Applications of blood biomarkers of traumatic brain injury across the severity spectrum – a focus on pathophysiological mechanisms

by

Alex P. Di Battista

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

© Copyright by Alex P. Di Battista 2017
Applications of blood biomarkers of traumatic brain injury across the severity spectrum – a focus on pathophysiological mechanisms

Alex P. Di Battista

Doctor of Philosophy

Institute of Medical Science
University of Toronto

2017

Abstract

INTRODUCTION. Traumatic Brain Injury (TBI) is a global health concern. Spanning mild cognitive disturbances to unsurvivable trauma, the clinical challenges of TBI range from acute life-saving predicaments to the management of chronic cognitive impairment. While the primary insult leading to injury is irreversible, the subsequent secondary manifestations are seemingly amenable to intervention. Yet, no effective therapies exist across the spectrum of brain injury, primarily owing to our inability to understand the multifaceted, complex, secondary manifestations that occur after the primary insult. While experimental animal research has been informative in this regard, our knowledge of these processes in humans is lacking. However, a growing body of evidence suggests blood biomarker analysis may be a useful tool to aid in the elucidation of important pathophysiological mechanisms across a range of severities in human TBI. OBJECTIVE. The aim of this thesis was to use blood biomarkers as a tool to advance knowledge of secondary injury processes across the severity spectrum of TBI. METHODS. To investigate moderate-to-severe injury, we evaluated patients over the first 24 h from hospital admission after isolated TBI. To investigate mild injury, we studied varsity athletes with a clinically diagnosed sport-related concussion (SRC), sampled from the subacute period throughout clinical recovery, and chronically up to years after injury. Using multiplex
immunoassay techniques, we evaluated a total of 58 blood biomarkers associated with numerous previously defined secondary injury processes, including central nervous system (CNS) injury, sympathetic nervous system (SNS) activity, inflammation, vascular injury and hemostasis.

**RESULTS.** In moderate-to-severe TBI, we found significant, dynamic pathological associations between SNS hyperactivity, inflammation, hemostasis, and vascular injury. In SRC, we found subacute alterations to numerous peripheral indices of CNS injury, inflammation and neuroinjury that persisted at medical clearance, as well as in ostensibly healthy athletes exposed to repetitive head impacts. **CONCLUSION.** Blood biomarkers are a useful tool to evaluate secondary injury processes across the spectrum of TBI. The results from these works necessitate future investigations into: (1) the potential therapeutic benefit of β-blockers acutely after moderate-to-severe injury, and (2) the interrelationships between chronic inflammation and CNS injury after SRC, and their potential involvement in neurodegeneration.
Acknowledgments

I would like to thank Dr. Andrew Baker and Dr. Shawn Rhind for their incredible support and guidance over the last 5 years. I am grateful to Dr. Rhind for his patience, positivity, and the many invaluable lessons he imparted on me. I thank Dr. Baker for the crucial role he played in challenging me to become a better scientist.

To my family and friends, I would like to thank you for continual support and patience; graduate school would not be possible without you. In particular, to my parents, Paul and Sue Di Battista, who were my first and most important mentors, and who I owe everything to.
# Table of Contents

ACKNOWLEDGMENTS  

TABLE OF CONTENTS  

LIST OF ABBREVIATIONS  

LIST OF TABLES  

LIST OF FIGURES  

LIST OF APPENDICES  

CHAPTER 1 INTRODUCTION.

## 1 INTRODUCTION

1.1 CLINICAL OVERVIEW AND EPIDEMIOLOGY OF TRAUMATIC BRAIN INJURY

1.1.1 EPIDEMIOLOGY AND OVERVIEW

1.1.2 DEFINITION AND DIAGNOSIS

1.1.3 PROGNOSIS

1.2 TBI PATHOPHYSIOLOGY

1.2.1 PRIMARY INJURY

1.2.2 SECONDARY INJURY - CNS

1.2.2.1 Neurometabolic disruption

1.2.2.2 DAI

1.2.2.3 Neuroinflammation

1.2.2.4 Blood brain barrier disruption

1.2.2.5 Cerebral edema

1.2.3 SECONDARY INJURY – PERIPHERY

1.2.3.1 SNS dysregulation

1.2.3.2 Systemic inflammation

1.2.3.3 Coagulopathy and endotheliopathy

1.2.4 LIMITATIONS OF ANIMAL MODELS IN TBI

1.3 THE APPLICATION OF BLOOD BIOMARKERS TO TBI
2.4.4 6-MONTH NEUROLOGICAL OUTCOME 75
2.4.5 MORTALITY 76
2.4.6 SECONDARY COMPLICATIONS CONTROLLED FOR INJURY SEVERITY 79
2.4.6.1 6-month GOSE 79
2.4.6.2 Mortality 79
2.4.7 NEUROLOGIC VS NON-NEUROLOGIC DEATH 81
2.4.8 COMBINED INFLAMMATORY SCORE AND PATIENT CHARACTERISTICS 81
2.5 DISCUSSION 83
2.6 CONCLUSION 88

CHAPTER 3 SYMPATHOADRENAL ACTIVATION IS ASSOCIATED WITH ACUTE TRAUMATIC COAGULOPATHY AND ENDOTHELIOPATHY IN ISOLATED BRAIN INJURY 89

3 SYMPATHOADRENAL ACTIVATION IS ASSOCIATED WITH ACUTE TRAUMATIC COAGULOPATHY AND ENDOTHELIOPATHY IN ISOLATED BRAIN INJURY 90

3.1 ABSTRACT 90
3.2 INTRODUCTION 91
3.3 METHODS 94
3.3.1 STUDY POPULATION AND DESIGN 94
3.3.2 BLOOD SAMPLE COLLECTION AND PROCESSING 94
3.3.3 HEMOSTATIC AND ENDOTHELIAL MARKER ANALYSIS 95
3.3.4 CATECHOLAMINE ANALYSIS 96
3.3.5 STATISTICAL ANALYSES 96
3.4 RESULTS 99
3.4.1 DEMOGRAPHICS AND CLINICAL CHARACTERISTICS 99
3.4.2 RELATIONSHIP BETWEEN BIOMARKERS AND 6-MONTH GOSE 99
3.4.3 COVARIANCE BETWEEN CATECHOLAMINES AND BIOMARKERS 102
3.5 DISCUSSION 104
3.6 CONCLUSION 108

CHAPTER 4 AN INVESTIGATION OF NEUROINJURY AND INFLAMMATORY BIOMARKERS AFTER SRC: FROM THE SUBACUTE PHASE TO CLINICAL RECOVERY 110
Chapter 4
AN INVESTIGATION OF NEUROINJURY AND INFLAMMATORY BIOMARKERS AFTER SRC: FROM THE SUBACUTE PHASE TO CLINICAL RECOVERY.

4.1 Abstract
4.2 Introduction
4.3 Methods
4.3.1 Participants
4.3.2 Sport Concussion Assessment Tool – 3 (SCAT3)
4.3.3 Blood Sample Collection
4.3.4 Blood Biomarker Analysis
4.3.5 Statistical Analysis
4.4 Results
4.4.1 Description of Participants
4.4.2 Blood Biomarker Profiles in Concussed Athletes
4.4.3 Relationship between Blood Biomarker Profiles and Participant Characteristics
4.5 Discussion
4.6 Conclusion

Chapter 5
ALTERED BLOOD BIOMARKER PROFILES IN ATHLETES WITH A HISTORY OF REPETITIVE HEAD IMPACTS.

5.1 Abstract
5.2 Introduction
5.3 Methods
5.3.1 Participants
5.3.2 Measures
5.3.3 Blood Sample Collection
5.3.4 Biomarker Analysis
5.3.5 Statistical Analyses
5.4 Results
5.4.1 Demographics and Clinical Characteristics
5.4.2 Systemic Inflammatory Marker Analysis
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>model prediction accuracy</td>
<td>Accur</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>AD</td>
</tr>
<tr>
<td>abbreviated injury score</td>
<td>AIS</td>
</tr>
<tr>
<td>adenosine monophosphate</td>
<td>AMP</td>
</tr>
<tr>
<td>D-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
<td>AMPA</td>
</tr>
<tr>
<td>autonomic nervous system</td>
<td>ANS</td>
</tr>
<tr>
<td>activated protein C</td>
<td>APC</td>
</tr>
<tr>
<td>amyloid precursor protein</td>
<td>APP</td>
</tr>
<tr>
<td>acute phase response</td>
<td>APR</td>
</tr>
<tr>
<td>activated partial thromboplastin time</td>
<td>aPTT</td>
</tr>
<tr>
<td>adenosine triphosphate</td>
<td>ATP</td>
</tr>
<tr>
<td>β2-adrenergic receptor</td>
<td>β2-AR</td>
</tr>
<tr>
<td>blood brain barrier</td>
<td>BBB</td>
</tr>
<tr>
<td>brain-derived neurotrophic factor</td>
<td>BDNF</td>
</tr>
<tr>
<td>break-down products</td>
<td>BDP</td>
</tr>
<tr>
<td>and calcium</td>
<td>Ca2⁺</td>
</tr>
<tr>
<td>compensatory anti-inflammatory response syndrome</td>
<td>CARS</td>
</tr>
<tr>
<td>cerebral blood flow</td>
<td>CBF</td>
</tr>
</tbody>
</table>
controlled cortical impact | CCI
chemokine ligand | CCL
cluster of differentiation | CD
confidence interval | CI
creatine kinase-BB isoenzyme | CKBB
central nervous system | CNS
cerebral perfusion pressure | CPP
Corticosteroid Randomization After Significant Head Injury | CRASH
corticotropin-releasing hormone | CRH
c-reactive protein | CRP
cerebral spinal fluid | CSF
chronic traumatic encephalopathy | CTE
computerized tomography | CTE
circumventricular organs | CVO
coefficient of variation | CVO
chemokine ligand | CXCL
diffuse axonal injury | DAI
damage/danger associated molecular pattern | DAMP
disseminated intravascular coagulation | DCI
d-dimer | DD
<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>diffusion tensor imaging</td>
<td>DTI</td>
</tr>
<tr>
<td>enzyme-linked immunoassay</td>
<td>ELISA</td>
</tr>
<tr>
<td>endothelial protein C receptor</td>
<td>EPCR</td>
</tr>
<tr>
<td>epinephrine</td>
<td>Epi</td>
</tr>
<tr>
<td>fibrin(ogen) breakdown products</td>
<td>FDPs</td>
</tr>
<tr>
<td>false discovery rate</td>
<td>FDR</td>
</tr>
<tr>
<td>fluid percussion injury model</td>
<td>FPI</td>
</tr>
<tr>
<td>Glasgow Coma Scale</td>
<td>GCS</td>
</tr>
<tr>
<td>glial fibrillary acidic protein</td>
<td>GFAP</td>
</tr>
<tr>
<td>Glasgow Outcome Scale</td>
<td>GOS</td>
</tr>
<tr>
<td>extended GOS</td>
<td>GOSE</td>
</tr>
<tr>
<td>hours</td>
<td>h</td>
</tr>
<tr>
<td>high mobility group box</td>
<td>HMGB</td>
</tr>
<tr>
<td>hypothalamic pituitary adrenal</td>
<td>HPA</td>
</tr>
<tr>
<td>intracellular adhesion molecule</td>
<td>ICAM</td>
</tr>
<tr>
<td>intracranial pressure</td>
<td>ICP</td>
</tr>
<tr>
<td>interferon</td>
<td>IFN</td>
</tr>
<tr>
<td>interleukin</td>
<td>IL</td>
</tr>
<tr>
<td>International Mission for Prognosis and Clinical Trial Design</td>
<td>IMPACT</td>
</tr>
<tr>
<td>international normalized ratio</td>
<td>INR</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>interferon-gamma induced protein</td>
<td>IP</td>
</tr>
<tr>
<td>interquartile range</td>
<td>IQR</td>
</tr>
<tr>
<td>inflammation score</td>
<td>IS</td>
</tr>
<tr>
<td>injury severity score</td>
<td>ISS</td>
</tr>
<tr>
<td>potassium</td>
<td>K⁺</td>
</tr>
<tr>
<td>locus coeruleus</td>
<td>LC</td>
</tr>
<tr>
<td>lipopolysaccharide</td>
<td>LPS</td>
</tr>
<tr>
<td>myelin basic protein</td>
<td>MBP</td>
</tr>
<tr>
<td>monocyte chemoattractant protein</td>
<td>MCP</td>
</tr>
<tr>
<td>macrophage-derived chemokine</td>
<td>MDC</td>
</tr>
<tr>
<td>macrophage inflammatory protein</td>
<td>MIP</td>
</tr>
<tr>
<td>matrix metalloproteinases</td>
<td>MMPs</td>
</tr>
<tr>
<td>magnetic resonance imaging</td>
<td>MRI</td>
</tr>
<tr>
<td>Meso-Scale Discovery</td>
<td>MSD</td>
</tr>
<tr>
<td>mild TBI</td>
<td>mTBI</td>
</tr>
<tr>
<td>norepinephrine</td>
<td>NE</td>
</tr>
<tr>
<td>nuclear factor-kappa B</td>
<td>NF-κB</td>
</tr>
<tr>
<td>N-methyl-D-aspartate</td>
<td>NMDA</td>
</tr>
<tr>
<td>nitric oxide</td>
<td>NO</td>
</tr>
<tr>
<td>neurogranin</td>
<td>NRGN</td>
</tr>
</tbody>
</table>
neuron specific enolase  NSE
odds ratio  OR
plasminogen activator inhibitor  PAI
post-concussion syndrome  PCS
partial least squares  PLS
partial least squares – discriminant analysis  PLS-DA
platelet  PLT
posterior probability  Pprob
peroxiredoxin  PRDX
pattern recognition receptors  PRRs
paraventricular nucleus  PVN
reactive oxygen species  ROS
s100 calcium binding protein  s100
serum amyloid A  SAA
subarachnoid hemorrhage  SAH
sport concussion assessment tool  SCAT
standard deviation  SD
syndecan  SDC
systemic inflammatory response syndrome  SIRS
sympathetic nervous system  SNS
proteolytic fragment of alpha-II spectrin

sport-related concussion

thrombin activatable fibrinolysis inhibitor

thymus and activations regulated chemokine

thrombin–antithrombin complexes

traumatic brain injury

tissue factor

transforming growth factor

tissue factor pathway inhibitor

toll-like receptor

thrombomodulin

tumor necrosis factor

tissue plasminogen activator

ubiquitin c-terminal hydrolase

vascular adhesion protein

vascular cell adhesion molecule

visinin-like protein

von Willebrand factor

SNTF

SRC

TAFI

TARC

TAT

TBI

TF

TFG

TFPI

TLR

TM

TNF

tPA

UCH

VAP

VCAM

VILIP

vWF
List of Tables

Table 2.1 Demographic and Clinical Characteristics for TBI patients.

Table 2.2 SNS and inflammatory marker correlations.

Table 2.3 Binomial logistic regression models assessing the ability of inflammatory markers to predict poor patient outcome, controlling for injury severity.

Table 2.4 Clinical characteristics according to dichotomized inflammatory scores.

Table 3.1 Demographic and Clinical Characteristics of TBI patients.

Table 4.1 Athlete demographics and characteristics.

Table 4.2 Biomarker values across groups.

Table 5.1 Athlete demographics and characteristics.

Table 5.2 List of biomarkers analyzed.
List of Figures

**Figure 1.1** Movement of biomarkers from the CNS to the peripheral blood after TBI.

**Figure 1.2** SNS mediated systemic inflammation after TBI.

**Figure 1.3** Potential drivers of coagulopathy after TBI.

**Figure 2.1** Plasma cytokine and chemokine concentrations in moderate and severe TBI patients sampled over 24 h.

**Figure 2.2** Plasma cytokine and chemokine concentrations in TBI patients stratified according to 6-month GOSE.

**Figure 2.3** Plasma cytokine and chemokine concentrations in TBI patients stratified by mortality.

**Figure 2.4** Plasma cytokine and chemokine concentrations in TBI patients according to cause of death.

**Figure 3.1** Partial Least Squares – Discriminant Analysis of patient outcome.

**Figure 3.2** Partial Least Squares analysis of covariance between the SNS and markers of endotheliopathy, coagulopathy, and inflammation.

**Figure 4.1** Plasma biomarker concentrations in athletes with a sport-related concussion measured sub-acutely and at medical clearance.

**Figure 4.2** Covariance between plasma biomarkers and SCAT3 total symptoms sub-acutely in athletes with a sport-related concussion.

**Figure 5.1** Biomarker covariance with concussion history in athletes.

**Figure 5.2** Covariance between biomarkers and head injury characteristics in male athletes.

**Figure 6.1** Potential secondary injury processes related to poor patient outcome acutely after moderate-to-severe TBI.

**Figure 6.2** Biomarker signatures of the subacute and recovery phases of SRC.
List of Appendices

**Appendix 2.1** Percentage of samples within detection limit for all circulating cytokines and chemokines analyzed.

**Appendix 2.2** Circulating concentrations of cytokines and chemokines in all TBI patients (GCS 3-13) within 24 hours of hospital admission.

**Appendix 3.1** Plasma concentrations of biomarkers.

**Appendix 3.1** Biomarker covariance with injury severity

**Appendix 5.1** Biomarker values in athletes according to concussion history

**Appendix 5.2** Biomarker values in athletes stratified by collision sport participation
Chapter 1
Introduction.

The following two papers are referenced throughout this chapter:


The lead author contributed to the conceptual framework of the paper, was responsible for analysis and interpretation of the literature, drafted the manuscript, and contributed to its critical revision.


The lead author contributed to the manuscripts conceptual framework, analyzed and interpreted the data, drafted the manuscript, and contributed to its critical revision.
1 Introduction

1.1 Clinical overview and epidemiology of Traumatic Brain Injury

1.1.1 Epidemiology and overview

Traumatic Brain Injury (TBI) is a leading cause of death and disability in adults worldwide, and the number one cause of death among traumatic injuries (Bruns and Hauser 2003, Gao and Zheng 2015); global estimates suggest TBI leads to the hospitalization and mortality of up to 10 million people per year (Hyder, Wunderlich et al. 2007). Spanning a range of injury severities, the incidence of severe TBI in Canada is 11.4/100 000 (Zygun, Laupland et al. 2005), and approximately 500/100 000 for mild injuries (Ryu, Feinstein et al. 2009), with an 8% overall mortality rate (Tator, Bray et al. 2007, Fu, Jing et al. 2015). Hospitalization is significantly higher in males than females, and children and adults under the age of 40 account for almost 70% of emergency department and urgent care centre visits (Tator, Bray et al. 2007). However, recent Canadian statistics suggest an increasing trend in the hospitalization of severe TBI patients, and TBI in elderly patients (Fu, Jing et al. 2015, Fu, Jing et al. 2016). Similar to other parts of the developed world, the most common causes of TBI in Canada are falls and motor vehicle collisions (MVCs). Notably, the rate of injury due to MVC’s is decreasing, and the rate due to falls is increasing (Fu, Jing et al. 2015).

TBI is not only a problem in civilian populations but is also recognized as the “signature injury” among combat deployed military personnel (McCrea, Pliskin et al. 2008, Elder 2015). Prevalence ranges from approximately 4 – 23%, and has been reported at 5.2% in Canadian troops (Garber, Rusu et al. 2014). Unique to this population is the prevalence of blast-related
TBI, which is the primary mechanism in at least half of all reported TBI’s (Garber, Rusu et al. 2014). Furthermore, concussion is now recognized as a predominant concern among sport-related injuries – particularly those involving contact/collision (Collins, Grindel et al. 1999, Collins, Lovell et al. 1999, Bleiberg, Cernich et al. 2004, Aarli, Dua et al. 2006).

The total direct-cost associated with head injury is in excess of $150 million annually nation-wide (Tator, Bray et al. 2007), making TBI both a substantial health and economic burden. Yet, significant heterogeneity in both primary injury mechanisms and secondary injury sequelae have made characterizing and elucidating TBI pathophysiology difficult, resulting in impediments to clinical care and therapy (Saatman, Duhaime et al. 2008). Hence, there are currently no effective treatments for TBI, and a 100% failure rate in clinical trials for treatments in the acute stage of injury (Stein, Geddes et al. 2015). In order to impact therapy, solidify diagnosis and classification, and ultimately improve patient outcome, a better understanding of TBI pathophysiology in humans is needed.

1.1.2 Definition and diagnosis

While there is no clear universal definition, TBI results from an external force causing variable alterations in brain function and/or pathophysiology, with a wide range of severities (Menon, Schwab et al. 2010, Stein, Geddes et al. 2015). Severe injury may result in altered levels of consciousness, seizure, coma, edema and various neurological deficits, while mild TBI (mTBI) may only manifest in minor neuropsychological and behavioral alterations with no gross structural injury (Bruns and Hauser 2003). The vast majority (70-90%) of injuries are considered mild, although severe TBI is fatal in 30-50% of cases, with rates increasing in Canada (Hyder, Wunderlich et al. 2007, Fu, Jing et al. 2015). Survivors of severe TBI commonly suffer lifelong disability (Zygun, Laupland et al. 2005, Turgeon and Lauzier 2012, Roozenbeek, Maas et al.
2013), and it is now appreciated that the chronic effects of even mTBI can substantially impact health and quality of life, and may be related to the development of neurodegenerative disorders (Omalu, Bailes et al. 2011, Fakhran, Yaeger et al. 2013, Lee, Hou et al. 2013, Barrio, Small et al. 2015, Mendez, Paholpak et al. 2015).

Diagnostic criteria for TBI vary from country to country and within different hospitals/clinical institutions. According to the World Health Organization, initial classification of TBI made upon hospital arrival is based on the patients’ level of consciousness; an unconscious patient is deemed to have a severe TBI, while a conscious patient is classified as having a “mild” injury (Aarli, Dua et al. 2006). This initial stratification is overly simplistic, requiring additional classification. Hence, the Glasgow Coma Scale (GCS) has become the most commonly used injury severity classification scale (Maas, Stocchetti et al. 2008). The GCS was originally developed to assess alterations in consciousness using three variables: motor responsiveness, verbal performance, and eye opening (Teasdale and Jennett 1974). A GCS of 3-8 indicates a severe TBI, 9-12 is considered moderate, and 13-15 is classified as mild (Teasdale and Jennett 1974, Aarli, Dua et al. 2006). Despite its universal acceptance, the GCS has been widely criticized due to concerns in reliability and interpretation, and a noted variance in results depending on the user and time of assessment (Green 2011). Furthermore, GCS is compromised in the acute classification of severe TBI due to the commonplace use of sedation, intubation and ventilation in the ambulatory and hospital setting (Maas, Hukkelhoven et al. 2005). The sensitivity of the GCS is particularly troublesome in mTBI where patients may have varied symptomology, yet present with the highest possible GCS of 15 (Ruff, Iverson et al. 2009). However, in patients diagnosed in the hospital setting, the GCS is not typically used in isolation, but rather in conjunction with a computerized tomography (CT) scan to reveal possible morphological injuries due to bleeding, bruising and swelling (Aarli, Dua et al. 2006). These
scans also aid in differentiating between focal and diffuse injuries, and are evaluated using one or more of a variety of classifications indices, with the Marshall CT classification system being the most widely implemented, and the Rotterdam score among the most recent - and hence still undergoing validation (Marshall, Marshall et al. 1992, Maas, Hukkelhoven et al. 2005, Saatman, Duhaime et al. 2008). To date, the CT scan remains the diagnostic method of choice in the acute care setting (Maas, Hukkelhoven et al. 2005).

Defining and diagnosing mild TBI is a large source of disagreement among both clinicians and scientists (Di Battista, Rhind et al. 2013). While mTBI may exist on the same spectrum as severe injury, its clinical presentation is often harder to identify and characterize. Confusion also exists regarding nomenclature, as mTBI is often used interchangeably with the term “concussion”. There is considerable dispute regarding the synonymy of these terms, as some feel they are distinct clinical entities, while others believe they are interchangeable terms (Di Battista, Rhind et al. 2013, Sharp and Jenkins 2015). The 2012 Zurich consensus statement on concussion in sport recommends a separation between concussion and mTBI, with the distinction being that concussion presents with no abnormalities on imaging (McCrory, Meeuwisse et al. 2013, Sharp and Jenkins 2015); in contrast, the American Academy of Neurology does not acknowledge this difference (Giza, Kutcher et al. 2013, Sharp and Jenkins 2015). An agreement on classification is clearly needed to help unify and standardize diagnostic and therapeutic strategies.

1.1.3 Prognosis

Early outcome prediction after TBI is highly valued for intensivists, neurosurgeons and neurologists (Gao and Zheng 2015), as it can help guide current treatment options as well as experimental therapeutic strategies (Roozenbeek, Lingsma et al. 2012, Gao and Wu 2015).
Clinicians prognosticate using several pre- and peri-injury clinical characteristics and demographic information (age, injury severity, extracranial complications, sex, pre-existing co-morbidities), alongside brain imaging. In recent years, the International Mission for Prognosis and Clinical Trial Design (IMPACT) (Maas, Marmarou et al. 2007, Marmarou, Lu et al. 2007) and Corticosteroid Randomization After Significant Head Injury (CRASH) (Collaborators, Perel et al. 2008) clinical trial, have collectively provided the best evidence regarding early outcome prediction after TBI. These groups have identified simple prognostic models based on readily available clinical characteristics that accurately predict patient outcome at 6-months post-injury according to the Glasgow Outcome Scale (GOS), and have been externally validated in numerous follow-up studies (Roozenbeek, Chiu et al. 2012, Roozenbeek, Lingsma et al. 2012, Lingsma, Andriessen et al. 2013, Gomez, de-la-Cruz et al. 2014). Findings from the IMPACT analysis showed that within the first few hours after hospital admission, age, the motor score component from GCS, and pupillary reactivity accurately predict patient outcome at 6 months (Steyerberg, Mushkudiani et al. 2008); as expected, increased age, lower motor component GCS score, and decreased pupil reactivity portended an unfavorable outcome (Steyerberg, Mushkudiani et al. 2008). Furthermore, outcome prediction improved upon the addition of secondary insults (hypoxia, hypotension) and CT scan characteristics (Steyerberg, Mushkudiani et al. 2008). Finally, the most accurate outcome prediction model included these indices, as well as blood glucose and hemoglobin (Steyerberg, Mushkudiani et al. 2008). Outcome predictors identified by CRASH were similar, although this trial included extracranial injury in its core model, and also evaluated mTBI (GCS 13-15) (Collaborators, Perel et al. 2008). These models are not without limitations; both CRASH and IMPACT results were subsequently found to overestimate mortality and unfavorable outcome in TBI patients treated with intracranial pressure (ICP)-targeted therapy (Olivecrona and Koskinen 2012, Olivecrona and Olivecrona...
2013). Hence, even with the use of multi-predictor models acquired through large, externally validated studies, early prognostication will remain imperfect until a complete pathophysiological understanding of early secondary injury mechanisms is elucidated.

The GOS is the most widely used outcome classification tool in TBI (Jennett and Bond 1975, Wilson, Pettigrew et al. 1998, King, Carlier et al. 2005). Introduced by Jennet and Bond in 1975, the GOS was intended as a global metric to categorize grouped patient outcome (Jennett and Bond 1975). However, it was often adopted as an individual assessment tool, and has been criticized for its broadly defined categories, limited interrater reliability, and focus on physical impairments (Jennett and Bond 1975, Pettigrew, Wilson et al. 1998, Levin, Boake et al. 2001). It has even been suggested that the inability of the GOS to detect small but relevant treatment effects may be a contributing factor to the numerous failed phase III clinical trials (Weir, Steyerberg et al. 2012). In 1981, Jennett introduced the extended GOS (GOSE), which included further categorical stratification for conscious survivors (Jennett, Snoek et al. 1981). There is some evidence that the GOSE may provide increased prognostic power as compared to the GOS (Weir, Steyerberg et al. 2012), although both scales have been validated as generally accurate prognostic indices for TBI patients (Wilson, Pettigrew et al. 1998, Nissen, Jones et al. 1999, Levin, Boake et al. 2001), and have particularly benefited from the implementation of a structured interview for administration (Pettigrew, Wilson et al. 1998, Wilson, Pettigrew et al. 1998).

Early indicators of prognosis in mTBI and concussion are less clear, and are often outside the scope of the GOS for classification. Available evidence suggests younger age, a history of previous concussion, and number/duration of initial symptoms, are correlated with prolonged recovery after sport concussion (Cancelliere, Hincapie et al. 2014). In a non-sport mTBI
population, high pre-injury educational level, low levels of post-traumatic stress, no acute nausea/vomiting, extracranial injuries and pain early after injury have been associated with a 90% chance of full return-to-work (Stulemeijer, van der Werf et al. 2008); conversely, prolonged symptomology has been associated with pre-injury mental disorders, life stressors, and female sex (Hadanny and Efrati 2016). An important distinction between prognosis in moderate-to-severe TBI and mTBI/concussion is that the latter has no current externally validated clinical model, but rather attempts to predict the length of time the patient is symptomatic or is unable to return to life activities such as sport participation and work. Prolonged symptomology is generally classified as post-concussion syndrome (PCS), which in itself is a constellation of relatively non-specific symptoms within the domains of cognition (memory, attention, concentration), somatic issues (headache, dizziness, light and noise sensitivity, insomnia, etc.), and behavioural/psychological disturbances (depression, irritability, anxiety) (Hadanny and Efrati 2016). PCS is poorly understood, and is a common source of disagreement and inconsistency within the literature (Carroll, Cassidy et al. 2004, McMahon, Hricik et al. 2014). In addition, PCS symptoms have been identified in non-brain injured patients (Meares, Shores et al. 2008), and while it has been shown that the majority of mTBI patients (80-90%) recover within 7-10 days (McCrory, Meeuwisse et al. 2013), others have found symptom persistence in anywhere from 40 – 80% of subjects at 12-months post-injury (Roe, Sveen et al. 2009, McMahon, Hricik et al. 2014). To further complicate matters, recent evidence from studies assessing professional athletes have shown that concussions, and even sub-concussive impacts, may contribute to the etiology of neurodegenerative diseases including chronic traumatic encephalopathy (CTE) and dementia (Omalu, Bailes et al. 2011, Lee, Hou et al. 2013, Small, Kepe et al. 2013, Barrio, Small et al. 2015). Taken together, a better understanding of the underlying pathophysiology across the full spectrum of TBI is needed to elucidate the
contributions of pre-injury factors, primary injury mechanisms, and biological secondary injury manifestations, to long-term patient outcome.

1.2 TBI pathophysiology

TBI is categorized into two components, the mechanical external forces acting on the brain causing the initial insult, termed “primary injury”, and the secondary physiological and biomolecular manifestations that follow, termed “secondary injury” (Greve and Zink 2009). Secondary injury begins and predominates from the moment of impact, and unlike primary injury, is seemingly amenable to treatment. Hence, it is on the latter that clinicians and scientists have focused their therapeutic strategies and research efforts. Unfortunately, the complexity and diversity of the numerous secondary complications after injury have proven difficult to elucidate, resulting in a lack of efficacious therapeutic options.

1.2.1 Primary injury

The external forces causing primary injury fall into numerous mechanistic classifications, including rapid acceleration/deceleration, direct impact, and blast wave-induced cerebral pressure disturbances (Maas, Stocchetti et al. 2008). Beyond this initial classification, TBI is pathoanatomically classified as “closed” or “penetrating”, and spatially categorized as “focal” or “diffuse” (Maas, Steyerberg et al. 2007, Maas, Stocchetti et al. 2008). There is considerable overlap between the initial mechanism and the subsequent pathoanatomic injury type; most focal injuries result from direct impact, while acceleration/deceleration is often associated with diffuse injury (Saatman, Duhaime et al. 2008). Typically, focal injuries include primary vascular trauma causing bleeding on the surface of the brain, within the brain, or in the cortical grey matter (i.e., contusion/hematoma, laceration, and hemorrhage); these are normally diagnosed through
imaging (Werner and Engelhard 2007, Meaney, Morrison et al. 2014), and are not typical in mTBI (Werner and Engelhard 2007, Meaney, Morrison et al. 2014). Conversely, diffuse injury classically encompasses swelling and diffuse axonal injury (DAI), the latter of which is the most commonly observed in mTBI (Werner and Engelhard 2007). It is important to note that beyond the macroscopic injuries resulting from primary injury, microscopic injuries also occur, and include microporation of membranes, ion channel disruption, conformational changes in proteins, and general tissue microtrauma to vessels and the brain parenchyma (Sheriff and Hinson 2015).

1.2.2 Secondary injury - CNS

Secondary injury is initiated at the moment of injury (Werner and Engelhard 2007) and subsequently develops over the following hours and days (Maas, Stocchetti et al. 2008). While these processes are initiated to instigate repair, they often contribute to further brain dysfunction, swelling and ischemia (Maas, Stocchetti et al. 2008, Saatman, Duhaime et al. 2008, Greve and Zink 2009). It has been estimated that up to 90% of patients who die as a result of TBI display ischemic cell changes, and hypotension and hypoxia are present in more than a third of patients (Greve and Zink 2009). Secondary injury processes include central nervous system (CNS) excitotoxicity, disruption of neurotransmitter release, free radical generation, calcium-mediated damage, mitochondrial dysfunction, blood brain barrier (BBB) disruption, neurovascular injury and inflammation (Werner and Engelhard 2007, Maas, Stocchetti et al. 2008).

1.2.2.1 Neurometabolic disruption

Mechanical trauma to the brain parenchyma leads to a cascade of neurometabolic events that propagate further tissue injury. The obvious limitation of accessing human brain tissue in
living persons has resulted in the extensive reliance upon experimental animal model research to further our mechanistic understanding of these processes. These works have generated hypotheses regarding the events proceeding mechanical injury that not only contribute to pathological cellular and molecular signaling sequelae, but manifest in tangible clinical symptomology - and are accumulating supportive evidence in humans (Bergsneider, Hovda et al. 1997, Bergsneider, Hovda et al. 2000, Giza and Hovda 2014, Jalloh, Carpenter et al. 2015, Jalloh, Carpenter et al. 2015, Barkhoudarian, Hovda et al. 2016).

Upon injury, mechanical trauma to neuronal tissue, particularly as a result of shearing forces, causes microporation of the lipid cellular membranes. This has the dual consequence of allowing glutamate release from damaged cells, as well as propagating an ion flux; potassium ($\text{K}^+$) leaves the cell, and calcium ($\text{Ca}^{2+}$) enters (Takahashi, Manaka et al. 1981, Katayama, Becker et al. 1990). Once released, glutamate binding to activated $\text{N}$-methyl-$\text{D}$-aspartate (NMDA) and $\text{D}$-amino-$3$-hydroxy-$5$-methyl-$4$-isoxazolepropionic acid (AMPA) receptors serves to depolarize and trigger ion channels, often referred to as “spreading depression” (Goforth, Ellis et al. 1999, Spaethling, Le et al. 2012, Giza and Hovda 2014, Seifert and Shipman 2015).

Activation of these energy-demanding channels quickly depletes intracellular adenosine triphosphate (ATP) stores, forcing the cell to fulfill its energy requirements through glycolysis (Bergsneider, Hovda et al. 1997, Xing, Hua et al. 2009, Carpenter, Jalloh et al. 2015, Jalloh, Carpenter et al. 2015). To make matters worse, this hypometabolic state is compounded by the acute hypoperfusion and subsequent hypoxia common in the acute period after TBI (Bouma, Muizelaar et al. 1991, Menzel, Doppenberg et al. 1999, Longhi, Pagan et al. 2007, Park, Bell et al. 2008, Veenith, Carter et al. 2016). In addition, intracellular $\text{Ca}^{2+}$ is sequestered in the mitochondria in an attempt to rid it from the intracellular space (Giza and Hovda 2014, Seifert and Shipman 2015), resulting in mitochondria dysregulation and altered oxidative metabolism.
(Lifshitz et al. 2004; Sullivan et al. 1999); intracellular redox is affected, leading to the
only do increases in intracellular Ca\(^{2+}\) cause dysfunction in the mitochondria, but they also
disrupt protein phosphorylation, microtubule formation and enzyme function (Greve and Zink
2009). In axons in particular, intracellular Ca\(^{2+}\) can cause proteolytic damage to subaxolemmal
spectrin and cytoskeletal proteins (Buki and Povlishock 2006). Finally, mitochondrial
dysfunction can contribute to cerebral edema through increased lactate production
(Barkhoudarian, Hovda et al. 2016).

Interestingly, these processes have been associated with specific clinical symptomology;
axonal dysfunction has been correlated to slowed cognition, and altered neurotransmission may
be related to both slowed cognition and reaction time (Giza and Hovda 2014). Of particular
importance to return-to-play guidelines after sport-related concussion (SRC), the energy crisis
may represent a time of increased vulnerability to subsequent injury (approx. 10 days), impacting
the severity of post-concussive symptomology (Giza and Hovda 2014). Hence, the culmination
of the acute neurometabolic response to TBI is potential widespread neuronal
damage/dysfunction that is secondary to the initial mechanical trauma, carrying numerous
possible clinical implications.

