal cord injury. Spared tissue was measured using Eriochrome Cyanine stained sections from 13.6 mm rostral and 13.6 mm caudal to the site of injury. (A) The total spared tissue in percent, relative to the entire area of the spinal cord of the same section was calculated for the 40 (red), 20 (blue), 10 cm (green), and SHAM (black) groups. (B) The total amount of tissue sparing (cumulative percent) was calculated using the area under the curve of total spared tissue (%) for the 40 (red), 20 (blue), 10 cm (green), and SHAM (black) groups. Asterisks indicate sparing for which significant differences were found between groups with ANOVA. Data is presented as means ± SEM for n = 4 animals per group. SCI, spinal cord injury.

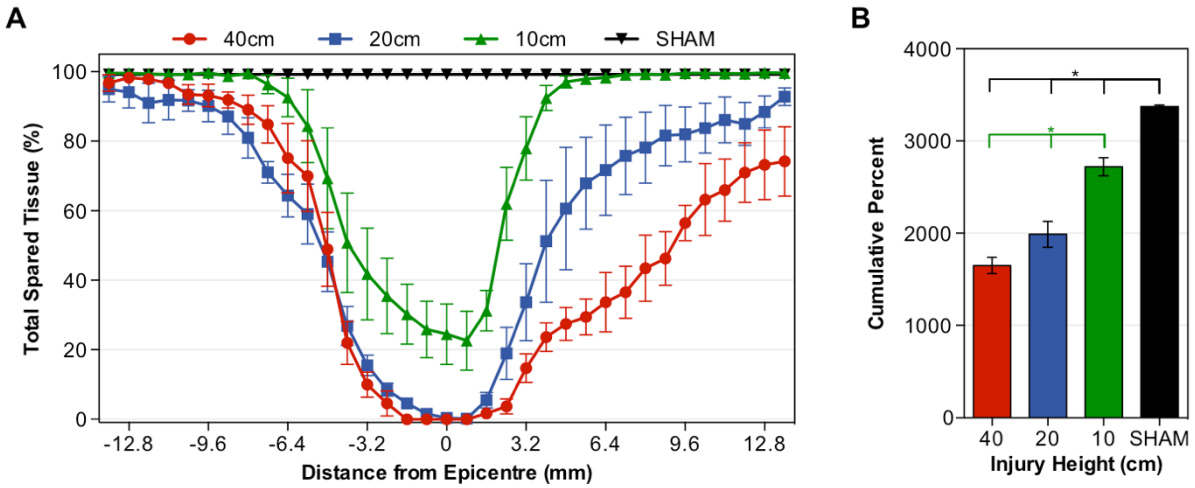


Figure 3.4 Bioinformatic pipeline results. (A) Bioinformatic Pipeline. Raw reads were assessed for quality using FastQC before and after adapter trimming with the Cutadapt package. Trimmed reads were mapped to the porcine (*Sus scrofa*) reference genome using the miRDeep2 mapper. Aligned reads were then mapped to the miRBase microRNA database using the miRDeep2 module. MiRDeep2 gives a matrix list of known and novel microRNAs. (B) Detected microRNAs. 314 out of the total 411 known *Sus scrofa* microRNAs (white) were identified across all serum (red, 314 microRNAs) and CSF (blue, 280 microRNAs) samples, while 14 novel microRNAs (grey) that do not yet exist in the *Sus scrofa* microRNA database were identified. (C) Number of microRNA reads for all samples. 60 serum samples (red) had over the cut-off of 50,000 microRNA reads, only 4 CSF (blue) samples had above 50,000 reads, and 11 CSF samples had over 10,000 microRNA reads.

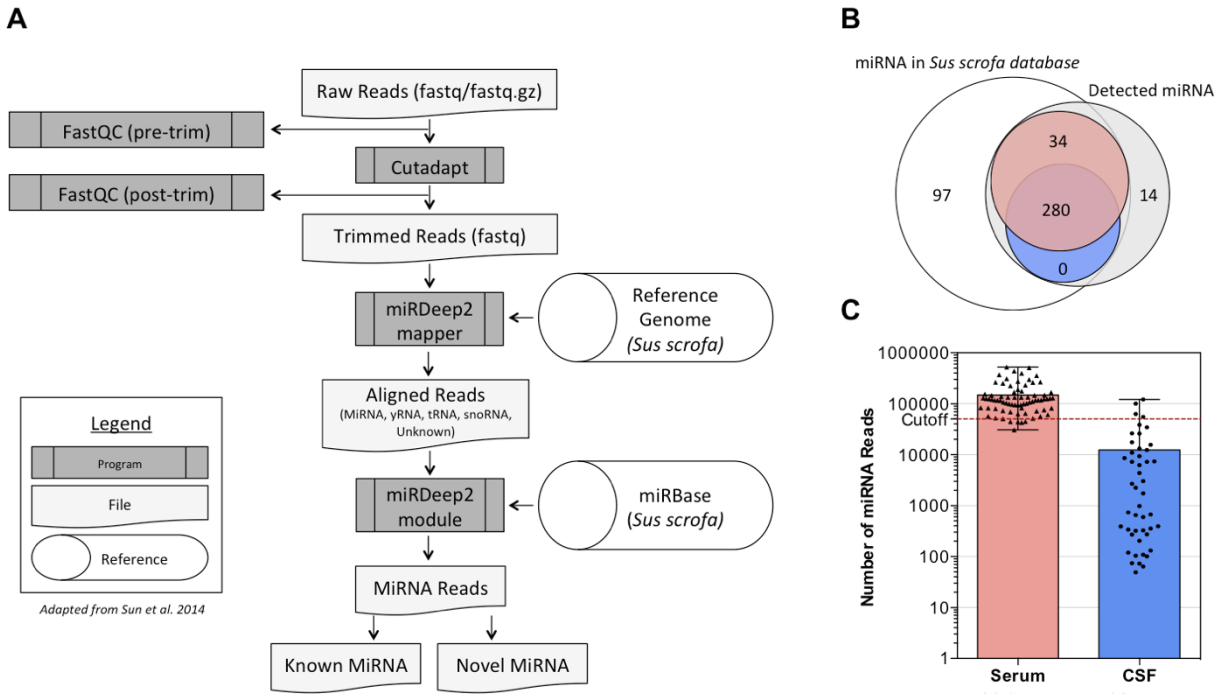


Figure 3.5 Spearman correlation of subsets of read counts to a total of 1.7 million reads. Spearman correlation (green) of subsets of randomly selected reads to a total of 1.7 million reads, starting with 1,000 reads and incrementally increasing by thousands to a total of 300,000. Number of microRNAs detected (blue) using randomly selected reads, starting with 1,000 reads and incrementally increasing by thousands to a total of 300,000.

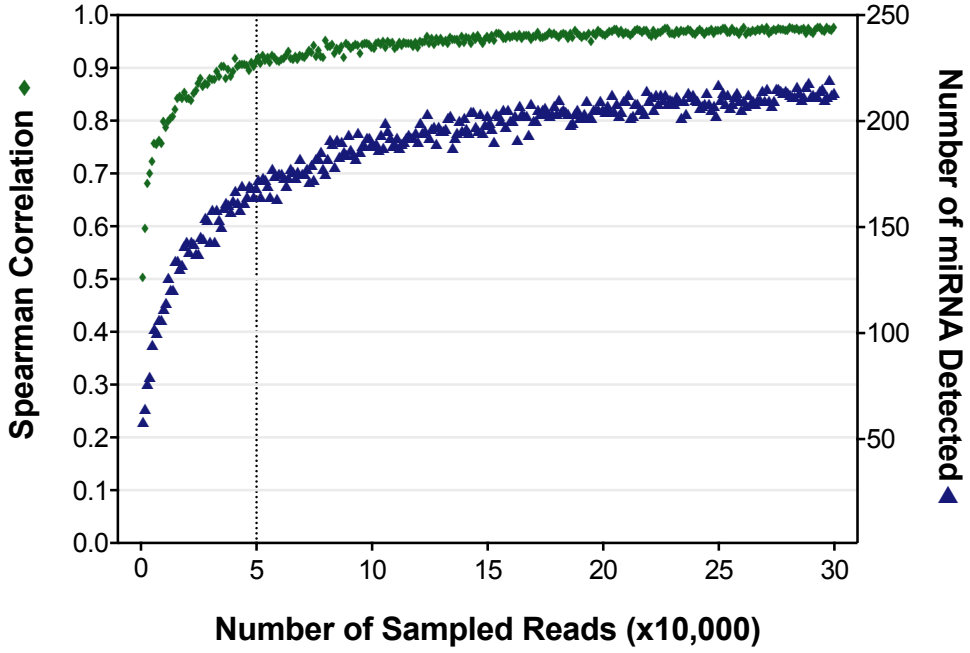


Figure 3.6 Correlation of serum and CSF average microRNA counts for all detected genes.

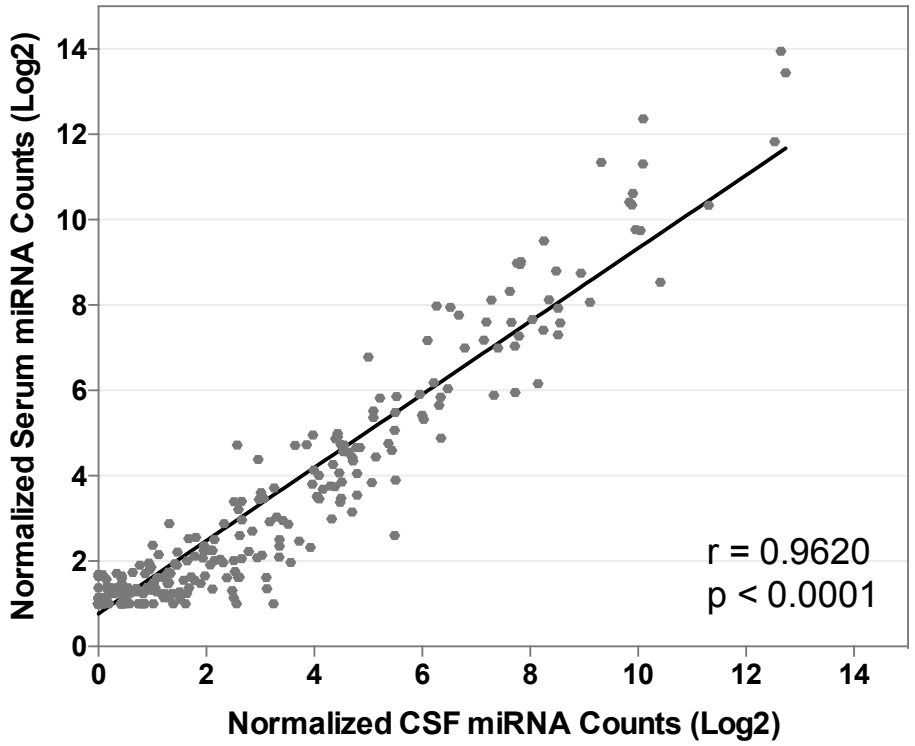


Figure 3.7 Venn diagram showing severity-dependent dysregulated serum microRNAs. Venn diagram showing numbers of deregulated serum microRNAs after severe (40 cm, red), moderate (20 cm, blue), mild (10 cm, green) injury, or SHAM surgery (Non-CNS injury control, black). Dysregulated microRNAs were determined by multiple t-tests, while adjusting the p-value using the Benjamini-Hochberg method.

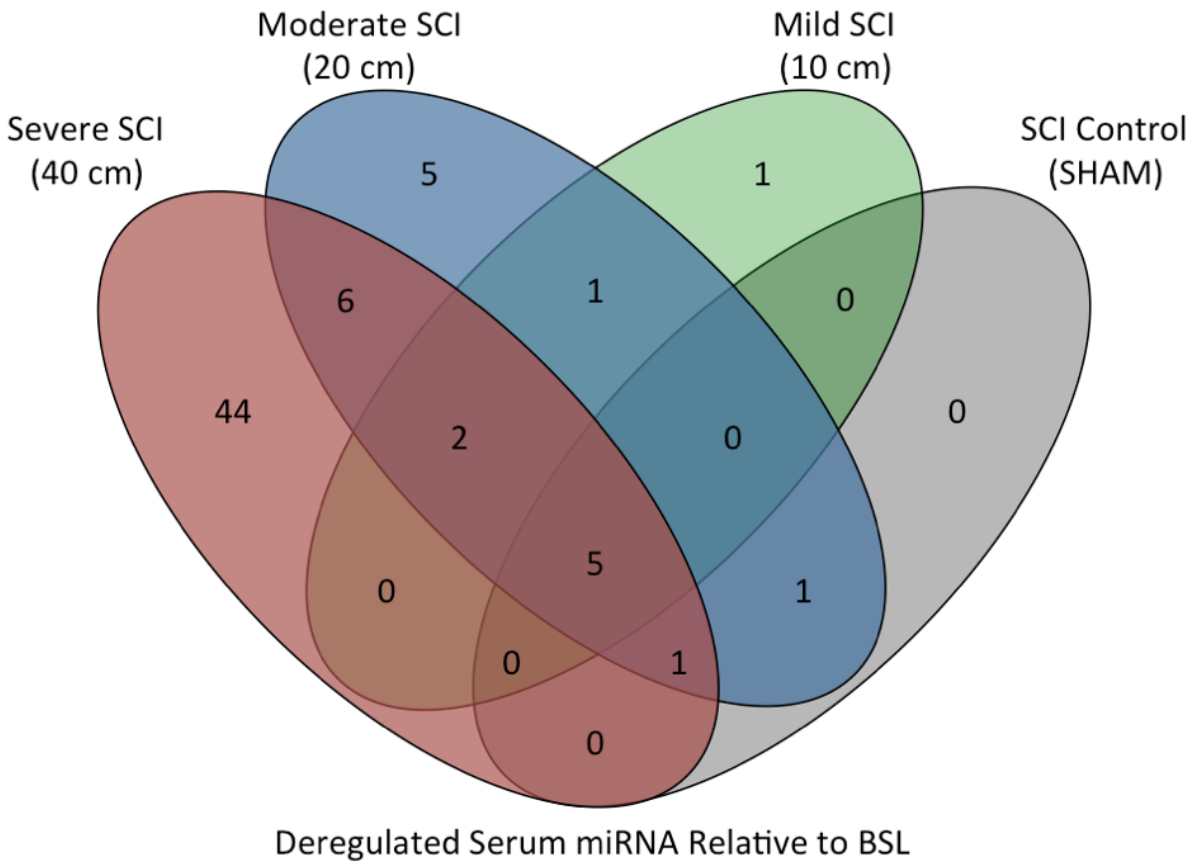


Figure 3.8 Effect of injury severity on global microRNA expression. Smoothed lines showing the trend in global microRNA expression at BSL (solid lines), and at 1, 3, and 5 days post injury (dashed lines) in each of the (A) 40 cm, (B) 20 cm, (C) 10 cm, and (D) SHAM groups. Data points represent the smoothed read counts for the top 100 microRNA genes for n = 4 animals per group and time point (see table 1).

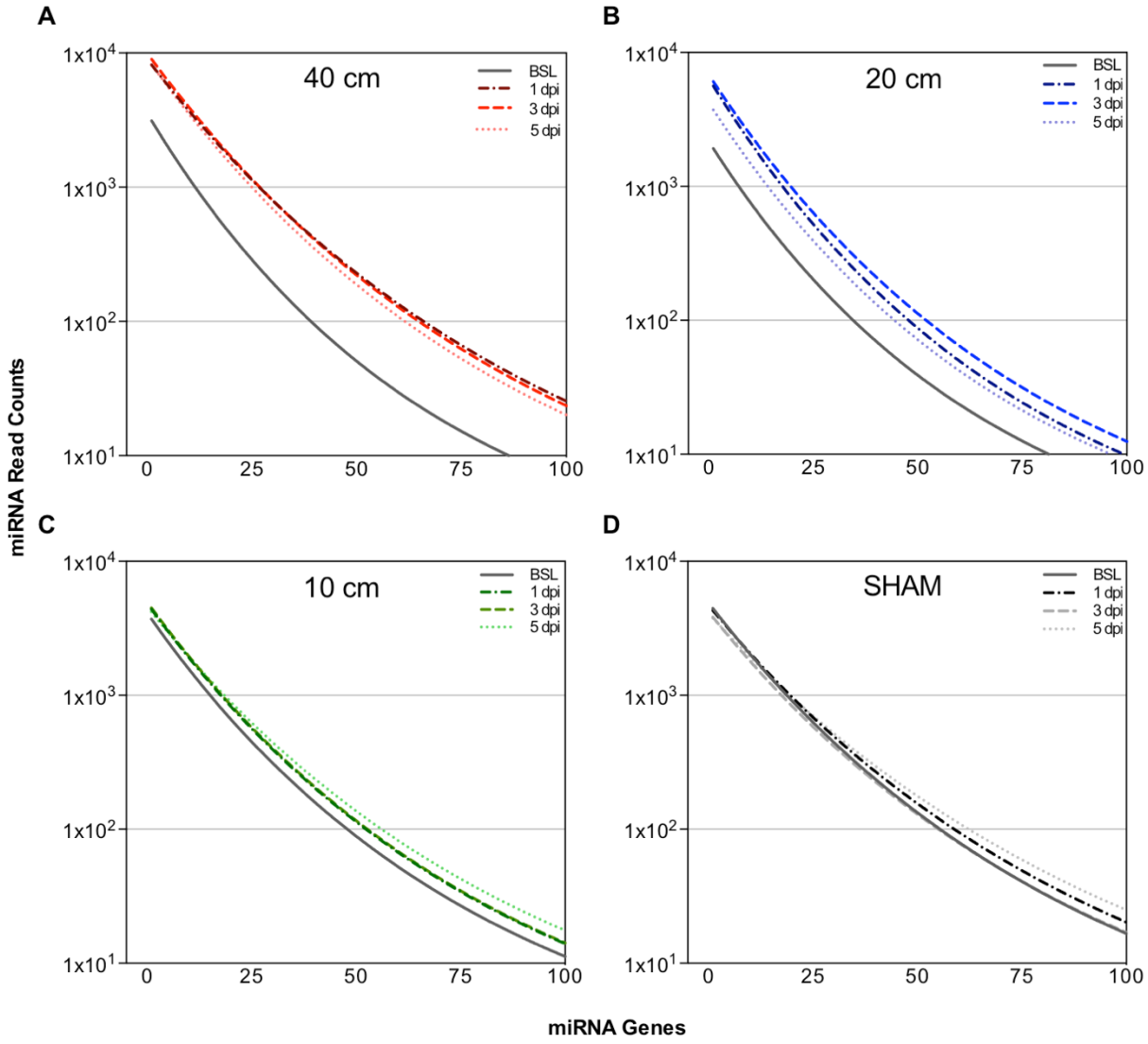


Figure 3.9 Effect of injury severity on total systemic microRNA. (A) The total number of normalized microRNA counts in the 40 cm (red), 20 cm (blue), 10 cm (green), and SHAM (black) groups. Data points represent the average of the normalized total microRNA reads for n = 4 animals per group and time point (see Table 1). B. The cumulative microRNA expression over time in the 40 cm (red), 20 cm (blue), 10 cm (green), and SHAM (black) groups. Asterisks indicate significant differences determined by ANOVA. Data is presented as means ± SEM.