1.2.2.2 DAI

Over the last 70 years, much attention has been paid to the trauma-related damage of
white matter axons. Initially observed after severe head injury, some patients displayed
prolonged loss of consciousness and profound morbidity in the absence of macroscopic lesions
(Rand and Courville 1946, Johnson, Stewart et al. 2013, Smith, Hicks et al. 2013). Microscopic
histological staining uncovered “bulbing” or swelling concentrated around midline white matter
tracts, such as the brainstem and corpus callosum (Rand and Courville 1946, Nevin 1967, Gentleman 1996, Johnson, Stewart et al. 2013, Smith, Hicks et al. 2013). In the following years, technological advances in immunohistochemical analysis led to the identification and characterization of what we now know as DAI, but is more accurately depicted as multi-focal axonal dysfunction throughout the brain parenchyma.

DAI is among the most common secondary manifestations in all TBI severities, and is notably present in up to 50% of severe TBI patients (Meythaler, Peduzzi et al. 2001). It typically occurs as a result of rotational shearing forces caused by rapid acceleration/deceleration of the brain (Gennarelli, Thibault et al. 1982), and also encompasses the secondary progressive biochemical injuries that ensue. While it was originally thought that severe TBI caused complete axonal disconnection, it is now believed that “primary axotomy” is a rare occurrence; DAI likely presents as an initial axonal disruption that progresses to disconnection and Wallerian degeneration, with unmyelinated axons being particularly susceptible (Tang-Schomer, Patel et al. 2010, Johnson, Stewart et al. 2013, Smith, Hicks et al. 2013). This ultimately renders the neuron incapable of communication with neighboring neurons, hence patients with DAI often have poor outcomes (Maas, Stocchetti et al. 2008).

Axons are particularly susceptible to injury due to their viscoelastic structure and anisotropic arrangement (Gennarelli, Thibault et al. 1982, Johnson, Stewart et al. 2013). In addition, while the elasticity of axons allows substantial deformation without structural damage, when mechanical force is applied rapidly, axons become brittle and more susceptible to damage (Gennarelli, Thibault et al. 1982, Johnson, Stewart et al. 2013). Initial damage quickly transitions into a progressive secondary axotomy (Christman, Grady et al. 1994), as microtubule disassembly occurs at breakage points to facilitate gradual relaxation and recovery of the axon.
(Smith, Meaney et al. 2003, Johnson, Stewart et al. 2013). However, this comes at the cost of impeding axonal transport, resulting in the accumulation of transport proteins such as amyloid precursor protein (APP) (Tang-Schomer, Patel et al. 2010, Tang-Schomer, Johnson et al. 2012). Accumulating proteins can then be visualized as “axonal varicosities”, with larger accumulations known as “axonal bulbs” (Johnson, Stewart et al. 2013). This process of axonal swelling is thought to occur within 2 hours of injury (Gentleman, Roberts et al. 1995), subsequently manifesting into continual swelling, and finally progressing towards degeneration (Johnson, Stewart et al. 2013). Additionally, recent evidence suggests that DAI may continue for years after TBI (Johnson, Stewart et al. 2013), with the accumulation of proteins evolving into plaques and contributing to neurodegenerative disorders such as Alzheimer’s disease (AD) and CTE (Tran, LaFerla et al. 2011, Tran, Sanchez et al. 2011).

In recent years, advanced imaging techniques such as diffusion tensor imaging (DTI) have been employed to help characterize both the acute and long term sequelae of DAI in humans (Smith, Hicks et al. 2013). Furthermore, experimental research has uncovered a number of potential therapeutic strategies including cytoskeleton stabilization, ion homeostasis, protease inhibition, mitochondria protection and free radical scavenging (Smith, Hicks et al. 2013). However, due to its complexity, future research efforts aimed at furthering our pathophysiological understanding of DAI are still needed.

1.2.2.3 Neuroinflammation

The local inflammatory response to TBI plays a crucial part in facilitating secondary injury, but is paradoxically necessary for tissue repair and regeneration (Finnie 2013). While animal model research has provided the foundation for our understanding of neuroinflammation both acutely and chronically after TBI (Chiu, Liao et al. 2016), human studies using post-mortem
brain tissue (Johnson, Stewart et al. 2013, Smith, Gentleman et al. 2013), biomarkers in fluids proximal to the brain such as parenchymal microdialysate and cerebral spinal fluid (CSF) (Kossmann, Stahel et al. 1997, Morganti-Kossmann, Hans et al. 1999, Helmy, Carpenter et al. 2007, Helmy, Carpenter et al. 2011), and neuroimaging (Albrecht, Granziera et al. 2016), have been crucial in translating experimental findings to an improved clinical understanding of these processes.

Indeed, common to the inflammatory response to any tissue injury, the primary purpose of the neuroinflammatory response to TBI is to bring plasma and its components to the site of injury, with the goal of tissue repair (Medzhitov 2008). Experimental and subclinical evidence has shown that primary injury stimulates a local inflammatory response to both vascular and parenchymal damage, involving at least three processes; 1) The activation of the contact/plasma kallikrein-kinin system, 2) the inflammatory actions of damage/danger associated molecular patterns (DAMPs) on resident CNS immune cells, and 3) the recruitment of peripheral leukocytes to the injured brain (Albert-Weissenberger, Mencl et al. 2014, Albert-Weissenberger, Mencl et al. 2014, Plesnila 2016). While this response may be both reparative and toxic in the acute phase, it is becoming increasingly evident that prolonged inflammation after TBI is primarily maladaptive, and may lead to neurodegeneration (Blaylock and Maroon 2011, Heneka, Carson et al. 2015).

Within minutes of injury, damage to the cerebrovasculature exposes the plasma to negatively charged surface molecules (Albert-Weissenberger, Mencl et al. 2014). A series of proteolytic events are then initiated that result in vasodilation, platelet activation, coagulation and the release of bradykinin (Wu 2015). The components comprising this response (coagulation factors XII and XI, prekallikrein, and high molecular weight kininogen) are referred to as the
Contact or plasma kallikrein-kinin system (Albert-Weissenberger, Mencl et al. 2014, Wu 2015, Plesnila 2016). As a consequence of contact activation, the BBB may become “leaky”, facilitating the recruitment of peripheral leukocytes to the brain (Albert-Weissenberger, Mencl et al. 2014). In addition, both the proinflammatory effects of bradykinin and the coagulopathic sequelae of injury contribute to altered cerebral blood flow, edema and further tissue damage (Langhauser, Göb et al. 2012, Albert-Weissenberger, Mencl et al. 2014).

Contact activation in response to parenchymal tissue injury is quickly followed by inflammation. Cell-fragments and intracellular molecules such as s100 calcium binding protein (s100)-B, high mobility group box (HMGB)-1 protein and ATP, are released into the extracellular space as a result of mechanical damage to structures including neuronal bodies and axons (Davalos, Grutzendler et al. 2005, Thundyil and Lim 2015). These DAMP molecules then initiate inflammatory signaling through pattern recognition receptors (PRRs) - primarily Toll-like receptors (TLRs) - located on the surface membranes of most CNS cells (Matzinger 1994, Kigerl, de Rivero Vaccari et al. 2014, Thundyil and Lim 2015, DiSabato, Quan et al. 2016). Accumulating evidence suggests the initial DAMP-mediated neuroinflammatory response to injury is predominantly facilitated by microglial cells (Hu, Leak et al. 2015).

Microglia are the most abundant resident immunological cell in the CNS, sharing functional lineage homology to tissue macrophages (Perry and Teeling 2013). They serve many physiological roles in the CNS, including the ridding of toxic substances, synaptic pruning, and immune surveillance (Nimmerjahn, Kirchhoff et al. 2005). In response to trauma, within hours microglia shift from their surveying, ramified state, to an activated one via ligand-receptor interactions with DAMP molecules (Hanisch and Kettenmann 2007, Kumar and Loane 2012). Activation encompasses several beneficial processes such as the removal of glutamate,
neurogenesis, angiogenesis, and axonal remodeling, but also mediates potentially damaging actions including the production of cytokines, chemokines, reactive oxygen species (ROS), and the physical isolation of injured areas of the brain from healthy tissue (Hanisch and Kettenmann 2007, Kumar and Loane 2012).

Microglial activation is complex, in part, due to its dimorphic activated states, termed “M1” and “M2”; M1 microglia are generally considered pro-inflammatory and detrimental to the brain parenchyma chronically, while M2 microglia are considered anti-inflammatory and reparative (Kumar and Loane 2012, Hu, Leak et al. 2015). This is likely an oversimplification, as numerous sub-phenotypes of both M1 and M2 microglia have been identified (Lynch 2009), and evidence exists suggesting the M1 phenotype is not always damaging, and may help with the early clearance of cell debris (Kim, Kim et al. 2009, Hu, Leak et al. 2015). Acutely after TBI, microglia recruited to the site of injury display an M2 phenotype, although prolonged activation (~ 1 week) results in a switch towards an M1 phenotype, primarily in white matter (Perego, Fumagalli et al. 2011, Hu, Li et al. 2012, Wang, Zhang et al. 2013). However, it has also been suggested that concurrent recruitment of numerous, overlapping microglial phenotypes to the injury site occurs post-injury (Morganti, Riparip et al. 2016).

Among its many effects, microglia activation and the resultant release of inflammatory mediators is responsible for initiating and sustaining the activation of astrocytes (Chiu, Liao et al. 2016). Astrocytic activation comprises cell proliferation and hypertrophy, and an increase in intermediate filaments including glial fibrillary acidic protein (GFAP) (Kumar and Loane 2012). Again, astrocyte activation results not only in reparative processes via the release of factors such as brain-derived neurotrophic factor (BDNF), but can be detrimental through the formation of “glial scars”. Glial scarring occurs as hypertrophic astrocytes surround brain lesions, and in an
attempt at creating an extracellular matrix, create a physical and chemical barrier inhibiting axonal regeneration (Wilhelmsson, Li et al. 2004, Cafferty, Yang et al. 2007, Zhang, Hu et al. 2010, Kumar and Loane 2012, Kou and VandeVord 2014). As a result, connections to neuronal networks both proximal and distal to the original insult are impaired, possibly leading to cognitive deficits (Pearn, Niesman et al. 2016). However, to add further complexity, it has been suggested that glial scarring, like inflammation itself, is a necessary process facilitating axon repair, and its detrimental consequences may be due to insufficient or excessive scarring beyond reparation (Rolls, Shechter et al. 2009). Taken together, it is evident that microglial activation is a central facilitator of post-TBI neuroinflammation, although its specific pathophysiology remains elusive.

The neuroinflammatory response to TBI is not only reserved for resident CNS cells; peripheral leukocytes can infiltrate the brain and contribute to neuroinflammation (Perry and Teeling 2013). Cytokines, chemokines, and DAMP molecules released from the CNS may facilitate the recruitment of peripheral leukocytes to the injured site across a BBB that has been disrupted through mechanical trauma, the production of inflammatory mediators and/or ROS, or across areas of the CNS without BBB protection such as the circumventricular organs (CVO) (Maness, Kastin et al. 1998, Chodobski, Zink et al. 2011, Pittman and Kubes 2013). However, it is locally produced chemokines from damaged neurons, microglia and astrocytes, along with upregulated adhesion molecules on the cerebrovascular endothelium, that primarily facilitate the extravasation of peripheral leukocytes into the CNS (Chodobski, Zink et al. 2011). Experimental findings have identified neutrophils as the first responding peripheral immune cells to migrate towards the brain after TBI (Gyoneva and Ransohoff 2015), initially accumulating in the cerebrovasculature and subarachnoid space, entering the brain around 1 day after injury (Gyoneva and Ransohoff 2015). At 3-5 days, neutrophil accumulation typically subsides and
monocytes become the predominant peripherally-recruited cell in the brain, where they become indistinguishable from locally produced microglia (Gyoneva and Ransohoff 2015). Chemokine ligand (CCL)-2, otherwise known monocyte chemoattractant protein (MCP)-1, is among the most influential mediators aiding in monocyte recruitment (Semple, Bye et al. 2010, Semple, Frugier et al. 2010), while interleukin (IL)-8, otherwise known as chemokine ligand (CXCL)-8, preferentially recruits neutrophils (Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005) (Semple, Bye et al. 2010). Yet, the effect of peripheral leukocyte recruitment to the brain is unclear; experimental studies using genetic knockouts to modify chemokine signaling have found a general reduction in neuronal damage, although it is unclear if ameliorating leukocyte recruitment in the acute phase has any effect on recovery (Semple, Bye et al. 2010, Semple, Frugier et al. 2010) (Plesnila 2016). In humans, elevations in chemokines in the CSF after TBI have been associated with BBB damage (Kossmann, Stahel et al. 1997), and elevated blood concentrations have been associated with poor outcome (Rhodes, Sharkey et al. 2009); a causative relationship in humans has yet to be determined.

A growing body of evidence suggests chronic neuroinflammation after TBI is pathological and may mediate neurodegeneration. (Loane, Kumar et al. 2014, Gupta and Sen 2016). This is not only a concern for those having sustained a moderate or severe TBI, but possibly for those with repeated mTBI or concussive injuries, such as military members and collision-sport athletes (Blaylock and Maroon 2011, Omalu, Bailes et al. 2011, Stein, Alvarez et al. 2014). While the exact role of neuroinflammation in facilitating neurodegeneration is still unclear, Blaylock and Maroon (2011) have highlighted a potential mechanism based on the concept of immunoexcitotoxicity. Briefly, this principle rests on the synergistic, toxic effect of excitotoxins and activated microglia. Based on experimental findings that showed microglial activation and proinflammatory cytokine production cannot damage neurons without
concomitant glutamate release (Bal-Price and Brown 2001, Shijie, Takeuchi et al. 2009), it was hypothesized that chronically activated microglia may synergistically act with excitotoxins to facilitate neuronal degeneration. Specifically, inflammatory cytokine receptors, particularly tumor necrosis factor (TNF)-α receptors, aid AMPA receptor trafficking to the cell surface, rendering them sensitive to glutamate stimulation (Blaylock and Maroon 2011). At the same time, the release of TNF-α impairs glutamate reuptake, further contributing to this feed-forward cycle (Blaylock and Maroon 2011). In humans, evidence of microglial activation has been found for years after a single TBI (Johnson, Stewart et al. 2013), and both post-mortem and living, in-vivo examinations of athletes sustaining repetitive head injuries have shown evidence of CTE (Omalu, Bailes et al. 2011, Small, Kepe et al. 2013). However, specific evidence of immunoexcitotoxicity in humans, and its causal relationship to chronic neurodegeneration after TBI have yet to be identified.

1.2.2.4 Blood brain barrier disruption

The cerebrovasculature serves not only as an energy supply, but as a protective shield which guards the brain from pathogens and potentially harmful molecules found in the peripheral circulation (Chodobski, Zink et al. 2011). This interface, consisting of vascular and non-vascular parenchymal cells, is known as the BBB. TBI results in both mechanical and biochemical perturbations to the surrounding BBB, with far-reaching consequences including coagulopathy, the promulgation of neuroinflammation, and the facilitation of brain edema (Chodobski, Zink et al. 2011). While predominantly studied using animal models, BBB dysfunction is present and common in human TBI (Rodriguez-Baeza, Reina-de la Torre et al. 2003, Saw, Chamberlain et al. 2014).
The BBB consists of brain endothelial cells, tight junction proteins including claudins and occludins, astrocyte processes and pericytes (Abbott, Ronnback et al. 2006). Different from those in the peripheral vasculature, brain endothelial cells have an increased abundance of mitochondria, and lack both fenestrations and lipid-carrying caveolae (Prakash and Carmichael 2015). Under physiological conditions, the BBB serves as the gatekeeper to the brain, surveying the peripheral circulation, selectively allowing the passage of certain molecules including water and glucose via processes ranging from passive diffusion to active, channel-mediated transportation (Abbott, Ronnback et al. 2006, Chodobski, Zink et al. 2011, Alves 2014, Blanchette and Daneman 2015). After TBI, however, mechanical and functional alterations take place which alter the integrity and permeability of the BBB and contribute to secondary injury (Neuwelt, Abbott et al. 2008). In rat models of brain injury, BBB permeability displays a biphasic peak occurring early within 4-6 h and again at 2-3 days (Shapira, Setton et al. 1993, Baldwin, Fugaccia et al. 1996, Baskaya, Rao et al. 1997, Hicks, Baldwin et al. 1997); the temporal kinetics have yet to be elucidated in human TBI.

The mechanical shearing forces of TBI can damage the cerebrovasculature, and may specifically mediate BBB permeability through the disruption of tight junction complexes, the widening of intercellular spaces, changes to the luminal morphology within vessels, cellular swelling, and increased vascular permeability (Rodriguez-Baeza, Reina-de la Torre et al. 2003, Sangiorgi, De Benedictis et al. 2013). As previously mentioned, the first subsidiary event following injury to the cerebrovasculature is the rapid activation of the coagulation cascade, which serves to temporarily reduce cerebral blood flow (Schroder, Muizelaar et al. 1998, Chodobski, Zink et al. 2011). The subsequent generation of thrombin, and infiltration of proteins such as fibrinogen and albumin, collectively alter the activation status of surrounding astrocytes and microglia (Chodobski, Zink et al. 2011) (Nimmerjahn, Kirchhoff et al. 2005) (Abbott,
Ronnback et al. 2006). From this point onward, interactions between glial and vascular cells, and between glial cells, predominate the biological pathology underlying BBB dysfunction (Chodobski, Zink et al. 2011).

The activation of glial cells by infiltrating proteins such as thrombin, fibrinogen and albumin, results in the release of cytokines including IL-1β, TNF-α, and IL-6 (Rochfort and Cummins 2015). These cytokines are then able to increase BBB permeability through producing ROS, forming actin stress fibers and intercellular gaps on the endothelium, decreasing the expression of junctional proteins, and increasing the expression of adhesion molecules (Hess, Bhutwala et al. 1994, Stanimirovic, Shapiro et al. 1997, Stanimirovic, Wong et al. 1997). Additionally, transforming growth factor (TFG)-β may also increase BBB permeability through its effects on tight junction proteins (Chodobski, Zink et al. 2011). However, possibly the most influential role of cytokines on BBB permeability is their ability to increase chemokine production on astrocytes and the cerebrovascular endothelium, thus dramatically increasing the ability for peripheral leukocytes to enter the CNS (Williams, Holman et al. 2014) (Fuentes, Durham et al. 1995, Yao and Tsirka 2014). In addition, chemokines may directly increase BBB permeability through inducing the formation of actin stress fibers and redistribution of tight junction proteins on the cerebrovascular endothelium (Stamatovic, Shakui et al. 2005).

Beyond cytokines, glutamate, ROS and matrix metalloproteinases (MMPs) may all influence BBB after TBI (Chodobski, Zink et al. 2011). Indeed, oxidative stress causes peroxidation of membrane fatty acids on the BBB, and glutamate can facilitate ROS production through upregulating nitric oxide (NO) (Mayhan 1996, Mayhan and Didion 1996, Parfenova, Basuroy et al. 2006, Lau and Tymianski 2010). In addition, MMPs found in cerebrovascular endothelial cells, astrocytes, microglia and neurons, can disrupt BBB integrity through the
degradation of tight junction complexes (Rosenberg, Cunningham et al. 2001, Rosenberg, Sullivan et al. 2001, Abdul-Muneer, Pfister et al. 2015). Interestingly, invading neutrophils are able to damage the BBB through all 3 previously described processes; they secrete glutamate, MMP9, and can produce large amounts of ROS (DiStasi and Ley 2009).

While BBB dysregulation has not been the focus of concussion and mTBI research, there is indeed the possibility that milder forms of head injury may perturb the cerebral vasculature. Animal modelling of blast wave injury has shown disruption of tight junction proteins (Abdul-Muneer, Schuetz et al. 2013), and in humans BBB disruption has been identified in up to 40% of football players (Marchi, Bazarian et al. 2013, Weissberg, Veksler et al. 2014).

BBB dysregulation is a complex, multi-faceted component to secondary injury after TBI. Yet, common to all facets of secondary injury, BBB disruption may be important to tissue repair. While initial increases in permeability may be corollary to mechanical injury, the second, prolonged increase in BBB permeability may repair the neurovascular unit through neovascularization (Prakash and Carmichael 2015). Yet, the BBB remains a promising therapeutic target, as substantial evidence has shown that its disruption propagates pathological neuroinflammation and contributes to both vasogenic and cytotoxic edema (Rosenberg, Cunningham et al. 2001, Cunningham, Wetzel et al. 2005, Abbott, Ronnback et al. 2006, Chodobski, Zink et al. 2011, Alves 2014).

1.2.2.5 Cerebral edema

Cerebral edema is the pathological elevation in the fluid mass contained in the brains’ interstitial space (Stokum, Gerzanich et al. 2016). Accounting for up to 50% of mortality in TBI (Donkin and Vink 2010), edema is of great concern to clinicians as it perturbs ICP and cerebral
blood flow (CBF), and leads to ischemia (Stokum, Gerzanich et al. 2016). Edema is best understood in terms of its specific progressive phases: cytotoxic, ionic and vasogenic.

The neurometabolic disruption occurring resulting from mechanical trauma is the predominant force leading to the first phase of edema – cytotoxic edema (Simard, Kent et al. 2007, Stokum, Gerzanich et al. 2016). It is important to understand that cytotoxic edema is not “true” edema, in that it does not result in any tissue swelling; there is no net gain in fluid within the brain, but rather a rearrangement of the fluid already present causing the cells to swell (Simard, Kent et al. 2007, Stokum, Gerzanich et al. 2016). It is initiated by injury-related perturbations in ion transport that result in a pathological influx of Na$^+$ into the cells (Walcott, Kahle et al. 2012). This process is preferential to astrocytes and vascular endothelial cells, and occurs within minutes of injury (Stokum, Gerzanich et al. 2016).

Ionic edema then arises due to cytotoxic edema, as ion and water flux into the cells creates an absence of these molecules in the extracellular space. Higher Na$^+$ in the vascular versus interstitial compartments results in the formation of a gradient across the BBB (Simard, Kent et al. 2007). Hence, osmotic pressure leads to an influx of water and low-molecular ions into the parenchyma across an intact BBB, causing tissue swelling (Simard, Kent et al. 2007, Stokum, Gerzanich et al. 2016).

The third progression of edema is referred to as vasogenic edema, where hydrostatic and osmotic pressures allow plasma protein extravasation into the parenchyma across a disrupted BBB (Durward, Amacher et al. 1983, Durward, Del Maestro et al. 1983, Simard, Kent et al. 2007, Stokum, Gerzanich et al. 2016). Together, ionic and vasogenic edema are referred to as “transvascular” edema, as unlike cytotoxic edema, they manifest into brain tissue swelling due to fluid influx across the cerebrovasculature (Stokum, Gerzanich et al. 2016). Consequently,
parenchymal tissue pressure is elevated above the pressure of surrounding capillaries, leading to a decrease in CPP and subsequent ischemia. In a feed-forward process, ischemic damage may propagate further cytotoxic edema, reinitiating the cycle (Stokum, Gerzanich et al. 2016).

1.2.3 Secondary injury – Periphery

In addition to its effects in the CNS, secondary injury has detrimental consequences extending beyond the brain. This may occur due to the dysregulation of two, interrelated processes: 1) the sympathetic nervous system (SNS), and 2) the inflammatory response (Lu, Goh et al. 2009, Jaerve and Muller 2012, Schwulst, Trahanas et al. 2013, Hinson, Rowell et al. 2015). Perturbations to these systems after TBI result in three clinically relevant and commonly observed sequelae: 1) the facilitation of further CNS injury, 2) coagulopathy and endotheliopathy, and 3) the development of sepsis and organ failure. In view of this, non-neurologic organ dysfunction has been identified in up to 89% of severe TBI patients (Zygun, Kortbeek et al. 2005), coagulopathic abnormalities may be present in 10-97% of TBI patients (Harhangi, Kompanje et al. 2008), and sepsis has been found in up to 75% of severe TBI patients (Corral, Javierre et al. 2012).

1.2.3.1 SNS dysregulation

TBI causes an acute increase in SNS activity that is observable through large increases in circulating catecholamine levels (Hortnagl, Hammerle et al. 1980, Clifton, Ziegler et al. 1981, Hamill, Woolf et al. 1987). The pleiotropic effects of SNS dysregulation make it both a complex clinical concern and an attractive therapeutic target. Indeed, multiple clinical investigations have found that beta blocker therapy improves patient outcomes (Arbabi, Campion et al. 2007, Cotton, Snodgrass et al. 2007). While the majority of these studies were retrospective, recent prospective
studies have yielded similar, positive results (Ko, Harada et al. 2016, Murry, Hoang et al. 2016).

Yet, the downstream mechanistic actions of SNS dysregulation after isolated TBI, and its effects on the inflammatory and coagulopathic response, are undetermined.

**Figure 1.1 - Passage of biomarkers from the CNS to the peripheral blood after TBI.**

1) Immediately after TBI, damage to parenchymal structures such as neurons and astroglial cells results in the extracellular release of injury-related molecules including, but not limited to, s100 calcium binding protein B (s100B), glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE). This is accompanied by a local inflammatory response, which results in an upregulation of inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. 2) In the classical view, TBI causes damage/dysregulation to the blood brain barrier (BBB) through numerous processes such as mechanical shearing forces, disruption of tight junction complexes, widening of intercellular spaces, changes in luminal morphology within vessels, and injury-related reactive oxygen species (ROS) production. This increases BBB permeability and allows passage of brain-borne injury molecules and inflammatory mediators into the peripheral blood. 3) More recently, it has been suggested that CNS biomarkers are primarily transported into the periphery after TBI through the lymphatic system. In this view, the cerebral spinal fluid (CSF) travels through the brain parenchyma along periarterial spaces, interchanges with the interstitial fluid (ISF) via astrocytic aquaporin (AQP)-4 channels, and clears along the perivenule spaces. From here, collected waste products and potential injury biomarkers enter the blood through the perineural lymphatics or arachnoid granulations.
The biological stress system is composed of the SNS and hypothalamic pituitary adrenal (HPA) axis (Elenkov, Wilder et al. 2000). Its physiological role is to maintain homeostasis via the regulation of numerous physiological functions including heart rate, vascular tone, metabolism, temperature and immune function (Elenkov, Wilder et al. 2000). The SNS is broadly comprised of two central components, the corticotropin-releasing hormone (CRH) system, and the locus coeruleus (LC) – norepinephrine (NE) autonomic nervous system (ANS) (Elenkov, Wilder et al. 2000, Dunser and Hasibeder 2009). The CRH system is primarily located in the paraventricular nucleus (PVN) of the hypothalamus, while the LC-NE ANS is located in the brain stem (Elenkov, Wilder et al. 2000). During times of stress, in order to preserve tissue oxygenation and organ integrity, both systems are activated via a reverbatory feedback loop (Elenkov, Wilder et al. 2000). This occurs because the CRH system can activate the LC-NE ANS via projections running from the PVN to the hindbrain, and because catecholaminergic fibers from the LC-NE ANS reach the PVN (Elenkov, Wilder et al. 2000). Activation of the SNS triggers massive local and systemic catecholamine release; NE release occurs via activation of LC-NE ANS nerve terminals innervating the CNS, peripheral organs and vasculature, while activation of the CRH system results in ACTH mediated epinephrine (Epi) release from the adrenal medulla (Elenkov, Wilder et al. 2000). Notably, while some NE is secreted from the adrenal medulla, the largest source of peripheral NE during the stress response is the intestinal tract (40% of total systemic NE release), due to its rich sympathetic innervation (Lyte 2004, Dunser and Hasibeder 2009).

The stress response after TBI is dramatic, resulting in a massive catecholamine surge in the periphery (Hortnagl, Hammerle et al. 1980, Clifton, Ziegler et al. 1981, Hamill, Woolf et al. 1987, Woolf, Hamill et al. 1987). These increases are associated with poor outcome (Clifton, Ziegler et al. 1981, Hamill, Woolf et al. 1987), and correlate with elevations in blood pressure,
temperature and heart rate (Hortnagl, Hammerle et al. 1980, Clifton, Ziegler et al. 1981, Clifton, Robertson et al. 1983). While the mechanisms are still unclear, sympathetic activation and catecholamine discharge appear to be initiated by an increase in ICP (Masuda, Sato et al. 2002, Woiciechowsky and Volk 2005, Hinson and Sheth 2012). It is also possible that the local neroinflammatory response to TBI contributes to adrenergic activity, as cytokines IL-1β, -6, and TNF-α can stimulate both the SNS and HPA axis (Elenkov, Wilder et al. 2000, Tracey 2002).

While initial SNS activity may protect the BBB and benefit cerebral perfusion pressure (CPP) (Harik and McGunigal 1984, Steiner, Johnston et al. 2004), dramatic hyperactivation of the SNS in the systemic circulation appears to portend unfavorable outcome (Clifton, Ziegler et al. 1981, Hamill, Woolf et al. 1987, Woolf, Hamill et al. 1987). How this occurs is uncertain, although a number of possibilities exist, such as the potentially pathological influence of the SNS on immune function (Elenkov, Wilder et al. 2000), and its mediating role in endothelial injury and coagulopathy (Johansson and Ostrowski 2010, Ostrowski, Berg et al. 2013). However, the implications of SNS hyperactivity after isolated brain injury remain unclear.

Early studies in subarachnoid hemorrhage (SAH) and burn trauma observed a potential therapeutic benefit from β-blocker treatment. This suggests that modulating SNS activity may improve patient outcomes, possibly by reversing its associated systemic consequences such as arrhythmia, pulmonary edema, end organ dysfunction, and immunosuppression (Neil-Dwyer, Walter et al. 1978, Ballard-Croft and Horton 2002, Ballard-Croft, Maass et al. 2002). Due to the similarity in secondary injury sequelae, and the previously identified inverse relationship between catecholamine levels and patient outcome (Clifton, Ziegler et al. 1981, Hamill, Woolf et al. 1987), it follows that β-blocker therapy may have the same beneficial effect in TBI. In view of this, a number of retrospective studies have found that β-blocker treatment after TBI reduces
mortality due to secondary injury (Cotton, Snodgrass et al. 2007, Inaba, Teixeira et al. 2008, Salim, Hadjizacharia et al. 2008, Schroeppe1, Fischer et al. 2010). However, the mechanisms by which this occurs are currently unknown, particularly regarding their potentially protective effect on the brain and extracranial organs, their influence on hemostasis, and modulation of immune function. Furthermore, additional prospective treatment studies with early β-blocker administration are required. Indeed, recent preliminary work has provided optimistic results; Murry and colleagues found that early propranolol treatment after TBI did not cause bradycardia and hypotension (Murry, Hoang et al. 2016) and may be associated with improved outcome, while Ko and colleagues found early propranolol treatment significantly improved patient survival (Ko, Harada et al. 2016).

1.2.3.2 Systemic inflammation

Accumulating evidence within the last 30 years has shown that inflammatory mediators are elevated in the peripheral blood after TBI (Goodman, Robertson et al. 1990, Kossmann, Hans et al. 1996, Kossmann, Stahel et al. 1997, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014). Indeed, beyond facilitating both injury and repair processes in the CNS, peripheral inflammation is a common theme to a number of post-TBI sequelae, including coagulopathy, infection and peripheral organ dysfunction (Catania, Lonati et al. 2009, Lu, Goh et al. 2009, Anthony and Couch 2014). However, studies evaluating peripheral inflammatory indices have typically included a limited number of cytokines and chemokines, often without consideration of their pathophysiological implications. In addition, despite positive results from a phase II trial using the anti-inflammatory drug N-acetylcysteine in military soldiers with mild, blast-related injury (Hoffer, Balaban et al. 2013), only scant evidence exists evaluating the role

The source of inflammatory mediators found extracranially after TBI is unclear, although it is likely that there are multiple contributing factors. Experimental animal and human data has produced at least three possible mechanisms leading to an increase in systemic cytokine and chemokine concentrations acutely after TBI: 1) the SNS mediated acute phase response (APR); 2) interactions between circulating immune cells and catecholamines; and 3) passage from the CNS into the periphery (Kossmann, Hans et al. 1995, Woiciechowsky, Schoning et al. 1999, Catania, Lonati et al. 2009, Helmy, Carpenter et al. 2011).

The immediate neuroinflammatory response to brain injury results in elevated CNS concentrations of cytokines IL-1β and TNF-α (Woodroofe, Sarna et al. 1991, Arvin, Neville et al. 1995, Helmy, Carpenter et al. 2011, Helmy, De Simoni et al. 2011). Both IL-1β and TNF-α have been shown to increase the release of hepatic APR proteins via stimulation of noradrenergic nerve terminals (De Simoni, Sironi et al. 1990, Campbell, Perry et al. 2005). This results in elevated levels of several inflammatory mediators in the blood, including C-reactive protein (CRP), IL-6, -8 and TNF-α (De Simoni, Sironi et al. 1990, De Simoni and Imeri 1998, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005).

In addition to the APR, peripheral leukocytes have the ability to respond to circulating catecholamines due to the presence of adrenergic receptors expressed on their cell-surface membranes (Elenkov, Wilder et al. 2000). The nature of this interaction is unclear, and may be pro- or anti-inflammatory based on the catecholamine ligand, the adrenergic receptor subtype, and the leukocyte subpopulation (Uotila 1996, Elenkov, Wilder et al. 2000). This signaling may then result in cytokine/chemokine production from leukocytes via nuclear factor-kappa B (NF-
κB) translocation or cyclic adenosine monophosphate (AMP)-dependent processes (Uotila 1996, Bierhaus, Wolf et al. 2003). Indeed, evidence of this mechanism has been observed in human TBI; Woicieszowsky and colleagues found that acute elevations of IL-10 in the periphery after injury were derived from circulating monocytes via Epi signaling on leukocyte β2-adrenergic receptors (β2ARs) (Woiciechowsky, Asadullah et al. 1998).

The increased permeability of the BBB after TBI has led to the hypothesis that cytokines found in the periphery after injury have “leaked” out of the CNS (Kossmann, Hans et al. 1995, Kossmann, Stahel et al. 1997, Helmy, Carpenter et al. 2011). Evidence to support this comes from studies that have found a greater post-injury CSF:blood ratio of cytokines including IL-6 and -8 (Kossmann, Hans et al. 1995, Kossmann, Stahel et al. 1997). More recently, others have used the same approach, but instead of CSF, utilized brain extracellular fluid (Helmy, Carpenter et al. 2011). Similarly, in these studies, numerous inflammatory cytokines and chemokines were suggested to originate in the brain due to a higher extracellular fluid:plasma ratio (Helmy, Carpenter et al. 2011).

While it is plausible that BBB disruption may allow passage of inflammatory mediators from the CNS into the periphery, recent research has found that brain to blood biomarker gradients occur irrespective of the BBB, but rather as a result of clearance via the newly identified glympathic system (Iliff, Lee et al. 2013, Iliff, Chen et al. 2014, Plog, Pierre et al. 2014). Current evidence of this approach has been found using indices of neuronal and axonal damage such as s100B and GFAP (Plog, Pierre et al. 2014). However, future studies will be required to extend these new findings to human subjects and inflammatory mediators.

It is likely that the systemic inflammatory response after TBI is multi-faceted, and that elevated concentrations of cytokines and chemokines in the blood after injury occur as a
summation of numerous contributory processes. However, more experimentation is required to evaluate the consequence(s) of altered systemic inflammation after TBI, with specific reference to how these indices contribute to various secondary injury processes and affect patient health. Furthermore, research is needed to evaluate the systemic inflammatory response after mTBI and concussion, as this response has not yet been characterized.
Figure 1.2 – SNS-mediated systemic inflammation after TBI. In response to injury, the sympathetic nervous system (SNS) system is activated by an increase in intracranial pressure (ICP) and possibly through the local release of inflammatory cytokines. This can facilitate systemic inflammation in multiple ways. 1) Activation of the corticotropin-releasing hormone (CRH) system causes the pituitary gland to release adrenocorticotropic hormone (ACTH), which acts on the adrenal medulla to secrete epinephrine (Epi) into the blood. 2) Epi can then modulate the inflammatory response by interacting with beta2-adrenergic receptors ($\beta_2$-AR) on circulating leukocytes, leading to the production of inflammatory mediators such as interleukin (IL)-10. 3) Concurrent to CRH activity, the locus coeruleus (LC) - norepinephrine (NE) autonomic nervous system (ANS) is also activated. This leads to the stimulation of SNS efferent nerves, resulting in increased peripheral NE concentrations via diffusional clearance of NE from the synaptic gap and synaptic spillover. Once in the blood, NE can interact with circulating leukocytes via adrenergic receptors, and modulate inflammation. 4) Activation of SNS nerve terminals can also stimulate organs such as the liver to release chemokines, possibly through activation of resident macrophages and/or hepatic inflammatory transcription factors.
1.2.3.3 Coagulopathy and endotheliopathy

The regulation of hemostasis is a long-standing problem in trauma (Attar, Boyd et al. 1969, Laroche, Kutcher et al. 2012, Davenport and Brohi 2016, Yuan, Sun et al. 2016). While the incidence varies, when present, hemostatic perturbations consistently lead to poor outcome in TBI patients (Harhangi, Kompanje et al. 2008). The pathophysiological mechanisms are unclear, although there are at least three contributory processes facilitating the interrelated vascular and hemostatic perturbations observed after brain injury: 1) tissue factor (TF) release; 2) thrombin-mediated activation of protein C; and 3) SNS hyperactivity (Johansson and Ostrowski 2010, Wafaisade, Lefering et al. 2010, Cohen, Call et al. 2012, Laroche, Kutcher et al. 2012). Importantly, inflammation is a likely contributor to all theorized coagulopathy mechanisms.

Hemostasis is the physiological response to vascular trauma initiated to help arrest blood flow while preventing thrombosis, and involves the balance of prothrombotic, anticoagulant, and fibrinolytic processes (Schafer 2007). The initial steps after injury include vasoconstriction, the formation of a platelet plug, clot formation, and clot breakdown (Schafer 2007). Clot formation occurs via two pathways: 1) the extrinsic pathway, resulting from external trauma and involving TF and Factor VII, and 2) the intrinsic pathway, which is initiated by platelets and contact with blood activation surfaces such as the basal lamina (Schafer 2007, Halpern, Reilly et al. 2008). To finalize clot production, both pathways meet in what is known as the common pathway, involving factors I, II, V and X (Schafer 2007, Halpern, Reilly et al. 2008). The culmination of these events is a fibrin clot, created by the formation of fibrin from fibrinogen, via the actions of thrombin (Schafer 2007). Endogenous antithrombotic systems then take over to prevent excessive clot formation. This is achieved through a process referred to as fibrinolysis, which
acts primarily through plasmin to break down clots into various fibrin(ogen) breakdown products (FDPs) (Schafer 2007, Halpern, Reilly et al. 2008).

After trauma, vasoconstriction and platelet activation occur immediately alongside the TF-induced, extrinsic coagulation cascade (Gando, Wada et al. 2013, Dobson, Letson et al. 2015). This process is hypothetically no different in isolated TBI, as the brain is a rich source of the potent, pro-coagulant TF; damage to the cerebral vasculature and parenchymal cells such as astrocytes, results in the release of TF into the circulation (Goodnight, Kenoyer et al. 1974, Gando, Nanzaki et al. 1999, Stein and Smith 2004). However, it is unclear whether the initial hemostatic response after TBI results in a pro- or anti-coagulant phenotype, as evidence for both exists (Dobson, Letson et al. 2015). The initial response to injury may be pro-coagulant, leading to the consumption of clotting factors and evolving into a hyperfibrinolytic “bleeding” phenotype (Gando, Saitoh et al. 2008, Gando, Wada et al. 2012, Gando, Wada et al. 2013). However, others argue that tissue hypoxia immediately after injury diverts thrombomodulin from cleaving fibrinogen, and instead activates the highly anticoagulant, activated protein C (APC) pathway, resulting in pathological hypo-coagulation (Brohi, Singh et al. 2003, Cohen, Brohi et al. 2007, Cohen, Call et al. 2012). The complexity of the hemostatic response itself, semantic disagreements among experts, and the inability to clinically evaluate hypercoagulability, all contribute to our inability to understand these processes in TBI (Joseph, Aziz et al. 2014, Dobson, Letson et al. 2015).

Ostrowski, Johannason & colleagues have identified another potential contributor to pathological hemostasis in the fields of trauma and sepsis (Johansson and Ostrowski 2010). This group has suggested that SNS-mediated damage to the vascular endothelium after trauma directly influences fluid phase (blood) coagulopathy (Johansson and Ostrowski 2010).
Specifically, it has been postulated that vascular endothelial damage causes a procoagulant tissue response that is counterbalanced by hypocoagulation in the fluid phase (Johansson and Ostrowski 2010). Evidence for this has come from studies in extracranial trauma, TBI, and sepsis, which have identified correlations between circulating catecholamine levels and indices of glycocalyx and endothelial damage acutely after injury (Johansson, Stensballe et al. 2011, Johansson, Sorensen et al. 2012, Ostrowski and Johansson 2012, Genet, Johansson et al. 2013, Ostrowski, Berg et al. 2013, Johansson, Henriksen et al. 2016). While SNS-mediated endothelial damage activates the clotting cascade, these authors have proposed that a pro- to anti-coagulative change in phenotype occurs via the release of heparin-like molecules from the damaged glycocalyx (Ostrowski and Johansson 2012). It has subsequently been hypothesized that both extracranial trauma and TBI share this common mechanism (Genet, Johansson et al. 2013). However, a detailed evaluation of SNS mediated endothelial/glycocalyx damage and its downstream effects on coagulopathy has not been conducted in isolated TBI.

The aforementioned hypotheses of TBI-induced hemostatic perturbations are intimately connected to the systemic inflammatory response. Indeed, evidence supports the direct effect of inflammatory molecules on the propagation of coagulopathy and vice versa; specific components of the coagulation cascade can directly influence the production of inflammatory mediators (van der Poll 2001, Levi and van der Poll 2010, van der Poll, de Boer et al. 2011). Hence, communication between coagulation and inflammation is bidirectional. For example, the bolus injection of the pro-inflammatory cytokine TNF-α in human subjects induces a pro-coagulant response (van der Poll, Buller et al. 1990); conversely, the injection of the anti-inflammatory mediators IL-6 and IL-10 leads to an anti-coagulant response (van der Poll, Jansen et al. 1994, Stouthard, Levi et al. 1996). This process likely reflects the ability of cytokines to induce or inhibit TF production on endothelial and monocytic cells, and may also be influenced by the
effect of complement proteins on the clotting cascade (van der Poll 2001, van der Poll, de Boer et al. 2011). In addition, several coagulation proteases have the ability stimulate the production of inflammatory mediators. Thrombin can induce IL-6 and IL-8 production from both monocytes and endothelial cells, and both Factor Xa and fibrin can stimulate the production of IL-6 and IL-8 from monocytes (Levi and Opal 2006, Levi and van der Poll 2010). Notably, APC has been shown to inhibit inflammation on both the vascular endothelium and leukocytes via interactions with the endothelial protein C receptor (EPCR) and the protease activated receptor (PAR)-1 (Mosnier, Zlokovic et al. 2007).

Our current understanding of the relationship between coagulopathy and inflammation stems primarily from research in extracranial trauma and sepsis (van der Poll 2001, Levi and van der Poll 2010). While the presence of coagulopathy, endotheliopathy and inflammation has been discretely observed in isolated TBI patients, the pathophysiological mechanisms governing these processes remain elusive.
Figure 1.3 - Potential drivers of coagulopathy after TBI. 1) The traditional hypothesis of coagulopathy after TBI is predicated on the known abundance of tissue factor (TF) in the brain. After injury, TF is released from the CNS where it drives the external clotting cascade. This, in turn, causes widespread disseminated intravascular coagulation throughout the body, leading to the consumption of clotting factors, hyperfibrinolysis, and a resultant increase in bleeding. Inflammation may contribute to this process via circulating monocytes, which are capable of secreting TF via interactions with cytokines and vice versa; TF can cause monocytic cytokine production. 2) The activated protein C (APC) hypothesis posits that TBI + hypoperfusion results in the diversion of thrombin from cleaving fibrinogen towards the activation of an anticoagulant complex including thrombomodulin (TM) and protein C. The APC pathway inhibits thrombin generation by inactivating clotting factors Va and VIIIa, and promotes hyperfibrinolysis. This pathway also serves to inhibit inflammation on vascular endothelial cells and leukocytes through its actions with the endothelial protein C receptor (EPCR), and the protease activated receptor (PAR)-1. 3) Recent research has identified the sympathetic nervous system (SNS) as a potential driver of coagulopathy in TBI. In this hypothesis, it is suggested that the injury-induced SNS surge causes endothelial injury and procoagulant activity in the tissue phase. This is counterbalanced by a hypo-coagulative response in the fluid phase, primarily facilitated by damage to the endothelial glyocalyx and release of heparin sulfate.
1.2.4 Limitations of animal models in TBI

Undoubtedly, animal modelling of TBI has been instrumental in shaping our understanding of its acute and chronic pathophysiological sequelae, and has laid the foundation for numerous therapeutic strategies. Specifically, rodent models of brain injury have been essential to the elucidation of the neuroinflammatory response to injury (Chiu, Liao et al. 2016), the role of chemokines in peripheral leukocyte recruitment (Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005) (Semple, Bye et al. 2010, Semple, Bye et al. 2010, Semple, Frugier et al. 2010), and the relationship between CNS injury and its numerous secondary biomolecular cascades (Chesselet and Carmichael 2012, Chiu, Liao et al. 2016, DiSabato, Quan et al. 2016, Plesnila 2016). Most importantly, animal experimentation has provided the blueprint for translation into human investigation. However, there are important incongruences between animal and human brain injury. This disparity can be seen in both the re-creation of the primary insult as well as the secondary biomolecular cascades that follow; it is logical to assume that this may be a contributory factor to the paucity of clinical treatments for TBI. While animal experimentation will continue to be essential to brain trauma research moving forward, understanding its limitations will be important for clinical translation.

While the 1980’s saw a variety of large and small animal species employed for TBI research, within the last 30 years the rodent has been the primary species of choice (Morganti-Kossmann, Yan et al. 2010). The four most-often used models are the fluid percussion injury model (FPI), the controlled cortical impact (CCI) model, the weight-drop model, and the blast tube model (Morganti-Kossmann, Yan et al. 2010). Briefly, FPI consists of a pressurized pulse of saline solution on the exposed skull (Lindgren and Rinder 1965), while CCI is similar but uses an air pressure-mediated rigid impactor in replace of fluid (Lighthall, Dixon et al. 1989, Chen,
Constantini et al. 1996); both models are typically used to recreate focal injury. While changing the position of the fluid pulse to the midbrain from the lateral position in FPI can cause a more diffuse injury (Singleton, Zhu et al. 2002), the most commonly employed model to encapsulate diffuse TBI is the weight-drop technique (Feeney, Boyeson et al. 1981, Foda and Marmarou 1994, Marmarou, Foda et al. 1994). Lastly, the rodent blast model uses pressurized air or explosives that travel through a metal tube and make contact with the animal while their body is restrained (Cernak 2005); this mimics the nature of diffuse TBI commonly seen in military personnel (Cernak 2005).

The use of these and other animal models has been essential to our pathophysiological understanding of TBI, but there are several caveats. For instance, FPI may accurately depict mild, focal injuries, but does not recreate the skull fractures commonly seen in moderate-to-severe TBI (Hardman and Manoukian 2002). Furthermore, the injection of the fluid pulse in FPI does not directly relate to the brain impact observed in human TBI (Deselms, Maggio et al. 2016), as fluid characteristics largely vary according to brain geometry and species (O'Connor, Smyth et al. 2011); slight manipulations to the intensity of the fluid pulse may result in drastic changes to injury severity (Lighthall, Dixon et al. 1989, Nilsson, Hillered et al. 1990). In addition, CCI models may not be suited for milder brain injuries as they produce intracranial contusions even with minimal impact; human mTBI often presents without anatomical damage (Dewitt, Perez-Polo et al. 2013). Lastly, most weight-drop models do not reproduce the frontal impact commonly seen in many mild injuries, and animal blast models can vary between labs due to the discrepancy over where to place the animal in the shock tube (Kilbourne, Kuehn et al. 2009, Xiong, Mahmood et al. 2013).
Beyond the specific shortcomings of animal modelling of TBI, there are important general discrepancies. For instance, brain geometry, craniospinal angle, gyral complexity and white/grey matter ratio varies between rodents and humans (Morganti-Kossmann, Yan et al. 2010, O'Connor, Smyth et al. 2011, Xiong, Mahmood et al. 2013, Deselms, Maggio et al. 2016). The ability to repair after injury also differs. For example, it is difficult to induce a coma in rodents, thereby hindering the capability of recreating severe TBI (Morganti-Kossmann, Yan et al. 2010). Additionally, beyond the differences between the rodent and human response to trauma, there is also considerable variation within different rodent strains (Fox, LeVasseur et al. 1999, Tan, Quigley et al. 2009, Reid, Rolfe et al. 2010). Furthermore, there is no standardized injury severity assessment in any animal model of TBI that mimics the GCS scoring system (Xiong, Mahmood et al. 2013). The resultant variability makes it difficult to evaluate pathophysiological similarities/differences across varying injury severities.

Lastly, given the importance of the inflammatory response to brain injury, it is important to consider the differences between the rodent and human immune systems. For instance, there is a large disparity in leukocyte subsets: human blood is “neutrophil rich”, accounting for approximately 50-70% of circulating leukocytes, while rodent blood is “lymphocyte rich”, comprising 75-90% of circulating leukocytes (Mestas and Hughes 2004). Rodents and humans also vary according to immunoglobulin isotype expression, as well as the function and expression of TLRs (Mestas and Hughes 2004). It is unclear if/how these immunological disparities contribute to functional differences in TBI pathophysiology, and as such, further investigation is warranted.
1.3 The application of blood biomarkers to TBI

1.3.1 Biomarker overview

In 2001, the National Institute of Health, Biomarkers Definitions Working Group provided a biomarker description that is still widely applicable today:

‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention’ (Biomarkers Definitions Working 2001).

This potentially encompasses a multitude of measures, including but not limited to, physiological (blood pressure, pulse), biological (blood/salivary/urine analytes) or anatomical (imaging) indices (Biomarkers Definitions Working 2001, Fuentes-Arderiu 2013). Indeed, there are a wide variety of applications for biomarkers; they can be used as tools to diagnose the presence or absence of pathology, to stage and classify severity, to indicate prognosis, and for the prediction and monitoring of treatment responses (Biomarkers Definitions Working 2001). Importantly, the application of biomarkers to any of the aforementioned categories has the prerequisite condition that the biomarker itself must in some capacity reflect the biological mechanism(s) of the disease/injury in question; hence, biomarkers can also be used as a primary tool to investigate pathophysiological mechanisms.

The ultimate status of a biomarker is that of a surrogate – a replacement for a clinical endpoint that reflects how the patient feels, functions or survives (Biomarkers Definitions Working 2001, Strimbu and Tavel 2010, Di Battista, Rhind et al. 2013, Fuentes-Arderiu 2013). Among the best examples of this is the use of cluster of differentiation (CD)-4 cell counts as a substitute for death and opportunistic infections in HIV studies evaluating antiviral agents (Lagakos and Hoth 1992, Deyton 1996, Pozniak 1998). However, single surrogate biomarkers
often only partially and/or indirectly reflect disease process, and may not be essential to the primary pathophysiology (Strimbu and Tavel 2010). Furthermore, there may be an incomplete understanding of the pathophysiology of the disease in question, leading to a possible disconnect between the biomarker and clinical endpoint it seeks to replace (Strimbu and Tavel 2010). Evidence of this has come from the cardiac arrhythmia suppression trial, where the suppression of ventricular arrhythmias was erroneously used as a replacement for sudden death after myocardial infarction; the pharmacological targeting of ventricular ectopy led to an increase in mortality due to shock and arrhythmia (Echt, Liebson et al. 1991, Fleming and DeMets 1996, Strimbu and Tavel 2010). Hence, the rarity of a single biomarker to achieve surrogate endpoint-status and wholly reflect the pathophysiological response of the condition in question, provides compelling support for the use of multiple markers, particularly when the primary research goal is to illustrate pathophysiology.

Taken together, and as pointed out by Strimbu and Tavel (2010) within the context of surrogate endpoints, the role of the biomarker should be to reduce uncertainty about the relationship between it and the clinical endpoint in question, acting as a “stand-in” instead of a replacement, and subject to ongoing re-evaluation of its utility. Indeed, the paradigm of reducing uncertainty via cautious, critical interpretation and reappraisal extends beyond the use of biomarkers as clinical surrogates, and can be applied across all biomarker applications, including those that evaluate their relationships to disease processes.

1.3.2 Peripheral blood as a source for biomarkers in TBI

There are inherent limitations in the study of human TBI, primarily stemming from the difficulty in accessing the CNS of living human beings. Neuroimaging and CSF analysis offer the advantage of proximity, but present a number of limitations (Di Battista, Rhind et al. 2013).
Furthermore, there are numerous important processes that occur extracranially after TBI which greatly affect patient health.

Neuroimaging has the potential to directly measure anatomical and functional abnormalities occurring after TBI (Albrecht, Granziera et al. 2016). However, while conventional neuroimaging techniques are of benefit when used diagnostically in moderate-to-severe TBI, they are often not sensitive enough to detect abnormalities in mTBI and concussion, and have been relatively limited in their ability to inform pathophysiology (Betterman and Slocomb 2012, Papa 2012, Di Battista, Rhind et al. 2013). Furthermore, magnetic resonance imaging (MRI) scans are difficult to conduct in cases of severe injury where patients are unstable, and concerns have been raised regarding the radiation exposure to subjects during CT scans (Brenner and Hall 2007, Papa, Robinson et al. 2008, Fazel, Krumholz et al. 2009). Similarly, as another proximal measurement tool, CSF fluid may contain quantifiable, brain-borne analytes released from the brain in response to injury. However, CSF analysis is a relatively invasive procedure, and can present difficulties in patient recruitment (Papa 2012, Di Battista, Rhind et al. 2013).

In view of the aforementioned analytical approaches, peripheral blood sampling is relatively cheap, non-invasive, and can provide meaningful pathophysiological information regarding disease processes and treatment efficacy (Di Battista, Rhind et al. 2013). Specifically, the detection and quantitation of specific molecules in the blood may inform the presence/absence and importance of ill-defined secondary injury mechanisms in human TBI. For example, CNS-injury molecules such as s100B (Jonsson, Johnsson et al. 2000, Ghanem, Loir et al. 2001, Michetti and Gazzolo 2002, Ingebrigtsen and Romner 2003), as well as many inflammatory mediators, have a very short half-life (minutes) in the peripheral blood, and are
often undetectable in the circulation of healthy individuals (Zhou, Fragala et al. 2010). Thus, the presence or elevated concentration of such markers, beyond the temporal window of physiological clearance, may provide supportive evidence of an ongoing pathophysiological response by the body.

Blood biomarker analysis has displayed the capability to inform processes relating to numerous TBI-related pathologies, including neuroendocrine communication between the CNS and the periphery, BBB disruption, vascular injury and hemostasis (Kossmann, Stahel et al. 1997, Woiciechowsky, Asadullah et al. 1998, Genet, Johansson et al. 2013). Furthermore, in conditions such as endotheliopathy and coagulopathy, the peripheral blood often serves as a direct measure of pathophysiology due to the proximity and direct involvement of the blood in mediating these processes. The following will highlight the main contributions of blood biomarker analysis to the field of adult, human TBI, with particular emphasis on the elucidation of secondary injury pathophysiology.

1.3.2.1 CNS injury

The mechanical trauma causing TBI may damage parenchymal tissue, leading to the release and passage of specific molecules into the blood stream. These indices, commonly originating from damaged or dysregulated neuronal or glial cells, are not typically exposed to the extracellular environment in any appreciable quantity unless there has been a breach to the integrity and/or function of brain cells (Matzinger 1994, Matzinger 2002, Thundyil and Lim 2015). However, there are several assumptions that have plagued the utility of brain injury markers for diagnosis, prognosis, and pathophysiological staging in TBI. Indeed, as indirect measures sampled distal to the site of injury, there are concerns regarding sensitivity and specificity. In view of this, the mechanism(s) underlying the appearance of CNS-specific
biomarkers in the peripheral blood are still unclear, and their relationship to TBI may be confounded by non-injury related factors such as exercise and/or pre-injury mental health conditions (sport-concussion and mTBI specific), or in some cases, may be overestimated due to post-processing issues such as hemolysis (Tolan, Vidal-Folch et al. 2013). However, a number of important contributions have been made through the evaluation of CNS injury biomarkers in the peripheral blood after TBI.

s100B is the most widely studied biomarker in TBI. It is predominantly found in the cytoplasm of astroglial cells (Donato and Heizmann 2010) and is thought to be secreted/released acutely from the CNS into the blood as a result of tissue injury (Sen and Belli 2007, Donato and Heizmann 2010). Numerous studies have found acute elevations in the blood of patients across all injury severities (Regner, Kaufman et al. 2001, Hayakata, Shiozaki et al. 2004, Chen and Zhu 2005, Schulte, Podlog et al. 2014). Furthermore, beyond its potential diagnostic capabilities, s100B has been correlated with poor patient outcome in moderate-to-severe TBI (Nylen, Ost et al. 2008, Yates 2011, Di Battista, Buonora et al. 2015, Rodriguez-Rodriguez, Egea-Guerrero et al. 2016), BBB dysfunction (Kanner, Marchi et al. 2003), cognitive deficits (Studer, Goeggel Simonetti et al. 2015), and abnormal CT scans (Kanner, Marchi et al. 2003, Laribi, Kansao et al. 2014, Wolf, Frantal et al. 2015). However, s100B can be found extracranially in several tissues including cardiac, bone, skin, liver and muscle (Jackson, Sales et al. 2001, Egea-Guerrero, Revuelto-Rey et al. 2012, Michetti, Corvino et al. 2012). In addition, elevated levels have been found in the blood of non-head injured patients (Unden, Bellner et al. 2005, Routsi, Stamatakis et al. 2006), as well as healthy individuals after exercise (Hasselblatt, Mooren et al. 2004).

GFAP is the principal intermediate filament protein in astrocytes, and like s100B, has been widely studied across the spectrum of TBI. It is thought that reactive astrocyte gliosis or
damage to astrocytes results in the release of GFAP from the CNS after injury (Okonkwo, Yue et al. 2013). Indeed, elevated blood concentrations of the protein have been observed acutely across all TBI severities (Pelinka, Kroepfl et al. 2004, Pelinka, Kroepfl et al. 2004, Honda, Tsuruta et al. 2010, Metting, Wilczak et al. 2012, Papa, Brophy et al. 2016), and related to poor patient outcome (Nylen, Ost et al. 2006, Wiesmann, Steinmeier et al. 2010, Di Battista, Buonora et al. 2015). While not definitive, GFAP may be superior to s100B as a TBI biomarker due to higher brain specificity. For example, in mild TBI, GFAP is less susceptible to extracranial trauma, and is better at detecting intracranial lesions compared to s100B (Papa, Silvestri et al. 2014). In severe injury, it outperforms s100B in diagnostic sensitivity and specificity (Honda, Tsuruta et al. 2010), and is not affected by extracranial trauma (Pelinka, Kroepfl et al. 2004). It has been suggested that GFAP may be more accurately evaluated in the blood by measuring its breakdown products (BDP); in the limited number of investigations of GFAP-BDP in TBI patients, positive correlations have been identified with injury severity, structural abnormalities, and the need for neurosurgical intervention (Papa, Lewis et al. 2012, Okonkwo, Yue et al. 2013).

Neuron specific enolase (NSE) is a predominantly neuronal enzyme involved in glycolysis (Begaz, Kyriacou et al. 2006). Increased blood concentrations have been observed in mild and severe TBI patients (Honda, Tsuruta et al. 2010), and have been correlated to poor outcome in the latter (Chabok, Moghadam et al. 2012, Cheng, Yuan et al. 2014). However, the application of NSE in TBI biomarker research has suffered from its presence in erythrocytes and susceptibility to post-processing hemolysis and extracranial injury (Johnsson, Blomquist et al. 2000, Thelin, Jeppsson et al. 2016). In addition, similar to s100B, elevations in circulating NSE have been found acutely after a bout of physical activity (Stalnacke, Tegner et al. 2004, Shahim, Tegner et al. 2014). Notably, NSE has been assessed chronically in athletes exposed to repetitive
head injury; elevated serum levels were found in boxers for up to two months after their last match (Zetterberg, Tanriverdi et al. 2009).

Myelin basic protein (MBP) is among the most abundant proteins in the CNS, located in myelin. Its presence in the circulation after TBI is thought to relate to axonal damage (Thomas, Rabow et al. 1979). However, concern has been raised regarding the usefulness of MBP in the acute period after injury; it is not immediately detectable in the blood, only appearing after a period of approx. 2-3 days post-injury (Berger, Adelson et al. 2005, Di Battista, Rhind et al. 2013).

Recently, a number of previously less-well studied indices of CNS damage have been investigated across the spectrum of TBI, with promising results. Ubiquitin C-terminal hydrolase (UCH) – L1 is a cytoplasmic protein found specifically in neurons, and may represent neuronal damage and disruption of the BBB after TBI (Di Battista, Rhind et al. 2013). Elevated serum levels of UCH-L1 have been identified after both mild and severe injury (Brophy, Mondello et al. 2011, Papa, Brophy et al. 2016). Additionally, the proteolytic fragment of alpha-II spectrin (SNTF) may reflect axonal damage (Johnson, Stewart et al. 2016), and has shown potential diagnostic and prognostic utility in mTBI (Siman, Giovannone et al. 2013, Siman, Shahim et al. 2015).

The long-term effects of brain injury are unclear, and evidence suggests a possible relationship between TBI and chronic neurodegeneration (Blaylock and Maroon 2011, Fakhran, Yaeger et al. 2013, Mendez, Paholpak et al. 2015). In view of this, tau is a microtubule associated protein located in axons (Mandelkow and Mandelkow 2012), and when pathologically phosphorylated, has been implicated in the etiology of neurodegenerative disorders such as AD and CTE (Mandelkow and Mandelkow 2012). In recent years, tau has been identified as a
quantifiable analyte in the peripheral blood (Bulut, Koksal et al. 2006, Zetterberg, Smith et al. 2013, Wang, Li et al. 2016). Given both its location and pathophysiological relevance to brain pathology, tau may have two-fold applicability in TBI as both a measure of 1) axonal damage, and 2) neurodegeneration.

While not universally supported (Bazarian, Zemlan et al. 2006, Bulut, Koksal et al. 2006), elevated blood tau levels have been found acutely after both mild and severe TBI (Liliang, Liang et al. 2010, Shahim, Tegner et al. 2014, Shahim, Linemann et al. 2016, Wang, Li et al. 2016), and for up to 3 years after the last self-reported TBI in military members (Olivera, Lejbman et al. 2015). Ambiguity in the literature regarding the utility of tau as an indicator of brain injury possibly owes to its numerous molecular antibody targets. Indeed, tau can be measured as total-tau (Olivera, Lejbman et al. 2015), phosphorylated-tau (theoretically active) (Puvenna, Engeler et al. 2016) or fragmented-tau (Shahim, Linemann et al. 2016). In view of the latter, Shahim et al found the tau-C fragment, but not tau-A, was elevated in concussed hockey players compared to pre-season baseline samples (Shahim, Linemann et al. 2016). To add further complexity, it is unclear how tau travels from the CNS to the plasma; it may be released across a dysregulated BBB in a similar vein to other CNS injury markers such as s100B or GFAP, or may be transported in exosomes, therefore requiring specific analytical considerations (Stern, Tripodis et al. 2016).

Taken together, these findings are consistent with the hypothesis that TBI causes immediate damage or dysregulation to parenchymal tissue, resulting in the appearance of related molecules in the peripheral blood where they can be analytically evaluated. These findings span the entire injury spectrum, and in severe TBI, biomarkers are often correlated with injury severity and poor patient outcome. There is also supportive evidence of a relationship between
clinically measured structural brain injury and an increase in neuronal/glial damage markers in the circulation. Furthermore, biomarkers may be used to evaluate the relationship between TBI and chronic neurodegeneration. Notably, results from these studies also support pathophysiological congruence between CNS damage incurred across all injury severities.

1.3.2.2 SNS activity

Activation of the SNS results in immediate adrenal and terminal secretion of catecholamines into the blood, and thus their measurement may be considered a relatively direct proxy of adrenergic function. This contrasts with the measurement of peripheral indices of CNS damage, which represent indirect correlates of a process occurring distal to the point of observation. Hence, blood biomarkers of SNS activity hold a potential comparative advantage in TBI research.

As previously mentioned, a number of studies have identified immediate, dramatic elevations of both Epi and NE in the peripheral blood of moderate-to-severe TBI patients, correlated with both injury severity and poor patient outcome (Hortnagl, Hammerle et al. 1980, Nayak, Mohanty et al. 1980, Hamill, Woolf et al. 1987, Woolf, Hamill et al. 1987, Woolf, Hamill et al. 1988). While no studies have evaluated catecholamine levels in mTBI, Hamill, Woolf et al. (1987) observed “slightly elevated or normal” NE and Epi levels in patients presenting with a GCS >11, suggesting adrenergic perturbations exist across all severities of brain injury.

1.3.2.3 Inflammation

Unlike CNS-specific biomarkers, inflammatory mediators can be released from a variety of sources in response to brain injury (Woiciechowsky, Asadullah et al. 1998, Catania, Lonati et
al. 2009), and can be used to reflect secondary, extracranial sequelae such as sepsis, peripheral organ dysfunction, endothelial injury and coagulopathy. Attempts at characterizing inflammation after TBI require careful interpretation and analysis, as the inflammatory response is highly pleiotropic and redundant, and is likely involved in every aspect of secondary injury.

The earliest inflammatory biomarker investigation in TBI was carried out by Goodman, Robertson et al. (1990). These authors identified elevations in the cytokine TNF-α in severely injured patients for up to 5 days post-trauma (Goodman, Robertson et al. 1990). Since then, elevations in a number of peripheral blood cytokines, primarily IL-1β, -6, and -10, have been observed acutely after moderate-to-severe TBI (Kossmann, Hans et al. 1995, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014), related to both injury severity and poor outcome (Tasci, Okay et al. 2003, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014). Notably, however, some investigators have found no relationship between cytokines and patient outcome (Dziurdzik, Krawczyk et al. 2004, Venetsanou, Vlachos et al. 2007, Stein, Lindell et al. 2011), and have even suggested that lower IL-10 levels acutely after injury are associated with an increased risk of infection (Dziurdzik, Krawczyk et al. 2004). However, the vast majority of studies which have evaluated the relationship between acute systemic inflammatory mediators and outcome in isolated TBI have included small patient sample sizes (n <30) (Shimonkevitz, Bar-Or et al. 1999, Pleines, Morganti-Kossmann et al. 2001, Stein, Lindell et al. 2011), a small number of analytes (Seekamp, van Griensven et al. 2002, Dziurdzik, Krawczyk et al. 2004, Stein, Lindel et al. 2012, Sohrevardi, Ahmadinejad et al. 2013), or have focused on patient survival as the only outcome measure (Arand, Melzner et al. 2001, Dziurdzik, Krawczyk et al. 2004).
To uncover the relationship between inflammatory mediators and patient outcome, it is first necessary to elucidate the associated pathophysiological mechanisms. A number of studies have made progress in this regard. For example, elevated circulating IL-10 has been associated with an increase in ICP, SNS activity, and a decrease in monocyte major histocompatibility complex II expression after brain injury (Woiciechowsky, Asadullah et al. 1998, Shimonkevitz, Bar-Or et al. 1999). In addition, elevations in IL-6, -8 and TNF-α have been associated with increased ICP (Hergenroeder, Moore et al. 2010, Stein, Lindell et al. 2011), and IL-6 has been correlated with the appearance of circulating APR proteins acutely after injury (Kossmann, Hans et al. 1995).

The assessment of circulating chemokines after TBI has predominantly centered around IL-8 and MCP-1 (Rhodes, Sharkey et al. 2009, Buonora, Yarnell et al. 2015, Di Battista, Buonora et al. 2015). Increases in MCP-1 levels have been observed across the spectrum of TBI (Semple, Bye et al. 2010, Buonora, Yarnell et al. 2015), although IL-8 has only been investigated in moderate-to-severe injury (Kossmann, Stahel et al. 1997, Seekamp, van Griensven et al. 2002, Kushi, Saito et al. 2003, Rhodes, Sharkey et al. 2009). Indeed, elevated concentrations of both analytes have been associated with poor outcome after severe TBI (Rhodes, Sharkey et al. 2009, Ferreira, Regner et al. 2014). However, only IL-8 has displayed covariance with pathophysiological measures; Stein, Lindel et al. (2012), observed a positive relationship between serum IL-8 levels and both cerebral hypertension and hypoperfusion.

Few studies have evaluated peripheral inflammatory biomarkers in the acute and subacute phases after mTBI, or chronically across the entire spectrum of injury. However, those that have, support dysregulated inflammation that extend across all severities, continuing for months and possibly years. For example, perturbations in peripheral blood mononuclear cell
gene expression have been observed in the acute and subacute periods following sport concussion (Gill, Merchant-Borna et al. 2016, Merchant-Borna, Lee et al. 2016), and elevated serum CRP and coated-platelet levels (an inflammatory correlate) have been observed for up to 9 years after mTBI (Prodan, Vincent et al. 2014, Su, Xu et al. 2014). Furthermore, in severe injury, in a recent study by Kumar, Boles et al. (2015), the authors found elevations in IL-1β, -6, -8, -10 and TNF-α for over 3 months post-injury in patients.

In summation, results of investigations using human biomarkers in TBI support a marked systemic inflammatory response in the acute period that may persist for months. Furthermore, evidence suggests systemic inflammation is a pathological event contributing to poor outcome, possibly by exacerbating CNS injury (Hergenroeder, Moore et al. 2010, Stein, Lindel et al. 2012). However, these findings have not been thoroughly evaluated using a broad number of mediators, in a large cohort of isolated TBI patients. Furthermore, regarding specific pathophysiology, it appears that SNS hyperactivity may stimulate circulating monocytes to produce IL-10, possibly leading to susceptibility to infection (Woiwiechowsky, Asadullah et al. 1998, Woiwiechowsky, Schoning et al. 1999), and there may be a relationship between peripheral IL-6 and the APR (Kossmann, Hans et al. 1995). Lastly, while systemic inflammation may be detectable after milder forms of injury, scant evidence exists characterizing this response.

1.3.2.4 Coagulopathy & endotheliopathy

The blood is the primary biological compartment for investigating coagulopathy and vascular damage. As standard clinical indices of coagulopathy are biased towards detecting hypocoagulation (Hemker, Al Dieri et al. 2004, Park, Martini et al. 2009), biomarkers capable of signifying all facets of dysregulated hemostasis are potentially advantageous. However, in TBI, it is admittedly difficult to position these processes to the neurovascular unit, as they are
theoretically occurring across the entire systemic circulation. Yet, the investigation of such processes is of merit, because perturbations to hemostasis and the vasculature can facilitate damage to the brain and/or peripheral organs, irrespective of anatomical location (Huber, Dorn et al. 1993, Zygun 2005, Catania, Lonati et al. 2009).

Numerous studies have identified elevated pro-coagulant and pro-fibrinolytic factors in the blood after TBI (Keimowitz and Annis 1973, Goodnight, Kenoyer et al. 1974, Hulka, Mullins et al. 1996, Scherer and Spangenberg 1998, Yokota, Naoe et al. 2002, Nekludov, Antovic et al. 2007). Specifically, elevated circulating TF and thrombin-anti-thrombin (TAT) complexes, indicative of excessive thrombin generation, have been identified immediately after moderate-to-severe injury (Kearney, Bentt et al. 1992, Gando, Nanzaki et al. 1999, Nekludov, Antovic et al. 2007). Furthermore, elevated D-dimer (DD) and other FDPs, along with decreased $\alpha_2$-plasmin inhibitor has been observed, suggesting excessive fibrinolysis (van der Sande, Veltkamp et al. 1978, Hulka, Mullins et al. 1996, Gando, Nanzaki et al. 1999, Nekludov, Antovic et al. 2007). Indeed, a number of these pro-coagulant indices have also been correlated to poor patient prognosis (van der Sande, Veltkamp et al. 1978, Selladurai, Vickneswaran et al. 1997, Kuo, Chou et al. 2004), and it has been suggested that SNS activity and/or the APR may mediate coagulopathic disturbances, as evidenced by the covariation of coagulation indices with circulating NE and IL-6, respectively (Kearney, Bentt et al. 1992, Nekludov, Antovic et al. 2007). Additionally, acute elevations of circulating APC have also been identified post-TBI, suggesting a possible early hypocoagulant response without the consumption of clotting factors (Cohen, Brohi et al. 2007).

Increased concentrations of indices of endothelial activation and damage have been found in the peripheral blood after TBI (Yokota, Naoe et al. 2002, Genet, Johansson et al. 2013,
Acute increases in circulating thrombomodulin (TM) and von Willebrand factor (vWF), which reflect endothelial damage and activation, respectively, have been observed after isolated TBI (Yokota, Naoe et al. 2002). Additionally, elevated concentrations of intracellular adhesion molecule (ICAM)-1 have also been found (Wahlstrom, Olivecrona et al. 2014). More recently, syndecan (SDC)-1, an analogue of glycocalyx injury, was identified in the circulation of TBI patients at concentrations comparable to non-head trauma patients (Genet, Johansson et al. 2013).

Blood biomarker investigations into hemostatic and endothelial abnormalities after isolated TBI have provided supportive evidence consistent with coagulopathy and disruption of the vascular endothelium. When this dysfunction occurs, patients are at risk of poor prognosis. Yet, while speculation exists, it is unclear how the inflammatory and neuroendocrine responses to trauma influence hemostasis and vascular biology after isolated brain injury. Furthermore, although substantial evidence exists supporting early TF-mediated thrombosis and consumptive coagulopathy after head injury, contrary findings have been presented, and thus, clarification is needed. Lastly, given the dynamic nature of the hemostatic response, it is unclear how coagulation phenotypes may change over time in the acute period following injury.

1.4 Rationale

TBI is a substantial societal health concern worldwide, with a vast range of clinical severities spanning mild to fatal. While the causal events leading up to TBI are irreversible, secondary cellular and molecular manifestations occurring post-trauma significantly contribute to patient health and are seemingly amenable to intervention. Yet, there are currently no effective clinical treatments for secondary neurological injury after TBI, and it has been repeatedly stated
that a poor understanding of its complex, heterogeneous nature is the primary impediment hindering therapeutic advances. Elucidation of the fundamental pathophysiological mechanisms of secondary injury, across the entire severity spectrum of TBI, is needed to inform future experimental and clinical therapeutic strategies and improve patient care.