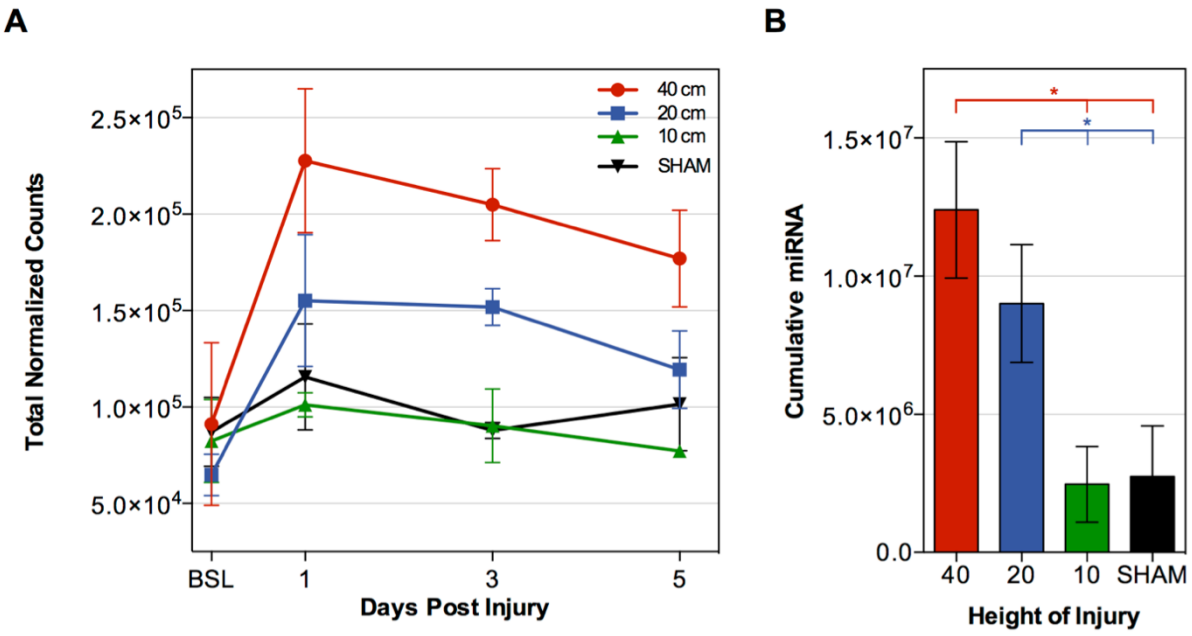


Figure 3.10 The diagnostic accuracy of significantly deregulated serum microRNAs for spinal cord injury severity. Accuracy was assessed using the area under the receiver operator curve of the 10 microRNAs with the highest diagnostic accuracy (area under the curve (AUC)) and smallest p-value, at 1- and 3-days post injury, to distinguish between (A) SCI and SHAM (non-CNS injury control). The AUC's were miR-133a-5p (AUC = 0.95), miR-378 (AUC = 0.91), miR-378b-3p (AUC = 0.90), miR-365-3p (AUC = 0.89), miR-133b (AUC = 0.89), miR-10b (AUC = 0.88), miR-885-5p (AUC = 0.88), miR-130a (AUC = 0.88), miR-100 (AUC = 0.88), and miR-208b (AUC = 0.87). (B) Severe and mild SCI (40 vs 10 cm), the AUC's were miR-423-3p (AUC = 1.00), miR-425-5p (AUC = 1.00), miR-486 (AUC = 1.00), miR-100 (AUC = 0.97), miR-10b (AUC = 0.94), miR-378 (AUC = 0.94), miR-204 (AUC = 0.92), miR-22-5p (AUC = 0.92), miR-378b-3p (AUC = 0.92,), and miR-125b (AUC = 0.91). (C) Severe and moderate SCI (40 vs 20 cm), the AUC's were miR-130a (AUC = 0.98), miR-744 (AUC = 0.98), miR-425-5p (AUC = 0.97), miR-130b (AUC = 0.95), miR-423-3p (AUC = 0.95), miR-125b (AUC = 0.92), miR-152 (AUC = 0.92), let-7i (AUC = 0.89,), miR-100 (AUC = 0.88), and miR-30b-5p (AUC = 0.88). (D) Moderate and mild SCI (20 vs 10 cm), the AUC's were miR-486 (AUC = 0.86,), miR-10b (AUC = 0.85), miR-100 (AUC = 0.82), miR-301 (AUC = 0.82), miR-378 (AUC = 0.81), miR-133a-5p (AUC = 0.79), miR-126-5p (AUC = 0.79), miR-30b-5p (AUC = 0.79), and miR-378b-3p (AUC = 0.79).

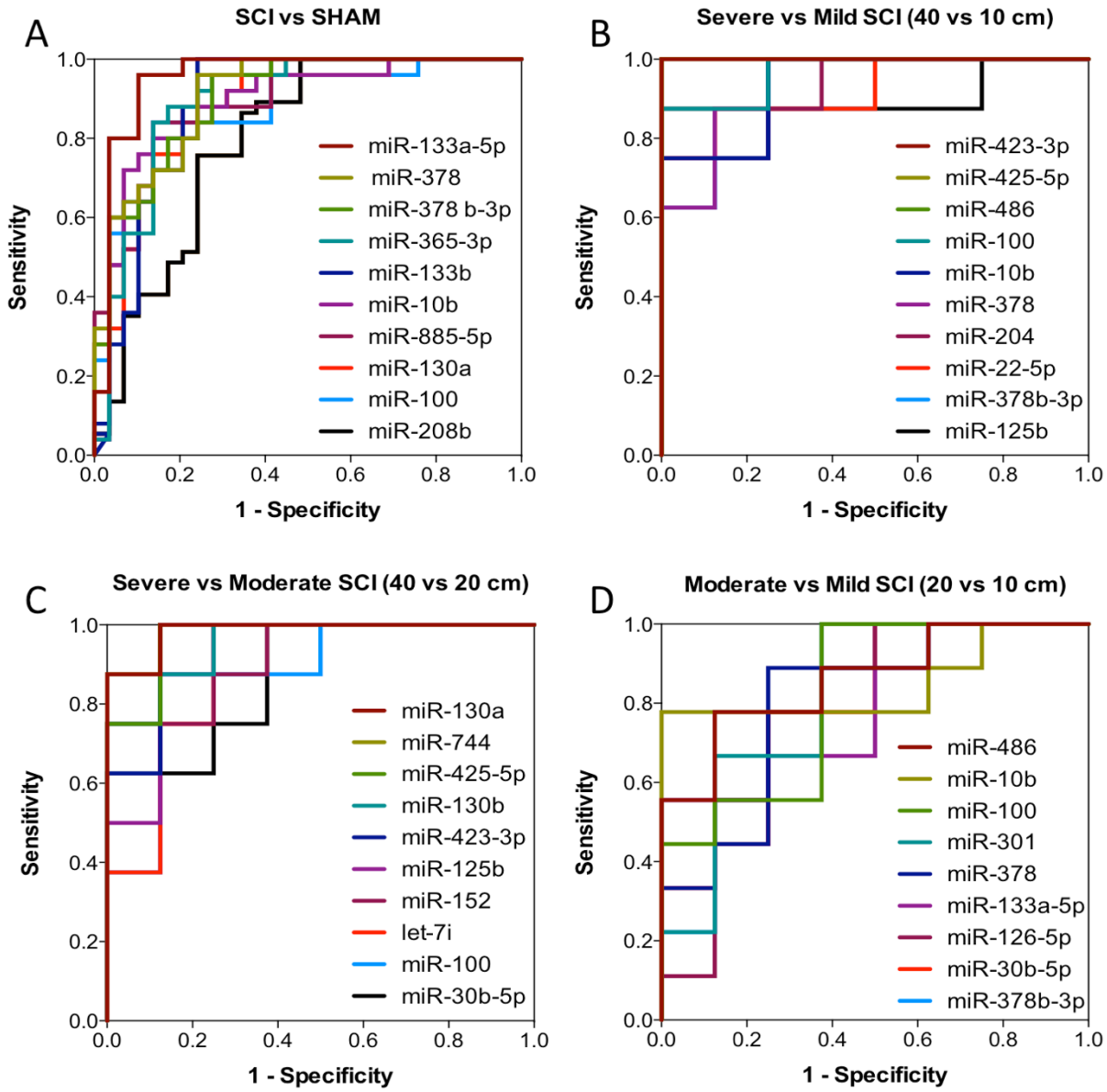

A
SCI vs SHAM
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
miR-133a-5p
miR-378
miR-378 b-3p
miR-365-3p
miR-133b
miR-10b
miR-885-5p
miR-130a
miR-100
miR-208b
B
Severe vs Mild SCI (40 vs 10 cm)
Sensitivity
1 - Specificity
miR-423-3p
miR-425-5p
miR-486
miR-100
miR-10b
miR-378
miR-204
miR-22-5p
miR-378b-3p
miR-125b
C
Severe vs Moderate SCI (40 vs 20 cm)
Sensitivity
1 - Specificity
miR-130a
miR-744
miR-425-5p
miR-130b
miR-423-3p
miR-125b
miR-152
let-7i
miR-100
miR-30b-5p
D
Moderate vs Mild SCI (20 vs 10 cm)
Sensitivity
1 - Specificity
miR-486
miR-10b
miR-100
miR-301
miR-378
miR-133a-5p
miR-126-5p
miR-30b-5p
miR-378b-3p

Figure 3.11 Correlations between total serum microRNA levels and porcine thoracic injury behaviour scores. Correlation between 12-week post-injury PTIBS scores and the total microRNA counts at (A) Baseline, before injury (BSL), (B) 1-day post injury, (C) 3 days post injury, and (D) 5 days post injury. The global upregulation in microRNAs at 1- and 3-days post injury is correlated strongly with the behavioral recovery at 12 weeks post injury.

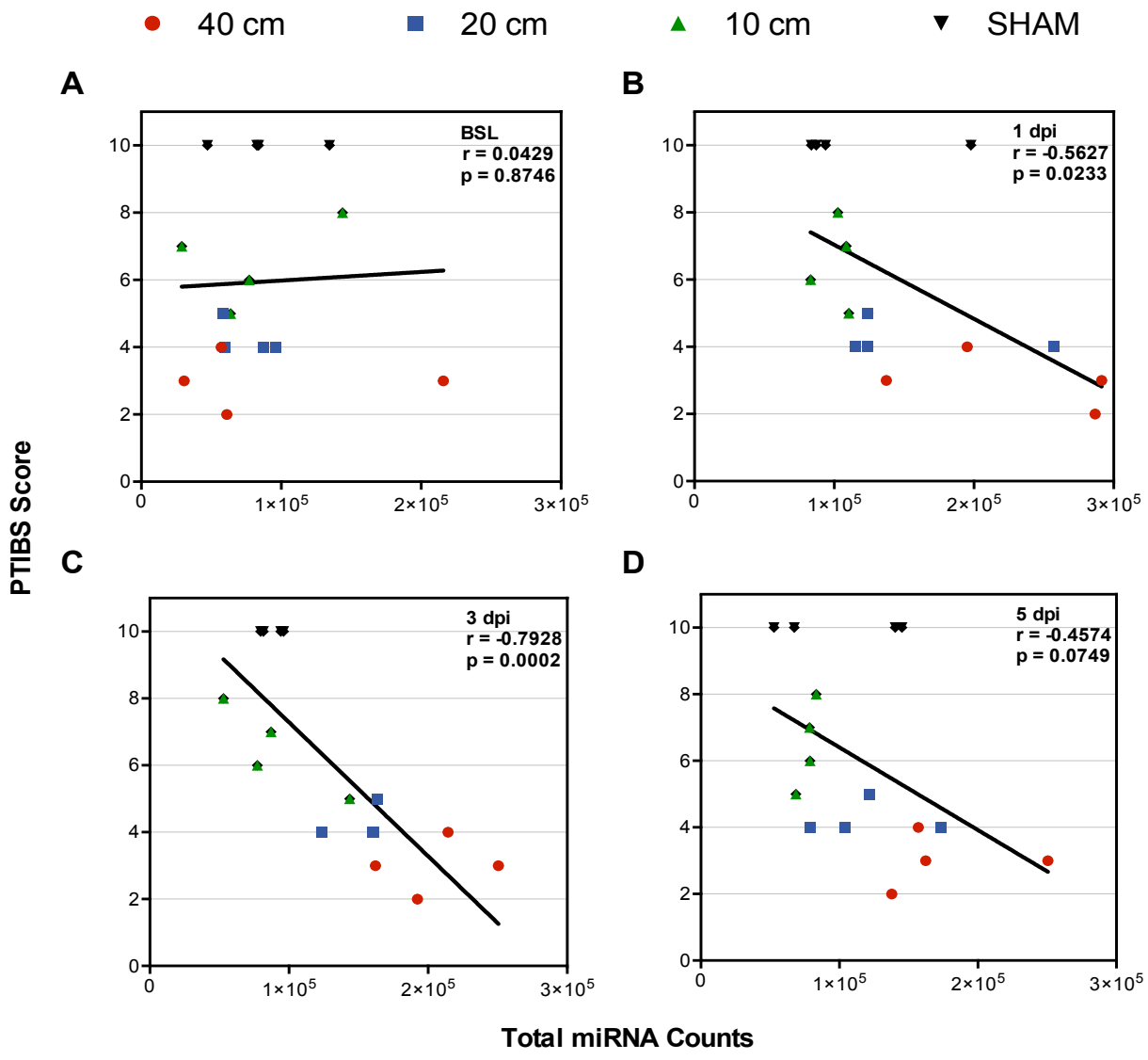


Figure 3.12 Correlations between total microRNA expression levels and total percent spared tissue. Correlation between total percent spared tissue and the total microRNA counts at (A) baseline before injury (BSL), (B) 1-day post injury (dpi), (C) 3 days post injury, and (D) 5 days post injury. The global upregulation in microRNA is correlated strongly with the extent of tissue damage at 1- and 3-days post injury in particular.

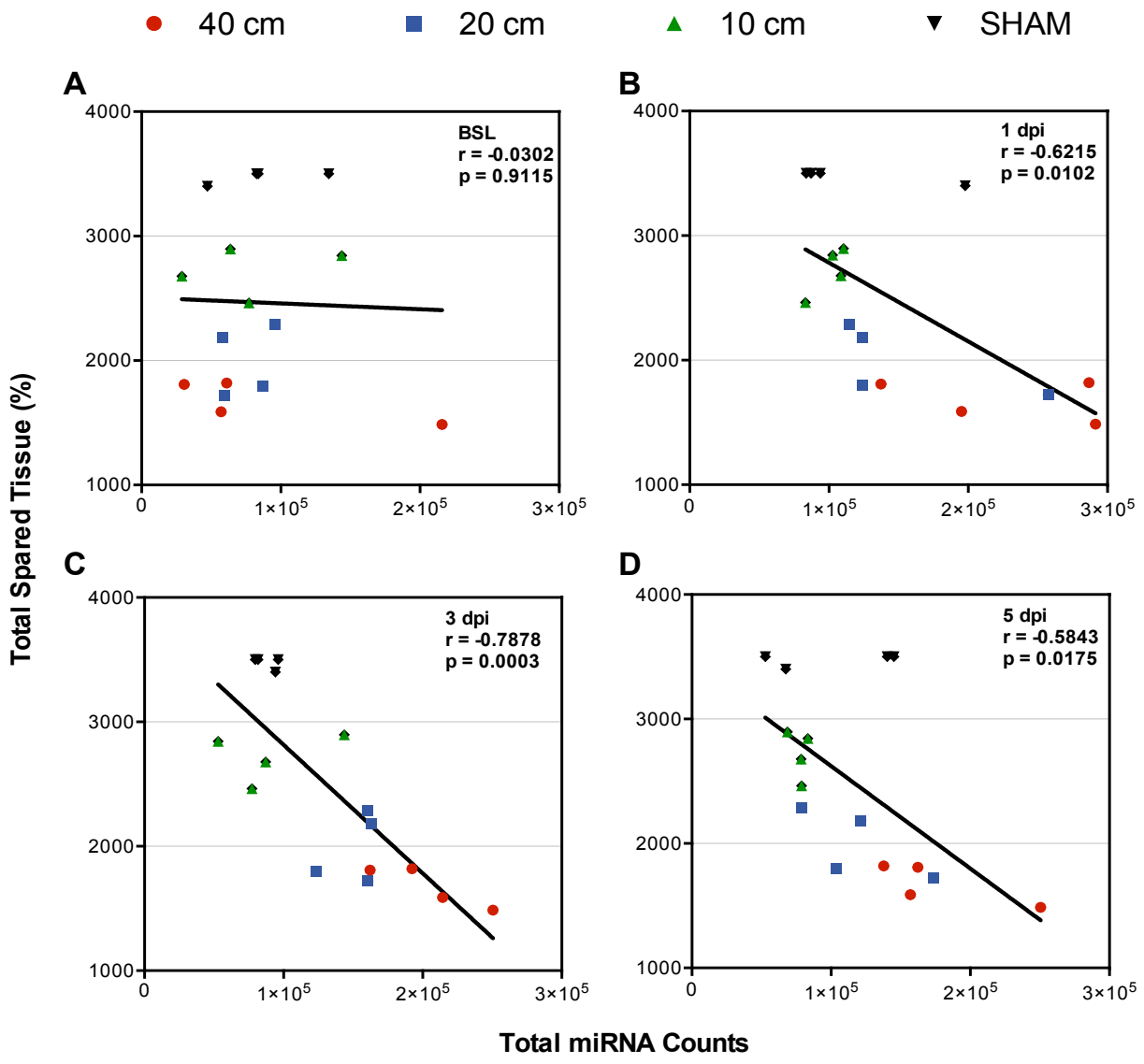
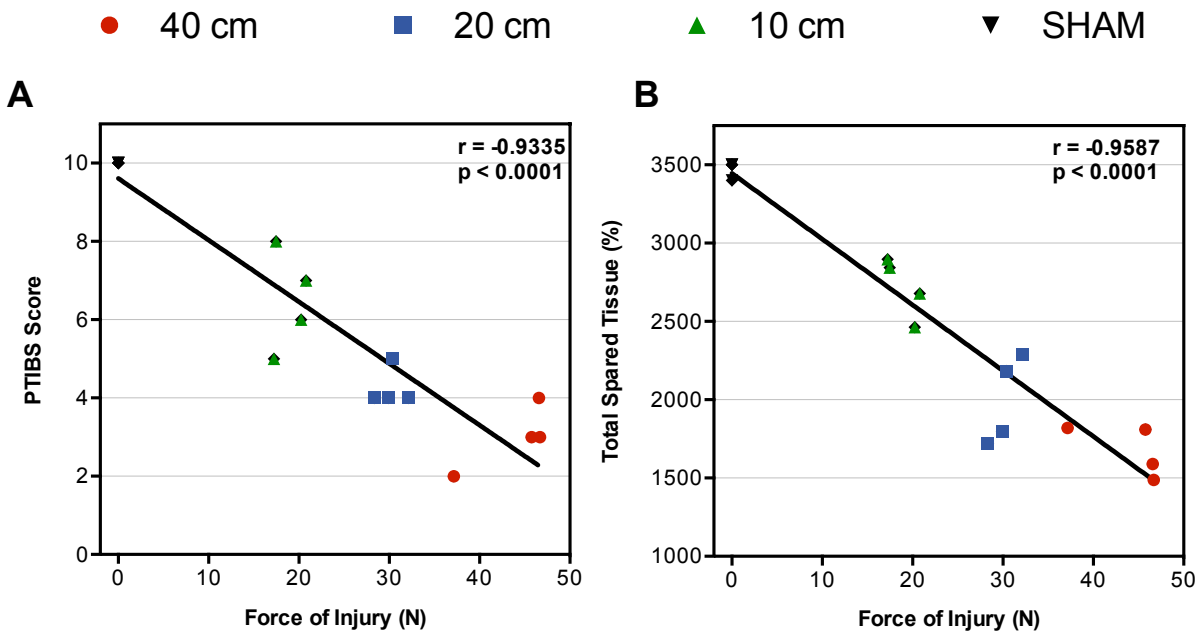


Figure 3.13 Correlation between Force of Injury (N) and outcome parameters. Correlation between Force of injury and (A) Porcine Thoracic Injury Behaviour Scores at 12 wpi and (B) Total percent spared tissue. These figures simply demonstrate that for our pig model of thoracic SCI, the initial force of the contusion impact (dictated by the height of the weight drop) is strongly correlated to the degree of hindlimb locomotor impairment as measured by PTIBS (A) and the amount of tissue damage that occurs in the spinal cord (B).



Chapter 4: MicroRNA biomarkers in human patients with acute traumatic spinal cord injury

4.1 Introduction

SCI is a devastating condition, often resulting in life-long disability[312-315]. The SCI patient population comprises an extremely heterogeneous group that differ with regards to age, injury mechanism, location, severity, and genetic background. Although a small number of acute SCI clinical trials have been conducted; none have yet shown convincing efficacy[17]. As a consequence, neuro-restorative treatment options for acute SCI patients are currently limited.

One major obstacle to the clinical testing and validation of novel SCI treatments is the reliance on the functional neurologic assessment, a test performed according to the ISNCSCI. The ISNCSCI examination requires patients to 1) demonstrate motor function in the upper and lower extremities, 2) report pin prick and light touch skin sensitivity throughout the body and 3) undergo an evaluation of voluntary anal contraction and deep anal pressure sensation. This evaluation is clearly challenging to perform in the acute setting, particularly in patients who have multiple injuries, brain trauma, or who are intoxicated or sedated pharmacologically. This inability to establish a standardized, functional baseline injury limits the pool of "recruitable" patients who are eligible for clinical trials. An additional challenge for clinical trials is that even when an ISNCSCI examination can be performed and a "baseline" AIS grade assigned, there is considerable variability in the spontaneous neurologic recovery of acute SCI patients. As a

consequence, studies must recruit large numbers of patients to achieve sufficient statistical power to detect any improvement in neurologic function[16].

Biological markers of SCI that objectively stratify the severity of cord damage could greatly expand the size and scope of clinical trials and enable the testing of novel therapies for acute SCI. For example, such biomarkers may be able to predict neurologic recovery over time with greater precision, sensitivity and reproducibility than the standard clinical examination, reducing the number of patients needed to sufficiently power clinical trials. Towards realizing this goal, there has been considerable efforts to establish CSF and serum biomarkers after acute SCI. We previously showed that the inflammatory cytokines IL-6, IL-8, and MCP-1, and other proteins such as tau, S100β, and GFAP were significantly different between AIS grades in acute SCI patients and these differences could be used to both stratify injury severity and to predict neurologic outcome and were actually more accurate in predicting neurologic recovery than AIS grade[25, 84].

In this study, we used NGS to profile microRNAs in the CSF and serum to test the premise that such microRNAs could serve as biomarkers of injury severity after acute traumatic SCI in human patients. While CSF sampling requires invasive collection techniques, serum is far less invasive and may serve as an important surrogate for CSF. MicroRNAs are short, ~22 nucleotide long non-coding RNAs (whose biological roles include post-transcriptional regulation of gene expression) that have emerged as attractive biomarker candidates owing to their stability in biological fluids, their conservation between humans and model mammals, and their tissue specificity[1-4]. A number of microRNAs have been implicated in SCI and diverse neurological processes[5-13]. We have

previously shown that significant severity-dependent changes in microRNA expression are detectable in porcine serum following traumatic SCI[4]. In this study, human CSF and serum samples were collected from acute SCI patients, and NGS was performed on the microRNA populations to determine if changes in microRNA expression could be used as biomarkers of injury severity. To our knowledge, this represents the largest microRNA profiling dataset in the CSF and serum of human patients with SCI.

4.2 Material and methods

4.2.1 Clinical trial enrolment

Individuals with an acute SCI (Table 4.1) were enrolled into this prospective, multi-center, observational study if they met the following inclusion criteria: 1) AIS grade A, B, or C upon presentation; 2) spinal injury between C1 and L1; 3) within 24 hours of injury; and 4) the ability to provide a valid, reliable neurological examination. In general, AIS A indicates individuals with complete loss of motor function and sensation below the level of injury, AIS B indicates individuals with complete loss of motor function with some preserved sensation, and AIS C indicates individuals with some preserved motor function and sensation. Patients were excluded if they had a concomitant brain injury, major trauma to the chest, pelvis, or extremities that required invasive intervention (e.g. chest tube, internal or external fixation), or were too sedated or intoxicated to provide a valid neurological examination. For the "negative," non-SCI control group, five adult subjects undergoing routine lumbar decompressions and/or fusions were included and had no current or past history of SCI or myelopathy (Table 4.2).

Table 4.1 Clinical characteristics of the cohort of 39 SCI patients. In summary, of the 39 patients, there were 24 AIS A, 8 AIS B, and 7 AIS C at baseline. There were 31 males and 8 females, and the mean age was 42.9 (median age = 40) with a standard deviation of 17.6. A total of 22 were cervical level injuries.

	Initial baseline examination					***6-month follow-up examination***				
Sex	*Level*	*AIS*	*UEMS*	*LEMS*	*MS*	*Level*	*AIS*	*UEMS*	*LEMS*	*MS*
Male	C1	A	24	0	24	C8	A	47	0	47
Male	C3	A	0	0	0	C2	A	2	0	2
Male	C5	A	22	0	22	C6	A	23	0	23
Male	C6	A	24	0	24	C6	A	27	0	27
Male	C7	A	46	0	46	C8	A	50	0	50
Male	L1	A	50	12	62	L3	A	50	20	70
Male	T3	A	50	0	50	T3	A	50	0	50
Male	T3	A	50	0	50	T3	A	50	0	50
Male	T5	A	50	0	50	T4	A	50	0	50
Male	T7	A	50	0	50	T5	A	50	0	50
Male	T9	A	50	0	50	T8	A	50	0	50
Male	T11	A	50	0	50	C7	A	50	0	50
Male	C4	A	20	0	20	C4	B	30	0	30
Female	C4	A	10	0	10	C4	B	17	0	17
Female	C6	A	23	0	23	C6	B	26	0	26
Male	L1	A	50	11	61	L3	B	50	26	76
Male	L1	A	50	2	52	L3	B	50	20	70
Male	T4	A	50	NA	50	T4	B	50	NA	50
Male	T12	A	50	2	52	L1	B	50	5	55
Male	C4	A	14	1	15	C3	C	16	15	31
Male	C7	A	43	0	43	C7	C	50	0	50
Male	T11	A	50	0	50	T11	C	50	2	52
Female	T12	A	50	0	50	T12	C	50	1	51
Male	C4	A	32	0	32	C4	D	34	28	62
Male	C4	B	13	0	13	C4	B	22	0	22
Male	C5	B	8	0	8	C5	B	15	0	15
Male	C5	B	46	0	46	C6	C	44	2	46
Male	C5	B	47	0	47	T1	C	50	7	57
Female	C4	B	12	0	12	C5	D	24	47	71

	Initial baseline examination					*6-month follow-up examination*				
Sex	*Level*	*AIS*	*UEMS*	*LEMS*	*MS*	*Level*	*AIS*	*UEMS*	*LEMS*	*MS*
Male	C5	B	17	0	17	C6	D	47	50	97
Male	L1	B	50	0	50	L2	D	50	29	79
Male	C4	B	44	0	44	C4	D	36	23	59
Male	C4	C	11	1	12	C4	D	22	39	61
Female	C4	C	19	26	45	C6	D	45	50	95
Female	C5	C	12	29	41	C5	D	42	49	91
Female	C5	C	20	24	44	C5	D	48	50	98
Male	L1	C	50	22	72	L2	D	50	45	95
Female	T3	C	50	2	52	T3	D	50	50	100
Male	T5	C	50	27	77	T5	D	50	44	94

Table 4.2 Demographics of the negative control patients. The non-SCI negative control group consisted of CSF samples collected from 1 female and 4 males who underwent surgical intervention not related to acute SCI. The mean age was 55.4 (median age = 51) with a standard deviation of 11.1 years.

Sex	***Age***	***Diagnosis***	***Surgical procedure***
Male	60	L5-S1 Spinal Stenosis	L5-S1 Hemilaminectomy/Foraminotomy
Male	72	L3-L5 Spinal Stenosis	L3-L5 Laminectomy
Female	51	1º: L5-S1 Disc Protrusion; 2º: T2-L4 Scoliosis	L5-S1 Laminectomy/Discectomy
Male	51	L5-S1 Disc Protrusion	L5-S1 Laminectomy/Discectomy
Male	43	L4-L5 Disc Protrusion	L4-L5 Laminectomy/Discectomy

4.2.2 Neurological evaluation

The severity of neurological impairment was graded according to the ISNCSCI, with motor scores recorded separately in the upper and lower extremities. All baseline testing and the assigning of the baseline AIS grade (A, B, or C) was conducted by research study nurses. The ISNCSCI examination was conducted again at 6 months post injury to determine whether AIS grade had improved ("AIS grade conversion"), and the extent of total motor score improvement.

4.2.3 Cerebrospinal fluid and serum collection and processing

For enrolled patients, an intrathecal catheter (PERIFIX® Custom Epidural Anesthesia Kit, B. Braun Medical Inc. Bethlehem, PA) was inserted in the lumbar spine at L2/3 or L3/4 using a standard aseptic technique. The catheter was advanced 15 – 20 cm from the entry point on the skin surface and kept in place for 5 days. The catheters were inserted prior to surgery with the patient log-rolled to the right side, and the spinal column protected in a neutral position by a physician. CSF samples of 3 – 4 mL were drawn at the time of catheter insertion, and then in the subsequent postoperative period approximately three times each day (discarding the first 1 mL of CSF aspirated from the line). For the non-SCI control group, CSF and serum samples were obtained from five adult subjects undergoing routine lumbar decompressions and/or fusions were collected. Sample processing was performed immediately at the patient's bedside by the research study nursing team. The CSF samples were centrifuged at 1000 xg for 10 minutes, and the supernatant was then dispersed into 200 µL aliquots and immediately frozen in an ethanol-dry ice bath and stored at −80 °C. Blood samples were incubated at room temperature for 25 minutes, then centrifuged at 10,000 xg for 5 minutes. The serum supernatant was collected and dispersed into 200 µL aliquots and immediately frozen in an ethanol-dry ice bath and store at −80 °C.

4.2.4 RNA isolation and sequencing

Total RNA was isolated from CSF or serum (n = 386 total) samples using the miRVana PARIS RNA Isolation kit (ThermoFisher, Cat#: AM1556) according to the manufacturer's instructions, incorporating the modifications of Burgos et al.[288] for increasing yield. Total RNA was re-suspended in 7 μL of RNAse free water and used for library preparation with Illumina's TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA). Small RNA (composed of a number of RNA biotypes such as microRNAs, tRNAs, piRNAs, snoRNAs, and yRNAs) concentrations are measured using the Bioanalyzer 2100 in order to normalize the library concentration loaded on the sequencer. Libraries were individually barcoded with 48 Illumina-provided index barcodes so that samples could later be demultiplexed. Libraries were pooled and sequenced on the Illumina HiSeq 2500, generating single-end 36 base pair reads.

4.2.5 Post-sequencing analysis pipeline

Sequencing reads were processed using the CAP-miRSeq[289] bioinformatic pipeline. Read quality was assessed using FastQC before and after trimming adapter sequences and low quality 3′ bases. Adapter sequences were trimmed with Cutadapt and reads shorter than 17 nucleotides were discarded. Trimmed reads were first aligned using Bowtie where the pipeline conducts two alignment processes: one used internally for miRDeep2[316] to quantify and predict novel microRNAs and another for all RNA quantification and data visualization. The CAP-miRSeq pipeline generates a summary for each sample's alignment statistics and number of microRNAs detected with reports of raw counts for known microRNAs of all samples in matrix format.

Samples with a total sum of mapped microRNA read counts less than 50,000 reads per sample were removed from analysis.