Experimental animal models continue to be an invaluable tool in shaping our basic understanding of TBI. However, there are important anatomical and physiological differences that limit their applicability to human brain injury. In view of this, analytical techniques including neuroimaging and CSF biomarker analysis have the potential to provide proximal information on structural, functional and biochemical alterations to the human brain. However, these methods are constrained by issues such as feasibility and cost. Furthermore, they are limited in their ability to infer injury-related processes that may occur extracranially; processes that are often crucial to patient outcome. Indeed, peripheral blood biomarker analysis is comparatively inexpensive and non-invasive, offering the potential to evaluate large cohorts at numerous time-points. Moreover, the assessment of biomarkers identified in the peripheral blood of TBI patients may elucidate pathophysiological mechanisms related to both CNS and non-CNS injury.

The acute period after moderate-to-severe TBI represents a critical window for clinical intervention. It is marked by continuous pathological SNS activity, hemostatic perturbations, and widespread inflammation. However, the etiology and coordination between these processes is unclear. Hence, it is not fully understood how they contribute to patient outcome. Given the bi-directional relationship between the SNS and immune system, their separately identified relationships to poor outcome after TBI, and promising results of β-blocker therapy in improving patient outcomes, it follows that SNS hyperactivity may mediate pathological inflammation.
acutely after injury. In addition, given the accumulating evidence supporting the role of the SNS in facilitating coagulopathy and vascular endothelial damage after non-head trauma and sepsis, these mechanisms may be evident and important in isolated TBI.

Although placed at the “mild” end of the TBI spectrum, both its frequency and potential long-term implications render concussion an important concern in medical science. Investigating SRC as a subset of mTBI offers the potential to evaluate brain injury in a relatively homogeneous population of young, ostensibly healthy adults. Furthermore, athletic populations are at specific risk of chronic, repetitive head injury, an emerging concern due to its possible relationship to neurodegenerative diseases such as AD and CTE. However, despite the relevance of inflammation in moderate-to-severe TBI, experimental evidence in animal models, and accruing evidence of the involvement of inflammation in chronic neurodegenerative disorders, there are only scarce reports supporting the involvement of inflammation in human mTBI, and even fewer in SRC. While ample evidence exists supporting the detection of CNS injury-specific analytes in the peripheral blood after mTBI/concussion, it is unclear if systemic inflammatory perturbations are detectable.

1.5 Aims and Hypotheses

1.5.1 Aims

The aim of this study was to employ blood biomarker analysis as a tool to support, advance, and generate hypotheses of secondary injury pathophysiology across the severity spectrum of human TBI.
1.5.2 Hypotheses

1.5.2.1 Global hypotheses

Biomarkers employed across the severity spectrum of TBI offer value in determining if, and when pathophysiological mechanisms are present and relevant, in calibrating their relative significance to patient outcome, and for discovering potentially relevant avenues for future investigation.

1.5.2.2 Specific hypotheses

1. Acutely after isolated, moderate-to-severe TBI, perturbations in circulating markers of inflammation, hemostasis and vascular endothelial injury covary with poor patient outcome and SNS hyperactivity.

2. Biomarkers of CNS injury and inflammation are detectable in the peripheral blood after sport-related concussion, and are related to injury characteristics.

1.5.2.3 Secondary hypotheses

Regarding specific hypotheses #1:

1. **Within 24 h of isolated, blunt, moderate-to-severe TBI, elevations in systemic cytokine and chemokine concentrations portend poor patient outcome and correlate with SNS hyperactivity.**

To test this hypothesis, a panel of relevant circulating cytokines and chemokines, as well as plasma catecholamines, were assessed in isolated TBI patients by immunoassay at four time points within the first 24 h after hospital admission. These analytes were quantified to evaluate possible interrelationships between key inflammatory mediators and 6-month patient outcome, and SNS activity.
2. **Circulating biomarkers of coagulopathy and endotheliopathy are present acutely after isolated TBI, and correlate with poor patient outcome and SNS hyperactivity.**

To test this hypothesis, levels of immunoreactive cytokines, chemokines, catecholamines, and biomarkers of hemostasis and vascular endothelial injury, were evaluated at two time points within 24 h of hospital admission in isolated TBI patients. Specifically, multivariate statistics were employed to assess the covariance between circulating markers of coagulopathy, endotheliopathy, inflammation, and both SNS activity and patient outcome at 6 months.

Regarding specific hypothesis #2:

3. **Biomarkers of CNS injury and inflammation are detectable in the peripheral blood in the subacute phase after sport-related concussion, and are correlated with injury symptoms.**

To test this hypothesis, circulating levels of cytokines, chemokines and CNS-injury specific molecules were analyzed in the blood of athletes within 7 days of a sport-related concussion, and throughout medical clearance. These indices were evaluated against matched healthy controls, and were assessed for covariance with the presence and severity of reported symptoms.

4. **A history of multiple concussions or repetitive head impacts is correlated with detectable perturbations in systemic biomarkers of inflammation and CNS injury.**

This hypothesis was tested by quantifying a broad spectrum of cytokines and chemokines, as well as a number of CNS-injury specific analytes in the circulation of healthy varsity athletes. These biomarkers were evaluated to determine possible covariance with previous concussion history and participation in sports with an elevated risk for repetitive head trauma.
Chapter 2
Inflammatory cytokine and chemokine profiles are associated with patient outcome and the hyperadrenergic state following acute brain injury.

The components of this chapter were published as follows:


The lead author contributed to the manuscripts conceptual framework, performed all cytokine/chemokine immunoassay experiments, statistically analyzed and interpreted the data, drafted the manuscript, and contributed to its critical revision.
Inflammatory cytokine and chemokine profiles are associated with patient outcome and the hyperadrenergic state following acute brain injury.

2.1 Abstract

BACKGROUND. Traumatic brain injury (TBI) elicits intense sympathetic nervous system (SNS) activation with profuse catecholamine secretion. The resultant hyperadrenergic state is linked to immunomodulation both within the brain and systemically. Dysregulated inflammation post-TBI exacerbates secondary brain injury and contributes to unfavorable patient outcomes including death. OBJECTIVES. The aim of this study was to characterize the early dynamic profile of circulating inflammatory cytokines/chemokines in patients admitted for moderate-to-severe TBI, to examine interrelationships between these mediators and catecholamines, as well as clinical indices of injury severity and neurological outcome. METHODS. Blood was sampled from 166 isolated TBI patients (aged 45±20.3 y; 74.7% male) on admission, 6-, 12-, and 24-h post-injury and from healthy controls (N=21). Plasma cytokines [interleukin (IL)-1β, -2, -4, -5, -10, -12p70, -13 tumor necrosis factor (TNF)-α, interferon (IFN)-γ], and chemokines [IL-8, Eotaxin, Eotaxin-3, IFN-γ-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, -4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus activation regulated chemokine (TARC)] concentrations were analyzed using high-sensitivity electrochemiluminescence multiplex immunoassays. Plasma catecholamines [epinephrine (Epi), norepinephrine (NE)] were measured by immunoassay. Six-month neurological outcome was assessed using the Extended Glasgow Outcome Scale (GOSE) dichotomized as good (>4) or poor (≤4) outcomes. RESULTS. Patients showed altered levels of IL-10 and all chemokines assayed relative to controls. Significant differences in a number of
markers were evident between moderate and severe TBI cohorts. Elevated IL-8, IL-10, and TNF-α, as well as alterations in 8 of 9 chemokines, were associated with poor outcome at 6 months. Notably, a positive association was found between Epi and IL-1β, IL-10, Eotaxin, IL-8 and MCP-1. NE was positively associated with IL-1β, IL-10, TNF-α, Eotaxin, IL-8, IP-10 and MCP-1.

CONCLUSIONS. Our results provide further evidence that exaggerated SNS activation acutely after isolated TBI in humans may contribute to harmful peripheral inflammatory cytokine/chemokine dysregulation. These findings are consistent with a potentially beneficial role for therapies aimed at modulating the inflammatory response and hyperadrenergic state acutely post-injury.

2.2 Introduction

Growing experimental and clinical evidence indicates that inflammation is an integral component to the pathogenesis of secondary injury after TBI (Lu, Goh et al. 2009, Hinson, Rowell et al. 2015). Its effects are not limited to the brain parenchyma, as systemic inflammation is a noted consequence of TBI, and can impact patient outcome by exacerbating cerebral tissue injury and contributing to systemic complications such as nosocomial infection and multiple organ failure (Catania, Lonati et al. 2009, Lu, Goh et al. 2009, Anthony and Couch 2014). While potentially harmful, bi-directional neuroimmune communication between the CNS and the periphery is also required for neuronal repair and regeneration (Morganti-Kossmann, Rancan et al. 2002). The duality of this process is highlighted by a number of failed clinical trials aimed broadly at reducing or inhibiting inflammation (Bergold 2016). Hence, a better understanding of the underlying mechanisms governing the inflammatory response to isolated TBI in humans can help guide future therapeutic strategies and improve patient outcome.
In an effort to restore vital homeostasis in the face of TBI, activation of the SNS results in a massive secretion of catecholamines (Epi, NE) into the periphery as part of the generalized host stress response to trauma (Hortnagl, Hammerle et al. 1980, Clifton, Ziegler et al. 1981, Clifton, Robertson et al. 1983, Hamill, Woolf et al. 1987, Woolf, Hamill et al. 1987, Woolf, Hamill et al. 1988, Desborough 2000). Moreover, we recently demonstrated in a large group of moderate-to-severe TBI patients, that both NE and Epi are elevated in a dose-dependent fashion according to injury severity, and that prolonged elevation of NE and Epi throughout the first 24 h after hospital admission is highly correlated with adverse patient outcomes (Da Luz, Capone Neto et al. 2015).

It has been hypothesized that early SNS activation after TBI may influence the inflammatory response both locally and systemically. This occurs prototypically as a response to elevations in cerebral IL-1β concentrations and subsequent initiation of both the local and systemic acute phase response (Campbell, Wilcockson et al. 2002, Wilcockson, Campbell et al. 2002, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005). In addition, trauma-induced activation of NE terminals in peripheral organs such as the liver and spleen may lead to the systemic release of inflammatory mediators into the circulation (Catania, Lonati et al. 2009, Li, Li et al. 2011, Chu, Li et al. 2013). It is also possible that elevated concentrations of peripheral catecholamines can differentially alter cytokine/chemokine production in circulating lymphocytes (Shimonkevitz, Bar-Or et al. 1999): NE and Epi interact with α- and β-ARs expressed on leukocytes and other tissues, influencing the production of inflammatory mediators from these cells (van der Poll, Coyle et al. 1996, van der Poll and Lowry 1997, Woiciechowsky, Asadullah et al. 1998, Bierhaus, Wolf et al. 2003). Furthermore, β-blocker therapy in human TBI has been associated with improved outcome (Schroeppel, Fischer et al. 2010, Schroeppel, Sharpe...
et al. 2014), and a number of animal studies using pharmacological blockade of β-adrenergic receptors have shown concomitant attenuation of the inflammatory response and improved outcome after treatment (Rough, Engdahl et al. 2009, Ley, Clond et al. 2012, Xu, Yu et al. 2015). However, no previous clinical studies have evaluated the relationship between the SNS and systemic inflammation in human isolated TBI patients.

Elevations in several cytokines, including IL-1β, -6, -10 and TNF-α, have been identified in the circulation of TBI patients within hours of injury (Goodman, Robertson et al. 1990, Maier, Schwerdtfeger et al. 2001, Tasci, Okay et al. 2003, Chiaretti, Genovese et al. 2005, Venetsanou, Vlachos et al. 2007, Santarsieri, Kumar et al. 2015). However, their relationship to patient survival and outcome is less clear, likely owing to the heterogeneous nature of both primary brain injury (i.e., focal vs. blunt trauma, extracranial complications etc.), and the complexity of secondary injury processes. Additionally, correlations have been found between early elevations in peripheral cytokines and poor patient outcome (Kushi, Saito et al. 2003, Gopcevic, Mazul-Sunko et al. 2007, Rhodes, Sharkey et al. 2009, Schneider Soares, Menezes de Souza et al. 2012), although others have found no association (Venetsanou, Vlachos et al. 2007, Stein, Lindell et al. 2011), or have even identified inverse relationships between cytokines and risk of infection after injury (Dziurdzik, Krawczyk et al. 2004). Moreover, chemokines appear to play an important role in TBI pathophysiology (Jaerve and Muller 2012), though human studies to date have focused predominantly on IL-8 and MCP-1 (Rhodes, Sharkey et al. 2009, Buonora, Yarnell et al. 2015, Di Battista, Buonora et al. 2015) and require further characterization.

Therefore, the purpose of this study was to: 1) identify the temporal profile of a panel of circulating cytokines and chemokines acutely after injury in both moderate and severe isolated blunt TBI patients; 2) to identify possible interrelationships between circulating catecholamines
and cytokines post-injury; 3) to evaluate these markers in patients stratified according to 6-month neurological outcome and mortality.

2.3 Methods

2.3.1 Ethics Statement

The study protocol complied with the ethical guidelines of the Declaration of Helsinki of 1975 and was approved by the Research Ethics Boards and Institutional Review Boards of the participating hospitals. All patients’ families received a comprehensive description of the study and gave written informed consent for their relatives’ participation. In the absence of a substitute decision maker, consent was delayed in accordance with the Tri-Council Policy Agreement for Research in Emergency Health Situations (Article 2.8); delayed written consent for participation in the study was subsequently obtained from next-of-kin or, where possible, directly from the patient once they had recovered sufficiently. Informed consent for a single blood sample was also obtained from healthy (control) volunteers.

2.3.2 Patients and Controls

Potential study participants were admitted to Sunnybrook Health Sciences Centre (Toronto, ON, Canada), St. Michael’s Hospital (Toronto, ON, Canada), and Los Angeles County + University of Southern California (USC) Medical Center (Los Angeles, CA). Upon admission, patients with an isolated TBI, defined by a GCS score <13 and a non-head abbreviated injury score (AIS) \( \leq 2 \), were considered for inclusion. Patients with an elapsed time between trauma and hospital admission >3 hours (h), with a penetrating brain injury, <16 years of age, pregnant, taking β-blockers, lacking vital signs prior to admission or clinically brain dead on admission,
were excluded. A healthy control group with no history of brain injury was included for a single blood donation for analysis of the selected panel of soluble inflammatory markers.

2.3.3 Study Design and Procedures

Upon hospital admission, clinical and demographic data were obtained from eligible patients: Demographics – age and gender; Clinical information – mechanism of injury, elapsed time from the trauma to the emergency room, injury severity score (ISS) and AIS head; Neurological status – level of consciousness categorized by the GCS, pupil size and reactivity, seizures, alcohol level; Clinical status – blood pressure, tracheal intubation, spontaneous vs. mechanical ventilation, oxygen saturation, temperature; Medical history – past medical history, present medications including β-blockers and anticoagulants. Routine laboratory exams and imaging were also completed upon admission, including chest radiography and CT scans. All significant clinical events during the first 24 h were recorded, including, but not limited to, sepsis/infection, organ failure, any medical treatments administered, surgical procedures, and any other significant changes in clinical parameters. Organ failure was defined by the following criteria, 1) Arterial hypoxemia - PaO2/FiO2 <300, 2) Acute oliguria - urine output <0.5 mL/kg/h for at least 2 h despite adequate fluid resuscitation, 3) creatine increase - >0.5 mg/dL or 44.2 µmol/L, 4) coagulation abnormalities – international normalized ratio (INR) > 1.5 or activated partial thromboplastin time (aPTT) > 60s, 5) Ileus - absent bowel sounds, 6) Thrombocytopenia - platelet count < 100,000 µ/L, 7) Hyperbilirubinemia - plasma total bilirubin >4 mg/dL or 70 µmol/L.

For patients who died, the cause of death was recorded and classified as TBI-related or non-TBI related. Upon hospital discharge, at 28 days, and at 6-months, patient outcome was assessed by GOSE.
2.3.4 Blood Sample Collection

Venous blood samples were drawn as soon as possible after admission to the trauma room or emergency department, and again at 6-, 12-, and 24-h post-injury. Samples were drawn into either 10-mL K$_2$EDTA (with 4mM sodium metabisulfite [Na$_2$S$_2$O$_5$]) or 10-mL sodium heparin vacutainers (Vacutainer, Becton Dickinson, Rutherford, NJ). Specimens were immediately centrifuged at 1600 x g for 15 minutes at 4°C, the plasma supernatant was then separated into six (1-2 mL) aliquots and frozen at -70°C until subsequent analysis.

2.3.5 Multiplex Cytokine and Chemokine Measurements

Immunoreactive plasma levels of 9 cytokines and 9 chemokines were analyzed with Meso-Scale Discovery (MSD) 96-Well MULTI-SPOT® Ultra-Sensitive Human Immunoassay Kits, using electrochemiluminescence detection on an MSD Sector Imager™ 6000 with Discovery Workbench software (version 3.0.18) (MSD®, Gaitherburg, MD, USA). Cytokines were measured using the TH1/TH2 10-plex kit, which included 9 markers (excluding IL-8): interferon (IFN) - γ, IL-1β, -2, -4, -5, -10, -12p70, -13 and TNF-α. Chemokines were measured using the Chemokine 9-plex, which also included 9 markers: Eotaxin, eotaxin-3, macrophage inflammatory protein (MIP)-1β, thymus and activations regulated chemokine (TARC), interferon-gamma induced protein (IP)-10, IL-8, MCP-1, macrophage-derived chemokine (MDC), MCP-4. All assays were performed according to manufacturer’s instructions, in duplicate, and without alterations to the recommended standard curve dilutions. Briefly, samples were thawed on ice, and added to a 96-well MULTI-SPOT® plate coated with capture antibodies in a spatially distinct fashion. SULFO-TAG® labelled detection antibodies were then added to each of the wells to complete the sandwich format, and a read buffer was added to alter the
chemical environment for electrochemiluminescence. The subsequent reaction resulted in the emission of light from the labelled analytes, which was then quantified to approximate the concentration (pg/mL) of each protein present in the sample.

2.3.6 Catecholamine Measurements

Plasma Epi and NE levels were determined from duplicate samples using a competitive enzyme immunoassay method according to the manufacturer’s instructions (Bi-CAT EIA, ALPCO Diagnostics, Salem, NH). Briefly, plasma Epi and NE were extracted by using a cis-diol-specific affinity gel, acylated and then derivatized enzymatically into N-acylmetanephrine and N-acylnormetanephrine, respectively. Antibody bound to the solid-phase catecholamines were detected by an anti-rabbit IgG-peroxidase conjugate using tetramethylbenzidine as a substrate. Quantification of unknown samples was achieved by comparing their absorbance with a reference curve prepared with known standard concentrations included in the kit.

2.3.7 Statistical Analysis

Demographic and clinical parameters are expressed as the mean ± standard deviation (SD) unless otherwise stated, while blood marker concentrations are graphically displayed as the median and interquartile range. Comparison of inflammatory marker levels between TBI patients at each sampled time point and healthy control subjects was performed using a Kruskal-Wallis analysis of variance, with Dunn’s multiple comparisons post-hoc test. To assess 6-month neurological outcome, patients were dichotomized into favorable (GOSE 5-8) and unfavorable (GOSE 1-4) outcomes. Mortality was assessed by stratifying patients into two groups, “survivors” and “non-survivors”. Death was further stratified as either “neurologic” or “non-neurologic”. Differences in cytokine and chemokine concentrations between moderate and
severe TBI patients, unfavorable and favorable outcome, survivors and non-survivors, and 
neurologic and non-neurologic death, were assessed by Mann-Whitney U. To identify possible 
correlations between catecholamines and inflammatory marker concentrations, pooled data over 
all time points was evaluated by spearman’s ρ. To generate an aggregate inflammation score 
(IS), peak quartile rank scores from each marker associated with both unfavorable outcome and 
survival were summed (Kumar, Boles et al. 2015, Santarsieri, Kumar et al. 2015). For each 
marker, values >75th percentile were given a score of 4, values between the 50th and 75th 
percentile were given a score of 3, values between the 25th and 50th percentile were given a score 
of 2, and values <25th percentile were given a score of 1. Where lower concentrations of a 
marker was associated with unfavorable patient outcome, the scoring system was reversed, hence 
values <25th percentile were given a score of 4, while values >75th percentile were given a score 
of 1. Quartile scores of each marker were added, resulting in an aggregate score; 6 markers were 
included, allowing for a score ranging from 6-24. Patients were then dichotomized into high vs. 
low inflammation categories based on the median aggregate IS (Grund and Sabin 2010). In 
addition, to assess the ability of cytokines and chemokines to predict poor patient outcome and 
death while controlling for injury severity, a multivariate binomial logistic regression analysis 
was employed. All markers were quartiled in order to standardize unit increases for statistical 
comparison. Each individual marker was added independently to a model containing GCS and 
AIS head scores. The binary dependent outcome variables were 6-month GOSE or survival. 
Furthermore, biomarker data was not statistically analyzed or graphically displayed unless at 
least 50% of the samples analyzed were within the detection range of the assay, or included 
replicate values with a coefficient of variation (CV) < 25%. Statistical significance in all 
analyses was indicated by a p-value of ≤0.05. All data were analyzed using GraphPad Prism 
Version 6.0d (GraphPad Inc, CA, USA) and Stata Version 13.1 (StataCorp, TX, USA).
Table 2.1 Demographic and Clinical Characteristics for TBI patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All TBI Patients (n=166)</th>
<th>Moderate TBI (n=33)</th>
<th>Severe TBI (n=133)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.8 ± 20.3</td>
<td>49.5 ± 19.7</td>
<td>44.9 ± 20.4</td>
</tr>
<tr>
<td>Male gender – n (%)</td>
<td>124 (74.7)</td>
<td>23 (69.7)</td>
<td>101 (75.9)</td>
</tr>
<tr>
<td>Clinical Characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to ED (min)</td>
<td>77.6 ± 63.5</td>
<td>74.7 ± 64.8</td>
<td>78.3 ± 63.4</td>
</tr>
<tr>
<td>ISS score</td>
<td>24.4 ± 11.8</td>
<td>18.8 ± 11.5</td>
<td>25.9 ± 11.5</td>
</tr>
<tr>
<td>AIS head</td>
<td>4.1 ± 1.1</td>
<td>3.5 ± 1.2</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>GCS</td>
<td>5.9 ± 3.0</td>
<td>10.5 ± 1.3</td>
<td>4.7 ± 1.9</td>
</tr>
<tr>
<td>Marshall score – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>28 (16.9)</td>
<td>11 (33.3)</td>
<td>17 (12.8)</td>
</tr>
<tr>
<td>II</td>
<td>76 (45.8)</td>
<td>16 (48.5)</td>
<td>60 (45.1)</td>
</tr>
<tr>
<td>III</td>
<td>13 (7.8)</td>
<td>1 (3.0)</td>
<td>12 (9.0)</td>
</tr>
<tr>
<td>IV</td>
<td>27 (16.3)</td>
<td>3 (9.1)</td>
<td>24 (18.0)</td>
</tr>
<tr>
<td>Evacuated mass lesion</td>
<td>21 (12.6)</td>
<td>2 (6.1)</td>
<td>19 (14.3)</td>
</tr>
<tr>
<td>Non-evacuated mass lesion</td>
<td>1 (0.6)</td>
<td>0 (0.0)</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>Pre-injury comorbidities – n (%)</td>
<td>45 (27.1)</td>
<td>14 (42.4)</td>
<td>31 (23.3)</td>
</tr>
<tr>
<td>Outcomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality – n (%)</td>
<td>45 (27.1)</td>
<td>1 (3.0)</td>
<td>44 (33.0)</td>
</tr>
<tr>
<td>Neurosurgery performed – n (%)</td>
<td>47 (28.3)</td>
<td>5 (15.1)</td>
<td>42 (31.6)</td>
</tr>
<tr>
<td>Sepsis/infection – n (%)</td>
<td>42 (25.3)</td>
<td>7 (21.2)</td>
<td>35 (26.3)</td>
</tr>
<tr>
<td>Organ failure – n (%)</td>
<td>18 (10.8)</td>
<td>0 (0.0)</td>
<td>18 (13.5)</td>
</tr>
<tr>
<td>6-month GOSE</td>
<td>4.0 ± 2.5</td>
<td>5.7 ± 2.1</td>
<td>3.6 ± 2.5</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, traumatic brain injury; ED, emergency department; ISS, injury severity score; AIS, abbreviated injury scale; GCS, Glasgow Coma Scale; GOSE, Glasgow Outcome Scale Extended.

Unless otherwise stated, results are expressed as mean ± standard deviation (SD).

2.4 Results

2.4.1 Demographics and clinical characteristics

Table 2.1 summarizes the demographic, clinical, and outcome data for the 166 (33 moderate, 133 severe) patients analyzed in the study. Subjects were predominantly male (n = 124, 74.7%), with an average age of 45.8 ± 20.3 years. The majority of patients had an unfavorable outcome at 6 months, classified as a GOSE score of 1-4 (n = 102, 61.4%). Eighteen patients (10.8%) developed organ failure, and there were 45 (27.1%) deaths. Among the 21
healthy control subjects, 71.4% were male (n = 15), and the mean age was 32.7 ± 7.8 years (data not shown).

2.4.2 Plasma concentrations of inflammatory markers in Moderate and Severe TBI patients

Plasma concentrations of cytokines and chemokines stratified according to moderate (GCS 9-12) and severe (GCS 3-8) TBI are shown in Figure 2.1. IL-10 was the only cytokine altered in patients compared with healthy controls – median admission IL-10 levels were 5- and 9-fold higher, respectively, in moderate and severe TBI patients, and were significantly elevated at all time points (Figure 2.1, panel A). Furthermore, at 6, 12, and 24 h after hospital admission, mean IL-10 levels in severe TBI patients were significantly elevated (~2-fold at admission, 6, and 24 h) compared to moderate TBI patients (Figure 2.1, panel A). Please see Appendix 2.1 and Appendix 2.2 for individual biomarker assay detectability and concentrations in TBI patients, respectively.
Figure 2.1 – Plasma cytokine and chemokine concentrations in moderate and severe TBI patients sampled over 24 h. Cytokines interleukin (IL)-10, tumor necrosis factor (TNF)-α (A & B, respectively), and chemokines Eotaxin, Eotaxin-3, interferon-gamma induced protein (IP)-10, IL-8, monocyte chemoattractant protein (MCP)-1, -4, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC) (C-K, respectively) in moderate (GCS 9-12, n=33, open squares) and severe (GCS 3-8, n=133, closed squares) TBI patients within the first 24 h of hospital admission vs. healthy control subjects (no TBI, n=21, open circles). Lines represent the median and interquartile range. * = p<0.05 vs. healthy controls by Kruskal-Wallis. †p<0.05 vs. moderate TBI by Mann-Whitney U test.
Significant alterations in all 9 chemokines were detected in TBI patients dichotomized by injury severity, vs. healthy controls (Figure 2.1, panels C-K). The most dramatic increase was seen in IL-8: patient concentrations peaked at 6 h after hospital admission and were nearly 3.5-fold higher in those with severe TBI vs. healthy control subjects (median concentrations, 12.2 vs. 3.5 pg/mL, respectively) (Figure 2.1, panel F). In addition, at admission and 6 h, mean IL-8 levels were significantly higher in patients with severe TBI compared to those with moderate TBI (Figure 2.1, panel F). Significant increases in MCP-1 concentrations in severe vs. moderate TBI patients were also identified (6 h) (Figure 2.1, panel G). Conversely, IP-10 and MDC concentrations were decreased in both moderate and severe TBI patients compared with controls (Figure 2.1, panels E & I, respectively), while eotaxin was decreased in severe TBI patients only (Figure 2.1, panel C). MCP-4 levels were significantly elevated on admission in moderate TBI patients compared with healthy subjects, but were significantly lower in severe TBI patients compared with healthy subjects at 12 and 24 h (Figure 2.1, panel H). Furthermore, MCP-4 levels were significantly lower in severe TBI patients at all sampled time points compared to moderate TBI patients (Figure 2.1, panel H). Eotaxin levels were elevated in severe TBI patients at admission, but like MCP-4, were significantly decreased at 12 h and 24 h (Figure 2.1, panel C).

2.4.3 Correlation between SNS and inflammatory markers

Pooled concentrations of NE and Epi were associated with a number of inflammatory markers acutely after TBI (Table 2.2). IL-1β and IL-10 were positively correlated to Epi, with IL-10 displaying the strongest correlation ($r = 0.44, P < 0.01$) (Table 2.2). NE concentrations were positively related to IL-1β, IL-10 and TNF-α (Table 2.2). Similar to Epi, NE was most strongly correlated to IL-10 ($r = 0.45, P < 0.01$). In addition, IL-1β displayed a stronger positive association with NE ($r = 0.28, P < 0.01$) than with Epi ($r = 0.11, P < 0.03$) (Table 2.2).
Chemokines Eotaxin, IL-8 and MCP-1 were positively related to both NE and Epi, while IP-10 was associated with NE only (Table 2.2). The strongest relationship between catecholamines and chemokines was found between IL-8 and NE ($r = 0.39, P < 0.01$); IL-8 also displayed the highest correlation of any chemokine with Epi ($r = 0.35, P < 0.01$) (Table 2.2).

**Table 2.2 SNS and inflammatory marker correlations**

<table>
<thead>
<tr>
<th>Cytokines (pg/mL)</th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.11</td>
<td>0.28</td>
</tr>
<tr>
<td>IL-5</td>
<td>-0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.44</td>
<td>0.45</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.00</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemokines (pg/mL)</th>
<th>Epinephrine</th>
<th>Norepinephrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td>IP-10</td>
<td>-0.00</td>
<td>0.10</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.23</td>
<td>0.33</td>
</tr>
<tr>
<td>MCP-4</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>MDC</td>
<td>-0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>-0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>TARC</td>
<td>-0.05</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Abbreviations: SNS, sympathetic nervous system; IL, interleukin; TNF-α, tumor necrosis factor - alpha; IP-10, interferon-gamma induced protein - 10; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP-1β, macrophage inflammatory protein – 1β; TARC, thymus and activation regulated chemokine.

* $= p < 0.05$ via spearman’s $p$
Figure 2.2 - Plasma cytokine and chemokine concentrations in TBI patients stratified according to 6-month GOSE. Cytokines interleukin (IL)-1β, -10, tumor necrosis factor (TNF)-α (A-C, respectively), and chemokines IL-8, monocyte chemoattractant protein (MCP)-1, macrophage-derived chemokine (MDC) (D-F, respectively) in TBI patients with unfavorable (GOSE 1-4, n=102) vs. favorable (GOSE 5-8, n=61) 6-month neurological outcome. Lines represent the median and interquartile range. *p<0.05 vs. favorable outcome by Mann-Whitney U test.

2.4.4 6-month neurological outcome

Within 24 h of hospital admission, significant differences in plasma levels of 3 cytokines and 3 chemokines were observed between patients with favorable and unfavorable 6-month neurological outcome (Figure 2.2, Panels A-C & D-F, respectively). IL-10 displayed the most profound difference of all cytokines assessed: concentrations at 6 h were near 3-fold higher in patients with unfavorable vs. favorable 6-month outcome (median concentrations, 13.3 vs. 4.5 pg/mL, respectively), and were significantly elevated at all sampled time points (Figure 2.2, Panel B). In addition, both IL-1β and TNF-α concentrations were elevated at 6 and 12 h in patients with unfavorable vs. favorable outcome (Figure 2.2, panels A & C, respectively). IL-8
levels were significantly elevated at all sampled time points, with a peak difference at 6 h (13.6 vs. 8.7 pg/mL in unfavorable vs. favorable outcome, respectively), while MCP-1 concentrations were significantly elevated at admission and 24 h in patients with unfavorable vs. favorable outcome (Figure 2.2, panels D & E, respectively).

2.4.5 Mortality

Alterations in inflammatory marker concentrations were observed in patients who lived vs. those who died; differences were noted in 3 cytokines and 8 chemokines (Figure 2.3, panels A-C & D-J, respectively). IL-10 levels were elevated at 6, 12, and 24 h after admission in patients who died compared to those who lived. In non-survivors, IL-1β concentrations were 2-fold higher at 12 h (median concentrations, 0.9 vs. 0.4 pg/mL, respectively), and IL-10 concentrations were 4-fold higher at 6 h (median concentrations, 28.1 vs. 6.5 pg/mL, respectively) (Figure 2.3, panels A & B, respectively). Similar to 6-month neurological outcome, IL-8 levels displayed the greatest elevation of all chemokines assessed in patients who died vs. those who lived (Figure 2.3, panel D). IL-8 concentrations were significantly elevated at all sampled time points, with the greatest disparity at 6 h, where patients who died had concentrations >2-fold higher than those who lived (median concentrations, 23.9 vs. 9.3 pg/mL, respectively) (Figure 2.3, panel D). Conversely, MCP-4, MDC and TARC concentrations at 24 h after hospital admission were significantly lower in TBI patients who died compared to survivors (Figure 2.3, panel G, H & J, respectively).
Figure 2.3 - Plasma cytokine and chemokine concentrations in TBI patients stratified by mortality. Cytokines interleukin (IL)-1β, -10, tumor necrosis factor (TNF) -α (A–C, respectively), and chemokines IL-8, interferon-gamma producing protein (IP)-10, monocyte chemoattractant protein (MCP) -1, -4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), Eotaxin (D–K, respectively) in TBI patients who died (n=45) vs. those who lived (n=119). Lines represent the median and interquartile range. *p<0.05 vs. lived by Mann-Whitney U test.
Table 2.3 Binomial logistic regression models assessing the ability of inflammatory markers to predict poor patient outcome, controlling for injury severity.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Admission</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>6</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P value</td>
<td>OR</td>
<td>P value</td>
<td>OR</td>
<td>P value</td>
<td>OR</td>
</tr>
<tr>
<td><strong>Unfavorable 6-month GOSE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.84</td>
<td>0.489</td>
<td>1.84*</td>
<td>0.002</td>
<td>1.65*</td>
<td>0.009</td>
<td>0.91</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.18</td>
<td>0.376</td>
<td>1.76*</td>
<td>0.005</td>
<td>1.57*</td>
<td>0.019</td>
<td>1.48*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.49†</td>
<td>0.024</td>
<td>1.61*</td>
<td>0.008</td>
<td>1.50*</td>
<td>0.021</td>
<td>1.30</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.66*</td>
<td>0.007</td>
<td>1.48*</td>
<td>0.030</td>
<td>1.44*</td>
<td>0.036</td>
<td>1.33</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.42</td>
<td>0.052</td>
<td>1.09</td>
<td>0.603</td>
<td>1.24</td>
<td>0.213</td>
<td>1.39</td>
</tr>
<tr>
<td>MDC</td>
<td>1.10</td>
<td>0.586</td>
<td>0.90</td>
<td>0.515</td>
<td>1.45</td>
<td>0.037*</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.02</td>
<td>0.935</td>
<td>1.06</td>
<td>0.825</td>
<td>1.85*</td>
<td>0.038</td>
<td>1.15</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.19</td>
<td>0.417</td>
<td>2.82*</td>
<td>&lt;0.001</td>
<td>2.09*</td>
<td>0.003</td>
<td>2.20*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.58*</td>
<td>0.027</td>
<td>1.77*</td>
<td>0.007</td>
<td>1.58*</td>
<td>0.033</td>
<td>1.19</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.31</td>
<td>0.168</td>
<td>1.89*</td>
<td>0.005</td>
<td>1.84</td>
<td>0.010*</td>
<td>1.37</td>
</tr>
<tr>
<td>IP-10</td>
<td>3.11*</td>
<td>&lt;0.001</td>
<td>2.06*</td>
<td>0.002</td>
<td>1.59*</td>
<td>0.030</td>
<td>1.19</td>
</tr>
<tr>
<td>MCP-1</td>
<td>1.26</td>
<td>0.230</td>
<td>1.46</td>
<td>0.069</td>
<td>1.57*</td>
<td>0.035</td>
<td>0.91</td>
</tr>
<tr>
<td>MCP-4</td>
<td>1.43</td>
<td>0.090</td>
<td>1.49</td>
<td>0.065</td>
<td>0.99</td>
<td>0.964</td>
<td>0.57*</td>
</tr>
<tr>
<td>MDC</td>
<td>1.03</td>
<td>0.864</td>
<td>0.91</td>
<td>0.620</td>
<td>1.14</td>
<td>0.529</td>
<td>0.71</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>1.13</td>
<td>0.544</td>
<td>1.27</td>
<td>0.243</td>
<td>1.29</td>
<td>0.221</td>
<td>0.89</td>
</tr>
<tr>
<td>TARC</td>
<td>0.95</td>
<td>0.801</td>
<td>1.00</td>
<td>0.991</td>
<td>0.77</td>
<td>0.231</td>
<td>0.56*</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>1.90*</td>
<td>0.005</td>
<td>1.90*</td>
<td>0.007</td>
<td>1.33</td>
<td>0.208</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Abbreviations: OR, odds ratio; GOSE, Glasgow outcome scale extended; IL, interleukin; TNF-α, tumor necrosis factor - alpha; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; IP-10, interferon-gamma induced protein - 10; MIP-1β, macrophage inflammatory protein - 1β; TARC, thymus and activation regulated chemokine.

Models were controlled for admission GCS and AIS Head scores.

All blood biomarker concentrations were standardized by quartiles. A one-unit increase is equivalent to a 25% increase in biomarker concentration.