4.2.6 Reverse transcription and pre-amplification

2 µL of isolated RNA was used for qPCR analysis. Reverse transcription (RT) was performed using the Taqman Advanced microRNA cDNA Synthesis Kit followed by a pre-amplification step before undergoing the real-time PCR reaction. Before RT, a polyA addition was performed on the 3' end using a 3 µL reaction mixture per sample containing 0.5 µL of 10x polyA Buffer and ATP, 0.3 µL of polyA enzyme and 1.7 µL of RNase-free water. The 5 µL reactions were incubated in a thermocycler for 45 minutes at 37 ˚C followed by 10 minutes of heat inactivation at 65 ˚C. An adaptor ligation addition was then performed to lengthen the 5' end by adding a ligation reaction mixture to the 5 µL polyA tailing reaction, containing 3 µL of 5x DNA Ligase Buffer, 4.5 µL of 50% PEG 8000, 0.6 µL of 25x Ligation Adaptor, 1.5 µL of RNA Ligase, and 0.4 µL of RNase-free water. This 15 µL reaction was incubated at 16 ˚C for 60 minutes. The resulting modified microRNAs were then subject to a universal RT reaction using a 15 µL RT reaction mix containing 6 µL of 5x RT Buffer, 1.2 µL of 25 mM dNTP mix, 1.5 µL of 20x Universal RT Primer, 3 µL of 10x RT Enzyme mix, and 3.3 µL of RNase-free water. RT was performed for 15 minutes at 42 ˚C followed by 5 minutes at 85 ˚C.

5 µL of the resulting cDNA was then subject to a pre-amplification reaction to increase the cDNA product for all the microRNAs. The RT product was incubated with 25 µL of 2x miR-Amp Master Mix, 2.5 µL of miR-Amp Primer Mix and 17.5 µL of RNase-free water at 95 ˚C for 5 minutes, followed by 18 cycles of 3 seconds of 95 ˚C and 30 seconds 60 ˚C treatment, and finally 10 minutes

at 99 ˚C before holding at 4 ˚C. The number of cycles were modified from the Taqman recommendations of 14 cycles to 18 cycles in order to accumulate sufficient microRNA cDNA for the qPCR reaction.

4.2.7 Taqman real time PCR

Three technical replicates of each cDNA sample were used for RT-PCR. The PCR reaction mix was prepared by mixing 10 µL of 2x Taqman Fast Advanced Master mix with 4 µL of RNase free water along with the addition of 1 µL of the appropriate specific 20x Taqman Advanced microRNA Assay. A 1:10 dilution was created for the cDNA sample product from the RT reaction, 5 µL of which was used for each of the replicate reactions. The 15 µL PCR Mix was added to each well of a Microamp Optical 96-Well Reaction plate to which 5 µL of diluted cDNA samples were mixed. An additional 3 wells were used as non-template controls where 5 µL of RNase-free water was mixed with the 15 µL of PCR mix. The plates were sealed with MicroAmp clear adhesive film and the PCR reaction was carried out under the default thermal-cycling conditions in the StepOnePlus Applied Biosystems machine (95 ˚C for 20 seconds followed by 40 cycles of 95 ˚C for 1 second and 60 ˚C for 20 seconds).

4.2.8 Specific microRNA assays for cerebrospinal fluid and serum samples

Taqman Advanced microRNA Assays were used as part of the RT-PCR reaction mix to detect the specific microRNA from the cDNA samples. Endogenous controls were identified from our NGS data to select microRNAs with the most stable levels across all samples. Two microRNA candidates (hsa-miR-10b-5p and hsa-miR-133a-3p) were detected in both CSF and serum and two endogenous controls (hsa-miR-30d-5p and hsa-miR-222-3p) were processed in parallel.

4.2.9 Statistical analysis

To determine if microRNA expression was significantly altered following SCI, the R package, DESeq2[317] was used to perform differential expression analysis on CSF and serum samples from SCI patients at all 5 time points (1 – 5 days post injury), relative to control patients. CSF and serum microRNAs were tested for differential expression through DESeq2 using the Wald test (absolute fold-change > 1.5), with Benjamini-Hochberg adjustment for multiple testing (FDR < 0.1) as per the DESeq2 vignette[317]. An FDR < 0.1 was used here to cast a wider net in determining candidate differentially expressed microRNAs for the sake of discovery. To determine microRNAs whose abundance monotonically increased or decreased with baseline AIS grade, we fit a linear regression model using DESeq2.

For diagnostic and prognostic performance of microRNAs, we focused our attention on the CSF sample drawn at the 24-hour post-injury time point (as in our previous work[25, 84, 318]). We selected 24 hours because this represents a time point at which most acute SCI patients would likely be admitted to a trauma center, and a CSF sample could be obtained for biomarker analysis. Multi-microRNA panels for discrimination between AIS grades and for prediction of improvement status were identified using sparse partial least-squares discriminant analysis (sPLS-DA) via the "mixOmics" R package[319]. This method was chosen because it includes variable selection (ie., microRNA selection). For each analysis, leave-one-out cross-validation was used to determine optimal sPLS-DA hyperparameters (number of components, number of microRNAs kept per component) and estimate out-of-sample performance. All analyses were conducted using R (version 3.3.0).

4.3 Results

44 patients were included for the final data analyses including: 24 AIS A, 8 AIS B, 7 AIS C, (demographics and clinical data are shown in Table 4.1), and 5 non-SCI control patients (Table 4.2). The primary mechanism of injury among both men and women was sports accidents (41%) followed by falls (31%). The age of patients ranged from 19 – 74 years old (mean = 42.9, median = 40, sd = 17.96), and females representing 20.5% of the cohort. CSF and serum samples were also collected from 5 non-SCI "control" individuals who were undergoing lumbar laminectomy/discectomy surgery. The age range of the non-SCI control patients were 43 – 72 years old (mean = 55.4, median = 51, sd = 11.1). In total, 193 samples of CSF were obtained and analyzed between days 1 and 5 post injury (116 CSF samples from AIS A, 37 from AIS B, and 35 from AIS C). In total, 193 samples of serum were obtained and analyzed between days 1 and 5 post injury (117 serum samples from AIS, 36 from AIS B, and 35 from AIS C).

4.3.1 MicroRNA detection in cerebrospinal fluid and serum

MicroRNA libraries were created from total RNA isolated from CSF or serum. Total RNA concentrations in CSF are reported to be between 15 – 30 ng/mL, while serum contains 10 – 60 ng/mL[288]. Interestingly we identified a severity dependent increase in small RNA concentration within the CSF in the first 24 hours following SCI, which returned to baseline by 3 days post injury (Figure 4.1). A logistic regression analysis showed that small RNA concentrations at 24 hours in the CSF were significantly correlated with motor score recovery ($R^2 = 0.258$, $p = 0.0016$). In contrast, there was no severity-dependent increase in small RNA concentration within the serum samples.

The relatively low concentration of total RNA necessitated the protocol modifications described by Burgos et al.[288] for Illumina's TruSeq Small RNA Library Preparation, which requires 1 ug of total input RNA. This modification enabled the generation of sequencing libraries for 188 CSF and 188 serum samples. Libraries were sequenced to generate short single-end reads, which were processed using the CAP-miRSeq bioinformatics pipeline. We detected a total of 1593 unique microRNAs in the CSF and 1711 unique microRNAs in the serum which were expressed in one or more time points. 253 microRNAs were detected only in the CSF, and 135 microRNAs were detected only in the serum. For identifying significantly differentially expressed microRNAs, we only considered samples with a total microRNA read count per sample above 50,000, and an average read count for any individual microRNA of over 5 counts.

4.3.2 Identification of microRNAs related to spinal cord injury

In total, 190 CSF microRNAs and 19 serum microRNAs were significantly altered post-SCI, compared to control patients (absolute fold-change > 1.5 and FDR < 0.1;Figure 4.2), with 11 that were common to both fluid types. In the CSF of patients with the most severe SCI (the AIS A group), at days 1, 2, 3, 4, and 5 post injury, a total of 84, 75, 73, 78, and 69 microRNAs were found to be upregulated and 26, 19, 19, 25, and 18 microRNAs were found to be downregulated, respectively. In the AIS B group, a total of 51, 59, 30, 22, and 22 microRNAs were found to be upregulated at days 1, 2, 3, 4, and 5, respectively, and 9, 12, 8, 9, and 10 microRNAs were found to be downregulated at days 1 through 5 post injury. In the AIS C group, a total of 39, 52, 26, 15, and 17 microRNAs were found to be upregulated and 6, 16, 8, 14, and 8 microRNAs were found to be downregulated at days 1 through 5 post injury. The overlap of these differentially expressed

CSF microRNA between days 1 – 5 are shown in Figure 4.3A showing an enrichment of differentially expressed microRNAs in the AIS A group. 28 of these CSF differentially expressed microRNAs were identified in all three SCI groups at 24 hours post injury, with their fold-change and adjusted p-value results shown in Table 4.3.

In general, within the serum of injured patients, there were far fewer microRNAs that were differentially expressed relative to control patients. In AIS A patients, a total of 12, 2, and 1 microRNAs were found to be upregulated and 5, 3, and 0 microRNAs were found to be downregulated at days 1 through 3 post injury, while no microRNAs were significantly altered in serum after 3 days post injury. In the serum of AIS B patients, 1 microRNA was upregulated at 3 days post injury, and in the serum of AIS C patients, 1 microRNA was upregulated, and 1 microRNA was downregulated at 2 days post injury. There were no serum microRNAs that were differentially expressed following SCI in all three AIS groups. The overlap of these differentially expressed serum microRNAs between days 1 – 5 are shown in Figure 4.3B. As previously mentioned, one of the advantages of microRNAs as biomarkers, is the fact that they are highly conserved, which allows one to compare results across species. By way of example, over 50% of the microRNAs altered in human CSF or serum in this study were also reported to change following SCI in a porcine model of SCI[4], or in various rat, mouse, and zebrafish models of SCI[10, 131, 146, 148, 181] (Figure 4.3C).

Table 4.3 Top differentially expressed cerebrospinal fluid microRNAs at 24 hours post injury. Included in the table are the microRNA ID's, log2 fold change (log2FC), and adjusted (Benjamini-Hochberg) p values (Adj. p value) for the comparison of AIS A, B, or C, versus control patients. A positive log2FC value indicates upregulated microRNAs relative to control patients, whereas a negative log2FC value indicates downregulated microRNAs. There were 28 CSF microRNAs that were significantly associated with SCI in all three AIS groups.

	AIS A		***AIS B***		***AIS C***	
MicroRNA	***log2FC***	***Adj. p value***	***log2FC***	***Adj. p value***	***log2FC***	***Adj. p value***
miR-219a-2-3p	7.20	2.38E-14	6.98	2.07E-10	6.00	1.79E-06
miR-9-5p	6.91	1.67E-15	5.91	1.89E-08	5.24	5.53E-06
miR-9-3p	6.32	2.89E-14	6.27	2.07E-10	5.16	3.40E-06
miR-129-5p	5.65	1.13E-08	4.90	9.22E-05	4.27	2.40E-03
miR-219a-5p	5.53	5.67E-08	5.66	2.99E-06	5.23	6.30E-05
miR-1246	5.24	2.30E-07	3.76	6.25E-03	4.80	3.09E-04
miR-760	4.77	2.02E-06	4.27	5.69E-04	3.37	2.85E-02
miR-410-3p	4.52	5.26E-08	4.03	1.36E-04	3.40	4.81E-03
miR-485-5p	4.52	2.64E-06	4.75	5.57E-05	3.98	3.04E-03
miR-323b-3p	4.46	1.18E-06	4.17	2.02E-04	4.13	5.14E-04
miR-124-3p	4.46	1.46E-04	4.22	3.99E-03	3.62	3.26E-02
miR-488-3p	4.18	3.17E-04	3.47	2.75E-02	3.61	2.85E-02
miR-320b	4.13	1.39E-05	4.30	2.02E-04	3.64	6.65E-03
miR-433-3p	4.06	3.33E-05	4.36	1.92E-04	3.83	3.62E-03
miR-1298-3p	4.04	1.70E-04	3.65	7.29E-03	3.44	2.62E-02
miR-211-5p	4.01	1.25E-03	4.43	2.33E-03	4.25	7.55E-03
miR-92b-3p	3.49	3.33E-05	3.62	3.54E-04	2.99	1.55E-02
miR-128-3p	3.31	4.55E-07	3.03	2.56E-04	2.22	4.30E-02
miR-1910-5p	2.87	4.99E-02	4.02	1.04E-02	4.54	4.40E-03
miR-10b-3p	2.85	2.76E-03	3.30	2.59E-03	2.88	2.85E-02
miR-3605-3p	2.84	2.09E-03	3.51	5.13E-04	2.94	1.61E-02
miR-125b-1-3p	2.63	3.17E-04	3.02	3.54E-04	2.63	7.55E-03
miR-584-5p	2.63	4.92E-03	3.47	5.69E-04	3.15	6.94E-03
miR-338-5p	2.51	3.22E-03	3.18	5.69E-04	2.64	2.21E-02
miR-21-3p	2.31	1.24E-03	2.39	5.49E-03	3.54	3.40E-06
miR-1307-5p	2.18	2.48E-02	2.45	3.65E-02	2.60	3.26E-02
miR-23b-3p	-2.05	1.40E-04	-2.30	3.54E-04	-2.42	3.31E-04
miR-195-5p	-2.61	2.48E-02	-3.82	2.20E-03	-4.11	1.98E-03

4.3.3 Altered microRNAs associated with injury severity

To determine if microRNA expression showed a trend across the AIS A, B, C, and control groups, a linear regression test was performed using DESeq2. In the CSF, 204 microRNAs were significantly associated with injury severity at 24 hours, with 139 microRNAs that showed a pattern of increasing expression with increasing injury severity, and 65 microRNAs showing a pattern of decreasing expression with increasing injury severity. The 24-hour CSF abundance profiles of the top nine microRNAs associated with injury severity, ranked by adjusted p-value are shown in Figure 4.4. The top microRNAs associated with injury severity are miR-9-5p (adjusted p = 1.74E-19), along with miR-181c-3p, miR-320a, miR-769, miR-9-3p, miR-219-2-3p, miR-432-5p, miR-128-3p, and miR-323a-3p.

In the serum, 83 microRNAs were significantly associated with injury severity at 24 hours, with 46 microRNAs that showed a pattern of increasing expression with increasing injury severity and 37 microRNAs showing a pattern of decreasing expression with increasing injury severity. The 24-hour serum abundance profiles of the top nine microRNAs associated with injury severity, ranked by adjusted p-value are shown in Figure 4.5. The top microRNAs associated with injury severity in serum are miR-192-5p (adjusted p = 1.95E-07), miR-133a-3p, miR-122-5p, miR-194-5p, miR-4792, miR-1246, miR-208b-3p, miR-499a-5p, and miR-148a-3p. While these microRNAs were considered differentially expressed by DESeq2, the clinical utility of the expression patterns of these microRNAs are limited due to the significant variability across patients. Attempts to create a predictive model of injury severity or neurological outcome using these serum microRNAs were unsuccessful.

4.3.4 Validation of next-generation sequencing by qRT-PCR

We sought to validate the differences in relative microRNA expression between AIS A, B, C, and control patients obtained from NGS, using specific assays for selected microRNAs. An ideal biomarker would be one that has relatively high expression, high differential expression, and low variance; for these reasons, we selected the top two ranking microRNAs detected in serum and CSF that met a set of criteria. First, we applied a percentile rank to all detected microRNAs based on read count data. Second, we applied a percentile rank to all detected microRNAs based on their fold-change compared to baseline. Third, we applied a percentile rank to all detected microRNAs based on their adjusted p-value. The two microRNAs in CSF and serum that scored the highest percentile rank sum across all three categories (scoring between 0-3) were selected for validation using qRT-PCR. Using this ranking method, it was determined the miR-10b-5p in the CSF and miR-133a-3p in the serum ranked highest. Endogenous control microRNAs were selected by using NormFinder[320] in order to determine the microRNAs with the greatest stability, which identified miR-30d-5p, and miR-222-3p in serum and CSF, respectively. Read counts, as measured by NGS, of CSF miR-10b-5p and serum miR-133a-3p are shown in Figure 4.6. We performed qRT-PCR on these two microRNAs in a subset of n = 7 AIS A patients, n = 6 AIS B, and C patients at 24 hours post injury, and n = 5 control patients, for a total of 24 samples. Relative microRNA levels of miR-10b-5p in CSF, as measured by qRT-PCR showed significant differences between AIS A, B, and C patients (Figure 4.7A), with higher levels in patients with more severe SCI, reflecting the same injury severity dependent pattern seen when utilizing NGS. The expression of miR-10b-5p were significantly higher in patients that were classified as AIS A, compared to AIS B, AIS C, and AIS D at 6 months post injury (Figure 4.7B), and interestingly, CSF expression of miR-10b-5p was significantly higher in patients that showed no neurological improvement at 6 months post

injury, compared to those that did (Figure 4.7C). Relative expression of miR-133a-3p in serum, as measured by qRT-PCR showed significant differences between AIS A, B, and C patients (Figure 4.8A), with higher levels in patients with more severe SCI. The relative expression of miR-133b-3p as measured by qRT-PCR showed significantly lower levels in patients that were classified as AIS D at 6 months post injury, compared to AIS C, but otherwise showed no differences between AIS groups at 6 months, and was not associated with neurological recovery at 6 months (Figure 4.8B, C).

4.3.5 Classifying baseline ASIA impairment grade using 24-hour post-injury microRNA expression

Because the baseline severity of neurological impairment, measured by the AIS grade, is currently the strongest clinical predictor of neurological outcome, the primary goal of this analysis was to evaluate whether microRNAs quantified by next-generation sequencing could differentiate among AIS A, B, and C severities of injury. Aside from actually taking biopsies of the injured spinal cord, the proximity of the CSF compartment to the injured spinal cord provides the most direct opportunity to monitor, at the molecular level, physiological responses to mechanical trauma to the spinal cord parenchyma and compare these metrics to AIS grade.

Based on the differential CSF expression of microRNAs across AIS groups at 24 hours post injury, a sparse partial least squares discriminative analysis (sPLS-DA) was performed to identify CSF microRNAs at 24 hours that could distinguish baseline AIS groups (Figure 4.9). For the cohort of 39 patients analyzed in this study, a sPLS-DA model including 70 microRNAs across two components resulted in the best observed classification performance in a 20 X 5-fold cross-

validation. Specifically, this model had a mean cross-validated area under the receiver operator characteristics curve (AUROC) of 0.75 for classifying AIS A patients vs. all others, 0.70 for classifying AIS B patients vs. all others, and 0.64 for classifying AIS C patients vs. all others.

4.3.6 Prognostic microRNA biomarkers for 6-month neurological improvement

Next, we determined whether AIS grade improvement at 6 months post injury could be predicted using CSF microRNA expression at 24 hours in AIS A patients (Figure 4.10A, B). Ultimately an improvement of at least one AIS grade by 6 months post injury was demonstrated in 24 patients out of the 39 (Table 4.1). We focused on the AIS A group in particular to determine whether microRNA profiles were different in those who remained AIS A and those who improved ("converted") to AIS B, C, or D. A sPLS-DA model including 30 microRNAs across two components resulted in the best observed classification accuracy in a 20 X 5-fold cross validation. Specifically, this model had a mean cross-validated AUROC of 0.75 in identifying AIS A patients that improved versus those that did not, at 6 months post injury. The microRNA with the heaviest contribution in this model, miR-423-3p was significantly higher in AIS A patients that showed improvement 6 months later, compared to those that did not (Figure 4.10C).

4.4 Discussion

Currently the development of novel therapies for acute SCI is severely hindered by the inability to clinically evaluate SCI patients in the acute injury period, the difficulty in accurately predicting neurological outcome, thus the challenge in recruiting sufficient numbers of subjects, and the lack of objective molecular measures of severity or outcome for monitoring treatment efficacy. The

baseline classification of neurological impairment according to the ISNCSCI standards is a fundamental component of the early assessment of acute SCI patients. Unfortunately, this examination is often very challenging, or impossible to perform in the acute setting. In a review of over 400 acute SCI patients admitted over a 4-year span, Lee and colleagues[15] reported that concomitant injuries and comorbidities would have made obtaining a valid ISNCSCI examination impossible in at least 30% of the patients, thus automatically excluding them from recruitment into an acute clinical trial. This assessment is important not only with respect to early communication with patients and their families but can also influence management decisions such as the urgency of surgical decompression[321].

In addition to these very practical issues, the lack of molecular-level observation and description of human pathophysiology after acute SCI is a broad limitation for translational research in a field that relies heavily on experimental animal models[322]. It is therefore imperative that a simple, unbiased, procedure be developed to assess acute SCI patients. This study represents the first description of the temporal microRNA patterns in CSF and serum in human patients with acute traumatic SCI. Here, we utilized NGS to profile the changes in microRNAs within the CSF and serum and to identify microRNAs that could potentially serve as biomarkers of injury severity in patients with acute SCI. Further, we sought to identify microRNAs that were associated with neurological recovery as measured by AIS grade improvement. We report that the CSF small RNA concentrations (including the total microRNA population) are moderately associated with motor score recovery and the pattern of microRNA expression is significantly altered within the CSF and serum following acute traumatic SCI, in a severity dependent manner. Further, CSF microRNA expression at 24 hours post injury within patients with AIS A injuries differs between those

patients who will, and those who will not convert to AIS B, C, or D injuries at 6 months post injury.

4.4.1 MicroRNAs related to spinal cord injury

In total, 190 microRNAs in the CSF of human SCI patients were found to be differentially expressed across all time points as compared to non-SCI controls (Figure 4.2), with 68 microRNAs common to all AIS grades, 26 common to the AIS A and B groups, and 50 microRNAs exclusive to the AIS A group (Figure 4.3A). The 28 microRNAs that were differentially expressed in all three SCI groups at 24 hours post injury are shown in Table 4.3. Many of the microRNAs found to be differentially expressed in human CSF have been implicated in neurological disorders including SCI, and have been previously shown to affect the secondary injury cascade following SCI. For example, miR-9, miR-219, miR-10b, and miR-21, amongst others, have been repeatedly shown to be involved in SCI processes and have been studied both as potential biomarkers and therapeutic targets. MiR-9 and miR-219 were proposed by Hachisuka et al[146] as promising serum biomarkers in a mouse model of contusive SCI. Consistent with this observation, we found that CSF expression of miR-9 and miR-219 were increased post-SCI in all three AIS groups at 24 hours post injury (Table 4.3) and was strongly associated with injury severity (Figure 4.4). Increases in miR-9 and miR-219 might be associated with the release of microRNAs from the destruction of myelin in the spinal cord[135]. MiR-10b (Table 4.3) is enriched in the spinal cord tissue[173] and may represent an important marker of destruction to the spinal cord parenchyma. MiR-21 has been repeatedly found to increase following SCI[10, 131, 134], and was found to be upregulated in neurons following cerebral ischemia[175]. MiR-21 likely plays a role in astrogliosis[134] and targets Faslg, a

TNFα family member and an important cell death-inducing ligand[175], underscoring its potential as both a biomarker and as a therapeutic target for SCI.

In total, 19 microRNAs were found to be differentially expressed in the serum of human SCI patients across all time points (Figure 4.2), 16 of which were specific only to the AIS A group. There were no serum microRNAs found to be differentially expressed in all three SCI groups (Figure 4.3B). One of the serum microRNAs that showed the most signification change in expression was miR-133a-3p, which showed a strong association with injury severity (Figure 4.5). Yu et al.[181] found that miR-133 is involved in functional recovery post-SCI in adult zebrafish. Two microRNAs, miR-208 and miR-499 show similar severity-dependent expression patterns in human serum (Figure 4.5). Boon et al.[188] showed that expression of miR-208 and miR-499 progressively declined following SCI and are thought to regulate skeletal muscle mass.

4.4.2 MicroRNAs related to pig spinal cord injury

We previously performed a comprehensive profiling of the serum microRNAs in a large animal, porcine model of traumatic thoracic SCI[4], using the porcine model of SCI previously described by Lee et al.[231, 323]. There, we described the acute microRNA expression changes within the first 5 days post injury and showed that these changes had strong correlations to behavioural and histological outcomes, 12 weeks post injury. Many of the microRNAs that were found to be differentially expressed in our porcine model of SCI, have also been found to be altered in the acute phases of human traumatic SCI. For example, we found that miR-133 shows severity dependent expression in the serum of human SCI patients at 24 hours post injury (Figure 4.5) and was significantly altered in a severity dependent fashion following traumatic SCI in pigs[4], with

expression of miR-133 reaching its highest between 1 – 3 days post injury. Serum miR-145-3p is significantly elevated post-SCI in human serum of AIS A patients, and was similarly upregulated in the serum of pigs, post-SCI, with expression correlating to injury severity[4]. In addition, miR-208b-3p and miR-499 show highly similar patterns related to injury severity in the serum of human SCI patients and were also altered in the serum of pigs[4]. In total 50% of the microRNAs that were found to be differentially expressed in a severity-dependent pattern in pig serum were also found to be differentially expressed in human patients after SCI. There were also notable differences between human and porcine data, most notably in that the serum microRNAs in human SCI patients did not show nearly as strong a relationship to injury severity, or neurological outcome. These differences may be due to the controlled experimental parameters and the greater homogeneity of subjects in the porcine study and is worthwhile to pursue. Nonetheless, there are promising, conserved similarities in the post-SCI microRNA expression patterns, which suggests the potential for further cross-species studies of microRNA biomarkers for injury severity.

4.4.3 Biomarkers to diagnose baseline injury severity

In the acute setting of SCI, it is impossible to accurately diagnose injury severity in up to 30% of patients, rendering them ineligible from clinical trials. The ability to objectively stratify an acute SCI patient's injury severity with quantitative biomarkers would facilitate the design and execution of clinical trials. Furthermore, microRNA biomarkers of SCI have the potential to serve as predictors of recovery. In addition to the post-SCI changes in microRNA expression described above, we identified a set of microRNAs that are diagnostic of baseline AIS grades. This set of 70 microRNAs had cross-validated AUC = 0.75 when classifying AIS A patients vs. all other AIS grades. While we were able to achieve better classification performance using a model consisting

of a much larger set of microRNAs, we felt the cost of using such a complex model would limit its utility. Further testing in an independent cohort of acute SCI patients will be necessary to validate (and potentially improve) the microRNA model.

4.4.4 Biomarkers to predict neurological outcome

One of the long-sought applications of biomarkers in acute clinical trials in SCI would be to improve prediction of neurological recovery. The early assignment of neurological impairment as AIS A, B, or C unfortunately leaves much uncertainty about the final neurologic outcome. The reasons for this include the variability in spontaneous neurologic recovery and the aforementioned limitations of the initial clinical assessment[16]. In addition to the post-SCI changes in microRNA expression described above, we identified a set of microRNAs that are prognostic of neurologic recovery, 6 months post injury in AIS A patients. Since it would be particularly useful to be able to identify those patients who are classified AIS A at baseline that are likely or not to recover, we sought to determine the accuracy in predicting which patients will improve by at least one AIS grade at 6 months post injury. This set of 30 microRNAs had cross-validated AUC = 0.75 when classifying AIS grade A patients who went on to improve at least 1 grade, at 6 months post injury, from those that did not. MiR-423, as one of the strongest prognosticators of neurologic improvement at 6 months post injury in humans, has been shown to regulate the activity of caspase-3, an apoptotic indicator, and may relate to neuronal apoptosis[324]. Mir-423 was also significantly altered in pigs post-SCI[4]. While the extensibility of this model outside of this cohort remains to be validated, it encourages our efforts to apply and refine this model for predicting outcome in acute SCI patients.

4.4.5 The importance of the baseline neurologic assessment and classification of injury severity in spinal cord injury

To date, there has been significant effort to expand upon the limited number of molecular observations in the field of SCI, TBI, and other acute and chronic neurologic conditions[1, 4, 8, 21, 25, 26, 32-35, 47, 84, 146, 158, 285, 286, 318, 325-331]. Specifically, with regards to acute traumatic SCI, Streijger et al.[318] identified a set of proteins that were diagnostic and prognostic of injury severity and neurological outcome through a targeted proteomic screen. Using metabolomic profiling, Wu et al.[325] identified 6 CSF metabolites that were potential biomarkers for the classification of injury severity in SCI patients. Kwon et al[25, 84] have shown improved diagnostic and prognostic utility using the set of proteins IL-6, tau, S100β, and GFAP within the CSF of patients with acute SCI, compared to the standard ISNCSCI assessment.

The CSF microRNAs found to be diagnostic and prognostic of injury severity in this study are promising and, with further validation, may lead to robust biomarkers for classifying acute traumatic SCI and predicting its trajectory. While each of these biomolecule subtypes are individually, promising, potentially greater impact may be realized through the application of unbiased machine learning algorithms to these comprehensive datasets. Indeed, the iteration of empirical testing, model building, and retesting could represent a powerful approach to refine the potential of microRNAs and other biomarkers as indicators of disease state and predictor of future neurologic recovery.

4.4.6 Limitations

There are a number of limitations that warrant consideration. First, although our cohort of 44 patients divided among AIS A, B, C injury severities and control patients, represents, to our knowledge, the largest series to undergo this type of next-generation sequencing of microRNA profiles in CSF or serum, it remains a relatively small cohort, and further validation in a larger cohort is warranted. Our "negative control" non-SCI group consisted of mainly patients who were older than our predominantly male SCI patients. With the small size of the entire cohort, it is unknown whether there were level of SCI, gender or age-related differences in the microRNA responses to injury. We also acknowledge that the relationship between very early microRNA changes and functional outcome at 6 months post injury may be clouded by variability in terms of the type, duration, and intensity of rehabilitation programs. In the search for useful biomarkers, however, we are definitely seeking biochemical changes that are so pronounced in the early stages of injury so as to overcome the effect size of this inevitable variability in post injury rehabilitation. Along the same lines, our analysis focused on the relationship between acute microRNA changes and neurological impairment, and we did not investigate other meaningful health-related quality of life measures (e.g., spinal cord independence measure, neuropathic pain). Finally, while a highlighted goal of this study was to identify microRNA patterns specifically in the blood as a surrogate for CSF that could discriminate between injury severities, we were unable to identify microRNAs that were diagnostic or prognostic of injury severity in this biological tissue. While there were a small set of microRNAs that correlated to baseline AIS grade, none performed adequately in identifying baseline classification or neurologic outcome. It would seem that CSF remains a unique and crucial biofluid with important biochemical changes relating to injury severity in acute traumatic SCI.

4.4.7 Future directions

Future work on microRNA biomarkers will focus on validating these findings in acute SCI patients who have been subsequently enrolled in our clinical initiative. Ultimately, our goal is to establish a panel of microRNA biomarkers that can be utilized as a tool for baseline injury severity diagnosis in clinical trials and for better prognoses of neurological outcome to reduce the variability due to spontaneous recovery. This would subsequently decrease the number of patients required for clinical trials to be completed. By characterizing the acute pathophysiological responses to traumatic SCI in humans, and establishing microRNA biomarkers, we hope to provide the field with tools that can facilitate our collective efforts to improve the lives of those with SCI. With the continuous emergence of new SCI therapies that are seeking validation in clinical trials, the field of SCI is in dire need of new approaches for classifying injury severity and improved methods of predicting outcome. MicroRNA biomarkers are promising solutions to this bottleneck in the pipeline of developing clinically relevant therapies for SCI.

4.5 Conclusions

Our study provides the first comprehensive description of the microRNA changes that occur in the CSF and serum during the early post-injury phase of acute SCI in human patients. In this study, we identified 28 CSF microRNAs that were differentially expressed in all three SCI severities, in addition to a set of CSF and serum microRNAs that showed severity-dependent changes which warrant further investigation and validation to determine their utility as potential biomarkers for SCI. We were not able to use serum microRNAs to discriminate baseline AIS grade or 6-month neurologic outcome. We were able to discriminate baseline AIS grade with reasonable

performance (AUC = 0.75, 0.70, 0.64 in AIS A, B, and C patients, respectively) using a model composed of the 24-hour patterns from 70 CSF microRNAs. We were also able to discriminate AIS grade improvement at 6 months post injury in AIS A patients (AUC = 0.75) using the 24-hour patterns of 30 CSF microRNAs.

Figure 4.1 Small RNA library concentrations. Time course of small RNA (inclusive of microRNA) library concentrations in CSF and serum. Mean small RNA library concentrations ± standard error of the mean for patients with injury severity AIS grade A (red, n = 24), B (blue, n = 8), C (green, n = 7), and control (black, n = 6), are shown at 24 hours, 48 hours, 3 days, 4 days, and 5 days post injury.

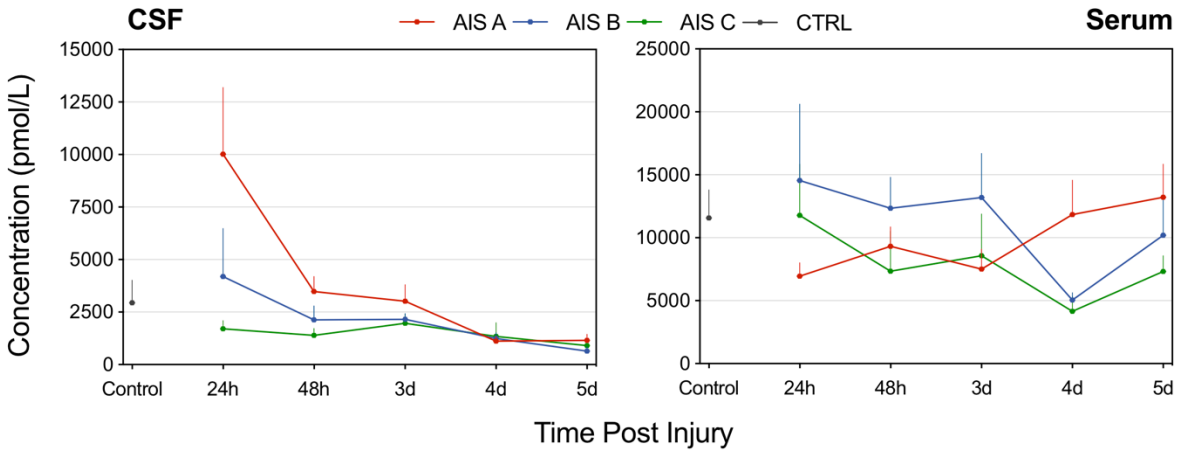


Figure 4.2 Number of differentially expressed microRNAs in cerebrospinal fluid and serum. Time course of the number of differentially expressed microRNAs, compared to control patients in CSF and serum. Number of differentially expressed microRNAs for patients with injury severity AIS grade A (red, n = 24), B (blue, n = 8), C (green, n = 7), are shown at 24 hours, 48 hours, 3 days, 4 days, and 5 days post injury. Values above zero correspond to the number of microRNAs that showed upregulation, while values below zero correspond to the number of microRNAs that showed down-regulation.