* = \( p < 0.05 \)
2.4.6 Secondary complications controlled for injury severity

2.4.6.1 6-month GOSE

When controlled for admission GCS and AIS head scores, and standardized according to ranked quartiles, cytokines IL-1β (peak, 6 h; OR [odds ratio], 1.84; 95% confidence interval [CI], 0.90 – 2.25) IL-10 (peak, 6 h; OR, 1.76; 95% CI, 1.25 – 2.70) and TNF-α (peak, 6 h; OR, 1.61; 95% CI, 1.13 – 2.29) were significant independent predictors of unfavorable 6-month outcome (GOSE 1-4) (Table 2.3). In addition, chemokines IL-8 (peak, admission; OR, 1.66; 95% CI, 1.15 – 2.39) and MDC (peak, 12 h; OR, 1.45; 95% CI, 1.02 – 2.05) were independent predictors of unfavorable outcome (Table 2.3).

2.4.6.2 Mortality

Controlled for admission GCS and AIS head scores, patient death was independently associated with 3 cytokines and 4 chemokines (Table 2.3). IL-10 was the greatest predictor of death of all cytokines analyzed (peak, 6 h; OR, 2.82; 95% CI, 1.63 – 4.87). However, the strongest predictor of death among chemokines as well as all evaluated inflammatory markers was IP-10 (peak, admission; OR, 3.11; 95% CI, 1.83 – 5.27) (Table 2.3).
**Figure 2.4 – Plasma cytokine and chemokine concentrations in TBI patients according to cause of death.** Cytokines interleukin (IL)-1β, -10, tumor necrosis factor (TNF)-α (A-C, respectively), and chemokines IL-8, interferon-gamma producing protein (IP)-10, monocyte chemoattractant protein (MCP) -1, and macrophage inflammatory protein (MIP)-1β D-G, respectively) in TBI patients who survived (n=119) vs. those who died by neurologic death (n=28) or by non-neuologic organ failure (n=17). Box’s represent the median and interquartile ranges, and whisker plot lines represent the range. *p<0.05 vs. patients who survived, and †p<0.05 vs. patients who succumbed to neurologic death, by Kruskal-Wallis.

### 2.4.7 Neurologic vs non-neurologic death

Differences in inflammatory biomarkers were observed between patients who lived vs. those who died by neurologic or non/neurologic organ failure. IL-1β was significantly elevated in patients who died by neurologic death vs. those who survived at 6, 12, and 24 h; no difference was found between those who died by organ failure and those who survived (Figure 2.4, Panel A). IL-10 levels were elevated in patients succumbing to both neurologic and non-neurologic death compared to those who survived, at all time points except admission (Figure 2.4, Panel B). Similarly, IL-8 concentrations were elevated in neurologic and non-neurologic death compared to patients who died at 6 h, but were elevated only in patients succumbing to neurologic death at all other sampled time points (Figure 2.4, Panel C). MCP-1 concentrations were elevated in neurologic death vs. survival at admission, 6, and 12 h, but were lower at 24 h in patients who died by non-neurologic organ failure vs. neurologic death (Figure 2.4, Panel F). In addition, MIP-1β concentrations were significantly lower in patients who died by non-neurologic organ failure vs. neurologic death at 6 and 24 h (Figure 2.4, Panel G).

### 2.4.8 Combined inflammatory score and patient characteristics

A combined IS was created in a subset of 76 patients using 3 cytokines (IL-1β, -10, and TNF-α) and 3 chemokines (IL-8, MCP-1, MDC) that were associated with both unfavorable patient outcome at 6 months and mortality. Compared to patients with low a IS (<15), patients
with a high IS ($\geq 15$) had significantly greater mean NE levels over 24 h from hospital admission, while Epi levels did not statistically differ (Table 2.4). Greater injury severity, as assessed by admission GCS, AIS head and ISS scores, was associated with a High IS. In addition, significantly more patients with an unfavorable 6-month GOSE score or who died had high IS scores (Table 2.4).

Table 2.4 Clinical characteristics according to dichotomized inflammatory scores.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Inflammation - Low (IS &lt; 15, n = 33)</th>
<th>Inflammation - High (IS $\geq 15$, n = 43)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catecholamine levels (pmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epi</td>
<td>1131.5 (656.0 – 1901.9)</td>
<td>1655.0 (733.2 – 4307.4)</td>
<td>0.110</td>
</tr>
<tr>
<td>NE</td>
<td>6064.3 (3081.0 – 18373.4)</td>
<td>12110.0* (7653.1 – 37939.8)</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>Injury severity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCS</td>
<td>7.0 (4.0 – 9.0)</td>
<td>3.0* (3.0 – 7.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>AIS head</td>
<td>4.0 (3.0 – 5.0)</td>
<td>5.0* (4.0 – 5.0)</td>
<td>0.006</td>
</tr>
<tr>
<td>ISS</td>
<td>20.5 (16.5 – 29.5)</td>
<td>29.5* (20.0 – 35.0)</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Outcome (n)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepsis/infection</td>
<td>13</td>
<td>9</td>
<td>0.079</td>
</tr>
<tr>
<td>Unfavorable 6-month GOSE</td>
<td>14</td>
<td>33*</td>
<td>0.002</td>
</tr>
<tr>
<td>Overall mortality</td>
<td>4</td>
<td>16*</td>
<td>0.014</td>
</tr>
<tr>
<td>Neurologic death</td>
<td>2</td>
<td>13*</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Abbreviations: IS, inflammation score; Epi, epinephrine; NE, norepinephrine; GCS, Glasgow coma scale; AIS, abbreviated injury scale; ISS, injury severity score; GOSE, Glasgow outcome scale extended.

Values described as the median and interquartile range unless otherwise stated.

IS variables consist of peak differences over 24 h in: IL-1β (6 h), IL-10 (6 h), TNF-α (6 h), IL-8 (6 h), MCP-1 (Admission), and MDC (12 h).

* Average catecholamine concentrations over 24 h.

*p < 0.05 vs. low inflammatory score by Mann Whitney $U$, or $Ch^2$, where appropriate.
2.5 Discussion

In this study, we demonstrated that following isolated TBI, acute alterations in systemic cytokine and chemokine levels were associated with unfavorable patient outcome and death. Our sample size of 166 patients is, to our knowledge, the largest to study to date to characterize the systemic inflammatory response in isolated TBI. A compelling finding of this work was the identification of a relationship between SNS activity and systemic inflammation acutely post-injury. In addition, our findings addressed the heterogeneous and complex nature of TBI, which has confounded our understanding of the pathophysiology and clinical relevance of post-traumatic systemic inflammation (Hinson, Rowell et al. 2015). In order to address this heterogeneity, our study population comprised isolated TBI patients with non-penetrating injuries, stratified according to injury severity, and sampled acutely at four time points over the first 24 h from hospital admission.

We found the anti-inflammatory IL-10 was the most prominently altered of all cytokines acutely after injury, and was positively correlated with injury severity. While this is in general agreement with previous findings (Shimonkevitz, Bar-Or et al. 1999, Hensler, Sauerland et al. 2000, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014), few studies have assessed IL-10 in isolated TBI patients (Hensler, Sauerland et al. 2000, Schneider Soares, Menezes de Souza et al. 2012). Furthermore, we are aware of only one previous study that evaluated IL-10 in isolated TBI patients dichotomized by injury severity, albeit in a population of 26 patients that assessed injury severity at 7-days post-injury (Woiciechowsky, Schoning et al. 2002). Also, in agreement with previous works, unfavorable patient outcome and mortality were associated with elevated peripheral concentrations of IL-1β, IL-10 and TNF-α (Tasci, Okay et al.
2003, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014, Kumar, Boles et al. 2015). Importantly, the relationships between cytokines and patient outcome were significant even after controlling for injury severity, suggesting a possible independent role in secondary injury pathogenesis. Moreover, our results provide evidence that certain cytokines may be associated with specific outcomes, for example, IL-1β and TNF-α were elevated only in patients who died from neurologic death, while IL-10 was higher in patients who died as a result of both neurologic and non-neurologic organ failure. Taken together, these findings not only reinforce the globally detrimental role of elevated IL-10 acutely after TBI, but also suggest that peripheral blood may be a viable source for biomarkers related to brain injury-specific outcomes.

Systemic concentrations of all 9 chemokines analyzed were significantly different in TBI patients compared to healthy subjects, and alterations in 8 of 9 chemokines were associated with poor patient outcome. In addition, we found Eotaxin-3, IL-8, MCP-1 and -4 were associated with injury severity. Similar to our cytokine analysis, when controlled for injury severity, 7 of 9 chemokines were still related to poor patient outcome, with increases in admission IP-10 levels displaying the strongest relationship to death of all inflammatory markers analyzed. Notably, while higher IP-10 levels in patients were independently associated with mortality on average, levels were lower in patients vs. healthy controls. The reasons for this are unclear, but it is possible that our total patient values were skewed by lower IP-10 concentrations in survivors vs. those who died. Furthermore, that a number of chemokines were lower in patients compared to controls, or lower in patients with unfavorable vs. favorable outcomes, may speak to the complex and divergent roles of chemokines in mediating secondary injury. Our findings are in general agreement with others who have identified elevations in systemic IL-8 and MCP-1 after TBI (Maier, Schwerdtfeger et al. 2001, Mussack, Biberthaler et al. 2002, Rhodes, Sharkey et al. 2009, Sohrevardi, Ahmadinejad et al. 2013, Buonora, Yarnell et al. 2015), and identified the
relationship between this and poor patient outcome (Kushi, Saito et al. 2003, Gopcevic, Mazul-Sunko et al. 2007, Di Battista, Buonora et al. 2015). However, to our knowledge no previous studies have characterized this diverse array of chemokines in the peripheral blood after human TBI. Interestingly, Helmy, Carpenter et al. (2011), evaluated a number of cytokines and chemokines in the brain extracellular fluid in 12 TBI patients post-injury. These authors concluded that amongst other inflammatory mediators evaluated, IL-8, MCP-1, IP-10 and MIP-1β were elevated in the brain extracellular fluid relative to plasma, and may thus be centrally produced after TBI. However, this study did not evaluate the plasma levels of these markers in comparison to healthy controls, and subsequently the relative systemic changes were not determined. Furthermore, Chen et al., (Chen, Wu et al. 2016), recently found serum levels of CXCL12 were significantly related to injury severity and patient death after isolated human TBI. Taken together, in view of these recent findings and those of the current study which support the deleterious involvement of IL-8 and MCP-1 in post-injury secondary pathology, as well as the independent association found between 7 different chemokines and poor patient outcomes, suggests a prominent role for systemic chemokines in acute secondary injury, and warrants further investigation.

We identified a number of specific correlations between systemic catecholamines and inflammatory mediators that are consistent with previous experimental findings. For example, Woiciechowsky, Asadullah et al. (1998), found that SNS activation after brain trauma results in the systemic release of IL-10. The authors showed that NE and Epi signaling through peripheral monocyte β2-adrenergic receptors caused an increase in circulating IL-10 concentrations, deactivation of circulating monocytes, and subsequent immunosuppression and infection (Woiciechowsky, Asadullah et al. 1998). That we found a strong association between both NE, Epi and IL-10 is consistent with the experimental findings of Woiciechowsky et al, and suggests
a mechanistic role for catecholamines in mediating IL-10 production in the acute period after isolated TBI. Additionally, it has been hypothesized that IL-1β may induce SNS activation after brain trauma (Saindon, Blecha et al. 2001, Kenney, Blecha et al. 2002, Woiciechowsky and Volk 2005): experimental studies have shown that both CNS and peripherally injected IL-1β has the ability to stimulate the SNS (Woiciechowsky, Schoning et al. 1999, Kenney, Blecha et al. 2002), which may then mediate leukocyte mobilization and the initiation of the hepatic acute phase response (Campbell, Wilcockson et al. 2002, Kenney, Blecha et al. 2002, Wilcockson, Campbell et al. 2002, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005). This is also consistent with the associations found between IL-1β and both NE/Epi in the current study. Furthermore, we identified a positive correlation between NE, Epi, and IL-8, and MCP-1. It has been suggested that the production and release of chemokines from the liver, particularly IL-8 and MCP-1, is an important component of the systemic acute-phase response after TBI (Campbell, Wilcockson et al. 2002, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005, Catania, Lonati et al. 2009), particularly in mediating the mobilization and recruitment of leukocytes to the brain (Campbell, Wilcockson et al. 2002, Campbell, Hughes et al. 2003, Campbell, Jiang et al. 2007, Giles, Greenhalgh et al. 2015). In a review by Catania and colleagues (2009), it was hypothesized that systemic chemokine production and release from the liver post-injury may be mediated by SNS activation, and specifically the activation of sympathetic nerve terminals and subsequent interaction between tissue macrophages and synaptic NE (Campbell, Hughes et al. 2003). In addition, NE has been found to interact with β-ARs on cultured peripheral blood mononuclear cells to produce MCP-1 (Takahashi, Tsuda et al. 2004), and Epi has been shown in multiple studies to potentiate lipopolysaccharide (LPS) induced IL-8 production in monocytes (Kavelaars, van de Pol et al. 1997, van der Poll and Lowry 1997). While the specific
mechanism(s) have yet to be determined, the results of the current study are supportive of catecholamine mediated chemokine production after isolated TBI.

While the pathogenesis of systemic inflammation after TBI remains uncertain, our results provide evidence consistent with a detrimental role for the innate immune system in the acute period after injury, and further supports the concept that SNS hyperactivity mediates this process. Indeed, the application of an inflammation-based prognostic score showed that the net inflammatory effect seen acutely after TBI is associated with poor patient outcomes, injury severity, and significant elevations in NE. Furthermore, our findings are generally consistent with the revised interpretation of the systemic inflammatory response syndrome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) noted after trauma and sepsis, which suggests that pro- and anti-inflammatory processes occur concurrently, not in a phase delayed manner as was previously hypothesized (Osuchowski, Welch et al. 2006, Gentile, Cuenca et al. 2012). We and others have consistently observed that the anti-inflammatory molecule IL-10 is among the earliest detectable mediators after trauma (Woiciechowsky, Schoning et al. 2002, Rizoli, Rhind et al. 2006, Ferreira, Regner et al. 2014), and is elevated concurrently with pro-inflammatory IL-1β, IL-8, TNF-α and numerous other chemokines in patients with poor outcomes. Notably, we did not find any association between acute inflammation and the onset of sepsis/infections, however, this study did not directly characterize cellular immune function; previous studies have reported that immunosuppression and subsequent infection after brain trauma may be related to impaired cellular immunity, including monocyte deactivation (Woiciechowsky, Asadullah et al. 1998, Rizoli, Rhind et al. 2006) and/or suppression of T cell function (Quattrocchi, Frank et al. 1990, Wolach, Sazbon et al. 2001).
The results of this study should be interpreted in the context of its limitations. Despite a well-controlled clinical design and moderately sized cohort of 166 TBI patients, a larger sample size would have allowed for further stratification of our patient population, particularly regarding variables such as sex and age, and isolated sepsis. Also, we were only able to assess the level of inflammatory markers in patients’ blood samples for the first 24 h post-injury, and a longer sampling period may have been advantageous to assess the potential for hyperadrenergic-mediated immunosuppression. In spite of these limitations, this investigation is one of the most comprehensive inflammatory studies in isolated human TBI to date. The results demonstrated a pronounced systemic cytokine/chemokine response acutely after isolated head injury, and suggest that this response is associated with the degree of SNS activation.

2.6 Conclusion

Several peripheral cytokines and chemokines are altered in the acute period after moderate and severe isolated TBI. These alterations are associated with unfavorable patient outcomes. Moreover, the systemic inflammatory response after TBI appears to be mediated, at least in part, by a profound trauma-induced hyperadrenergic state. In the present study, we found marked increases in circulating IL-10 and alterations in all chemokines assessed within 24 h of hospital admission. Poor patient outcome was associated with alterations in IL-1β, IL-10, and TNF-α, and all chemokines with the exception of eotaxin-3. Furthermore, circulating NE and Epi levels were positively correlated with IL-1β, IL-10, IL-8, Eotaxin and MCP-1. Future controlled clinical studies should continue to emphasize potential therapeutic interventions that modulate excessive SNS activation and inflammation, including treatment with selective adrenergic blocking agents.
Chapter 3
Sympathoadrenal Activation is Associated with Acute Traumatic Coagulopathy and Endotheliopathy in Isolated Brain Injury

The components of this chapter were published as follows:


The lead author contributed to the manuscripts conceptual framework, performed all cytokine/chemokine, and adhesion molecule immunoassay experiments, statistically analyzed and interpreted the data, drafted the manuscript, and contributed to its critical revision.
3 Sympathoadrenal Activation is Associated with Acute Traumatic Coagulopathy and Endotheliopathy in Isolated Brain Injury

3.1 Abstract

**BACKGROUND:** Acute coagulopathy after traumatic brain injury (TBI) involves a complex multifactorial hemostatic response that is poorly characterized. **OBJECTIVES.** To examine early posttraumatic alterations in coagulofibrinolytic, endothelial and inflammatory blood biomarkers in relation to sympathetic nervous system (SNS) activation and 6-month patient outcomes, using multivariate partial least squares (PLS) analysis. **METHODS.** A multi-center observational study of 159 adult isolated TBI patients admitted to the emergency department at an urban level I trauma center was performed. Plasma concentrations of 6 coagulofibrinolytic, 10 vascular endothelial, 19 inflammatory, and 2 catecholamine biomarkers were measured by immunoassay on admission and 24 hours post-injury. Neurological outcome at 6 months was assessed using the extended Glasgow outcome scale (GOSE). PLS discriminant analysis (PLS-DA) was employed to identify salient biomarker contributions to unfavorable outcome, while PLS regression analysis was used to evaluate the covariance between SNS correlates (catecholamines) and biomarkers of coagulopathy, endotheliopathy, and inflammation. **RESULTS.** Biomarker profiles in patients with an unfavorable outcome displayed pro-coagulation, hyperfibrinolysis, glycocalyx and endothelial damage, vasculature activation, and inflammation. A strong covariant relationship was evident between catecholamines and biomarkers of coagulopathy, endotheliopathy, and inflammation both at admission and 24 h post-injury. **CONCLUSIONS.** Biomarkers of coagulopathy and endotheliopathy are associated with
poor outcome after TBI. Catecholamine levels were highly correlated with endotheliopathy and coagulopathy markers within the first 24 h after injury. Further research is warranted to characterize the pathogenic role of SNS-mediated hemostatic alterations in isolated TBI.

3.2 Introduction

TBI is a major cause of death and disability in both civilian and military populations (Reid and Velez 2015). Although less common than mild head injuries (Garber, Rusu et al. 2014), moderate-to-severe TBI is often life threatening (Tien, Acharya et al. 2010), causing secondary injury complications that dramatically worsen patient outcome (Pannell, Brisebois et al. 2011). Despite wartime advances in damage control resuscitation and surgery (Tien, Beckett et al. 2015), trauma-associated coagulopathy in critically injured casualties remains an urgent concern both on and off the battlefield.

Acute traumatic coagulopathy is a global failure of the hemostatic system frequently reported in isolated TBI (Wafaisade, Lefering et al. 2010, Epstein, Mitra et al. 2014, Joseph, Aziz et al. 2014). The cited incidence of coagulopathy after TBI varies widely from 10–97% (Harhangi, Kompanje et al. 2008), but when present, coagulofibrinolytic derangements are strongly associated with increased mortality, transfusion requirements, organ failure, and hospital stay (Epstein, Mitra et al. 2014, Abdelmalik, Boorman et al. 2016). Both hypo- and hyper-coagulable states have been described (Maegele 2013). Unfortunately, the pathophysiological mechanisms of acute trauma-induced coagulopathy are not well defined, owing to the complex underlying biology, limitations of current laboratory diagnostic tests (Hemker, Al Dieri et al. 2004, Park, Martini et al. 2009), and a lack of consensus on clinical terminology (Dobson, Letson et al. 2015). Moreover, it is also unclear if coagulopathy in isolated TBI differs from multisystem trauma in either incidence (Harhangi, Kompanje et al. 2008, Cap
and Spinella 2011, Genet, Johansson et al. 2013) or pathogenesis (Genet, Johansson et al. 2013, de Oliveira Manoel, Neto et al. 2015). In order to improve patient care and advance therapeutic options, a better understanding of acute coagulopathy and how it contributes to poor patient outcomes is needed (Davenport and Brohi 2016).

Hemostasis involves the tightly regulated balance between coagulation and fibrinolysis that permits control of bleeding while preventing intravascular thrombosis (Versteeg, Heemskerk et al. 2013). Following injury, clotting is initiated when tissue damage exposes subendothelial TF that activates coagulation factors to produce thrombin and fibrin (Schafer 2007). The brain is a rich source of procoagulant TF (Goodnight, Kenoyer et al. 1974, Stein and Smith 2004), and while it is generally agreed upon that brain damage triggers elevated circulating TF levels and subsequent coagulopathy (Gando, Nanzaki et al. 1999, Stein and Smith 2004, Cohen, Brohi et al. 2007), there is no consensus of the sequelae that follow. Previous reports have suggested widespread TF release elicits disseminated intravascular coagulation (DIC), where consumption of clotting factors leads to excessive bleeding (Gando, Nanzaki et al. 1999, Stein and Smith 2004). However, others have refuted this, suggesting an immediate hypocoagulable response without the consumption of clotting factors, mediated by activation of protein C (Cohen, Brohi et al. 2007).

Recent studies in multisystem trauma suggest SNS activation drives coagulopathy through endothelial damage/dysfunction, particularly by glycocalyx disruption (Ostrowski and Johansson 2012, Johansson, Haase et al. 2014, Ostrowski, Haase et al. 2015). In addition, we recently demonstrated a link between SNS hyperactivity and inflammation in isolated TBI (Di Battista, Rhind et al. 2016). Indeed, the reciprocal role of inflammation in coagulopathy is well characterized (Levi and van der Poll 2010, van der Poll, de Boer et al. 2011); inflammatory
cytokines promote a procoagulant state through upregulation of TF-mediated thrombin production, and thrombin itself can be immunogenic in stimulating immune cells to secrete inflammatory mediators (Levi and van der Poll 2010, van der Poll, de Boer et al. 2011). Hence, the SNS can influence coagulation via multiple pathways, both directly and indirectly. However, there is currently limited understanding of the interrelationships between the SNS, endotheliopathy, inflammation and coagulation in the acute period after TBI.

Divergent findings in TBI-related coagulopathy have arisen, in part, from the routine use of ex-vivo clotting tests to assess coagulation abnormalities. The INR, aPTT, and platelet (PLT) count are the most commonly used diagnostic screens to identify coagulopathy (Talving, Benfield et al. 2009, Wafaisade, Lefering et al. 2010, Cap and Hunt 2015). However, these conventional tests measure independent features of the clotting process, do not assess the termination phase of coagulation, and are unable to identify hypercoagulation (Hemker, Al Dieri et al. 2004, Park, Martini et al. 2009, Dobson, Letson et al. 2015). In view of this, blood biomarkers can be informative in developing our understanding of secondary injury cascades after TBI (Papa 2012, Di Battista, Rhind et al. 2016), and several candidate markers, such as DD, SDC-1 and TM, have shown potential in characterizing posttraumatic coagulopathy (Yokota, Naoe et al. 2002, Ostrowski, Berg et al. 2013, Ostrowski, Gaini et al. 2015). Thus, a comprehensive assessment of biological mediators of coagulopathy and endotheliopathy may improve our understanding of the multifactorial hemostatic responses to injury (White, Contaifer et al. 2015).

The purpose of this study was to characterize peripheral blood biomarkers of coagulopathy and endotheliopathy acutely after isolated TBI, according to 6-month patient outcomes, using multivariate partial least squares (PLS) analysis. Furthermore, we sought to
evaluate the potential covariance between these markers and SNS correlates in order to provide evidence to support or refute sympathoadrenal hyperactivity as a potential mechanistic driver of coagulopathy.

3.3 Methods

3.3.1 Study population and design

As part of a larger prospective observational cohort study (Da Luz, Capone Neto et al. 2015), this *a priori* subgroup analysis enrolled 159 adult patients with newly acquired traumatic brain injury at three Level-1 Trauma Centers, from November 2011 to September 2013. Patients were included in the study according to conventional criteria for isolated blunt moderate-to-severe TBI, defined by a GCS score < 13, and a non-head AIS ≤ 2. For complete patient recruitment and clinical data collected please see our previous works (Di Battista, Buonora et al. 2015, Di Battista, Rhind et al. 2016). The study was approved by the local Research Ethics Committees and Institutional Review Boards of all participating institutions. All patients or legal representatives were informed of the study details and provided their consent. A group (n=27) of healthy donors free from any medications and without a history of brain injury were included as a control reference in all measurements. All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

3.3.2 Blood Sample Collection and Processing

Venous blood samples were drawn from each patient as soon as possible after admission to the emergency department and again at 24-post-injury. Specimens for soluble biomarker analyses were obtained from patients and controls using an evacuated tube collection system containing K$_2$-ethylenediaminetetraacetic acid, lithium-heparin and Na$_3$-citrate anticoagulants (BD-Vacutainer®, Becton Dickinson, Rutherford, NJ, USA). Anticoagulated blood was
centrifuged immediately after sample collection for 20 min at 2,000g to obtain platelet-poor plasma, after which the plasma was separated into aliquots and frozen at −80°C until further analysis.

3.3.3 Hemostatic and endothelial marker analysis

Plasma coagulation and fibrinolytic biomarkers were analyzed in duplicate using commercially available IMUBIND quantitative enzyme-linked immunoassay (ELISA) kits (Sekisui Diagnostics, LLC, Lexington, MA) for TF, tissue factor pathway inhibitor (TFPI), thrombin activatable fibrinolysis inhibitor (TAFI), thrombin–antithrombin complexes (TAT), plasminogen activator inhibitor (PAI-1), tissue plasminogen activator (tPA), and DD. Soluble endothelial-derived biomarkers SDC-1 and vascular adhesion protein-1 (VAP-1) were analyzed by quantitative ELISA (BioVendor, LLC, Asheville, NC). Absorbencies for all plates were read using an automated microplate photometer (Synergy 2 Multi-Mode Reader with Gen5™ Software; BIO-TEK Instruments, Winooski, VT). All test samples were analyzed in duplicates according to the manufacturer’s instructions.

Cytokine, chemokine and vascular injury marker analysis

Plasma concentrations (pg/mL) of selected cytokines, chemokines and vascular molecules were analyzed batchwise using MSD® 96-Well MULTI-ARRAY/-SPOT® Ultra-Sensitive Human Immunoassay Kits: TH1/TH2 10-plex for IFN-γ, IL-1β, -2, -4, -5, -10, -12p70, -13, and TNF-α; Chemokine 9-Plex for Eotaxin, Eotaxin-3, MIP-1β, TARC, IP-10, IL-8, MCP-1, MDC, MCP-4; Vascular Injury 1 and 2 for TM, ICAM-3, E-Selectin, P-Selectin, serum amyloid A (SAA), CRP, vascular cell adhesion molecule (VCAM)-1 and ICAM-1. Analytes were detected by electrochemiluminescence on an MSD Sector Imager™ 6000 (Gaitherburg, MD). All assays were performed in duplicate according to manufacturer’s instructions.
3.3.4 Catecholamine analysis

Plasma catecholamine (Epi and NE) concentrations (pmol/L) were determined from duplicate samples using a competitive ELISA method according to the manufacturer’s instructions (Bi-CAT EIA, ALPCO Diagnostics, Salem, NH). Absorbance was measured using a multi-detection microplate reader (PerkinElmer VICTOR 3, Waltham, MA).

3.3.5 Statistical Analyses

Clinical, demographic, and laboratory variables were dichotomized according to 6-month GOSE (favorable, GOSE 5-8; unfavorable, GOSE 1-4) and statistically compared by chi-square, independent students t-test, or Mann Whitney U, where applicable. Coagulopathy was calculated using standard laboratory measures INR, aPTT, and PLT. Patients were considered coagulopathic if they had an INR >1.3 or aPTT >38 s or PLT <100 000/µL (Abdelmalik, Boorman et al. 2016). Multivariate PLS analysis was employed to characterize the relationships between blood biomarkers and clinical outcomes. PLS is a supervised technique which identifies optimal combinations of predictor variables that co-vary with either binary (termed PLS-Discriminant Analysis (PLS-DA)) or continuous response variables (Wold, Sjostrom et al. 2001). A PLS-DA output provides model prediction accuracy (Accur) and posterior probability (PProb). Briefly, these indices measure how accurately a fitted model can predict a binary outcome based solely on predictor variables. Accur is evaluated by assigning each subject to the outcome group with the most similar mean PLS score; 1 = correctly predicted, and 0 = incorrectly predicted. This provides a simple, robust metric of prediction, which does not depend on a specific probability model. PProb is the likelihood of the PLS model identifying the correct outcome conditional on the observed subject scores under a Gaussian noise model (Bishop 2006). This provides an alternative probabilistic measure that accounts for uncertainty in the PLS model and observed data. When the response variables are continuous, a PLS regression output provides the
fraction of variance. Fraction of variance reflects the proportion of total inter-subject variability in biomarker data that is described by the PLS component of interest. A high fraction of variance for a single component indicates that subject variability is "1-dimensional", and well-described by a single latent variable. Conversely, a low fraction of variance indicates more complex inter-subject differences, and cannot be fully captured within a single PLS component. First, PLS-DA analysis was used to identify significant biomarkers in discriminating unfavorable vs. favorable patient outcome. Previous research has identified that age and injury severity are associated with patient outcome after TBI (Talving, Benfield et al. 2009, Epstein, Mitra et al. 2014, Abdelmalik, Boorman et al. 2016). Therefore, to account for the influence of these factors, we included age, ISS, AIS head, and GCS in our models. Second, to assess the role of the SNS in mediating acute pathophysiology, PLS was used to identify the covariance between SNS indices (NE and Epi) and biomarkers of coagulopathy and endotheliopathy. All variables were imputed for missing data using the k-means nearest-neighbour method (Armitage, Godzien et al. 2015) and rank-transformed to ensure robustness against non-normality in biomarker values. Significant variable loadings were derived by performing bootstrap resampling on subjects (1000 iterations) to obtain empirical p-values, which were subsequently corrected for multiple comparisons at a false discovery rate (FDR) = 0.05. For all plots, variable loadings are represented as bootstrap ratios (i.e. the bootstrapped mean / standard error), which are z-scored statistics reflecting the reliability of variable contributions. Descriptive and univariate statistics were completed using Stata Version 14.1 (StataCorp, TX, USA), and multivariate statistics were analyzed using in-house software developed for Matlab, Version R2015b (Matworks, Natick MA). Graphs were prepared using GraphPad Prism Version 6.0h (GraphPad Inc, CA, USA).
### Table 3.1 Demographic and Clinical Characteristics of TBI patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients (n = 159)</th>
<th>Six-month neurological outcome</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Favourable (GOSE &gt; 5) (n = 59)</td>
<td>Unfavourable (GOSE &lt; 5) (n = 100)</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.8 ± 20.3</td>
<td>36.2 ± 15.7</td>
<td>51.5 ± 20.6</td>
</tr>
<tr>
<td>Male gender – n (%)</td>
<td>118 (74.2)</td>
<td>47 (79.7)</td>
<td>71 (71.0)</td>
</tr>
<tr>
<td>Clinical Variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to ED (min)</td>
<td>77.8 ± 63.9</td>
<td>85.9 ± 67.4</td>
<td>73.0 ± 61.6</td>
</tr>
<tr>
<td>ISS score</td>
<td>24.3 ± 11.2</td>
<td>18.6 ± 9.6</td>
<td>27.8 ± 10.7</td>
</tr>
<tr>
<td>Head AIS</td>
<td>4.1 ± 1.1</td>
<td>3.6 ± 1.2</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>GCS</td>
<td>5.9 ± 2.9</td>
<td>7.2 ± 3.0</td>
<td>5.1 ± 2.6</td>
</tr>
<tr>
<td>Marshall score</td>
<td>2.7 ± 1.3</td>
<td>2.1 ± 1.2</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>Pre-injury comorbidities – n (%)</td>
<td>40 (25.2)</td>
<td>13 (22.0)</td>
<td>27 (27.0)</td>
</tr>
<tr>
<td>Neurosurgey performed – n (%)</td>
<td>46 (28.9)</td>
<td>10 (16.9)</td>
<td>36 (36.0)</td>
</tr>
<tr>
<td>Laboratory Variables – median (IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.0 (35.3 – 37.0)</td>
<td>36.2 (35.4 – 37.0)</td>
<td>36.0 (35.0 – 36.5)</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 (7.3 – 7.4)</td>
<td>7.3 (7.3 – 7.4)</td>
<td>7.3 (7.3 – 7.4)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>134.0 (120 – 160)</td>
<td>130.0 (120.0 – 155.0)</td>
<td>135.5 (120.0 – 162.0)</td>
</tr>
<tr>
<td>INR (normal &lt;1.3)</td>
<td>1.0 (1.0 – 1.3)</td>
<td>1.0 (1.0 – 1.1)</td>
<td>1.1 (1.0 – 1.2)</td>
</tr>
<tr>
<td>aPTT (normal &lt;38 s)</td>
<td>28.0 (25.3 – 31.4)</td>
<td>27.7 (25.0 – 29.4)</td>
<td>28.4 (25.8 – 33.3)</td>
</tr>
<tr>
<td>PLT (normal &gt;100)</td>
<td>218.0 (173.0 – 266.0)</td>
<td>238.0 (201.0 – 278.0)</td>
<td>196.0 (166.0 – 243.0)</td>
</tr>
<tr>
<td>*Coagulopathy – n (%)</td>
<td>20 (19.0)</td>
<td>0 (0.0)</td>
<td>20 (20.3)</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortality – n (%)</td>
<td>44 (27.7)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Neurologic – n (% of mortality)</td>
<td>27 (61.4)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Organ failure – n (% of mortality)</td>
<td>17 (38.6)</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, traumatic brain injury; GOSE, Glasgow Outcome Scale Extended; ED, emergency department; injury severity score; AIS, abbreviated injury scale; GCS, Glasgow Coma Scale; SBP, systolic blood pressure; INR, international normalized ratio; aPTT, activated partial thromboplastin time; PLT, platelet; S, seconds.

Results displayed as the mean ± standard deviation (SD), unless otherwise stated.

Unfavorable vs. Favorable outcome was computed for each characteristic by Mann-Whitney U or chi-squared, where appropriate. Significance was determined at p < 0.05, and displayed in bold.

*Patients were considered coagulopathic if they had admission INR scores >1.3 or aPTT >38 sec or PLT <100 000/µL. Coagulopathy data was available on a subset of 105 patients, and percentages were calculated from this number.
3.4 Results

3.4.1 Demographics and clinical characteristics

Patient demographic, clinical, and laboratory variables were dichotomized according to unfavorable and favorable 6-month neurological outcome and summarized in Table 3.1. A total of 159 patients were included in the study, the majority (n=100; 62.9%) of which had an unfavorable GOSE (1-4) prognosis at 6 months post-injury. Forty-four (27.7%) patients died, 61.4% of these by neurologic death, and 38.6% by non-neurologic organ failure. The median time to death was 4 days (range = 1 – 96 days) (data not shown). The study sample was predominantly male (n=118; 74.2%), with a mean age of 45.8 ± 20.3 years. Unfavorable outcome was associated with increased age and injury severity (ISS, AIS head, GCS, Marshall score). Coagulopathy, as defined by INR >1.3 or aPTT >38 sec or PLT <100 000/µL, was present in 19.0% percent of patients at admission, all of which had an unfavorable 6-month outcome. At 24 h, the percentage of patients who were coagulopathic increased to 36.2% (data not shown). Individually, aPTT did not differ in patients according to outcome.

3.4.2 Relationship between biomarkers and 6-month GOSE

Figure 3.1 depicts the PLS-DA analysis, indicating the combination of biomarkers that optimally distinguished favourable and unfavourable 6-month outcome. For TBI patient blood biomarker concentrations please see Appendix 3.1. Variable loadings represent the direction and magnitude of the biomarkers associated with unfavorable outcome. At admission, mean posterior probability of correct classification (PProb) of outcome was 0.71, and the mean classification accuracy (Accur) was 0.80. In addition to injury severity and age, elevations in 13 blood biomarker variables were significantly associated with unfavorable outcome at FDR = 0.05. The most salient marker at admission was TAT, followed by SDC-1, NE and tPA, which had higher
loading scores than all demographic and clinical indices (Fig. 3.1, Panel A). At 24 h, the pprob and accru were 0.67 & 0.75, respectively; 15 blood biomarkers were significantly associated with unfavorable outcome at FDR = 0.05 (Fig. 3.1, Panel B). Similar to admission, biomarkers with the highest loading scores were SDC-1, tPA, and NE. Contrary to admission TM, P-Sel, IL-10 and MDC (negative loading) were significant contributors to outcome at 24 h, although Epi and DD were no longer statistically significant at this time (Fig. 3.1, Panel B).
Figure 3.1 - Partial Least Squares – Discriminant Analysis of patient outcome. Biomarker and clinical/demographic predictors discriminating unfavourable from favourable 6-month outcome in TBI patients. Clinical and demographic markers include age, injury severity score (ISS), head abbreviated injury severity scale (AIS), and Glasgow coma scale (GCS) scores. Blood biomarkers include (1) sympathetic nervous system (SNS) biomarkers – epinephrine (Epi)
and norepinephrine (NE), (2) endotheliopathy biomarkers - E-selectin (E-sel), P-Selectin (P-Sel), intercellular adhesion molecule (ICAM)-1, -3, vascular cell adhesion molecule (VCAM)-1, vascular activation protein (VAP) -1, syndecan (SDC)-1, (3) coagulopathy biomarkers - thrombin anti-thrombin complex III (TAT), tissue factor (TF), tissue factor platelet inhibitor (TFPI), thrombomodulin (TM), tissue plasminogen activator (tPA), d-dimer (DD), plasminogen activation inhibitor (PAI)-1, (4) inflammation biomarkers – interleukin (IL)-6, tumor necrosis factor (TNF)-α, c-reactive protein (CRP), serum amyloid a (SAA), IL-10, -8, interferon producing protein (IP)-10, monocyte chemoattractant protein (MCP)-1, -4, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), eotaxin, and eotaxin-3 (ET-3). Blood biomarker contributions are displayed at admission (A) and 24 h (B). Bars represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. *Represents significance at FDR = 0.05.