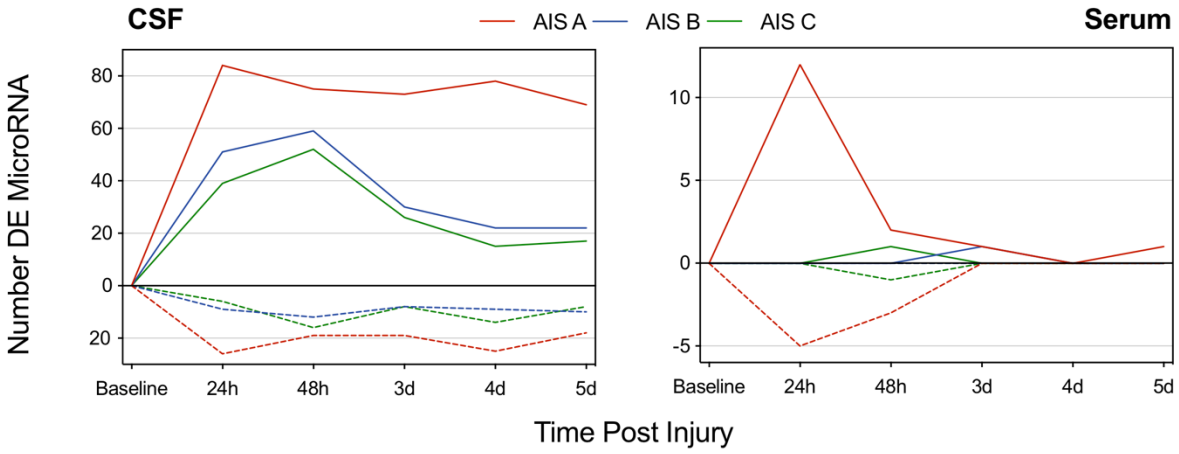


Figure 4.3 Venn diagrams of differentially expressed microRNAs at 24 hours post injury. (A) Differentially expressed microRNAs at 24 hours post injury in CSF of AIS A, B, and C patients. (B) Differentially expressed microRNAs at 24 hours post injury in human serum of AIS A, B, and C patients. (C) Differentially expressed microRNAs at 24 hours post injury in humans, pigs, and reported in literature.

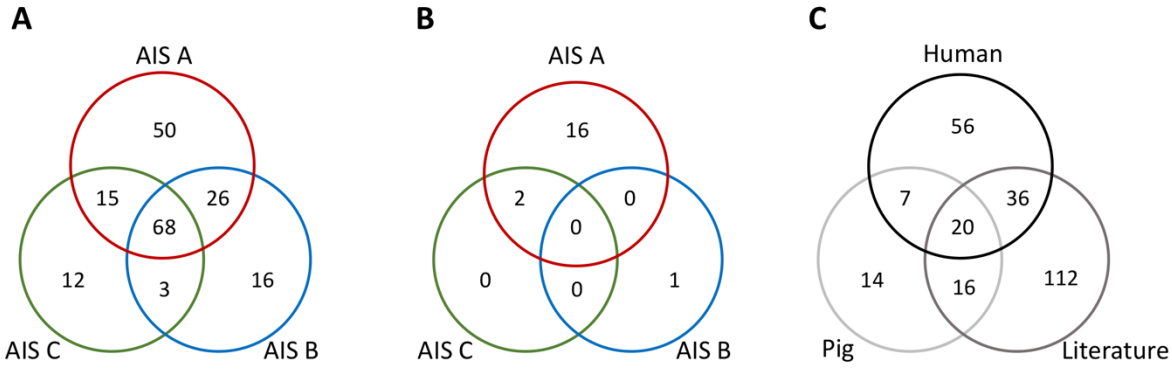


Figure 4.4 Cerebrospinal fluid microRNA expression levels of top nine microRNAs associated with AIS grade at 24 hours post injury. Expression profiles in CSF for the top nine microRNAs associated with AIS grade at 24 hours post injury. Mean normalized microRNA counts (± SEM) for patients with injury severity AIS grade A (n = 24), B (n = 8), C (n = 7), and control (n = 6) are shown at 24 hours post injury.

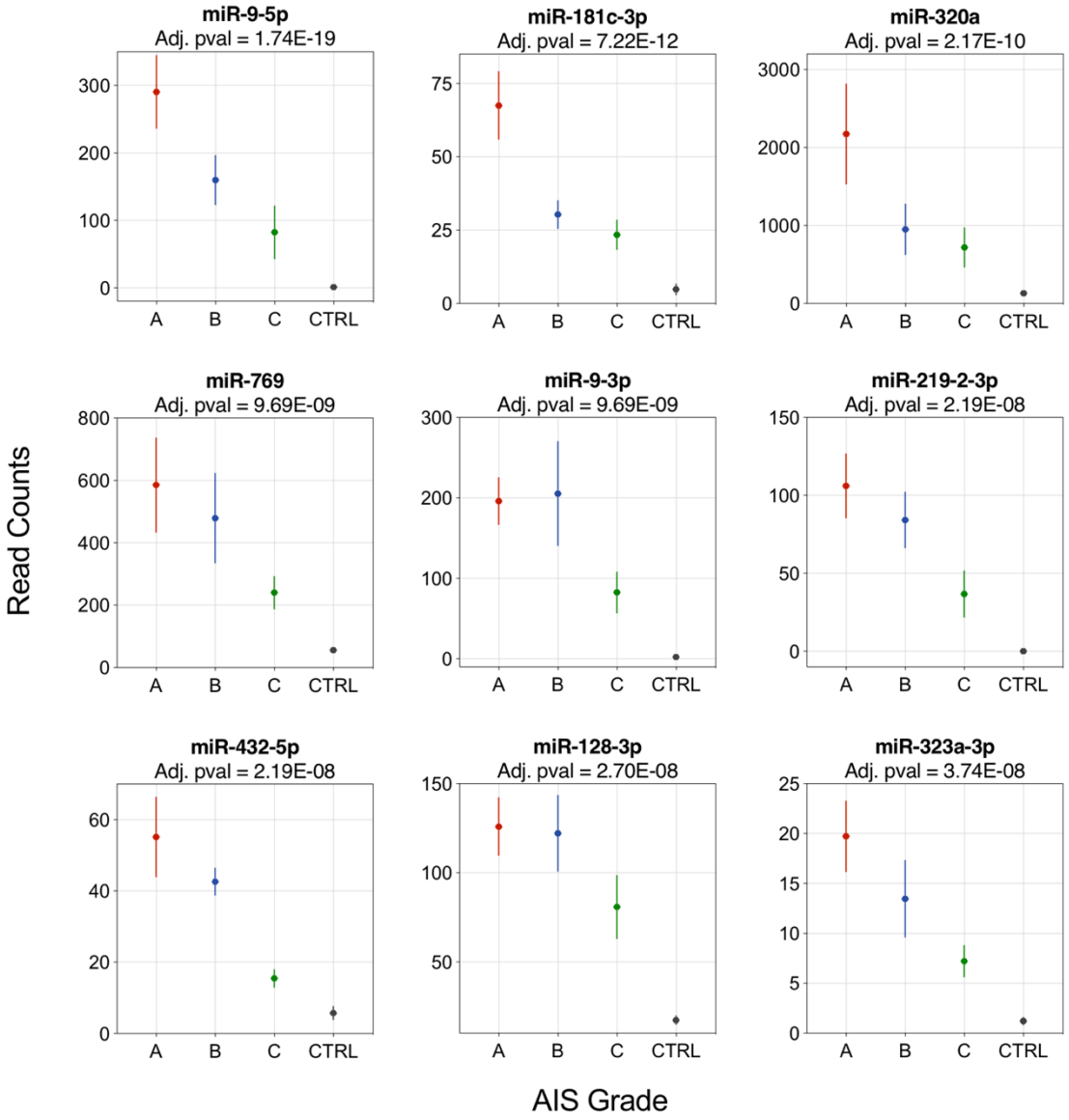


Figure 4.5 Serum microRNA expression levels of top nine microRNAs associated with AIS grade at 24 hours post injury. Expression profiles in serum for the top nine microRNAs associated with AIS grade at 24 hours post injury. Note that in contrast to the CSF, the relationship between serum microRNAs and injury severity is quite variable, with the most severely injured AIS A patients having in some cases the highest and in other cases the lowest values. Mean normalized microRNA counts (± SEM) for patients with injury severity AIS grade A (n = 24), B (n = 8), C (n = 7), and control (n = 6) are shown at 24 hours post injury.

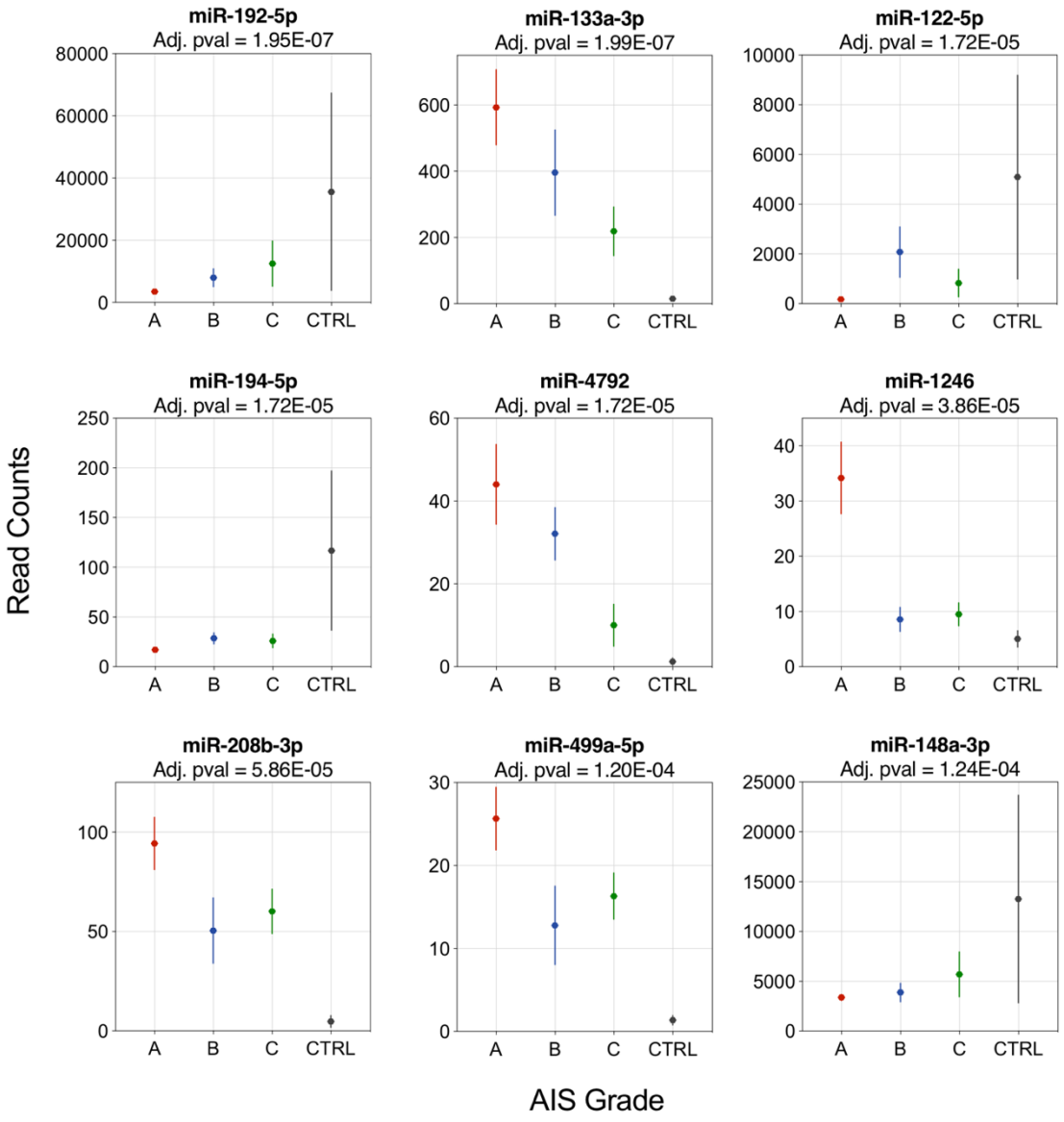

miR-192-5p
Adj. pval = 1.95E-07
miR-133a-3p
Adj. pval = 1.99E-07
miR-122-5p
Adj. pval = 1.72E-05
miR-194-5p
Adj. pval = 1.72E-05
miR-4792
Adj. pval = 1.72E-05
miR-1246
Adj. pval = 3.86E-05
miR-208b-3p
Adj. pval = 5.86E-05
miR-499a-5p
Adj. pval = 1.20E-04
miR-148a-3p
Adj. pval = 1.24E-04
A
B
C
CTRL
Read Counts
AIS Grade

Figure 4.6 Abundance levels of miR-10b-5p in cerebrospinal fluid and miR-133a-3p in serum. Mean sequencing read counts (± SEM) for patients with injury severity AIS grade A (red, n = 24), B (blue, n = 8), C (green, n = 7), and control (black, n = 6), are shown at 24 hours, 48 hours, 3 days, 4 days, and 5 days post-injury.

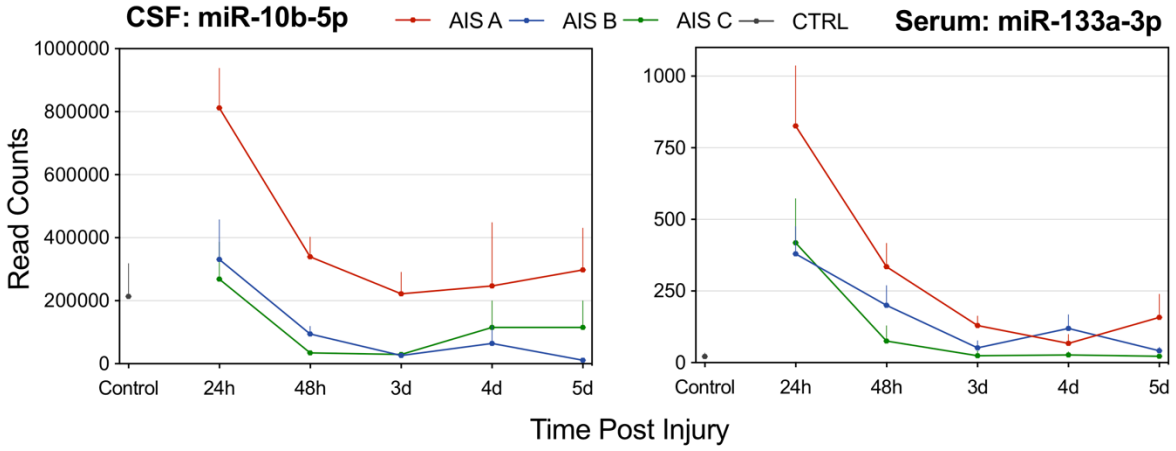


Figure 4.7 Relative levels of miR-10b-5p in cerebrospinal fluid at 24 hours post injury. (A) Mean log2 fold change (± SEM), relative to control patients are shown for patients with baseline injury severity AIS grade A, B, and C (n = 6 each). (B) Mean log2 fold change (± SEM), relative to control patients, are shown for patients with 6-month injury severity AIS grade A, B, C, and D. (C) Mean log2 fold change (± SEM), relative to control patients are shown for patients that show no neurological conversion ("No", red), and patients that do show neurological conversion ("Yes", blue). Asterisks indicate significant differences (Student's t-test, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****)).

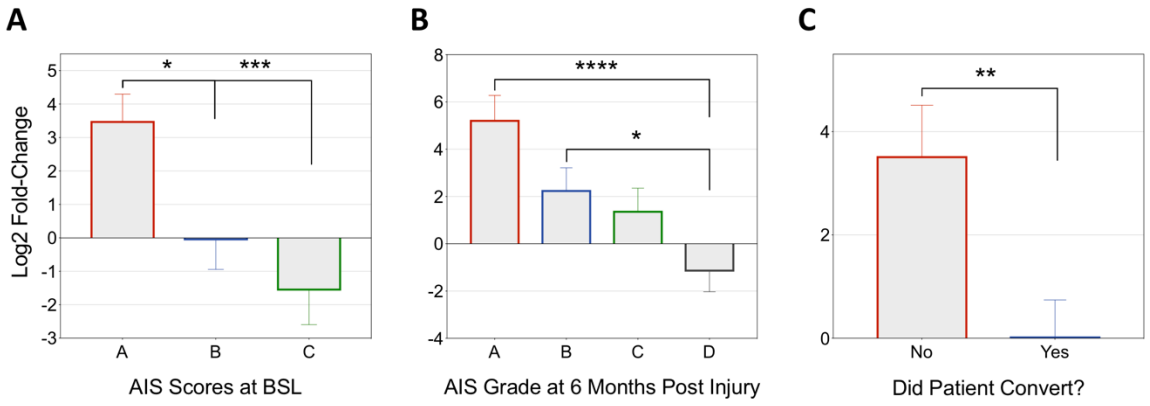


Figure 4.8 Relative levels of miR-133a-3p in serum at 24 hours post injury. Mean log2 fold change (± SEM), relative to control patients are shown for patients with baseline injury severity AIS grade A, B, and C (n = 6 each). (B) Mean log2 fold change (± SEM), relative to control patients are shown for patients with 6-month injury severity AIS grade A, B, C, and D. (C) Mean log2 fold change (± SEM), relative to control patients are shown for patients that show no neurological conversion and patients that do show neurological conversion. Asterisks indicate significant differences (Student's t-test, $p < 0.05$ (*), $p < 0.01$ (**)).