3.4.3 Covariance between catecholamines and biomarkers

A PLS analysis of the covariance between catecholamine levels (Epi and NE) and blood correlates of endotheliopathy, coagulopathy and inflammation, is visualized in Fig. 3.2. At admission, the fraction of variance explained for the relationship between catecholamines and blood biomarkers was 0.81. Fourteen biomarkers showed significant covariation with catecholamine levels at FDR = 0.05. The strongest associations were TAT, followed by TF, SDC-1, and tPA. At 24 h, the fraction of variance explained was 0.74, and 14 biomarkers significantly covaried with Epi and NE. The strongest associations were IL-10, SDC-1, TM, and IL-6 (Fig. 3.2, Panel A). At 24 h, NE displayed a stronger covariant relationship with the predictor biomarkers compared to Epi. Similar to admission, increases in NE and Epi covaried with increases in SDC-1, VAP-1, TAT, TF, IL-6, IL-10 and IL-8. Contrary to admission, increases in E- Selectin and P-Selectin, and TM significantly covaried with Epi and NE (Fig. 3.2, Panel B).
Figure 3.2 - Partial Least Squares analysis of covariance between the SNS and markers of endotheliopathy, coagulopathy, and inflammation. **SNS biomarkers** – epinephrine (Epi) and norepinephrine (NE); **endotheliopathy biomarkers** - E-selectin (E-sel), P-Selectin (P-Sel), intercellular adhesion molecule (ICAM) -1, -3, vascular cell adhesion molecule (VCAM)-1, vascular activation protein (VAP)-1, syndecan (SDC)-1; **coagulopathy biomarkers** - thrombin anti-thrombin complex (TAT), tissue factor (TF), tissue factor platelet inhibitor (TFPI), thrombomodulin (TM), tissue plasminogen activator (tPA), d-dimer (DD), plasminogen
activation inhibitor (PAI)-1; inflammation biomarkers – interleukin (IL)-6, tumor necrosis factor (TNF)-α, c-reactive protein (CRP), serum amyloid a (SAA), IL-10, -8, interferon producing protein (IP)-10, monocyte chemoattractant protein (MCP)-1, -4, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), eotaxin, and eotaxin-3 (ET-3). Blood biomarker contributions are displayed at admission (A) and 24 h (B). Bars represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. *Represents significance at FDR = 0.05.

3.5 Discussion

This study characterized blood biomarker profiles of coagulopathy and endotheliopathy after isolated TBI, and identified potential mechanistic links between sympathoadrenal activation and these pathologies using multivariate PLS analysis. There were two main findings: (1) unfavorable patient outcome after isolated blunt TBI was associated with acute elevations in circulating biomarkers of endotheliopathy and coagulopathy, (2) biomarkers of coagulopathy and endotheliopathy co-varied with indices of SNS activity. We evaluated patient biomarker profiles according to 6-month GOSE to accurately identify biological correlates of coagulopathy that were most influential in determining poor outcome. This is in contrast to patient stratification by “coagulopathic” or “non-coagulopathic” groups according to standard laboratory tests of coagulopathy; these assays are inherently designed to assess the hypocoagulable state only (Hemker et al. 2004; Kunio et al. 2012; Park et al. 2009), and may have skewed our results through the selective bias of patients with the greatest hypocoagulable profiles.

Our results are consistent with others who have correlated TBI with an immediate pro-coagulant, hyperfibrinolytic state (Gando, Nanzaki et al. 1999, Nekludov, Antovic et al. 2007). At hospital admission, we found the pro-coagulant markers TAT and TF, and the hyperfibrinolysis marker tPA strongly contributed to unfavorable outcome, while the anti-coagulant indices TFPI and TM were insignificant. While levels of TAT and TF remained significantly altered at 24 h, their relative contributions to poor outcome was overshadowed by
TM. This change may be supportive of the “DIC with a hyperfibrinolysis phenotype” hypothesis, which suggests an early consumption of clotting factors portends a later hypocoagulable state (Stein and Smith 2004, Gando, Wada et al. 2013, Dobson, Letson et al. 2015). Furthermore, that the two strongest coagulopathic predictors of poor outcome at 24 h were TM and tPA – both important components of the activated protein C pathway (Cohen, Brohi et al. 2007, Dobson, Letson et al. 2015) – also suggests the potential involvement of this mechanism in a progressive bleeding phenotype. However, it is important to note that the predominant biological function of soluble TM is not well defined; beyond its anti-coagulant effects, it is also a well-known marker of endothelial damage (Ishii, Uchiyama et al. 1991, Yokota, Naoe et al. 2002). Hence, we are unable to definitively conclude that the biomarker phenotype shifted from pro- to anti-coagulant in patients over 24 h. In addition, SDC-1, a key proteoglycan marker of endothelial glycocalyx degradation found elevated in major trauma (Johansson, Stensballe et al. 2011, Ostrowski and Johansson 2012), sepsis (Ostrowski, Berg et al. 2013), and acute myocardial infarction (Ostrowski, Pedersen et al. 2013), was a strong contributor to poor patient outcome at both admission and 24 h. While these results are supportive of glycocalyx degradation acutely after trauma, it is again difficult to interpret these results in terms of their contributory effects on hypo/hypercoagulation. Shedding of the glycocalyx into the circulation may contribute to a hypocoagulant state through endogenous heparanization (Ostrowski and Johansson 2012), although it may also increase circulating concentrations of DAMPs including hyaluronan fragments and heparin sulfate (Chignalia, Yetimakman et al. 2016). These DAMPS may then contribute to a hypercoagulant state through the production of inflammatory mediators and subsequent thrombin generation (Levi and van der Poll 2010); indeed, previous studies have shown glycocalyx disruption increases thrombin production (Nieuwdorp, van Haeften et al. 2006, Levi and van der Poll 2010). Nevertheless, that SDC-1 strongly co-varied with unfavorable
outcome at both admission and 24 h warrants future investigation on the role of the glyocalyx in TBI. Overall, our results are consistent with a relationship between poor outcome and biomarker indices of coagulopathy and endotheliopathy over the first 24 h of hospitalization in isolated TBI patients.

Evidence suggests SNS hyperactivity as a likely mechanistic driver of post-trauma coagulopathy (von Kanel and Dimsdale 2000, Johansson and Ostrowski 2010, Johansson, Stensballe et al. 2012). In the current study, 81% of the variance observed at hospital admission in SNS correlates NE and Epi was explained by variance in biomarkers of coagulopathy and endotheliopathy. The strongest covariate was TAT, followed by TF, SDC-1, and markers of hyperfibrinolysis (tPA, DD). The observed change in biomarker profiles towards an anti-coagulant state at 24 h was mirrored in our covariation analysis; at 24 h the explained variance was 74%, NE became the strongest catecholamine covariate, and was highly associated with increases in SDC-1, TM, and tPA. Indeed, Ostrowski and colleagues have produced a number of studies correlating sympathoadrenal activation to endothelial damage and indices of hyperfibrinolysis, and have identified an interrelationship between catecholamines and glyocalyx injury in multiple trauma modalities (Johansson, Sorensen et al. 2011, Ostrowski and Johansson 2012, Ostrowski, Berg et al. 2013, Ostrowski, Pedersen et al. 2013, Johansson, Haase et al. 2014, Ostrowski, Gaini et al. 2015, Ostrowski, Haase et al. 2015). That we found covariance between Epi, NE and correlates of glyocalyx damage (SDC-1), endothelial activation (E-Sel, VAP-1), and hyperfibrinolysis (tPA, DD), is consistent with the works by this group and further suggests similarities in SNS-mediated endotheliopathic and hyperfibrinolytic mechanisms in multisystem trauma and isolated TBI.
A complex, bidirectional relationship exists between SNS activity and both inflammation (Flierl, Rittirsch et al. 2008, Xu, Yu et al. 2015, Di Battista, Rhind et al. 2016) and coagulopathy (Levi and van der Poll 2010, van der Poll, de Boer et al. 2011). In the current study, poor outcome and heightened SNS activity co-varied with a pro-coagulant/pro-inflammatory biomarker phenotype on hospital admission that progressed towards an anti-inflammatory phenotype at 24 h post-injury due to the increased contribution of IL-10. In support of this, it has been shown that chemical sympathectomy diminishes inflammation, glycocalyx shedding and coagulopathy in rats (Xu, Yu et al. 2015). In addition, pro-inflammatory cytokines are capable of inducing coagulation through their ability to generate TF (Levi and van der Poll 2010, van der Poll, de Boer et al. 2011), and it follows that increases in counterregulatory anti-inflammatory mediators may contribute to a progressive shift from a pro- to anti-coagulative state. However, as coagulopathic mediators such as thrombin and TM may also induce pro- and anti-inflammatory responses, respectively (von Kanel and Dimsdale 2000, Conway 2012), the directionality of the relationship between coagulopathy and inflammation remains uncertain. Furthermore, it is possible that the covariance we observed between catecholamines and biomarkers of inflammation, coagulopathy and endotheliopathy was influenced by their mutual relationship to injury severity. However, we found that biomarkers co-varied with catecholamines to a greater extent than with indices of injury severity (Fraction of variance explained – 1) Epi, NE; 81% at admission, 74% at 24 h, vs 2) GCS, AIS head, ISS; 67% at admission, 68% at 24 h, Appendix 3.2). This finding, together with our outcome analysis which showed that individual biomarker contributions to unfavorable outcome often exceeded the contributions of traditional indices of injury severity (Fig 3.1), are consistent with a pathological relationship between SNS activation and indices of coagulopathy, endotheliopathy and inflammation.
There were several limitations of the current study. Despite evaluating multiple biomarkers spanning different facets of coagulopathy, using a well-controlled clinical design, the analysis of additional cofactors may have been helpful to fully assess hemostatic alterations. However, our study design was proscribed in terms of pre-analytical considerations and on the basis of available patients’ sample volume. Likewise, we were only able to assess the selected biomarkers at two time points (i.e., admission and 24 h post-injury), whereas a greater sampling frequency may have been valuable in evaluating potentially rapid kinetic changes in the acute biomarker profiles. Moreover, the ability to correlate our biomarker data with additional clinical laboratory indices such as thromboelastometry - capable of identifying hypercoagulable states - could have strengthened our findings. Furthermore, while our cohort was representative of the general trauma population regarding sex distribution, our sample size prevented the assessment of biomarker profiles between males and females. Indeed, previous studies have shown that trauma can elicit sex-specific immune and coagulopathic responses; females may be immunologically protected through the effects of the sex steroid 17β-oestradiol (Choudhry, Bland et al. 2007), while females with acute trauma coagulopathy may have worse outcomes compared to males (Brown, Cohen et al. 2012). Nonetheless, our results provide a novel in-depth assessment of coagulopathy in a large cohort of isolated TBI patients, and are consistent with the notion that this process is related to poor outcome and is mediated by sympathoadrenal hyperactivity.

3.6 Conclusion

In conclusion, biomarkers of coagulopathy and endotheliopathy are associated with poor outcome in isolated TBI patients. Patients with poor outcome exhibit increased circulating markers of glyocalyx and endothelial damage, vascular activation, inflammation, pro-coagulation and hyperfibrinolysis. Moreover, SNS activity as assessed by circulating catecholamines is
highly correlated with markers of endotheliopathy and coagulopathy within the first 24 hours after injury. Additional research is warranted to further characterize the pathogenic role of sympathoadrenal-mediated hemostatic alterations in isolated TBI.
Chapter 4
An investigation of neuroinjury and inflammatory biomarkers after sport-related concussion: from the subacute phase to clinical recovery

The components of this chapter were submitted to the Journal of Neurotrauma on October 7th, 2016 and are under review; they were also peer-reviewed by the Defense Research and Development Canada (DRDC) Publications Office prior to submission.

Di Battista, A.P., Rhind, S.G., Baker, A.J., Jetly, R., Debad, J.D., and Hutchison, M.G,

The lead author contributed to the manuscripts conceptual framework, performed all cytokine/chemokine immunoassay experiments, statistically analyzed and interpreted the data, drafted the manuscript, and contributed to its critical revision.
4 An investigation of neuroinjury and inflammatory biomarkers after SRC: from the subacute phase to clinical recovery.

4.1 Abstract

BACKGROUND: The inflammatory response after sport-related concussion (SRC) is not well defined. OBJECTIVES: The purpose of this study was to characterize a panel of blood biomarkers of inflammation and brain injury after SRC. METHODS: Thirty-eight interuniversity athletes from 16 sports were recruited (n = 19 SRC; n = 19 healthy matched-control). Blood samples were taken within one week of SRC and at medical clearance. Blood was also sampled from 87 healthy athletes before the start of the competitive season. Thirty-nine total blood biomarkers were evaluated by high-sensitivity immunoassay. RESULTS: At both subacute and medical clearance time points, circulating peroxiredoxin-6 concentrations were elevated in SRC athletes compared to healthy matched-controls. Interferon gamma-induced protein (IP) -10 and T-tau were higher at medical clearance compared to matched controls. Multiple inflammatory indices were inversely correlated with total reported symptoms subacutely in SRC athletes. In addition, numerous biomarkers varied in healthy athletes throughout the academic year. Taken together, blood biomarkers may be useful in elucidating secondary injury pathophysiology after SRC in the subacute period and throughout recovery. However, their implementation requires mindfulness of extracranial factors such as academic stressors and exercise, as well as the inherent heterogeneity of concussive injury.

CONCLUSIONS: Our findings are consistent with studies using other modalities (neuroimaging, electrophysiology), that have identified perturbations at clinical recovery, and further support the continued use of blood biomarkers in elucidating the biology of brain restitution after SRC.
4.2 Introduction

Concussion or mild traumatic brain injury (mTBI) spans all age groups and includes specialized sub-populations, such as athletes and military personnel (Voss, Connolly et al. 2015, Garber, Rusu et al. 2016). Despite the high prevalence and societal burden, the pathophysiology of concussive injury is unclear (Barkhoudarian, Hovda et al. 2016). A better understanding of the complex biological processes occurring after injury is needed to help guide therapy and management (Levin and Diaz-Arrastia 2015, Papa 2016).

Experimental animal studies have been important in identifying numerous secondary brain injury sequelae in the acute and subacute phase of injury, including local metabolic disruption, neuronal injury, and inflammation (Blaylock and Maroon 2011, Giza and Hovda 2014). In humans, advanced neuroimaging techniques have provided some support of these early findings (Bergsneider, Hovda et al. 1997, Bergsneider, Hovda et al. 2000, Jalloh, Carpenter et al. 2015, Barkhoudarian, Hovda et al. 2016, Wright, Trezise et al. 2016), yet our ability to comprehend secondary injury processes after sport-related concussion (SRC) remains challenging.

Peripheral blood has emerged as a viable, non-invasive tool to further our pathophysiological knowledge of secondary injury processes (Di Battista, Rhind et al. 2013, Woodcock and Morganti-Kossmann 2013, Di Battista, Rhind et al. 2016). Multiple lines of evidence suggest that protein markers reflecting brain injury and repair can be detected in the peripheral circulation through several mechanisms, including transport across a disrupted blood-brain barrier (BBB), glymphatic clearance (Chodobski, Zink et al. 2011, Woodcock and Morganti-Kossmann 2013, Alves 2014, Plog, Dashnaw et al. 2015), or aberrant neuroendocrine signaling (Catania, Lonati et al. 2009, Santarsieri, Kumar et al. 2015, Di Battista, Rhind et al.)
Alterations in various circulating indices of brain tissue damage such as glial fibrillary acidic protein (GFAP), s100 calcium-binding protein B (s100B), neuron specific enolase (NSE), αII-spectrin N-terminal fragment (SNTF) (Shahim, Tegner et al. 2014, Siman, Shahim et al. 2015, Papa, Brophy et al. 2016), and leukocyte inflammatory gene expression profiles, have been observed in the acute and sub-acute phases after SRC (Gill, Merchant-Borna et al. 2016, Merchant-Borna, Lee et al. 2016). Higher peripheral blood chemokine levels have also been identified chronically after SRC (Di Battista, Rhind et al. 2016), and chronically elevated circulating coated platelets and C-reactive protein have been observed after non-sport related mTBI (Prodan, Vincent et al. 2014, Su, Xu et al. 2014). However, no studies have evaluated soluble neuroinjury specific and inflammatory proteins concurrently in the acute and sub-acute phase after SRC. Hence, the purpose of this study was to advance our understanding of SRC pathobiology through the characterization of blood-based biomarkers related to brain injury and inflammation in the subacute period post-injury and at medical clearance. Concussed athletes were compared to healthy athletes individually matched on sex, age, sport participation, time of varsity season, and concussion history.

4.3 Methods

4.3.1 Participants

Thirty-eight interuniversity athletes were recruited between August 2014 and April 2016 from basketball (M/F), football (M), ice hockey (M/F), lacrosse (M/F), rugby (M/F), soccer (M/F), volleyball (M/F), and wrestling (M) (16 total sports). Nineteen athletes with a physician-diagnosed sport concussion were enrolled and assessed within the first week post-injury (average = 4 days, range = 2-8 days). The diagnosis was made by a staff physician from a single clinic, in
accordance to the definition set forth by the Concussion in Sport Group (McCrory, Meeuwisse et al. 2009). In addition, 19 healthy control athletes matched for age, sex, sport participation, previous concussion history, and time of season were enrolled; matched healthy control samples (“matched controls”) were recruited and sampled within close proximity of their injured counterparts, and then again approximately 3 weeks later to match the projected average recovery time for concussed athletes. Our baseline healthy controls (“baseline controls”) consisted of samples from 87 uninjured athletes taken before the start of the competitive season; data from this cohort was published previously (Di Battista, Rhind et al. 2016). Participants were excluded if they were suffering from a non-concussion related acute illness or injury, disclosed any inflammatory-related healthy conditions (i.e., seasonal allergies), or were taking any medications beyond birth control. Throughout the study, 3 healthy matched-control athletes and 2 concussed athletes were lost due to attrition and/or inability to acquire a blood sample. Blood collection and administration of the Sport Concussion Assessment Tool 3 (SCAT3) was performed by members of the research team. Medical history was obtained by the team’s therapist/trainer. All participants provided written informed consent prior to enrollment, and all study procedures were approved by the Health Sciences Research Ethics Board, University of Toronto (protocol reference # 27958).

4.3.2 Sport Concussion Assessment Tool – 3 (SCAT3)

The Sport Concussion Assessment Tool (SCAT) is the one of the most widely used tools to assist in the diagnosis, management, and prognosis of individuals with concussion. The SCAT3 is the most recent version and was developed at The 4th International Conference on Concussion in Sport in 2012. The SCAT3 is comprised of a symptom evaluation, cognition assessment by the Standardized Assessment of Concussion (SAC), neck examination, balance examination by the modified balance error scoring system (BESS), and a coordination exam.
symptom score is comprised of a 22-item post-concussion symptom scale using a seven-point Likert scale rating. Symptom severity is obtained by summing the rated symptom score for each symptom (McCrory, Meeuwisse et al. 2013). This symptom scale has been shown to be reliable and valid for the assessment of both symptom presence and severity (Galetta, Galetta et al. 2013, Guskiewicz, Register-Mihalik et al. 2013, Brown, Elsass et al. 2015).

4.3.3 Blood sample collection

Venous blood samples were drawn from athletes at the time of their cognitive assessment. Samples were drawn into a 10-mL K$_2$EDTA tube. At approximately one hour, specimens were centrifuged for 2 min using the PlasmaPrep 12™ centrifuge (Separation Technology Inc., FL, USA), and the plasma supernatant was aliquoted and frozen at -70°C until analysis. All samples were processed in the same manner.

4.3.4 Blood biomarker analysis

Thirty-nine biomarkers were evaluated using Meso Scale Diagnostics (MSD) 96-well MULTI-ARRAY® technology and run on a MSD® Sector Imager™ 6000 with Discovery Workbench software (version 3.0.18). The platform uses an array-based multiplex format with sensitive electrochemiluminescence detection. Sandwich immunoassays were used with capture antibody coated on arrays within plate wells, and detection antibodies were conjugated with electrochemiluminescent SULFO-Tag™.

Twenty-eight markers were analyzed using MSD V-PLEX® Human Immunoassay Kits (see Table 2 for complete list of markers analyzed). A prototype assay panel was used to quantitate 11 neuroinjury-associated biomarkers; this panel was developed at MSD in part through work supported by US Army Medical Research and Materiel Command (Contract No. W81XWH-13-C-0196). The panel included GFAP, s100B, neuron specific NSE, total tau (T-
tau), neurogranin (NRGN), creatine kinase-BB isoenzyme (CKBB), visinin-like protein (VILIP)-1, von Willebrand factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX)-6, and monocyte chemoattractant protein (MCP)-1.

4.3.5 Statistical analysis

Individual biomarker values were statistically evaluated only if they fell within the manufacturers’ recommended level of quantitation for each analyte, and displayed a coefficient of variance <25% between duplicate samples. A mixed-model analysis on log-transformed data was used to evaluate between-group (concussion vs. matched controls) and within-group (sub-acute vs. medical clearance) differences. A Kruskall-Wallis analysis of variance was employed to identify potential differences in biomarker levels between the aforementioned groups and baseline controls. Multivariate analysis was conducted by partial least squares (PLS) to evaluate the covariance between blood biomarkers and total symptoms in concussed athletes. PLS is a supervised technique used to objectively characterize the covariance between a set of predictor variables and response variables. PLS analysis yields the fraction of variance explained, reflecting the proportion of total inter-subject variability in biomarker data that is described by the PLS component of interest. Biomarkers were not included in the multivariate analysis if >30% of the data points were missing in any group. Missing biomarker values were imputed using the k-means nearest-neighbour method, and were rank-transformed to ensure robustness against non-normality. Significant biomarker loadings were identified by performing bootstrap resampling on subjects (1000 iterations) to obtain empirical p-values, which were then corrected for multiple comparisons at a false discovery rate (FDR) of 0.05. For PLS plots, variable loadings are represented as bootstrap ratios (i.e., the bootstrapped mean ± standard error), which are z-scored statistics reflecting the reliability of variable contributions. Univariate statistics comparing SCAT3 symptom scores between concussed athletes and matched controls were
calculated by Mann Whitney $U$. Descriptive and univariate statistics were completed using Stata Version 14.1 (StataCorp, TX, USA). Multivariate analyses were conducted using in-house software developed for Matlab, Version R2015b (Mathworks, Natick MA). All data were visualized using GraphPad Prism Version 6.0f (GraphPad Inc., CA, USA).

4.4 Results

4.4.1 Description of participants

Table 4.1 summarizes demographics and characteristics for SRC athletes. Median total symptoms and symptom severity was 7.5 (IQR 5 – 13) and 12.0 (IQR 5.3 – 22.6) respectively, at the time of medical evaluations; at the time of blood sampling, the median total symptoms reported by SRC athletes was 5 (IQR 4 -11), and median symptom severity was 8 (IQR 4 - 17). At the time of blood sampling, compared to healthy controls, concussed athletes had significantly higher total symptoms scores (median score = 5.0 vs. 3.0 respectively; $p = 0.04$, data not shown) but not symptom severity scores. Six of twenty-two SCAT3 symptoms were significantly elevated in concussed athletes, with “sensitivity to light” displaying the greatest significant difference (median score 1.0 vs 0.0; $p = 0.0009$, data not shown). There was a also a significant, positive correlation identified between total symptoms and days to medical clearance ($\rho = 0.63$; $p = 0.3$, data not shown).
Table 4.1 Athlete demographics and characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Concussed athletes (n = 19)</th>
<th>Matched healthy controls (n = 16)</th>
<th>Baseline preseason controls (n = 87)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19 (18 - 20)</td>
<td>19 (18 – 21)</td>
<td>19 (18 – 21)</td>
</tr>
<tr>
<td>Sex (n, % - male)</td>
<td>8 (42.1)</td>
<td>7 (43.7)</td>
<td>60 (69.0)</td>
</tr>
<tr>
<td>Sport (n, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basketball</td>
<td>2 (10.5)</td>
<td>2 (12.5)</td>
<td>17 (19.5)</td>
</tr>
<tr>
<td>Football</td>
<td>3 (15.8)</td>
<td>2 (12.5)</td>
<td>10 (11.5)</td>
</tr>
<tr>
<td>Ice hockey</td>
<td>3 (15.8)</td>
<td>3 (18.8)</td>
<td>13 (14.9)</td>
</tr>
<tr>
<td>Lacrosse</td>
<td>3 (15.8)</td>
<td>1 (6.3)</td>
<td>1 (1.1)</td>
</tr>
<tr>
<td>Rugby</td>
<td>6 (31.6)</td>
<td>5 (31)</td>
<td>15 (17.2)</td>
</tr>
<tr>
<td>Soccer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volleyball</td>
<td>2 (10.5)</td>
<td>2 (12.5)</td>
<td>18 (20.7)</td>
</tr>
<tr>
<td>Wrestling</td>
<td>--</td>
<td>--</td>
<td>4 (4.6)</td>
</tr>
<tr>
<td>Number of previous concussions n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6 (31.6)</td>
<td>8 (50)</td>
<td>52 (59.8)</td>
</tr>
<tr>
<td>1</td>
<td>7 (36.8)</td>
<td>1 (6.3)</td>
<td>17 (19.5)</td>
</tr>
<tr>
<td>2</td>
<td>4 (21.1)</td>
<td>5 (31.2)</td>
<td>11 (12.6)</td>
</tr>
<tr>
<td>3 or more</td>
<td>2 (10.5)</td>
<td>2 (12.5)</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Collision sport participation – n (%)</td>
<td>11 (57.9)</td>
<td>9 (56.3)</td>
<td>36 (41.4)</td>
</tr>
<tr>
<td>Medical History – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraines</td>
<td>2 (10.5)</td>
<td>1 (6.3)</td>
<td>4 (4.6)</td>
</tr>
<tr>
<td>Learning disability</td>
<td>1 (5.3)</td>
<td>1 (6.3)</td>
<td>2 (2.3)</td>
</tr>
<tr>
<td>Psychiatric disorders</td>
<td>0 (0)</td>
<td>2 (12.5)</td>
<td>8 (9.2)</td>
</tr>
<tr>
<td>Family history of psychiatric illness</td>
<td>4 (21.0)</td>
<td>7 (43.8)</td>
<td>22 (25.3)</td>
</tr>
<tr>
<td>SCAT3 symptoms at time of injury</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total symptoms</td>
<td>7.5 (5 – 16)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Symptom severity</td>
<td>12 (5.5 – 16)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>SCAT3 symptoms at time of sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total symptoms</td>
<td>5 (4 – 11)</td>
<td>3 (0.7 – 7)</td>
<td>3 (1 – 5)</td>
</tr>
<tr>
<td>Symptom severity</td>
<td>8 (4 – 15)</td>
<td>5 (0.7 – 9.2)</td>
<td>4 (1.7 – 8.2)</td>
</tr>
<tr>
<td>Days to sample from injury</td>
<td>4 (3 – 4.7)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Days to medical clearance</td>
<td>33 (12-95)</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Unless otherwise stated, results are reported as the median and interquartile range (IQR).

4.4.2 Blood biomarker profiles in concussed athletes

Among the analytes assayed, 20 of 39 biomarkers (5 of 19 cytokines, 8 of 10 chemokines, and 7 of 10 neuroinjury markers) were within quantifiable ranges in >50% of samples across all groups, and thus were included for statistical analysis. Biomarker
concentrations for all groups can be seen in Table 4.2; all biomarkers that displayed significant differences among groups are shown in Figure 4. Matched controls were compared to pre-season baseline controls in order to evaluate a) the variation due to the time of athletic/academic year the sample was acquired, and b) to assess the variability of blood biomarkers across different healthy samples. In view of the latter, biomarkers were also compared longitudinally within the matched control group in order to evaluate variability in biomarker values in the same individual at different time-points. Relative to the baseline control group, the SRC matched controls varied in a number of blood biomarkers; tau and PRDX-6 levels were lower in the matched controls compared to baseline controls, while NRGN levels were higher (Fig 4 A, D & C, respectively). Cytokines TNF-α and IL-16 were higher and lower in matched control samples compared to those sampled pre-season, respectively (Fig 4 F & H, respectively), while the chemokine MCP-4 was significantly higher (Fig 4 J). Within-group analysis of biomarkers in healthy matched athletes over time also revealed significant differences; s100B levels were significantly higher at the second time-point compared to the first, while vWF levels were significantly lower (Fig 4 B & E, respectively). Both MCP-1 and MCP-4 levels were higher at the second sampling time-point in matched control athletes, and significant elevations were also observed in the second sample for MIP-1α, TARC, and Eotaxin (Fig 4 I-N).
### Table 4.2 Biomarker values across groups

<table>
<thead>
<tr>
<th>Markers (pg/mL)</th>
<th>Baseline pre-season (n = 87)</th>
<th>3-7 days post-injury</th>
<th>Medical clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Matches Healthy (n = 16)</td>
<td>Concussed (n = 19)</td>
<td>Matches Healthy (n = 16)</td>
</tr>
<tr>
<td></td>
<td>Cytokines</td>
<td></td>
<td>Cytokines</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5.3 (4.0 - 9.1)</td>
<td>4.9 (3.7 - 11.2)</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-6</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-7</td>
<td>2.6 (2.0 - 3.7)</td>
<td>2.9 (1.9 - 3.9)</td>
<td>2.7 (2.1 - 3.2)</td>
</tr>
<tr>
<td>IL-10</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>121.9 (89.9 - 146.3)</td>
<td>113.2 (75.7 - 156.9)</td>
<td>131.6 (96.0 - 174.5)</td>
</tr>
<tr>
<td>IL-13</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-15</td>
<td>2.3 (2.0 - 2.7)</td>
<td>2.1 (1.8 - 2.4)</td>
<td>2.3 (1.9 - 2.7)</td>
</tr>
<tr>
<td>IL-16</td>
<td>253.6 (198.6 - 358.4)</td>
<td>190.8 (139.2 - 251.0)</td>
<td>205.1 (147.9 - 320.5)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>1.8 (1.6 - 2.2)</td>
<td>1.7 (1.5 - 2.4)</td>
<td>2.4 (2.1 - 3.0)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TNF-β</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VEGF</td>
<td>35.8 (27.6 - 55.6)</td>
<td>45.3 (25.5 - 56.8)</td>
<td>63.0 (42.3 - 82.0)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemokines</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>78.1 (62.3 - 96.6)</td>
<td>69.2 (53.9 - 102.2)</td>
<td>107.4 (67.6 - 127.6)</td>
<td>77.6 (57.1 - 108.4)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>22.2 (18.1 - 31.3)</td>
<td>20.6 (17.3 - 26.8)</td>
<td>21.1 (18.0 - 28.0)</td>
<td>22.1 (15.9 - 33.4)</td>
</tr>
<tr>
<td>IP-10</td>
<td>207.0 (156.7 - 258.5)</td>
<td>275.3 (172.8 - 334.8)</td>
<td>215.6 (190.7 - 281.0)</td>
<td>270.7 (237.1 - 318.5)</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.9 (1.5 - 2.8)</td>
<td>2.4 (1.7 - 2.9)</td>
<td>2.3 (2.1 - 2.9)</td>
<td>1.6 (1.5 - 2.9)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>86.8 (71.2 - 109.5)</td>
<td>80.7 (68.2 - 112.6)</td>
<td>92.7 (78.2 - 102.3)</td>
<td>93.1 (75.0 - 115.7)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>26.1 (19.4 - 38.3)</td>
<td>33.6 (26.1 - 48.7)</td>
<td>36.2 (31.6 - 42.1)</td>
<td>37.7 (22.0 - 50.1)</td>
</tr>
<tr>
<td>MDC</td>
<td>809.4 (706.3 - 997.3)</td>
<td>788.8 (727.6 - 870.3)</td>
<td>709.2 (620.7 - 780.8)</td>
<td>777.8 (634.9 - 1096.0)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>38.0 (30.6 - 49.9)</td>
<td>41.1 (25.0 - 58.2)</td>
<td>52.0 (37.2 - 63.9)</td>
<td>45.2 (34.0 - 49.6)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>35.4 (30.4 - 43.8)</td>
<td>39.7 (31.5 - 56.0)</td>
<td>50.9 (37.8 - 81.1)</td>
<td>48.2 (29.3 - 97.1)</td>
</tr>
<tr>
<td>TARC</td>
<td>42.2 (26.3 - 57.0)</td>
<td>35.1 (31.8 - 56.4)</td>
<td>39.7 (31.5 - 56.0)</td>
<td>50.9 (37.8 - 81.1)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neuroinjury Markers</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>s100B</td>
<td>697.0 (600.5 - 867.6)</td>
<td>570.3 (466.5 - 746.2)</td>
<td>630.3 (559.2 - 649.4)</td>
<td>647.3 (594.0 - 802.0)</td>
</tr>
<tr>
<td>GFAP</td>
<td>76.4 (63.4 - 99.1)</td>
<td>64.9 (59.9 - 84.7)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>NE (ng/mL)</td>
<td>1.5 (1.2 - 2.1)</td>
<td>1.5 (1.3 - 1.9)</td>
<td>1.4 (1.0 - 2.0)</td>
<td>1.5 (1.2 - 2.4)</td>
</tr>
<tr>
<td>Ng (ng/mL)</td>
<td>7.8 (4.6 - 11.9)</td>
<td>11.4 (6.1 - 21.0)</td>
<td>16.4 (7.2 - 19.6)</td>
<td>9.2 (5.4 - 28.8)</td>
</tr>
<tr>
<td>CKBB</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VILIP-1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tau</td>
<td>24.2 (19.0 - 33.2)</td>
<td>13.8 (11.7 - 20.2)</td>
<td>14.4 (13.0 - 17.9)</td>
<td>20.4 (16.8 - 23.4)</td>
</tr>
<tr>
<td>vWF (µg/mL)</td>
<td>37.7 (22.6 - 53.7)</td>
<td>41.7 (23.5 - 52.0)</td>
<td>30.4 (20.4 - 34.6)</td>
<td>35.7 (19.5 - 55.6)</td>
</tr>
<tr>
<td>BDNF (ng/ml)</td>
<td>0.9 (0.6 - 2.0)</td>
<td>1.3 (0.7 - 2.7)</td>
<td>1.3 (0.8 - 4.1)</td>
<td>1.7 (0.8 - 4.1)</td>
</tr>
<tr>
<td>PRDX-6 (ng/L)</td>
<td>26.4 (18.6 - 33.3)</td>
<td>29.3 (21.9 - 47.6)</td>
<td>20.7 (15.6 - 25.0)</td>
<td>24.6 (20.4 - 39.0)</td>
</tr>
</tbody>
</table>

Interleukin (IL) -1α, -1β, -2, -4, -5, -6, -7, -10, -12p40, -12p70, -13, -15, -16, -17A; tumor necrosis factor (TNF) -α, -β; granulocyte macrophage colony-stimulating factor (GM-CSF); vascular endothelial growth factor (VEGF); interferon-gamma (IFN-γ); interferon gamma-induced protein (IP) -10; monocyte chemoattractant protein (MCP) -1, -4; macrophage derived chemokine, (MDC); macrophage inflammatory protein (MIP) -1α, -1β; thymocyte- and activation-regulated chemokine (TARC); s100 calcium binding protein beta (s100B), glial fibrillary acidic protein (GFAP); neuron specific enolase (NSE); neurogranin (Ng); creatine kinase-BB isoenzyme (CKBB); visinin-like protein (VILIP-1); von Willebrand factor (vWF); brain derived neurotrophic factor (BDNF); peroxiredoxin (PRDX) -6.

All biomarkers are expressed as the median and IQR.

- **a** = Biomarkers were included if replicates had less than a 25% CV, were within the LLOQ and ULOQ, and had an inter-plate variance of less than 20% as measured by internal controls.

- **b** = pg/mL unless otherwise specified.
SRC athletes displayed several significant biomarker differences, both between groups as compared to matched control athletes, and within groups across time. Tau levels in SRC athletes at medical clearance were significantly elevated compared to samples within 1-week of injury and matched controls (Fig 4 A). In addition, subacute tau levels in SRC athletes were significantly lower than baseline controls, but not different from matched control athletes (Fig 4 A). PRDX-6 levels were significantly elevated sub-acutely after injury and at medical clearance compared to matched controls (Fig 4 D). Lastly, sub-acute vWF levels were elevated in SRC athletes compared to matched controls.

Differences were identified in inflammatory markers between SRC athletes and matched controls. TNF-α levels were significantly lower at medical clearance in SRC athletes compared to matched controls (Fig 4 F). Furthermore, chemokine IP-10 levels were elevated in SRC athletes at medical clearance compared to matched controls and baseline controls (Fig 4 K).
Figure 4.1 - Plasma biomarker concentrations in athletes with a sport-related concussion measured sub-acutely and at medical clearance. Neuroinjury markers: Tau, s100 calcium binding protein beta (s100B), neurogranin (NRGN), peroxiredoxin (PRDX)-6 and von
Willebrand factor (vWF); Cytokines: tumor necrosis factor (TNF)-α, interleukins (IL)-12p40, -16; Chemokines: monocyte chemoattractant protein (MCP)-1, -4, interferon gamma-induced protein (IP)-10, macrophage inflammatory protein (MIP)-1β, thymocyte- and activation-regulated chemokine (TARC), and eotaxin measured approx. one week post-injury and at medical clearance (N = 19; red circles), vs. matched control subjects for time of year, sex, age and sport (N = 16; green circles), and healthy athletes sampled prior to the beginning of the varsity season (N = 87; white circles). Circles and lines represent the median and IQR, respectively. \( ^a = p < 0.05 \) vs. matched control; \( ^b = p < 0.05 \) vs. 3-7 days post-concussion; \( ^c = p < 0.05 \) vs. pre-season healthy athletes.

4.4.3 Relationship between blood biomarker profiles and participant characteristics

PLS analysis of the covariance between SCAT3 symptoms and blood biomarkers in concussed athletes sampled sub-acute post-injury is shown in Figure 4.2. The model yielded a fraction of variance explained at 0.77. An increase in total symptoms reported was associated with a significant decrease in brain injury markers NSE, PRDX-6, and inflammatory chemokines MCP-1, MIP-1β and Eotaxin, corrected at FDR 0.05 (Figure 4.2). MCP-1 displayed the greatest inverse covariant relationship to total symptoms (Figure 4.2).
Figure 4.2 - Covariance between plasma biomarkers and SCAT3 total symptoms sub-acute in athletes with a sport-related concussion. Neuroinjury markers: s100 calcium binding protein beta (s100B), neuron-specific enolase (NSE), neurogranin (NRGN), Tau, von-Willebrand factor (vWF), brain-derived neurotrophic factor (BDNF), peroxiredoxin (PRDX)-6; Cytokines: interleukin (IL)-7, -12p40, -15, -16, tumor necrosis factor (TNF)-α; Chemokines: IL-8, monocyte chemoattractant protein (MCP)-1, -4, interferon gamma-induced protein (IP)-10, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymocyte- and activation-regulated chemokine (TARC), eotaxin and eotaxin-3. Bars represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. * = FDR < 0.05
4.5 Discussion

There were three main findings in the current study: 1) blood biomarker levels differed in SRC athletes sub-acute and at medical clearance compared to uninjured athletes, 2) greater symptom endorsement reported by athletes in the subacute phase after SRC was inversely related to blood biomarker levels, and 3) biomarker levels varied in uninjured athletes over the course of the varsity/academic year.

Recent evidence suggests that peripherally measured tau in the systemic circulation may reflect CNS tauopathy (Zetterberg, Wilson et al. 2013, Stern, Tripodis et al. 2016) or axonal damage (Rubenstein, Chang et al. 2015). In the current study, while we did not observe differences in tau levels between SRC athletes and matched controls in the subacute time period, they were significantly higher at medical clearance. While similar null findings have been found within 24 h after mTBI (Bulut, Koksal et al. 2006, Kavalci, Pekdemir et al. 2007), our findings are in contrast to others who have identified elevations in serum tau both immediately and in the subacute period after SRC (Shahim, Tegner et al. 2014). Study design may partly account for these discrepancies, as one of the aforementioned studies compared T-tau levels in concussed athletes to pre-season baseline levels as opposed to in-season matched controls, and only evaluated males participating in a single sport (ice hockey) (Shahim, Tegner et al. 2014). Furthermore, tau may have a biphasic secretion pattern, and it is possible that our chosen sampling times did not coincide with these peak periods (Shahim, Tegner et al. 2014).

It is unclear why we observed a gradual increase in peripheral tau levels after SRC that was not observed sub-acute. However, chronically elevated t-tau has been found in military soldiers with a history of reported or medically documented concussion up to 18 months post-deployment (Olivera, Lejbman et al. 2015), and elevated T-tau levels have been shown at one
month post-severe TBI (Rubenstein, Chang et al. 2015). Indeed, there are a number of similarities between SRC and military concussion; both populations are typically young, athletic, have the same physical and mental requirements for success in their fields, and are at risk for repetitive trauma. In addition, despite some reported differences in acute concussive symptoms and neurocognitive performance between military-related mild TBI/concussion and SRC (Luethcke, Bryan et al. 2011), a recent retrospective case series demonstrated that the natural history of recovery in blast-induced mild brain injury mirrors the pattern of recovery in SRC (Larres, Carr et al. 2016). Lastly, our findings are also in agreement with recent work from our group, which demonstrated that a history of multiple previous concussions and collision sport participation was associated with increased T-tau levels in the plasma of uninjured athletes (Di Battista, Rhind et al. 2016). Understanding if and how these long-term peripheral perturbations relate to the accumulation of tau in the brain will continue to be important, as the latter has been linked to chronic neurodegenerative disorders such as chronic traumatic encephalopathy and Alzheimer’s disease (McKee, Cantu et al. 2009, Omalu, Bailes et al. 2011, Small, Kepe et al. 2013, Zetterberg, Wilson et al. 2013, Barrio, Small et al. 2015).

Along with T-tau, we found elevated PRDX-6 in athletes with SRC compared to healthy athletes sub-acutely and at medical clearance. PRDX-6 is an antioxidant enzyme found primarily in astrocytes, and may protect neuronal membranes and mitochondria from damage due to lipid peroxidation (Manevich, Hutchens et al. 2014). PRDX-6 has been targeted in TBI due to the vulnerability of the brain to lipid peroxidation (Manevich, Hutchens et al. 2014), and was recently uncovered as a candidate TBI biomarker in a rodent model using unsupervised autoimmune screening (Buonora, Mousseau et al. 2015). Elevated PRDX-6 levels have been observed acutely across the spectrum of TBI in humans (Buonora, Yarnell et al. 2015), and the recovery of PRDX-6 activity within 24 h of severe TBI in humans has been associated with
improved recovery (Manevich, Hutchens et al. 2014). Notably, PRDX-6 may be released from platelets (Buonora, Mousseau et al. 2015) and peripheral levels may be influenced by exercise (Wadley, Aldred et al. 2016).

We also found elevated IP-10 levels at medical clearance in SRC athletes compared to matched controls. IP-10 is a chemokine with a number of functions, including leukocyte recruitment and the activation of T-cells, B-cells, macrophages and natural killer cells (Liu, Guo et al. 2011). In TBI, chemokines are important in mediating peripheral leukocyte recruitment to the brain to facilitate repair (Semple, Bye et al. 2010, Semple, Frugier et al. 2010, Jaerve and Muller 2012). We previously observed elevated levels of IP-10 in the acute period after moderate and severe TBI that were highly correlated with patient mortality (Di Battista, Rhind et al. 2016). However, there are no studies to our knowledge that have evaluated peripheral IP-10 protein levels sub-acute after SRC. While these initial findings are consistent with both our findings in acute moderate and severe TBI, and are generally supported by the known role of chemokines in brain injury (Catania, Lonati et al. 2009, Jaerve and Muller 2012), the specific role of IP-10 chronically after mild TBI/concussion has yet to be elucidated.

Notably, that we found perturbations in systemic biomarker levels at the time of medical clearance is consistent with a number of previous investigations that have identified physiological disturbances at clinical recovery; perturbations in functional and structural connectivity, as well as neurometabolism have been identified beyond cognitive symptom resolution in the brains of athletes after SRC (McCrea, Prichep et al. 2010, Chamard, Lassonde et al. 2013, Zhu, Covassin et al. 2015). In addition, our group recently showed that heart rate variability – an index of neuroendocrine function – was also perturbed at clinical recovery post-SRC (Hutchison, Mainwaring et al. 2016). It is unclear if these prolonged physiological
symptoms are a risk factor for future injury, or a by-product of brain restitution. However, our results are supportive of further investigation into the potential clinical application of employing blood biomarkers to evaluate physiological recovery in asymptomatic individuals.

Several inflammatory mediators, including MCP-1, MIP-1β and Eotaxin, as well as neuroinjury markers PRDX-6 and NSE, were inversely correlated with total symptoms reported by athletes in the subacute period after injury. In support of this, Gill and colleagues recently showed decreased inflammatory gene expression in the peripheral blood mononuclear cells of SRC athletes sub-acutely after injury (Gill, Merchant-Borna et al. 2016). This finding was reinforced by a subsequent study by the same group, which found that 73% and 85% of gene transcripts – including numerous inflammatory genes – were down-regulated in the acute and subacute period, respectively, after SRC (Merchant-Borna, Lee et al. 2016). Similar to our study design, in both investigations a healthy control group matched for age, gender, sport and exercise was included. However, while we observed decreased protein levels in the subacute period after SRC in symptomatic athletes, we did not see the global changes in protein expression that were noted by Gill and colleagues. The reasons for this are unclear, but it is likely that the complex interplay between gene transcription and protein synthesis is partly responsible for such discordant results (Greenbaum, Colangelo et al. 2003, Guo, Xiao et al. 2008). Nevertheless, our findings are consistent with these previous reports of a subacute immunosuppression after SRC.

Understanding how blood biomarkers relate to injury-induced processes in the brain is of paramount importance in order to further our pathophysiological knowledge of secondary injury in humans. The primary concerns regarding peripheral indices of CNS processes are 1) how blood biomarkers mechanistically link to brain injury/repair signaling, and 2) the potential confounding extracranial sources, if any, of such markers. Recent evidence suggests that in the
acute period after TBI, brain-derived biomarkers enter the circulation via glymphatic clearance, and that the disruption of glymphatic function may impede the clearance of such molecules into the circulation, hence making peripheral biomarker detection difficult after injury (Iliff, Wang et al. 2012, Iliff, Chen et al. 2014, Plog, Dashnaw et al. 2015, Plog and Nedergaard 2015). However, disruption of the BBB as well as neuroendocrine dysfunction may also alter peripheral biomarker levels after brain injury (Chodobski, Zink et al. 2011, Santarsieri, Kumar et al. 2015, Di Battista, Rhind et al. 2016). Indeed, alterations to hormones produced by both the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA)-axis can modulate peripheral immune function (Elenkov, Wilder et al. 2000, Pavlov and Tracey 2004, Bellavance and Rivest 2014), and Merchant-Borna et al. observed that at 7 days after SRC, gene transcription profiles in peripheral blood leukocytes reflected regulation of the HPA-axis (Merchant-Borna, Lee et al. 2016). In addition, we recently demonstrated that peripheral catecholamine release acutely after moderate and severe TBI was highly correlated to circulating levels of inflammatory cytokines and chemokines (Di Battista, Rhind et al. 2016). Indeed, that we observed lower levels of TNF-α in SRC athletes at medical clearance may support on-going neuroendocrine dysfunction; the activation of neural afferent and efferent pathways can inhibit cytokine production via cholinergic signaling, a mechanism that has specifically been shown to inhibit the production of TNF-α by macrophages (Tracey 2002, Tracey 2007, Tracey 2009). However, little evidence of these mechanisms exists in milder forms of TBI; while we found inflammatory alterations in symptomatic athletes after SRC, we did not evaluate their potential relationship to neuroendocrine disruption. Future studies are warranted to probe both the involvement of the neuroendocrine regulation after SRC, and its reciprocal influence on inflammation.

In view of the potential extracranial influences on peripheral biomarkers of SRC, we found many of the neuroinjury and inflammatory markers assayed were significantly altered
throughout the course of the athletic/academic season. This is consistent with the notion that biomarkers distal to the site of injury are subject to “noise” from extracranial biological perturbations, particularly in an athletic population where the influence of exercise and other academic/sport-related stressors are present. For example, it has recently been suggested that peripheral tau may be derived from muscle tissue as a result of strenuous exercise or muscle damage incurred throughout the season (Peskind, Brody et al. 2013). However, our results do not support this, as we found T-tau decreased over the course of the varsity/academic year in healthy athletes. While it is possible that academic-related stress and/or the chronic effects of exercise may alter systemic biomarker levels, this has not been clearly demonstrated in humans to date. In fact, Shahim and colleagues found that a “friendly” hockey game without any concussive injuries resulted in increased peripheral blood levels of the neuroinjury markers s100B and NSE, but not tau (Shahim, Tegner et al. 2014). Moreover, controlling for extracranial factors is especially pertinent when looking at peripheral cytokines and chemokines due to the inherent pleiotropicity of the inflammatory response.

This study was limited by a relatively small sample size. A greater number of participants would have allowed for sex stratification, as well as the ability to dichotomize collision-sport athletes and non-collision sport athletes in view of our previous findings that identified specific blood biomarker signatures according to sex and sport participation (Di Battista, Rhind et al. 2016). Furthermore, while the SCAT3 is a validated concussion assessment tool, additional clinical tests, including mental health and cognitive assessments would have aided the current correlative analysis between symptoms and blood biomarkers. Furthermore, this study was limited by our sampling period; the addition of earlier sample times may have aided in understanding the temporal kinetics of peripheral biomarkers after SRC. Finally, while a number of the biomarkers evaluated in this study, particularly those comprising the novel prototype
neuroinjury panel, have been experimentally and clinically associated with traumatic brain injury, it is important to note that a number of these markers, such as MCP-1 and vWF, are not exclusive to brain injury. However, despite these limitations, we were able to identify significant differences in peripheral blood biomarkers in athletes with a SRC compared to healthy athletes, and observed a significant inverse correlation between blood biomarker levels and total symptoms at the time of sampling.

4.6 Conclusion

Our findings support the application of blood biomarkers to inform secondary injury pathophysiology after SRC, from the subacute period throughout recovery. These findings are consistent with a growing body of research highlighting physiological perturbations at the time of clinical recovery. Notably, in the subacute phase of SRC there appears to be an inverse correlation between peripheral biomarkers and total symptoms reported. Lastly, future biomarker endeavors in sport concussion research should consider not only the complexity and heterogeneity of sampling populations, but also the potential impact of concurrent athletic/academic stressors present throughout the academic year.
Chapter 5
Altered Blood Biomarker Profiles in Athletes with a History of Repetitive Head Impacts

The components of this chapter were published as follows:


The lead author contributed to the manuscripts conceptual framework, performed all cytokine/chemokine immunoassay experiments, statistically analyzed and interpreted the data, drafted the manuscript, and contributed to its critical revision.
5 Altered Blood Biomarker Profiles in Athletes with a History of Repetitive Head Impacts.

5.1 Abstract

BACKGROUND. The long-term health effects of concussion and sub-concussive impacts in sport are unknown. Growing evidence suggests both inflammation and neurodegeneration are pivotal to secondary injury processes and the etiology of neurodegenerative diseases.

OBJECTIVES. In the present study we characterized circulating brain injury and inflammatory mediators in healthy male and female athletes according to concussion history and collision sport participation. METHODS. Eighty-seven university level athletes (male, n=60; female, n=27) were recruited before the start of the competitive season. Athletes were healthy at the time of the study (no medications, illness, concussion or musculoskeletal injuries). Dependent variables included 29 inflammatory and 10 neurological injury analytes assessed in the peripheral blood by immunoassay. Biomarkers were statistically evaluated using partial least squares multivariate analysis to identify possible relationships to self-reported previous concussion history, number of previous concussions and collision sport participation in male and female athletes. RESULTS. Multiple concussions were associated with increases in peripheral MCP-1 in females, and MCP-4 in males. Collision sport participation was associated with increases in tau levels in males.

CONCLUSION. These results are consistent with previous experimental and clinical findings that suggest ongoing inflammatory and cerebral injury processes after repetitive mild head trauma. However, further validation is needed to correlate systemic biomarkers to repetitive brain impacts, as opposed to the extracranial effects common to an athletic population such as exercise and muscle damage.
5.2 Introduction

Concern regarding the potential negative health impact of concussions and collision sport participation has led to an increased demand to delineate the pathophysiological mechanisms mediating long-term outcomes (Blennow, Hardy et al. 2012). Our current conceptual understanding of concussion pathophysiology consists of an acute disturbance of neurobehavioral function together with damage to neuronal and glial cells (Giza and Hovda 2014). Symptoms are commonly short-lived and self-limited, resolving within a span of days to weeks (McCrea, Guskiewicz et al. 2003, Giza, Kutcher et al. 2013, Broglio, Cantu et al. 2014); however, recent objective advances in neuroimaging and analytical biomarker assessment have documented underlying functional and structural abnormalities persisting beyond symptom resolution (Gosselin, Saluja et al. 2010, Murugavel, Cubon et al. 2014, Yuh, Hawryluk et al. 2014). Furthermore, evidence is now emerging that suggests concussion, as well as the repetitive head impacts that commonly occur in collision sport participation, may contribute to negative health outcomes such as CTE (Omalu, Bailes et al. 2011, Lee, Hou et al. 2013, Small, Kepe et al. 2013, Fakhran and Alhilali 2014, Barrio, Small et al. 2015, Mendez, Paholpak et al. 2015). However, our current understanding of these pathophysiological processes in humans is limited.

Inflammation is an important contributor to both repair and neurodegenerative processes after neurotrauma (Jaerve and Muller 2012, Anthony and Couch 2014, Balu 2014). Resident microglial cells and CNS invading peripheral immune cells facilitate the acute repair and regeneration of damaged brain tissue via the release of neurotrophic factors and scavenging of debris (Loane and Byrnes 2010, Blaylock and Maroon 2011, Loane and Kumar 2016). However, chronic inflammation may also exacerbate neuronal and glial cell injury, leading to further cellular degeneration and culminating in the deposition of neurofibrillary tangles and amyloid plaques (Blaylock and Maroon 2011). In view of this, human studies have found prolonged
neuroinflammation persisting for months to years after moderate and severe TBI (Gentleman, Leclercq et al. 2004, Ramlackhansingh, Brooks et al. 2011, Johnson, Stewart et al. 2013, Smith, Gentleman et al. 2013), and experimental evidence suggests these maladaptive processes may occur through the interaction of inflammatory mediators and glutamate receptors in the CNS (Stellwagen, Beattie et al. 2005, Takeuchi, Ikoma et al. 2006, Yawata, Takeuchi et al. 2008, Shijie, Takeuchi et al. 2009). Moreover, multiple head impacts may worsen these processes by priming microglial cells, leading to an exaggerated inflammatory reaction upon subsequent trauma (Blaylock and Maroon 2011, Perry and Holmes 2014).

Inflammation post-concussion is difficult to characterize due to practical limitations such as the inability to access tissue proximal to the site of injury, and the invasive nature of CSF acquisition (Di Battista, Rhind et al. 2013). Nevertheless, peripheral blood samples have the potential to provide meaningful information regarding inflammatory processes both in the CNS and periphery in response to brain injury, in a relatively cost-effective, non-invasive manner (Blaylock and Maroon 2011, Di Battista, Rhind et al. 2013, Muccigrosso, Ford et al. 2016). In view of this, recent evidence has shown that increased circulating CRP levels post-injury are associated with persistent post-concussive syndrome symptoms (Su, Xu et al. 2014), and coated platelet levels, an inflammatory correlate, are elevated in mild TBI patients up to 9 years post-injury (Prodan, Vincent et al. 2014).

Historically, one of the limitations in concussion research has been the lack of consideration for potential sex differences. Specifically, there has been a paucity of concussion research on females (Comper, Hutchison et al. 2010). Yet, available evidence suggests that females may be at a greater risk for concussion (Hootman, Dick et al. 2007, Dick 2009), report more symptoms post-concussion (Dick 2009, Brown, Elsass et al. 2015), and take longer to
recover (Dick 2009, Baker, Leddy et al. 2016). In addition, it is known that males and females display distinct immunological responses; women exhibit stronger cellular and humoral immune responses, are more prone to many autoimmune diseases, but are less susceptible to various of bacterial, viral, and fungal infections (Bouman, Heineman et al. 2005, Fish 2008). Therefore, the possibility exists that inflammatory related processes occurring chronically after concussion may have sex-specific pathological sequelae.

Thus, in this study we set out to examine a panel of systemic brain injury markers and inflammatory mediators in a sample of male and female athletes to characterize the relationship between these biological indices, concussion history, and collision sport participation.

5.3 Methods

5.3.1 Participants

Participants were recruited from University of Toronto intercollegiate “varsity” athletic teams between August 2014 and December 2015. A member of the research team provided an overview of the study and requested consent to obtain blood samples and use the SCAT3 results for research purposes. Medical history was obtained by the team’s therapist/trainer, followed by administration of the SCAT3. Sixteen teams (8 male, 8 female) were contacted for research purposes, including the following sports: basketball, baseball, field hockey, football, ice hockey, lacrosse, rugby, soccer, wrestling and volleyball. Athletes were excluded if they suffered from seasonal allergies, cold, infection, disclosed any inflammatory-related health conditions, were taking any medications other than birth control at the time of the study, or had musculoskeletal injuries (9 subjects). Study procedures were approved by the Health Sciences Research Ethics Board, University of Toronto (protocol reference # 27958), and all participants provided written informed consent prior to the study.
5.3.2 Measures

**Sport Concussion Assessment Tool 3 (SCAT3):** The SCAT3 combines aspects of several previously published concussion tools into eight components designed to assess concussion symptoms (number endorsed and severity), cognition (Sideline Assessment of Concussion or SAC and Maddocks questions), balance (firm conditions of the Balance Error Scoring System or BESS), GCS and neurological signs (physical signs, coordination) (Guskiewicz, Register-Mihalik et al. 2013). Each of the eight components are scored and recorded. The symptom score is comprised of a 22-item post-concussion symptom scale using a seven-point Likert scale rating. Symptom severity is obtained by summing the rated symptom score for each symptom (Guskiewicz, Register-Mihalik et al. 2013). This symptom scale has been shown to be reliable and valid for the assessment of both symptom presence and severity (Galetta, Galetta et al. 2013, Guskiewicz, Register-Mihalik et al. 2013, Brown, Elsass et al. 2015).

5.3.3 Blood Sample Collection

Venous blood samples were drawn from athletes after consent was obtained and prior to the beginning of the competitive varsity season. Samples were drawn into a 10-mL K$_2$EDTA (with 4mM sodium metabisulfite [Na$_2$S$_2$O$_5$]) or 4-mL non-additive (Vacutainer, Becton Dickinson, NJ, USA) tube. Within one hour, specimens were centrifuged at 1600 x g for 15 minutes at 4°C, and the plasma supernatant was aliquoted and frozen at -70°C until analysis.

5.3.4 Biomarker Analysis

Twenty-eight of the thirty-nine markers were analyzed using MSD® 96-Well MULTI-ARRAY/-SPOT® V-plex Human Immunoassay Kits purchased from MSD (MD, USA), and run on a MSD® Sector imager™ 6000 with Discovery Workbench software (version 3.0.18). A prototype assay panel of eleven additional neuroinjury markers including total tau, GFAP,
s100B, NSE, NRGN, CKBB, VILIP-1, vWF, BDNF, PRDX6, and MCP-1, was assessed by multiplexed immunoassay (Debad, Campbell et al. 2016).

5.3.5 Statistical Analyses

Demographic and descriptive statistics were completed on male and female athletes by student’s independent $t$-test Mann Whitney $U$, or $\chi^2$, where appropriate. For dichotomized analysis of collision vs. non-collision sports, collision sports were delineated as sports with purposeful contact as an inherent part of the game, and included men’s ice hockey, football, rugby, lacrosse, and women’s rugby (Meehan, Taylor et al. 2016). All other sports, including those where inadvertent contact may occur (soccer, basketball), were considered non-collision sports (Meehan, Taylor et al. 2016). For all analyses, individual biomarker values were excluded if they were above or below the manufacturers’ recommended level of quantitation for each analyte, or displayed a CV >25% between duplicates. Because multiple 96-well plates were analyzed, inter-plate variance was accounted for; plates were only included in the statistical analysis if the inter-plate variance was <20%, calculated from internal control samples acquired on each plate. Biomarkers were not included in the multivariate analysis if >30% of the data points were missing in any group. Multivariate analysis was conducted using a PLS-DA. PLS-DA is a supervised technique used to objectively characterize the covariance between a set of predictor variables and binary response variables (Wold, Sjostrom et al. 2001, Ballabio and Consonni 2013). A PLS-DA output provides model Accur and PProb. Briefly, these indices measure how accurately a fitted model can predict a binary outcome based solely on predictor variables. Accur is evaluated by assigning each subject to the outcome group with the most similar mean PLS score; 1 = correctly predicted, and 0 = incorrectly predicted. This provides a simple, robust metric of prediction, which does not depend on a specific probability model. PProb is the likelihood of the PLS model identifying the correct outcome conditional on the
observed subject scores, under a Gaussian noise model. This provides an alternative probabilistic measure that accounts for uncertainty in the PLS model and observed data. With numerous response variables, the PLS analysis yields the fraction of variance explained. Fraction of variance reflects the proportion of total inter-subject variability in biomarker data that is described by the PLS component of interest. In the current study, covariance between peripheral blood biomarkers (predictor variables) and both concussion history and collision sport participation (response variables) was assessed separately in male and female athletes. Missing biomarker values were imputed using the k-means nearest-neighbour method (Armitage, Godzien et al. 2015), and were rank-transformed to ensure robustness against non-normality. Significant biomarker loadings were identified by performing bootstrap resampling on subjects (1000 iterations) to obtain empirical p-values, which were then corrected for multiple comparisons at a FDR of 0.05. For PLS plots, variable loadings are represented as bootstrap ratios (i.e., the bootstrapped mean / standard error), which are z-scored statistics reflecting the reliability of variable contributions. Descriptive and univariate statistics were completed using Stata Version 14.1 (StataCorp, TX, USA). Multivariate analyses were conducted using in-house software developed for Matlab, Version R2015b (Matworks, Natick MA). All data were visualized using GraphPad Prism Version 6.0f (GraphPad Inc., CA, USA).

5.4 Results

5.4.1 Demographics and Clinical Characteristics

A total of 87 athletes were included in the study (male, n = 60; female, n = 27). Athlete characteristics and concussion history are listed in Table 5.1. Briefly, athletes were of similar age, and we observed no significant differences in medical history and SCAT3 symptoms at the time of the study. There were no differences between male and female athletes regarding concussion history, number of previous concussions, and days since last concussion. As
expected, a significantly higher proportion of males played in collision sports as compared to their female counterparts (63.9% vs. 7.4%, respectively).

Table 5.1 Athlete demographics and characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male (n = 60)</th>
<th>Female (n = 27)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>19.5 ± 2.0</td>
<td>19.5 ± 1.8</td>
<td>0.86</td>
</tr>
<tr>
<td>Concussion history – n (%)</td>
<td>23 (38.3)</td>
<td>12 (44.4)</td>
<td>0.55</td>
</tr>
<tr>
<td>Days since last concussion – median (IQR)</td>
<td>793 (420 – 1249)</td>
<td>552 (375.5 – 714.5)</td>
<td>0.170</td>
</tr>
<tr>
<td>Number of previous concussions</td>
<td>0.64 ± 1.0</td>
<td>1.1 ± 1.7</td>
<td>0.619</td>
</tr>
<tr>
<td>0 – n (%)</td>
<td>37 (61.7)</td>
<td>15 (55.6)</td>
<td></td>
</tr>
<tr>
<td>1 – n (%)</td>
<td>12 (20.0)</td>
<td>5 (18.5)</td>
<td></td>
</tr>
<tr>
<td>2 – n (%)</td>
<td>8 (13.3)</td>
<td>3 (11.1)</td>
<td></td>
</tr>
<tr>
<td>&gt; 3 – n (%)</td>
<td>3 (5.0)</td>
<td>4 (14.8)</td>
<td></td>
</tr>
<tr>
<td>Collision sport participation – n (%)</td>
<td>39 (65.0)</td>
<td>2 (7.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Medical history – n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Migraines</td>
<td>2 (3.3)</td>
<td>0 (0.0)</td>
<td>0.156</td>
</tr>
<tr>
<td>Learning disability</td>
<td>1 (1.7)</td>
<td>0 (0.0)</td>
<td>0.203</td>
</tr>
<tr>
<td>Depression/Anxiety or other psychiatric disorders</td>
<td>1 (1.7)</td>
<td>2 (7.4)</td>
<td>0.226</td>
</tr>
<tr>
<td>Family history of psychiatric illness</td>
<td>12 (20.0)</td>
<td>7 (25.9)</td>
<td>0.247</td>
</tr>
<tr>
<td>SCAT3 symptom scores</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total symptoms</td>
<td>3.4 ± 3.6</td>
<td>3.8 ± 3.0</td>
<td>0.350</td>
</tr>
<tr>
<td>Symptom severity</td>
<td>5.4 ± 7.0</td>
<td>6.0 ± 4.7</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Unless otherwise stated, results are reported as the mean ± standard deviation (SD).

Demographic and characteristic differences between male and female athletes were assessed by $\chi^2$, Mann-Whitney U, or independent student’s t-test, where appropriate.
Table 5.2 List of biomarkers analyzed.

<table>
<thead>
<tr>
<th>Markers (pg/mL)*</th>
<th>% Quantifiable*</th>
<th>Median (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>27.6</td>
<td>--</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>IL-2</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.1</td>
<td>--</td>
</tr>
<tr>
<td>IL-5</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.3</td>
<td>--</td>
</tr>
<tr>
<td>IL-7</td>
<td>59.8</td>
<td>2.6 (2.0 – 3.7)</td>
</tr>
<tr>
<td>IL-10</td>
<td>6.9</td>
<td>--</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>97.7</td>
<td>121.4 (93.1 – 146.2)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>IL-13</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>IL-15</td>
<td>100</td>
<td>2.3 (2.0 – 2.7)</td>
</tr>
<tr>
<td>IL-16</td>
<td>70.1</td>
<td>259.2 (198.5 – 369.0)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>1.1</td>
<td>--</td>
</tr>
<tr>
<td>TNF-α</td>
<td>96.5</td>
<td>1.8 (1.5 – 2.2)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>--</td>
</tr>
<tr>
<td>VEGF</td>
<td>87.3</td>
<td>36.5 (28.0 – 55.6)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>16.1</td>
<td>--</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>91.9</td>
<td>77.7 (62.7 – 94.3)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>69.3</td>
<td>22.2 (18.5 – 31.3)</td>
</tr>
<tr>
<td>IP-10</td>
<td>77.0</td>
<td>202.6 (159.7 – 257.1)</td>
</tr>
<tr>
<td>IL-8</td>
<td>82.8</td>
<td>1.9 (1.5 – 2.7)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>96.6</td>
<td>86.8 (72.4 – 109.4)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>94.2</td>
<td>26.5 (19.5 – 38.3)</td>
</tr>
<tr>
<td>MDC</td>
<td>98.8</td>
<td>807.2 (706.3 – 989.1)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>4.6</td>
<td>--</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>95.4</td>
<td>37.9 (30.3 – 49.6)</td>
</tr>
<tr>
<td>TARC</td>
<td>88.5</td>
<td>43.1 (27.3 – 55.5)</td>
</tr>
<tr>
<td><strong>Neuroinjury Markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s100B</td>
<td>85.0</td>
<td>707.1 (603.0 – 896.1)</td>
</tr>
<tr>
<td>GFAP</td>
<td>59.8</td>
<td>75.6 (63.2 – 98.1)</td>
</tr>
<tr>
<td>NSE (ng/mL)</td>
<td>100</td>
<td>1.5 (1.2 – 2.1)</td>
</tr>
<tr>
<td>Neurogranin (ng/mL)</td>
<td>100</td>
<td>7.8 (4.5 – 11.8)</td>
</tr>
<tr>
<td>CKBB</td>
<td>11.5</td>
<td>--</td>
</tr>
<tr>
<td>VILIP-1</td>
<td>8.0</td>
<td>--</td>
</tr>
<tr>
<td>Tau</td>
<td>98.8</td>
<td>24.0 (18.5 – 32.9)</td>
</tr>
<tr>
<td>vWF (µg/mL)</td>
<td>96.5</td>
<td>38.2 (23.3 – 53.5)</td>
</tr>
<tr>
<td>BDNF</td>
<td>100</td>
<td>856.4 (566.4 – 2022.7)</td>
</tr>
<tr>
<td>PRDX-6 (ng/mL)</td>
<td>100</td>
<td>26.4 (18.6 – 33.2)</td>
</tr>
</tbody>
</table>

Interleukin (IL)-1α, -1β, -2, -4, -5, -6, -7, -10, -12p40, -12p70, -13, -15, -16, -17A, tumor necrosis factor (TNF) -α, -β, granulocyte macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ), eotaxin, eotaxin-3, interferon gamma-induced protein (IP) -10, IL-8, monocyte chemoattractant protein (MCP)-1, -4, macrophage derived chemokine, (MDC), macrophage inflammatory protein (MIP)-1α, -1β, thymocyte- and activation-regulated chemokine (TARC),
s100 calcium binding protein beta (s100B), glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), creatine kinase-BB isoenzyme (CKBB), visinin-like protein (VILIP-1), von Willebrand factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX) -6.

* = all markers reported as pg/mL unless otherwise stated

^a^ = Biomarkers were included if replicates had less than a 25% CV, were within the LLOQ and ULOQ, and had an inter-plate variance of less than 20% as measured by internal controls.

“—” = below assay quantitation in >50% of samples analyzed.

5.4.2 Systemic inflammatory marker analysis

A list of all biomarkers with corresponding median values and the percent of samples detectable in the plasma for each analyte are listed in Table 5.2. No significant differences were identified between male and female athletes who did not participate in collision sports or who had no previous history of concussion (data not shown).

5.4.3 Multivariate analysis

PLS analysis of the covariance between peripheral blood biomarkers and athlete characteristics is shown in Fig 5.1. No individual biomarkers were significantly correlated to previous concussion history in either male (model PProb = 0.50, Accur = 0.48) or female (model PProb = 0.41, Accur = 0.35) athletes (Fig 5.1 A). Similarly, when further stratified, compared to athletes with no concussion history, athletes with one previous concussion displayed no significant differences in biomarker levels (males – model PProb = 0.47, Accur = 0.46; females – model PProb = 0.46, Accur = 0.46) (Fig 5.1 B). However, in athletes with multiple previous concussions vs. those with no previous concussions (males – model PProb = 0.53, Accur = 0.51; females – model PProb = 0.43, Accur = 0.44), female athletes had significantly higher MCP-1 (median conc.; 96.4 vs. 69.3 pg/mL) levels, while male athletes had significantly higher MCP-4 (median conc.; 48.3 vs. 26.1 pg/mL) (Fig 5.1 C). See Appendix 5.1 for plasma concentrations of
all biomarkers according to concussion history.

**Fig 5.1 - Biomarker covariance with concussion history in athletes.** Brain injury markers: s100 calcium-binding protein B (s100B), neuron specific enolase (NSE), Neurogranin (NRGN), tau, von Willebrand factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX) – 6; inflammatory markers: interleukin (IL) -12p40, -15, tumor necrosis factor (TNF) -α, IL-8, monocyte chemoattractant protein (MCP)-1, -4, interferon gamma induced protein (IP) -10, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), eotaxin. Blood biomarker contributions are displayed on the x-axis for males, and y-axis for females, in (A) healthy athletes with vs. without a history of concussion, (B) healthy athletes with a single previous concussion vs. no history of concussion, and (C) healthy athletes with multiple previous concussions vs. no history of concussion. Dots represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. FDR = 0.05.

PLS analysis of the covariance between systemic biomarkers, and both collision sport participation and previous concussion history in males is shown in Fig 5.2. Only collision sport participation significantly co-varied with increases in tau (median conc; 33.9 vs. 20.8 pg/mL in non-collision sport athletes). See Appendix 5.2 for plasma concentrations of all biomarkers according to collision sport participation.
Fig 5.2 - Covariance between biomarkers and head injury characteristics in male athletes. 

Brain injury markers: s100 calcium-binding protein B (s100B), neuron specific enolase (NSE), Neurogranin (NRGN), tau, von Willebrand factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX) – 6; inflammatory markers: interleukin (IL) -12p40, -15, tumor necrosis factor (TNF) -α, IL-8, monocyte chemoattractant protein (MCP)-1, -4, interferon gamma induced protein (IP) -10, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), eotaxin. Bars represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. FDR = 0.05.

5.5 Discussion

In this study, we identified differences in the systemic biomarker profiles of male and female athletes who sustained multiple previous concussions, and in males who participate in collision sports. We included blood samples from athletes with no inflammatory-related conditions, musculoskeletal injuries or concussion symptoms prior to the start of the competitive
season. To our knowledge, this is the first report to combine an array of brain injury-related and inflammatory indices chronically after sport concussion in male and female athletes.