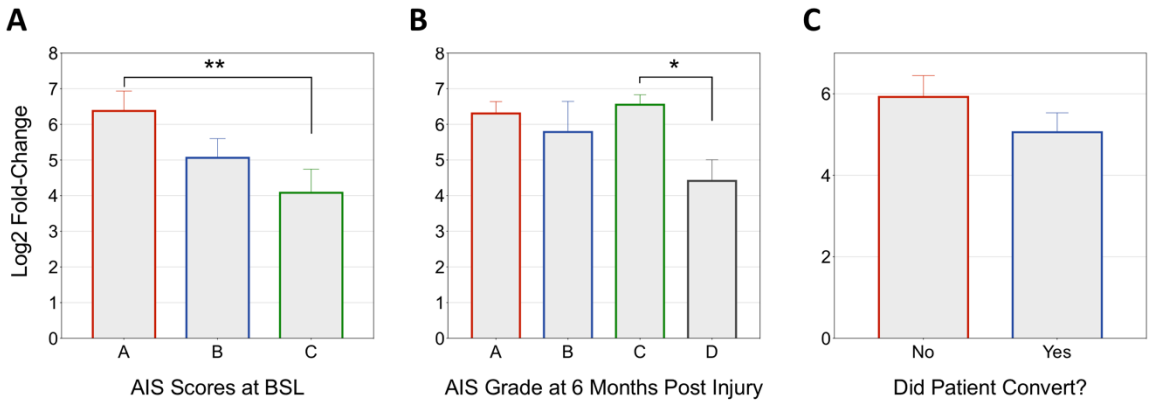


Figure 4.9 Diagnostic performance of microRNAs for baseline AIS grade. A) Using CSF microRNAs to discriminate between baseline AIS grades, a sparse partial least squares discriminant analysis (sPLS-DA) model including 70 microRNAs across two components had mean AUC in a 20 x 5-fold cross-validation of 0.75 [0.67, 0.82] when discriminating AIS A patients vs. all others (red), 0.70 [0.60, 0.80] for AIS B patients vs. all others (blue), and 0.64 [0.52, 0.77] for AIS C patients vs. all others (green). (B) The first and second components from the sPLS-DA model are plotted for all 24-hour sample data in 39 SCI patients.

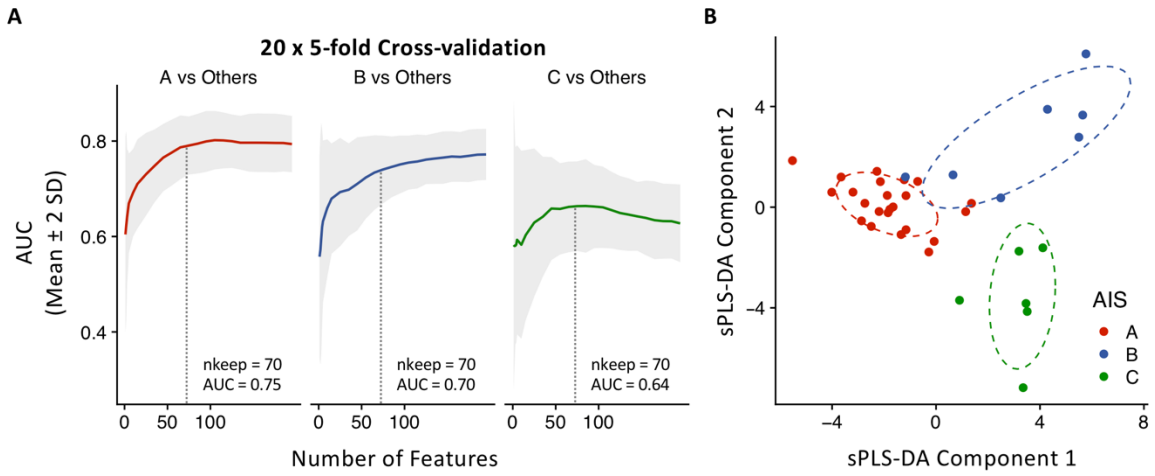
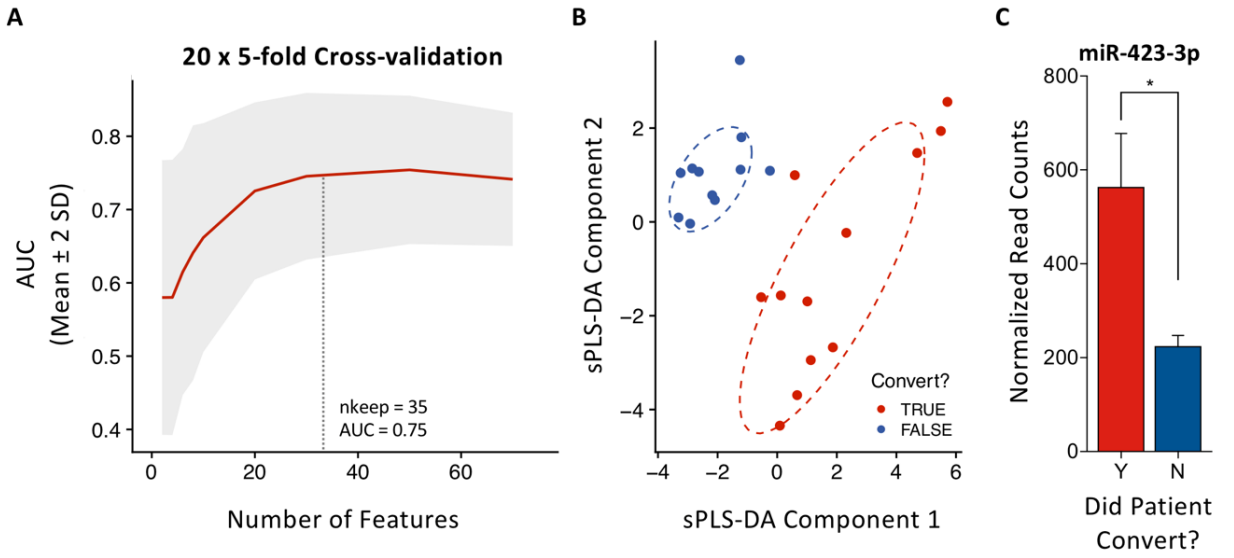


Figure 4.10 Prognostic performance of microRNAs for predicting neurological improvement in AIS A patients. (A) Using CSF microRNAs to discriminate between AIS A patients that showed AIS grade improvement at 6 months post injury versus those that did not, a sparse partial least squares discriminant analysis (sPLS-DA) model including 30 microRNAs across two components had mean AUC in a 20 x 5-fold cross-validation of 0.75 [0.63, 0.86]. (B) The first and second components from the sPLS-DA model are plotted for all 24-hour sample data in 24 AIS A SCI patients. (C) The read counts of the top microRNA, miR-423-3p in AIS A SCI patients that did convert (Y), compared to those that did not convert (N), p = 0.0108.



Chapter 5: General discussion

5.1 Summary

Each year, over 1,500 Canadians are paralyzed after suffering an acute traumatic SCI[332]. Beyond the devastating personal impact of this injury, the annual societal costs are massive – estimated at $2.67 billion in Canada alone[333]. Over the past two decades, a handful of therapies for acute SCI have been tested in large-scale clinical trials[334]. Unfortunately, none have demonstrated convincing efficacy, leaving us with no effective treatments to improve neurologic outcome after acute SCI. Our understanding of the complex cellular effects and their underlying mechanisms that take place after human SCI is limited. We have made significant progress through the study of rodent models of SCI, and many promising therapies have emerged that target the secondary injury responses after SCI[335-337]. However, direct observations describing these secondary injury responses in human SCI are limited. The lack of empirical biological research in human SCI makes it difficult to determine if the pathologic responses that are developed in targeted animal models are relevant to the human SCI condition[338]. This thesis aims to narrow the present knowledge gap and fundamentally change our understanding of the microRNA regulatory processes that occur after spinal cord injury by providing a comprehensive temporal characterization of the complex genetic alterations that occur after SCI in pigs and humans.

We first developed a large animal model of acute traumatic contusive SCI using Yucatan mini-pigs. In this model, we experimentally induced 3 injury severities along with a SHAM group that received a laminectomy but no injury. We showed that, by dropping a 50-gram weight from various heights, we could create 4 behaviourally and histologically distinct injury severities. We designed a technique to collect CSF and whole blood samples with minimally invasive techniques

from awake/moving animals to promote clinical relevance. We developed and optimized novel techniques to isolate nucleic acids from precious samples with low concentrations of RNA and performed highly technical and challenging library preparations for NGS. In order to analyze complex NGS data, we developed a bioinformatic pipeline to process the sequencing data and generate microRNA read counts for downstream analysis. As a result of our efforts, these important experimental processes are now established here at the University of British Columbia so they can be easily implemented and expanded upon by researchers in the future. As described in Chapter 3, we created the most comprehensive data set assembled to date describing the microRNA expression, over time, in a large animal model following acute traumatic contusive SCI. From this resource we identified a set of serum microRNAs that were diagnostic of SCI severity and predictive of behavioural and histological outcome – something that has never been shown before in SCI models and will contribute to the literature describing microRNA changes in CNS pathophysiology.

In Chapter 4, we sought to replicate the microRNA changes that we identified in pigs in Chapter 3, in clinical samples of CSF and serum collected from patients with acute traumatic SCI. Using the experimental techniques and bioinformatic infrastructure that we developed in Chapter 3, we profiled the CSF and serum microRNA in human SCI patients over 5 days following their SCI. This chapter aims to address a significant knowledge gap in the field, where there are no known descriptions of microRNA changes in human SCI patients with acute traumatic SCI over 5 days post injury. In Chapter 4, we identified commonalities between human and pig SCI, and identified a set of microRNAs that were diagnostic of baseline AIS classification, and predictive of motor score recovery and improvement status. By identifying common microRNA biomarkers of injury

severity in pigs and humans, we have sought to bridge the gap in the lack of outcome measures in preclinical models that can be used to evaluate therapies for SCI prior to embarking on highly challenging clinical trials.

5.2 The reality of microRNAs as biomarkers for spinal cord injury

Biomarkers will play an important role in facilitating the translation of acute SCI therapies[339, 340]. In the ideal scenario, biomarkers that accurately classify injury severity and precisely predict neurologic outcome, particularly in patients who cannot be functionally evaluated, will increase the number of patients that can be recruited for clinical trials and will reduce the number needed to complete them. Biomarkers that reflect the biological responses to SCI would also allow for improved patient stratification during enrollment and in principle will help define which patients would be the best candidates for (and most likely to benefit from) specific therapies depending upon their mechanisms of action. A challenge in the translation of novel therapies in many fields of biomedical research is the lack of direct biological correlates between the experimental and human condition[322, 341, 342]. Establishing these correlates between animal models and humans was specified as an important undertaking to promote bi-directional translation in the Institute of Medicine's workshop entitled "Improving the Utility and Translation of Animal Models for Nervous System Disorders"[343]. In the proceedings of this workshop, the *"*mismatch between endpoints used in preclinical animal studies and those used in trials*"* was communicated as an important contributing factor to the failure of clinical trials. In this context, the SCI field is no different. In the testing of novel therapies for SCI, standard outcome measures such as "hindlimb locomotor function" or "white matter sparing" in rodents, and "PTIBS" in pigs, are routinely used to determine a therapy's efficacy. These are not, however, measurable outcomes in humans and

therefore may have little relation to the human condition. Therefore, there is a "missing link" when translating a novel treatment into human SCI, in that the tools that are routinely used to determine the treatment's efficacy preclinically cannot be measured and/or are irrelevant in the injured human. Establishing biological markers that are measurable and predictive in both animal models and human patients with SCI will provide important guidance for the translation of novel treatments.

This thesis sought to accomplish two main goals:

1. **Identify CSF and/or serum microRNA biomarkers for the diagnosis and prognosis of acute traumatic spinal cord injury in humans.**
2. **Identify whether the same microRNA in humans are diagnostic and prognostic for SCI in the CSF and/or serum of a preclinical pig model of traumatic SCI.**

I will aim to address, with regards to these two goals, the context of this thesis, the limitations, and what 'next steps' to take to move towards the end goal of using microRNA biomarkers in the clinic for acute traumatic SCI.

5.2.1 Replication

MicroRNAs are increasingly being investigated as biomarkers for multiple diseases, yet over the past years, there has been a lack of replicable results. Within the field of SCI alone, various studies have described either general downregulation of microRNA[10], or upregulation[4, 131, 190] following injury to the spinal cord. This is likely in part, due to the difference in study design, animal models, tissues analyzed, in- and exclusion criteria, processing platforms, data processing and statistical methods[344-346]. Furthermore, often incomplete microRNA names or incomplete microRNA annotation result in microRNAs being referenced differently (e.g. miR-1 instead of miR-1-3p). In

this thesis, for example, the pig and human microRNA databases have significantly different numbers of annotated microRNAs (411 vs 2589 microRNA). Establishing reference standards and best practices for measuring microRNA and assessing RNA-seq accuracy, reproducibility, and robustness is needed if microRNAs are to ever reach the clinic. In addition to the challenges in study design, a large pitfall of RNA-seq studies is the small number of study subjects, likely due to the high cost of experiments. In an analysis of the effects of small sample sizes on microRNA profiling, Kok et al. created a data set that included 100 altered microRNA and found that in sample size of 5 versus 5, an average of only 47/100 microRNA were detected as altered, showing that a small samples size led to more than 50% false negatives[347]. In addition to missing true differentially expressed microRNA, a small sample size made it more difficult to detect microRNA that have small effect sizes (e.g. 1.2 – 1.4-fold change). This is pertinent to the current thesis, which had sample sizes of 24 AIS A, 8 AIS B, 7 AIS C, and 5 control patients, and n = 4 pigs per injury severity, suggesting a significant number of differentially expressed microRNA may not have been identified. In addition to the small sample size, the subjects in this thesis show large heterogeneity in enrollment institution, sex, age, injury level and mechanism. I highlight these limitations, to emphasize that, despite these shortcomings, we identified a high overlap in microRNA changes found in here in human SCI and pig SCI, and in other published studies of SCI in rodents (Figure 4.3). We also show a relatively high diagnostic and prognostic accuracy of microRNAs for injury severity and outcomes in humans and pigs after SCI. This suggests that with established best practices, increased sample sizes, and further validation, there is promise for the use of microRNA biomarkers for SCI in the future.

5.2.2 Biomarker discovery and development

Less than 10% of drugs that enter Phase I trials end up earning approval by the FDA[348], with efficacy being the greatest reason for attrition. Therefore, it is imperative to the future of industry and society at large that biomarkers continue to be developed which can provide metrics for drug exposure at the site of action, pharmacological activity, and optimal patient selection and stratification. The biomarker discovery and development pipeline require large investment of time and resources. This pipeline includes the early discovery phase, where candidate biomarkers are identified, followed by the development phase, which focuses on generalizability across large sample sizes, and standardization of assays. I claim this thesis has accomplished the initial discovery phase of biomarker development and has begun to address aspects of the development phase.

Using NGS to profile hundreds of microRNA candidates, we have successfully 'discovered' the microRNA that are actually expressed and detectable in CSF and blood in human SCI patients, and have identified a promising (yet likely not comprehensive, due to sample size) list of microRNAs that are altered due to SCI. While it will unlikely be feasible to continue using NGS as a point-of-care (POC) strategy for reasons I will discuss later, using NGS to perform unbiased profiling of CSF and serum microRNA was crucial to the discovery of microRNA biomarkers. Because pre-identified probes are not necessary for microRNA sequencing, NGS presented the opportunity to quantify by counts, microRNA that are actually present in CSF and serum in pigs (where no pre-designed probe sets existed, and the microRNA annotation was limited), as well as in humans. This is not insignificant – for example, a noteworthy study published in Scientific Reports performed microarray profiling of serum microRNAs for the diagnosis of human TBI[8]. In

this study, the microRNA that 'gave most stabilized expression both in control and TBI samples' was miR-202, and therefore miR-202 was chosen to normalize data. However, referring to the NGS data from this thesis (also in human serum), I have shown that human serum expression of miR-202 is **non-existent,** having zero counts in 188 serum samples. Contrary to microarray data, NGS provides count-level information on microRNA expression. Microarray data can only generate relative fluorescence units for microRNA expression, and has the potential give rise to misidentified 'stable' microRNA. Acknowledging the potential influence of different experimental protocols on the identification of expressed microRNAs, it is possible that the 'most stable' microRNA was one with no expression across samples at all, thus, having no variability. Moving forward, the SCI community will have the opportunity to refer to the NGS data set provided by this thesis in order to understand what microRNA are actually present in the CSF and serum of human and pigs, and which are altered after SCI.

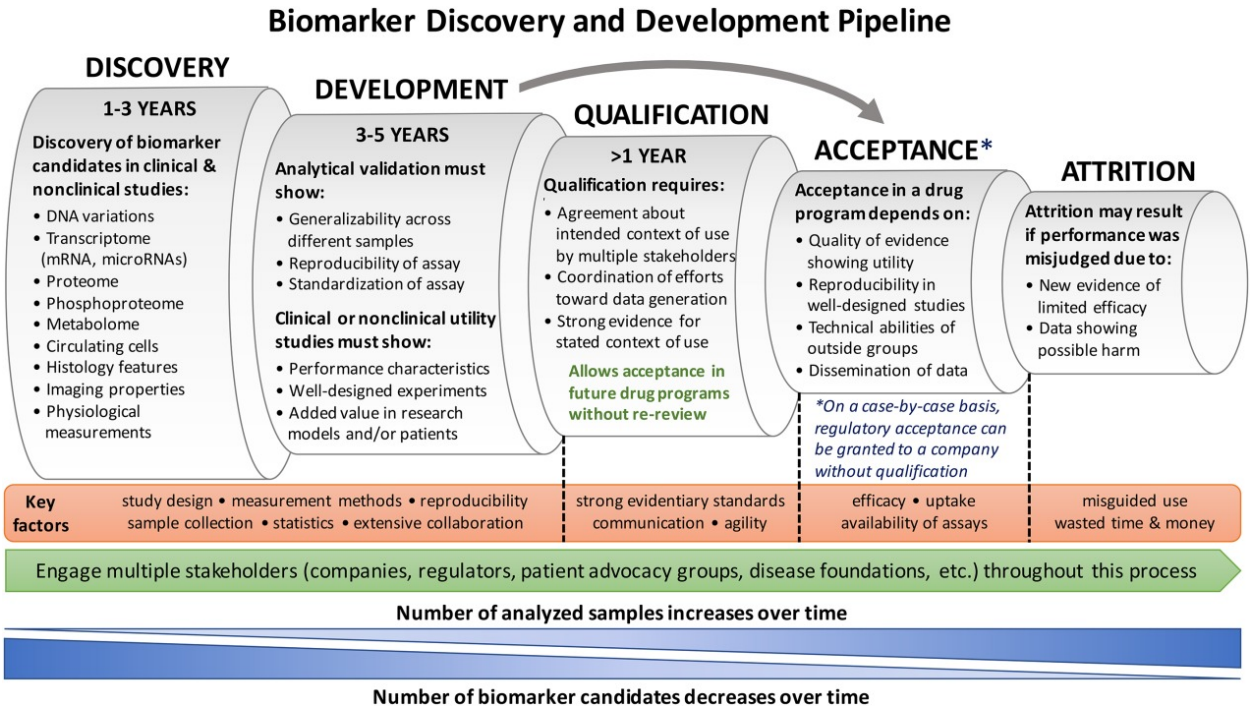
The work presented in this thesis has discovered a total of 95 differentially expressed microRNA in pig serum after SCI, and 204 microRNA differentially expressed in human CSF and serum after SCI. These present promising targets for biomarker development and this work represents the bulk of the discovery phase of the biomarker pipeline. Of course, we sought to actually identify whether these microRNA exhibit promising performance characteristics, an aspect of the development phase, but more work is needed to show generalizability, reproducibility, and standardization. In assessing the performance characteristics of pig microRNA, the top ten serum microRNAs were able to distinguish SCI vs non-SCI with an AUROC of 0.87 – 0.95, and the top three microRNA were able to distinguish severe SCI vs mild SCI with 100% sensitivity and specificity – this despite a sample size of n = 4 per group. In humans, we were able to create a model using data from 70

microRNA to classify baseline ISNCSCI classification (AIS A, B, and C) with an AUROC of 0.75 and a model using data from 35 microRNA to predict recovery in AIS A patients with an AUROC of 0.75. To put this into context, we can look to comparable microRNA biomarker studies in human patients from our neurotrauma colleagues in TBI. In the previously mentioned study of microRNA changes in serum from human TBI patients, with a sample size of n = 8 per group, Bhomia et al. identified ten microRNA that had an AUROC of between 0.66 – 0.86[8] for classifying patients with TBI versus uninjured patients. Of note is that one of their top serum microRNAs, miR-486 (AUC = 0.81), also showed 100% sensitivity and specificity after pig SCI and is the top contributor to the second component of the baseline classification model in human CSF. In a separate study of salivary microRNA biomarkers for mild TBI, with a sample size of n = 28 versus n = 16, a multivariate ROC curve analysis revealed a set of 5 microRNA that could distinguish TBI patients from non-TBI patients with an AUROC of 0.83[349]. This highlights that with larger sample sizes and models only designed to identify 'injury versus non-injury', the TBI field is performing only slightly better than the models presented in this thesis which aim to differentiate between 4 poorly defined injury severities, all of which share characteristics with each other.

Finally, while a model requiring the measurement of 70 microRNA may be impractical, it is not unreasonable. MammaPrint is an important example of a 70-gene signature test for early stage breast cancer that assesses the risk of a tumour metastasizing to other areas of the body, giving a binary "high-risk" or "low-risk" classification. Cleared by the FDA in 2007, MammaPrint uses tissue for microarray analysis of RNA isolated from tumor samples[350]. MammaPrint showed an 83% classification accuracy with a sample size of 78 patients. The international, prospective, randomized, phase 3 study for MammaPrint was conducted between 2007 and 2011, recruiting a

total of 6,600 patients across 112 institutions. With the small sample sizes used in this thesis, it is likely that a model with far fewer microRNA could exhibit a higher classification performance with more replicates.

Figure 5.1 Biomarker discovery and development pipeline. *Adapted from Gerlach et al*[348]



5.2.3 Point of care strategies

As mentioned previously, NGS technology is unlikely to be a practical POC strategy for microRNA profiling in order to classify SCI patients. NGS is costly, time consuming, and requires significant expertise in both sample preparation and bioinformatics. It will be imperative to validate the microRNA changes identified in this thesis, with a cheaper, more user-friendly, and faster technology if microRNAs are to make their way into the clinic for SCI classification. There are a number of promising approaches to develop better POC strategies for the utilization of microRNAs to classify SCI severity. While further work is needed to identify the source of the elevation in CSF microRNA that we see in human SCI patients, I believe it is likely due to a passive release of cellular microRNA from damaged neurons. We have identified early (24 hours post injury), severity-dependent increases in cDNA-converted small RNAs (Figure 4.1), which are comparable to uninjured CSF microRNA levels by 3 days post injury. Preliminary work using ThermoFisher's Qubit microRNA assay to quantify microRNA concentrations in RNA extracted from 1 mL of CSF suggests that microRNA concentrations at 24 hours post injury are significantly different between AIS grades, with higher levels in AIS A patients. In comparison to the time-consuming NGS library preparation methods, this microRNA quantitation assay can be performed in under 2 days. It may be possible that simply quantitating microRNA concentrations in a purified, or even unprocessed sample of CSF will provide sufficient information for classifying SCI severity as a measurement of spinal cord trauma.

There is no question that better POC technologies for microRNA detection are needed. Nearly 100 novel microRNA detection techniques have been published, aimed at developing POC techniques that can measure microRNAs in the field, often with unprocessed biofluids, as reviewed by

Gillespie et al.[351]. Nucleic acid sensing is on the frontier of diagnostic medicine, with many diseases being linked to altered microRNA expression. It is reasonable that with the data set provided in this thesis, and further validation, we will be strategically positioned for the development of POC strategies as microRNA detection technologies are developed in the near future. As a hypothetical example taken from MammaPrint, a custom glass microarray slide could be designed to determine the expression profile of the 70-microRNA signature described in this thesis. The profile then, would be fed through the 'MixOmics' sPLS-DA algorithm to categorically classify patients as having a severe, moderate, or mild SCI, and determine whether they are likely to improve or not at 6 months.

5.2.4 Using the pig model as a translational intermediary for clinical trials

Currently, no outcome measures exist in animal models that are also available in human SCI. We are restricted to extrapolating, for example, what 'paw placement', 'grooming', or 'weight bearing extensions' mean with regards to neurologic outcomes in a human patient. When we see improvements in behavioural parameters in preclinical animal models, we have little evidence that this means we can expect to see meaningful improvements in humans. Biomolecules that are measurable in both humans and animal models and are representative of SCI severity or response to SCI therapies would shift the way we conduct preclinical testing. We sought to identify common microRNAs in pigs and humans as a solution to this missing link in outcome measures between clinical and preclinical studies. While it has been noted that there were technical failures in identifying microRNA expression within the CSF of pigs, there still remains an abundance of promising data describing the microRNA expression in the serum of pigs after SCI, and in CSF and serum of human patients with SCI. What is particularly encouraging is, despite the small

sample sizes, different species, biofluids, injury mechanisms, and variability in technical sample preparation, there exists an undeniable overlap in altered microRNA in pigs and humans after SCI. A number of the microRNA that performed best at classifying injury severity in pigs, namely let-7i, miR-10b, miR-30b, miR-133, miR-208, and miR-486 composed some of the 70 microRNA that also contributed to the baseline AIS classification model in human SCI patients, or were significantly altered post-SCI in humans. Importantly, these microRNA are the very same microRNA that have been repeatedly identified as classifiers of TBI[8, 170, 349, 352, 353]. Let-7i is a potential regulator of S-100β and UCH-L1[354], miR-10b is enriched in the spinal cord tissue[173] and may represent an important marker of destruction to the spinal cord parenchyma, miR-30 targets programmed cell death 2-like (PDCD2L)[355], miR-133 is involved in functional recovery post SCI in adult zebrafish, suggesting roles in targeting RhoA, enhancing regrowth of the corticospinal tract, and promoting neuroprotection[181], miR-208 progressively declined following SCI in skeletal muscle and is thought to regulate skeletal muscle mass, therefore presence may indicate muscle atrophy as a result of SCI, and finally, miR-486 has a profound role in regulating SCI and TBI pathology, targeting NeuroD6, a protein the is important for neuronal differentiation and oxidative stress response. Interestingly, infusion of miR-486 into the spinal cords of uninjured mice produced SCI-like effects. There is clearly a set of microRNAs that are identifiable in biological fluids following neurotrauma-related events and further work must be conducted to elucidate these mechanisms.

The evidence provided in this thesis presents a strong argument for a shared set of microRNA changes induced by SCI in pigs and humans. Unfortunately, we were unable to successfully profile pig CSF microRNA but despite this, there are significant commonalities to the altered microRNAs

in both species that warrant further investigation. The microRNA that are altered after SCI fit within a CNS regulatory framework – the CSF microRNA that are altered after SCI are highly expressed within the CNS tissue, and are also known or predicted to be involved in the regulation of common neuronal processes. The challenge here is identifying if they are altered due to changes in the regulatory processes triggered by SCI, or if they are merely regulators of normal neuronal biology and are released due to the trauma to the cells they are known to be highly expressed in (neurons).

5.3 Limitations

Here, I summarize the overarching challenges with microRNA detection, and shortfalls of this thesis as described in length, above. With regards to microRNA detection, current technologies lack best practices and gold standards. There is significant variability and introduction of bias throughout the isolation of genomic material, sample preparation, PCR amplification, pipetting, and sequencing. There is no agreed-upon gold standard bioinformatic pipeline, and computational approaches vary widely across studies. MicroRNA sequencing data normalization strategies are adopted from the field of transcriptome sequencing, however these strategies are unlikely to be appropriate for microRNA data, where the number of genes detected are in the hundreds (versus tens of thousands for the transcriptome), introducing false positives and negatives.

Regarding the limitations in the study design of this thesis, there were small sample sizes – an unfortunate reality of most studies in the field of SCI and Neuroscience as a whole. A main objective of this study was to compare microRNA expression in the same biofluids between humans and pigs. Unfortunately, we had very low success with sample preparation of pig CSF that

was unable to be resolved, making comparisons between species very challenging. A second main objective of this study was to identify microRNAs in the blood that could classify severity, given that blood is a less invasively collected fluid compared to CSF. While human CSF microRNA profiles were promising in their ability to classify severity, human serum microRNAs performed poorly; unfortunately, it seems that CSF will continue to be an important biofluid in understanding and classifying SCI. Finally, NGS is inconvenient as a POC technology for microRNA detection and classification of SCI severity – new technologies will need to be validated in order to develop microRNA biomarkers that can be used in the clinic.

5.4 Future directions

This research has provided the groundwork for developing microRNA biomarkers for acute traumatic SCI. While the work here addresses the bulk of the 'discovery phase' of the biomarker development pipeline, much more work is needed before microRNA can be used as a point-of-care strategy for the diagnosis or prognosis of patients with acute traumatic SCI. A number of next steps must be taken to tackle the 'development', 'qualification', and 'acceptance' phases of the biomarker development pipeline in order to get microRNA biomarkers into the clinic, and are outlined below:

1. **What is the source of upregulated microRNA, and what are the biological effects of increases in microRNA within the CSF or serum after injury?**

Moving forward, it will be important to identify the biological source of microRNA that are detected in CSF after SCI. A rapid, acute increase in microRNA concentration is measured at 24

hours post injury in patients and in pigs after SCI. It is unknown whether these microRNA are packaged within exosomes with regulatory functions, or passively released from destroyed neurons or glial cells, in which they may or may not have regulatory functions. Future work should aim to address this, using methods that purify extracellular vesicles. Techniques to do this include differential centrifugation, density gradients, size exclusion chromatography, and affinity capture beads[356]. As mentioned previously, Turchinovich et al. has highlighted the disparity in opinions regarding the proportions of microRNAs that are free-floating in circulation and packaged in exosomes[112-114]. To investigate the source of CSF-derived microRNA, it may be valuable to refer to, or independently create a single-cell RNA-seq data set in order to identify microRNA expression profiles of individual cell types within the spinal cord. For example, miR-10 is the most abundant CSF-derived microRNA in human SCI patients, and it is known to be highly enriched in neurons; miR-219 is thought to be enriched in myelin; miR-150 and miR-133, are both highly severity dependent, are reportedly specific to muscle cells and/or myocytes[187, 193, 357]. Performing single-cell RNA-seq on the cellular populations within the spinal cord would provide the opportunity to elucidate the origin of these microRNA and provide evidence for the rationale of 'tissue specificity' to which we refer in our justification of microRNA biomarkers.

2. Are there other, non-invasive biofluids that might hold biomarkers that are promising to pursue for the classification of SCI severity such as saliva or urine?

This thesis aimed at identifying microRNA changes in CSF and serum, with the perspective that serum will contain microRNA that 'leaked' from the CSF due to a compromised blood-spinal cord barrier. Unfortunately, the microRNA expression in the blood had very high variability across

human subjects and was poor at classifying SCI severity. There is some evidence that suggests microRNAs isolated from urine samples may share more in common with CSF microRNA, compared to blood[106]. In order to address the objective of not relying on invasive collection of CSF, it may be imperative to look to other easily-accessible biofluids to identify microRNA changes that are able to classify SCI severity.

3. **What microRNA detection technologies appropriately meet the requirements for speed, expertise, and cost-effectiveness for POC diagnostics?**

As mentioned, better POC technologies for microRNA detection are needed. Recently, a DNA-gold nanoparticle based lateral flow nucleic acid biosensor was developed by Gao et al.[358]. This biosensor is designed for visual detection of microRNA in aqueous solutions and biological samples with low-cost and short analysis time. The biosensor was shown to be able to detect a minimum concentration of 60 pM and a maximum of 10 nM. To compare this to the concentration of human microRNAs in 1mL of CSF, AIS A patients had microRNA concentrations of over 450 pM, while the top 5 microRNAs make up 78% (~350 pM) of the total microRNAs detected and are composed of the very microRNAs that are highly altered after SCI in both humans and pigs. Therefore, a simple visual biosensor may be able to differentiate SCI severity based on the concentration differences in specific, highly expressed microRNA. As these technologies continue to emerge, the NGS data presented here will need to be validated in a platform that meets the appropriate POC requirements for SCI classification.

Ultimately, moving forward, the strategy I would propose to push these microRNA biomarkers into the domain of clinical utility is the following: Treating the current NGS data as a 'discovery dataset', continue to isolate total RNA from 1 mL of CSF from humans and pigs. This RNA contains enough material to aliquot into at least two assays: The first assay should aim to quantitate the concentration of microRNA in each sample, while the second assay should aim to quantitate a few (qRT-PCR, ddPCR) or a large panel of (microarray) all pre-identified, differentially expressed microRNA in order to validate the NGS data on a platform that is cheaper and less labour intensive than sequencing. At the same time, urine and/or saliva should be collected from SCI patients in order to continue the pursuit of biomarkers in non-invasive biofluids. These samples should go through the same discovery phase, using NGS to objectively identify altered microRNA. Finally, while there is no widely accepted quick and convenient method to detect microRNAs for POC purposes, the goals above are designed to strategically prepare for the inevitability of such a technology emerging in the near future.

5.5 Conclusions

In conclusion, the work presented in this thesis is an important contribution to the discovery of biomarkers for the diagnosis and prognosis of acute traumatic spinal cord injury and a needed description of the temporal microRNA changes that occur following SCI – a fundamental resource for the SCI community in the future. It also provides a framework of best practices for molecular biomarker discovery in the SCI field, for the future. These data will lay the foundation for critical research in further developing biomarkers for SCI, but also for developing potential therapies using microRNAs and altering their downstream targets – a field that is growing daily. Further work should aim to validate these data in human patients with SCI and other forms of CNS injury. As a

foundational data set, the microRNA changes that occur in pigs and humans described here should be used as a guiding resource for future studies investigating microRNA changes following SCI. As we collaborate and build on previous research, I hope that the work described here can be a small but important piece of the groundwork for the management and treatment of individuals who have suffered from SCI.

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