We found healthy female athletes with a reported history of multiple concussions had elevated blood MCP-1 levels, while males had elevations in MCP-4. Chemokines are important facilitators of peripheral immune cell migration to the CNS after injury (Jaerve and Muller 2012), and may contribute to BBB breakdown (Chodobski, Zink et al. 2011). Treatments aimed at alleviating inflammation after TBI by inhibiting chemokine recruitment to the brain have been successful in reducing cerebral damage and cognitive deficits in animals (Bao, Shultz et al. 2012, Webster, Wright et al. 2015). Furthermore, MCP-1 and MCP-4 levels are elevated acutely after moderate and severe TBI in humans, and correlated to poor patient outcome (Buonora, Yarnell et al. 2015, Di Battista, Rhind et al. 2016). While systemic chemokines have not been assessed chronically after concussion, these results are consistent with previous evidence of persistent inflammation months after mild TBI in both animals (Shultz, Bao et al. 2012, Muccigrosso, Ford et al. 2016) and humans (Prodan, Vincent et al. 2014). Admittedly, it is difficult to speculate whether these findings represent detrimental or reparative processes, as chemokines may also aid in neuronal repair and regenerative axonal sprouting (Jaerve, Schiwy et al. 2011, Jaerve and Muller 2012). Furthermore, it is unclear if MCP-1 and MCP-4 share overlapping or distinct biological actions in response to brain injury. While both molecules are involved in leukocyte recruitment, they may differ in their ability to stimulate other inflammatory mediators; for example, MCP-4 but not MCP-1 is responsible for mediating the production of chemokines IP-10 and the platelet derived chemokine ligand -5, during atherogenesis (Breland, Michelsen et al. 2010). Hence, further research is needed to elucidate both the biological sequelae and health consequences of elevated systemic chemokine levels after multiple concussions in males and females.
A second important finding was tau concentrations were higher in male athletes who participate in collision sports compared to non-collision sport athletes. Additionally, when assessed in conjunction with collision sport participation, previous concussion history became a non-significant contributor to biomarker variance. This suggests that the repetitive sub-concussive impacts associated with collision sport participation may elicit a greater biological response than reported concussion, and could have a distinct pathology. Concern regarding collision sport participation and the potential link to neurodegeneration has been highlighted in recent years as tau-laden plaque depositions have been identified in the brains of post-mortem (Omalu, Hammers et al. 2011) and living (Barrio, Small et al. 2015) former collision-sport athletes. We found collision-sport participation in male athletes was associated with a 62% increase in peripheral tau levels compared to males who participate in non-collision sports. Previous studies have also found elevated plasma and CSF tau levels in ostensibly non-concussed male boxers (Neselius, Brisby et al. 2012, Neselius, Zetterberg et al. 2013) and in military personnel who sustain multiple mTBI’s during deployment (Olivera, Lejbman et al. 2015). Regarding the latter, tau levels were elevated in soldiers with a self-reported history of concussion, and similar to the current study, participants were sampled within a time-frame of 3 months to 3 years post-injury (Olivera, Lejbman et al. 2015). While it is unclear if systemic tau is pathologically related to neurodegeneration or cerebral injury, recent findings have specifically identified plasma exosomal tau as a potential CTE biomarker in former professional athletes (Stern, Tripodis et al. 2016), and have detected associations between plasma tau and clinical conditions such as Alzheimer’s Disease (Blennow, Hampel et al. 2010, Zetterberg, Wilson et al. 2013) and mTBI (Bulut, Koksal et al. 2006). Taken together our results are consistent with these previous works, and suggest that systemic tau may be related to repetitive, sub-concussive impacts in male collision-sport athletes.
Brain-borne biomarkers may travel from the CNS into the periphery in at least two distinct fashions, through a disrupted/leaky BBB (Chodobski, Zink et al. 2011, Alves 2014), or via the glymphatic system (Iliff, Wang et al. 2012, Plog, Dashnaw et al. 2015). Regarding the latter, alterations to glymphatic function caused by clinical maladies including TBI and sleep deprivation, may attenuate the movement of proteins from the brain to the blood (Plog, Dashnaw et al. 2015, Plog and Nedergaard 2015). Yet, this process does not affect the passage of molecules across a leaky/damaged BBB (Plog, Dashnaw et al. 2015), and while the athletes evaluated in the current study were not concussed, repetitive head impacts may alter BBB integrity and increase permeability (Marchi, Bazarian et al. 2013, Zetterberg, Smith et al. 2013). Hence, it is plausible that the biomarkers we identified peripherally may be related to ongoing biological processes linked to repetitive head impacts (Broglio, Eckner et al. 2011, Erlanger 2015).

An important question stemming from these findings is how the observed elevations in these indirect peripheral measures may relate to the biological consequences of repetitive head trauma, as opposed to the effects of confounding factors common to an athletic population such as exercise and/or peripheral injury. We recognize numerous inflammatory mediators, including MCP-1, may be elevated in both the plasma and skeletal muscle for hours after a single bout of exercise (Peake, Suzuki et al. 2005, Deyhle, Gier et al. 2015). Although the time after the last exercise bout and duration/intensity were not recorded, this study was conducted during pre-season training, and we can therefore assume that all athletes (collision and non-collision) had been physical active within 72 h of blood sampling. Hence, any potential confounding effects of exercise are likely common to both groups of athletes. Furthermore, while our study design intentionally excluded athletes with musculoskeletal injuries, the physical demands of collision-sport participation may have the potential to influence biomarker concentrations. For example,
tau is expressed in extracranial rat tissues (Gu, Oyama et al. 1996), and in the muscle fibers of patients with inflammatory myopathy (Maurage, Bussiere et al. 2004, Nogalska, D'Agostino et al. 2011). As tau is released from neurons as a by-product of cell death (Avila, Simon et al. 2014), muscle damage/turnover may result in the extracellular release of tau. Hence, despite being sampled before the onset of the competitive season in athletes absent overt musculoskeletal injuries, we cannot rule out the effect of pre-season training. Future studies are needed to evaluate potential extracranial release of these biomarkers, particularly from damaged/injured muscle tissue.

Though we did not identify differences in a number of previously identified TBI inflammatory markers such as IL-1β, IL-6 and IL-10, these markers have typically been evaluated in the acute stages after severe TBI (Tasci, Okay et al. 2003, Schneider Soares, Menezes de Souza et al. 2012, Ferreira, Regner et al. 2014, Di Battista, Rhind et al. 2016); conversely, our cohort was ostensibly healthy, and the median time from last concussion was approximately two years (Table 1). Furthermore, while numerous cytokines have been found elevated for up to three months after severe TBI (Kumar, Boles et al. 2015), few studies have evaluated the chronic inflammatory response after concussion. Yet, Prodan and colleagues found platelet activation in previously concussed military personnel ranging from 6 months to 9 years post-injury (Prodan, Vincent et al. 2014), and in a follow-up study, identified a positive correlation between this inflammatory correlate and the number of concussions sustained (Prodan, Vincent et al. 2016). While these previous works evaluated military personnel and included mechanistically distinct blast-related concussion, the results are consistent with our findings, and suggest that biological perturbations resulting from multiple head injuries are evident systemically up to years after injury.
In the current study, the differences identified in biomarker signatures between male and female athletes after multiple concussions is supportive of the previously noted sex-differences in immunobiology (Bouman, Heineman et al. 2005, Fish 2008), and aligned with prior evidence of sex-differences in concussion recovery (Dick 2009, Broglio, Cantu et al. 2014, Baker, Leddy et al. 2016). Although it is difficult to speculate on the biological basis of these findings, the potency of male and female sex hormones to differentially mediate inflammatory responses represents a plausible explanation (Ramien, Taenzer et al. 2016). The sexually dimorphic neurochemical composition of the brain may contribute to divergent responses to brain injury (Cahill 2006), leading to a different complement of proteins appearing in the blood. However, in addition to the pleiotropic effects of systemic inflammatory indices, and chemokines in particular, the gap in sex-based inflammation research in TBI makes interpretation of our results difficult. Yet, these findings necessitate sex-stratification in future concussion study cohorts, as potentially distinct mechanisms mediating the long-term effects of multiple head impacts may exist.

A limitation of the study was the cross-sectional design, therefore, we lacked the ability to evaluate inflammatory marker levels prior to injury in the athletes with a history of concussion. Furthermore, a larger sample size with additional female athletes who participate in collision sport (i.e., rugby) would allow the evaluation of biomarkers in collision vs non-collision sports. As previously identified, we were unable to control for the potential confounding effects of exercise on biomarker levels; while the homogeneity of our population suggests both collision and non-collision sport athletes were presumably similar in their exercise habits, the ability to quantify the duration and intensity of exercise and how this may have affected any of the markers assessed would have strengthened our results. Finally, while the SCAT3 is the most utilized evaluation tool in the sport context, it is a crude measure of cognitive abilities, and we
recognize its comparative limitations to more advanced neuropsychological tests. However, despite these limitations, our results demonstrate potentially sex-specific systemic inflammatory alterations in athletes with multiple previous concussions, and in males who participate in collision sports.

5.6 Conclusion

Multiple previous concussions are associated with elevations in MCP-1 and MCP-4 in healthy female and male athletes, respectively. Furthermore, collision sport participation displays a greater covariance with systemic biomarkers compared to that of concussion history, and is specifically associated with increases in tau. Future studies are required to identify the source and biological relevance of systemic biomarkers in athletes who have sustained repetitive head trauma and who participate in collision sports to better understand and characterize the potential health consequences. Attention should be paid to sex differences, as well as extracranial sources of biomarkers related to muscle damage and exercise.
Chapter 6
Discussion, Conclusion, Future Directions
6 Discussion

6.1 Moderate-to-severe TBI

6.1.1 Acute systemic inflammation contributes to pathological outcomes

Secondary injury is marked by inflammation in both the CNS and periphery. While numerous studies have identified alterations in peripheral blood inflammatory mediators acutely after injury (Venetsanou, Vlachos et al. 2007, Schneider Soares, Menezes de Souza et al. 2012, Stein, Lindel et al. 2012, Ferreira, Regner et al. 2014, Santarsieri, Kumar et al. 2015), their relation to patient outcomes and secondary injury processes remain unclear. This likely owes to the inherent heterogeneity of TBI and resultant difficulty this presents to study design, as well as the complexity of the inflammatory response - specifically its ability to both heal and damage (Lenzlinger, Morganti-Kossmann et al. 2001, Jaerve and Muller 2012). In support of the latter, therapeutic attempts aimed at general suppression of inflammation have failed to improve patient outcomes (Bergold 2016). In order to advance the field, biomarker investigations need to go beyond undirected characterization, and evaluate previously demarcated experimental hypotheses related to pathophysiology.

In chapters 2 and 3, we set out not only to characterize the inflammatory response, but to calibrate its relative significance to poor patient outcome. To address injury heterogeneity, we employed a population with isolated, blunt, non-penetrating, moderate-to-severe brain trauma. In agreement with our first specific hypothesis (section 1.5.2.2), we found that multiple inflammatory mediators were associated with poor outcome and mortality. Specifically, patient death and unfavorable 6-month outcome were associated with a changing biomarker phenotype over the first 24 h from hospital admission, and inflammatory signatures varied according to the cause of death.
It has been suggested that trauma is accompanied by an acute inflammatory storm that is overcompensated by a subsequent anti-inflammatory response, culminating in immunosuppression (Osuchowski, Welch et al. 2006, Gentile, Cuenca et al. 2012). The possible clinical relevance of this process is susceptibility to infection and end-organ damage, two important and commonly observed sequelae of TBI (Zygun, Kortbeek et al. 2005, Catania, Lonati et al. 2009, Lu, Goh et al. 2009). However, current revisions to this theory suggest this may be an oversimplification, as both pro- and anti-inflammatory mediators are released concurrently after injury (Osuchowski, Welch et al. 2006, Gentile, Cuenca et al. 2012). Our results showed a phenotypic pro- to anti-inflammatory shift overtime. Indeed, while we found immediate perturbations in both pro- and anti-inflammatory mediators, when controlled for injury severity an interesting trend emerged: within the first 12 h from hospital admission, increases in proinflammatory cytokines IL-1β and TNF-α, as well as chemokines IL-8, IP-10, MCP-1 and eotaxin, were related to poor patient outcomes. However, from 6 h to 24 there was a phenotypic shift, as IL-10 became the preeminent cytokine correlated with patient outcome; by 24 h, IL-10 was the only cytokine associated with poor outcome and death alongside decreases in MCP-4 and TARC. These results suggest that while both pro- and anti-inflammatory mediators may be elevated at admission, it is the prolonged elevation of the anti-inflammatory IL-10, along with an insufficient chemokine response, that may contribute to pathological immunosuppression. This also supports the body of work by Woicichowsky and colleagues, who hypothesized that monocytic production of IL-10 is an important mechanistic contributor to brain injury through the promotion of infection (Woicichowsky, Asadullah et al. 1998, Woiciechowsky, Schoning et al. 1999, Woiciechowsky and Volk 2005). Additionally, that low levels of MCP-4 and TARC were associated with poor outcome is generally consistent with a
dualistic role for inflammation as both detrimental and beneficial throughout the acute phase of isolated TBI.

Results from chapter 2 highlighted the importance of chemokines in the acute phase after TBI. The impetus to evaluate peripheral chemokines came from extensive animal work which has shown their ability to aid in leukocyte migration to the brain after injury (Campbell, Wilcockson et al. 2002, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005, Campbell, Anthony et al. 2008, Semple, Bye et al. 2010, Semple, Frugier et al. 2010, Semple, Kossmann et al. 2010). Until now, only IL-8 and MCP-1 had been investigated in the peripheral blood after TBI (Mussack, Biberthaler et al. 2002, Seekamp, van Griensven et al. 2002, Gopcevic, Mazul-Sunko et al. 2007, Rhodes, Sharkey et al. 2009, Ferreira, Regner et al. 2014, Di Battista, Buonora et al. 2015). We found perturbations in all 9 assayed chemokines, in which 8 of 9 were associated with poor outcome or mortality. Indeed, elevated admission IP-10 levels were associated with a tripled risk of mortality when controlled for injury severity – this was the highest value found at any time point, in any marker evaluated.

Biomarker profiles differed between patients who died from neurological vs. non-neurological causes after isolated TBI. Cytokines IL-1β and TNF, as well as chemokines IL-8 and MCP-1, were associated with brain-related death, while IL-10 was associated with all-cause mortality. These results are supportive of experimental evidence which has identified IL-1β and TNF-α in the initiation of the APR after TBI (De Simoni, Sironi et al. 1990, De Simoni, De Luigi et al. 1993, Woiciechowsky, Schoning et al. 1999, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005), and consistent with the aforementioned role of IL-8 and MCP-1 in the pathological migration of leukocytes to the brain (Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005, Catania, Lonati et al. 2009, Semple, Kossmann et al. 2010). Conversely, while
IL-10 has been implicated pathologically in TBI (Woiciechowsky, Asadullah et al. 1998, Woiciechowsky and Volk 2005), it is logical to presume that such as powerful systemic immunosuppressant may have effects beyond the CNS, increasing the risk of infection and sepsis, culminating in the dysfunction of extracranial organs (Rossaint and Zarbock 2015).

In chapter 3 we found covariance between coagulopathy and inflammatory biomarkers. Furthermore, as hemostatic indices changed over a 24 h period from a pro- to anti-coagulopathic phenotype, the immunological profile became increasingly anti-inflammatory. Indeed, at hospital admission, poor outcome was associated with concomitant increases in numerous circulating pro-coagulant indices, as well as IL-6, TNF-α, IL-8 and MCP-1. However, at 24 h, as the hemostatic phenotype changed, elevated IL-10 levels became significantly correlated to patient outcome. Indeed, a pro-coagulant, pro-inflammatory; anti-coagulant, anti-inflammatory relationship has been noted in trauma and sepsis (Levi and van der Poll 2010, van der Poll, de Boer et al. 2011). Furthermore, these findings were supportive of our results from chapter 2 regarding the pathological consequences of prolonged elevations in IL-10, and suggest that its role may extend beyond immunosuppression to affect hemostasis.

Taken together, acute systemic inflammation appears to portend unfavourable outcome in isolated TBI patients. Pathological outcomes are generally related to early (admission) elevations in pro-inflammatory mediators, and a later (24 h) anti-inflammatory response that may mirror a pro- to anti-coagulant shift, respectively. While overlap exists, brain-related vs. non-neurologic deaths are associated with distinct biomarker signatures, inferring unique pathogenesis. Lastly, while understudied, chemokines represent an important subset of cytokines in secondary injury.
6.1.2 Dynamic coagulopathy in the acute phase of injury

The most prominent question regarding coagulopathy in TBI is whether acute hemostatic perturbations result in a pro- or anti-coagulopathic state. Knowledge of this has important implications on early, potentially lifesaving therapeutic strategies. As standard clinical tests are currently unable to present an unbiased account of hemostasis (Hemker, Al Dieri et al. 2004, Park, Martini et al. 2009), blood biomarkers represent proximal indices of both the clotting and fibrinolysis pathways, as well as vascular injury.

The results from chapter 3 supported our hypothesis that alterations in circulating indices of hemostasis and vascular injury found acutely after TBI are pathological. At hospital admission, patients with an unfavorable 6-month outcome displayed a procoagulant, hyperfibrinolytic phenotype, with elevations in indices of vascular endothelial and glycocalyx damage. These results are supportive of previous findings in TBI and general trauma, which suggest immediate procoagulant activity leads to DIC and consumption of clotting factors (Gando, Nanzaki et al. 1999, Stein and Smith 2004). Indeed, our results are also consistent with a mediating role for TF in driving this response (Goodnight, Kenoyer et al. 1974, Goodnight 1977, Gando, Nanzaki et al. 1999). At 24 h, there was an apparent shift in biomarkers, as the hypothetically anticoagulant TM became a significant contributor to poor outcome. This may support a progressive role for APC in mediating the observed progressive hypocoagulant phenotype, as TM is a crucial component to APC signaling. However, this differs from the previously delineated “APC hypothesis”, which is predicated upon early hypocoagulopathy without clotting (Cohen, Brohi et al. 2007) Our admission biomarker signature does not support this – elevations in a number of procoagulant indices, such as TF, TAT, as well as proinflammatory indices such as TNF-a, IL-6 and IL-8, were observed.
The “glycocalyx” hypothesis delineated by Ostrowski and colleagues is well-supported by our findings in chapter 3 (Johansson and Ostrowski 2010). Indeed, elevated concentrations of the glycocalyx damage marker SDC-1, as well as endothelial injury indices VAP-1 and TM, were associated with poor patient outcome at all time points. Furthermore, our findings are generally consistent with an evolving, pro- to anti-coagulant shift hypothesized by these authors in response to tissue injury (Johansson and Ostrowski 2010).

In summation, the results from chapter 3 demonstrate that numerous hemostatic disturbances previously identified in extracranial trauma also exist in isolated TBI. Acute alterations in a number of biomarkers of coagulopathy and endotheliopathy were associated with poor patient outcomes. While hyperfibrinolysis and endotheliopathy were present throughout the first 24 h from hospital admission, an immediate pro-coagulant phenotype became increasingly anti-coagulant at 24 h.

6.1.3 SNS hyperactivity as a key modulator of pathological secondary injury processes

The primary objective of both chapter’s 2 and 3 was to evaluate the hypothesis that early pathological inflammation and hemostatic perturbations were mechanistically driven by SNS hyperactivity. Indeed, this hypothesis was derived from numerous lines of evidence, detailed in sections 1.2.3.2, 1.2.3.3, 2.2, and 3.2.

Results from chapter 2 were consistent with the notion that associations between inflammatory cytokines, chemokines and poor patient outcomes may be facilitated by an early hyperadrenergic response to injury. Most notably, strong correlations were identified between circulating IL-10, IL-8, MCP-1, and both NE and Epi in patients. This is supportive of both the aforementioned hypothesis by Woicichowsky and colleagues regarding the relationship between
SNS activity and IL-10, as well as the large body of work suggesting that the production of IL-8 and MCP-1 from the liver after brain injury is SNS-mediated. It would also appear that the general association between SNS activity and inflammation is predominantly mediated by NE, which may be important in evaluating therapeutic adrenergic blocking targets.

The association between pathological inflammation and SNS hyperactivity in chapter 2 was further corroborated by our findings in chapter 3. We found a strong relationship between catecholamines and indices of glycocalyx damage, procoagulant and prohyperfibrinolytic indices. This is supportive of the “glycocalyx” hypothesis proposed by Ostrowski and colleagues (Johansson and Ostrowski 2010). While these authors suggest a hypocoagulant response in the fluid phase after injury, that we found an early procoagulant biomarker phenotype in the blood (fluid phase) does not negate the possibility that the observed procoagulant indices are reflective of clotting mechanisms occurring in response to tissue injury. Interestingly, at 24 h, similar to chapter 2, NE became the predominant catecholamine covariant with indices of vascular damage and coagulopathy. Again, in agreement with Ostrowski and colleagues, the increasing involvement of TM and IL-10 throughout the first 24 h after hospital admission, suggests there may be a hypo-coagulant response to the initial glycocalyx and endothelial injury caused by SNS hyperactivity.

The clinical relevance of these findings is not limited to the demarcation of SNS-related secondary injury processes after TBI, but also support the possibility that adrenergic blockade may have therapeutic benefit. Indeed, numerous investigations have observed that beta blockade can improve outcome after brain injury (Cotton, Snodgrass et al. 2007, Heffernan, Inaba et al. 2010, Ko, Harada et al. 2016, Murry, Hoang et al. 2016). A recent study by Xu and colleagues has offered insight into the mechanisms by which this may occur, and their results are largely
supported by our findings. These authors submitted a group of rats to post-trauma chemical sympathectomy (Xu, Yu et al. 2015). This elicited a protective effect that was associated with a decrease in indices of inflammation, glycocalyx injury, and hyperfibrinolysis (Xu, Yu et al. 2015). While this has not yet been replicated in TBI, our findings in chapters 2 and 3 show SNS-associated, pathological alterations in indices of each of the mechanisms evaluated by Xu and colleagues, and it is therefore logical to assume that pharmacologically targeting the SNS in isolated TBI may have the same beneficial effect.

Our results are strongly supportive of a diverse array of pathological implications stemming from excessive SNS activity in the acute period after trauma. These include, but are not limited to, an immediate dysregulated proinflammatory response including the activation of the hepatic APR. This may subsequently lead to a pathologically anti-inflammatory state, resulting in infection susceptibility and an increased risk of both neurologic and non-neurologic organ death. Furthermore, sympathetic hyperactivity may cause widespread vascular endothelial and glycocalyx injury and dysregulated hemostasis, possibly characterized by a shift from an early procoagulant to anticoagulant state.
Figure 6.1 Potential secondary injury processes related to poor patient outcome acutely after moderate-to-severe TBI. Results from chapters 2 and 3 are supportive of a dynamic acute biological response to isolated brain injury, involving SNS hyperactivity, inflammation and coagulopathy over the first 24 h. At hospital admission, SNS hyperactivity, via catecholamines Epi and NE, is associated with a hypercoagulant, proinflammatory biomarker phenotype. At 24 h, there is a change in the biological signature reflecting an increasing contribution of anti-inflammatory, hypocoagulant processes, co-varying predominantly with NE. Indices of hyperfibrinolysis and vascular injury persist throughout.
6.2 Sport-related concussion

6.2.1 Biomarkers have applicability to SRC research

Chapters 4 and 5 were designed to employ peripheral blood biomarkers as a tool to characterize secondary injury after SRC, similar to the manner in which they were used in moderate-to-severe TBI in chapters 2 and 3, for the purpose of generating hypotheses regarding secondary injury pathophysiology. Clearly, there are important differences to consider when evaluating blood biomarkers in concussion vs. more severe brain injuries. First, the anticipated biological perturbations are much lower. This possibly decreases the “signal-to-noise” ratio, increasing the importance of controlling for extracranial factors. This is particularly relevant to the immune system due to its inherent pleiotropicity. Indeed, in a varsity athlete population, the effects of exercise, musculoskeletal injuries, and other various stressors incurred throughout the academic year may confound indices otherwise related to brain injury. To account for this, in chapters 4 and 5 we employed a varsity athlete population, controlled for time of season, musculoskeletal injury, sport participation, sex, and age. Admittedly, while the majority of our healthy participants were within 72 h of their last exercise bout, we were unable to specifically delineate the exact time from last exercise, as well as the nature and duration. However, as we were collecting blood throughout the athletic season, we are reasonably confident that the possible effects of exercise on biomarker profiles were equally present in all analyzed groups, and thus did not affect our findings. Furthermore, as opposed to more severe injuries where patients are often admitted to the hospital within a short period of time, in SRC, injuries often take place during practices or games, and therefore athletes may not immediately seek medical attention. Hence, it can be difficult to recruit and evaluate participants close to the time of injury. In view of this, our most proximal sampling time among this population was between 2-8 days’ post-injury. Yet, in chapter 4 we identified perturbations in systemic indices of inflammation and
CNS injury in the subacute phase that extended through medical clearance, and in chapter 5 we found biomarker perturbations in athletes chronically up to years after their last concussion, or in those who were exposed to repetitive head impacts. Indeed, these results are in agreement with our specific hypothesis #2, and secondary hypotheses #3 and #4 (sections 1.5.2.2 & 1.5.2.3, respectively), and support the capability of peripheral blood biomarkers to detect biological perturbations that may reflect secondary injury processes after SRC.

6.2.2 Tau and chronic neurodegeneration

Growing evidence suggests that systemic tau is not only a correlate of axonal damage, but may reflect neurodegeneration (Zetterberg, Wilson et al. 2013, Stern, Tripodis et al. 2016). This hypothesis that has been supported in both sport and clinical populations such as head trauma and AD (Zetterberg, Wilson et al. 2013, Fyfe 2015, Olivera, Lejbman et al. 2015, Shahim, Linemann et al. 2016, Stern, Tripodis et al. 2016, Wang, Li et al. 2016). In view of this, our investigation of tau is of potential clinical interest, as we found alterations in circulating levels in two separate analyses (Chapters 4 & 5). In chapter 4 we found tau was elevated after a single SRC at clinical recovery (median time = 33 days), and in chapter 5 we found elevated levels of tau in healthy male athletes who participate in collision sports. Interestingly, in both cases, athletes displayed elevated tau levels in the absence of concussion symptomology at the time of sampling. That we identified elevated tau in two separate, brain-injury related scenarios, and that a growing body of evidence suggests tau is a useful proxy of neurodegeneration, warrants future examination into the potential relevance of these findings to athlete health. Additionally, that these findings occurred in the absence of clinical evidence of concussive injury, necessitates further investigation into the possible utility of blood biomarkers in informing return-to-play strategies in a sport-setting. Indeed, results from chapters 4 and 5 support possible ongoing dysnunction/reparation in “healthy” athletes; it is unclear if these
findings can be interpreted to suggest that athletes are susceptible to further injury and should refrain from participation, or if these perturbations have no bearing on future head insults. Furthermore, if elevated tau in the periphery is reflective of neurodegeneration, what does this say for the long-term brain health of participants in collision sports? Is there an optimal systemic tau cut-off at which point athletes are in danger of prospective health issues? However, before tau can be evaluated as a possible clinical adjunct in assessing athlete health, further experimentation evaluating all possible contributory factors to elevated systemic tau levels must be explored, including potential contributions from exercise and sub-clinical musculoskeletal injuries.

6.2.3 Chemokines in SRC

In chapter 4 we observed higher levels of IP-10 at medical clearance in SRC; in chapter 5, we found that multiple previous concussions were associated with higher levels of MCP-1 in females, and MCP-4 in males. Notably, and in line with our findings in chapter 2, these results are consistent with an important role for chemokines across all severities of TBI, in both the acute and chronic stages. However, it is unclear why chemokine levels are chronically elevated after SRC, and whether this is a maladaptive or reparative effect. It would appear that acute increases in systemic chemokines in severe TBI are detrimental through mediating pathological leukocyte recruitment to the CNS (Campbell, Wilcockson et al. 2002, Campbell, Hughes et al. 2003, Campbell, Perry et al. 2005, Semple, Bye et al. 2010, Semple, Sadjadi et al. 2016). Yet, whether they have a similar role in concussive injury is unclear, as chemokines may also facilitate the healing response by facilitating axonal sprouting (Jaerve, Schiwy et al. 2011, Jaerve and Muller 2012). However, results from both chapters 4 and 5 are supportive of future experimentation to elucidate the chronic role of chemokines after SRC.
In chapter 4, we identified an inverse correlation between chemokine levels and total symptoms reported in the subacute phase. While this supports our secondary hypothesis #3 (section 1.5.2.3), the nature of this relationship was slightly surprising. Yet, previous investigations into systemic immune function in the subacute phase after SRC are generally consistent with immunosuppression (Gill, Merchant-Borna et al. 2016, Merchant-Borna, Lee et al. 2016), and our own previous findings within a severe TBI cohort may also be supportive; in chapter 2, in contrast with our observations at hospital admission, we found evidence of immunosuppression at 24 h, highlighted by a decrease in multiple chemokine levels in patients with a poor outcome. Indeed, it follows that the results from chapter 4 may reflect immunosuppression in the subacute period as a compensatory response to an acute pro-inflammatory state that we did not capture in SRC, due to the timing of our first sample. Moreover, that we did not find these trends generally, but rather in the most symptomatic patients, speaks to the likely possibility that this perturbation is not as dramatic as is seen in severe injury, and may not be detectable across all patients using current analytical detection methods.

Taken together, our results from chapters 2, 4, and 5 are supportive of an important role for systemic chemokines in secondary injury across the spectrum of TBI. These results also support congruency between peripheral inflammatory pathophysiology in concussive injury and moderate-to-severe TBI, and provide evidence of both acute and chronic perturbations.
Figure 6.2 Biomarker signatures of the subacute and recovery phases of Sport-Related Concussion. The blood biomarker signature of the subacute period in sport-related concussion (SRC) is marked by elevations in circulating peroxiredoxin (PRDX)-6 concentrations and symptom-dependent decreases in numerous chemokines. At clinical recovery, elevations in PRDX-6 persist, however elevations in Total (T) – Tau, and interferon-γ produced (IP)-10 are observable, alongside lower tumor necrosis factor (TNF)-α concentrations. Possible contributory mechanisms include altered glymphatic clearance, blood brain barrier (BBB) disruption, and neuroendocrine dysregulation (not shown).
6.3 Conclusions

Blood biomarkers can be used to inform pathophysiological mechanisms across the spectrum of injury severity in human TBI. They can improve our knowledge of important secondary injury processes by supporting the presence/absence of previously delineated experimental hypotheses, through calibrating the relative significance of these processes to patient outcomes, and in generating new hypotheses to justify further clinical and experimental investigation. The four studies undertaken in this dissertation were supportive of the delineated hypotheses highlighted in sections 1.5.2.1, 1.5.2.2, and 1.5.2.3. Chapters 2 and 3 provided supportive evidence of previously defined hypotheses from animal model research and human studies in extracranial trauma; acutely after isolated moderate-to-severe TBI, inflammation, vascular injury and coagulopathy contribute to poor patient outcome, and covary with SNS hyperactivity. Chapters 4 and 5 provided results consistent with previous animal model experimentation regarding the potential role of inflammation in mild brain injury; from the subacute period throughout medical clearance, alterations in neuroinjury and inflammatory biomarkers are present in SRC athletes, and covary with symptom presentation. Furthermore, alterations in circulating chemokine levels are detectable for months-to-years after repetitive head trauma in athletes. In all 4 chapters, perturbations in novel biomarkers not previously evaluated in TBI provide grounds for future investigation and hypothesis generation; It would appear that several chemokines are involved in secondary injury after moderate-to-severe TBI. In particular, IP-10 may be an important molecule involved in secondary injury pathophysiology across all severities of brain injury. Lastly, it is possible that the chronic inflammatory response in SRC is sex-specific. Future biomarker investigations across the spectrum of TBI are warranted to investigate secondary injury mechanisms in both the acute and chronic phases. Careful evaluation of the relevant targets highlighted in these works may provide the rational basis for
the consideration of future therapeutic trials, including those that may augment SNS activity and/or inflammation.

6.4 Future directions

6.4.1 Moderate-to-severe TBI

There are numerous future studies that would help address the pertinent questions stemming from these works. For example, in view of the evidence of developing immunosuppression after moderate-to-severe TBI, a follow-on TBI biomarker study with blood samples outside of the 24 h period would be helpful in further characterizing this response, as it appears to have a progressive trajectory. Moreover, it is still unclear if immunosuppression after TBI is induced by the direct actions of IL-10, or as a consequence of immune anergy. Uncovering this mechanism may have relevant therapeutic consequences. While this may require a combination of experimental and clinical investigations, it would be useful to *ex-vivo* stimulate the blood of TBI patients with an immunogenic agent, both at hospital admission and at later time points, in order to document changes in immune function over time.

While our results from chapter 3 are supportive of an early pro-coagulant phenotype in the blood, it is unclear if this translates to clinical hypercoagulation. Indeed, Ostrowski and colleagues suggested that a pro-coagulant vasculature induces a hypo-coagulative fluid-phase response (Johansson and Ostrowski 2010), and hence it is uncertain if our findings reflect a possible pro-coagulant state in the blood or the vasculature. A follow-on study using similar biological indices, but with the addition of a more definitive measure of coagulopathy, such as thromboelastography, would be useful; thromboelastography, unlike commonly used indices of coagulopathy, can equally measure hypo- and hyper-coagulative states, as well as fibrinolysis (Park, Martini et al. 2009, Figueroa and Merriman-Noesges 2014, Massaro, Doerfler et al.)
2015, Rao, Laurie et al. 2016). If these measures were correlated to blood biomarkers of hemostasis, this would be an important validation for their utility in inferring the state of the fluid phase after brain trauma. Additionally, it may be useful to sample the CSF in future TBI coagulopathy studies, as it is still unclear whether these biomarkers, particularly those representing vascular injury, are primarily sourced from the cerebrovasculature, or widespread throughout the body.

The results from chapter 2 and 3 provide strong evidence of the involvement of the SNS in mediating a number of pathological sequelae after TBI. While there have been recent prospective reports on the utility and safety of beta-blocker therapy (Ko, Harada et al. 2016, Murry, Hoang et al. 2016), a clinical study which not only looks at patient outcome, but evaluates the effect of beta blocker therapy on biomarker indices of inflammation, vascular injury, and hemostasis, would help justify a future clinical trial. While there have been no effective clinical trials to date in TBI, some degree of success has been achieved through the CRASH-2 trial, which sought to evaluate the effectiveness of tranexamic acid (TA) to prevent excessive bleeding after general trauma (Crash-2 Collaborators 2011, Roberts, Shakur et al. 2013, Roberts, Prieto-Merino et al. 2014). The results of CRASH-2 have led to CRASH-3, where TA is currently being evaluated in TBI (Dewan, Komolafe et al. 2012, Munoz-Sanchez, Egea-Guerrero et al. 2012). In view of this, beta-blockers not only have the potential to alleviate coagulopathic disturbances, but can modulate inflammation; another proposed therapeutic mechanism of TA in CRASH-2 (Roberts, Prieto-Merino et al. 2014). Hence, the evidence of the potential therapeutic benefit of reversing dysfunctional hemostasis and inflammation after injury, along with optimistic results from preliminary retrospective and prospective investigations, warrant future beta-blocker studies in TBI, employing the biomarkers utilized in chapters 2 and 3 to quantitate their effects on these sequelae.
6.4.2 Sport-related concussion

Among the most salient immediate concerns regarding the applicability of blood biomarkers in SRC is their specificity to brain injury and/or repair. In continuing with these investigations, it will be important to evaluate and control for extracranial influences. Indeed, future studies utilizing varsity athletes will specifically need to control for exercise and the effect of an entire academic and sport season on biomarker profiles. Furthermore, it will be important to investigate potential biological perturbations in the acute phase (<48 h), particularly regarding indices of inflammation. This would be a useful adjunct to the works of chapters 4 and 5, and would improve our knowledge on the natural history of the inflammatory response to human concussive injury. Moreover, moving forward it will be important to compare biomarker signatures post-SRC in athletes who recovery within the normally identified trajectory, versus those who have continued symptomology. This would potentially inform important, maladaptive secondary injury processes.

It has been suggested that SRC may aid the study of military-related blast injury. Indeed, there are a number of similarities in these groups: youth, risk for repetitive trauma, and similar physical and mental requirements in their respective fields. In addition, preliminary evidence has suggested similar recovery trajectories in both populations (Larres, Carr et al. 2016). Indeed, to evaluate this hypothesis, a biomarker study comparing post-injury military and athlete populations would be informative; if similarities in biomarker profiles exist, this may have an important impact on the study of military-related mTBI, as subject recruitment in this cohort can be challenging.
6.4.3 Alternative analytical techniques

Beyond utilizing ELISA based technology to evaluate blood biomarkers, flow cytometry may be a powerful tool to investigate the systemic immune system in brain injury. Flow cytometry has the ability to inform leukocyte biology; circulating lymphocytes, monocytes, and granulocytes, along with their respective subsets, can be analyzed for relative changes to surface receptor expression, intracellular cytokine production, nuclear transcription factors, morphology, apoptosis, and other functional activities (Krutzik, Irish et al. 2004, Chattopadhyay, Hogerkorp et al. 2008, Litwin and O'Gorman 2011, Chattopadhyay and Roederer 2012). For example, flow cytometry may be useful in elucidating the mechanisms involved in chemokine-mediated leukocyte migration to the CNS after injury. The potential ability to quantitatively measure chemokine receptor biology on specific leukocyte subpopulations after injury represents an impactful contribution to the field, particularly in view of our results in chapters 2, 4, and 5, which further support the vast importance of chemokines in TBI pathophysiology. Furthermore, flow cytometry could be used to evaluate the hypothesized monocytic production of IL-10 post-injury, and to investigate the recently identified role of surface receptor CD11d in mediating macrophage and neutrophil recruitment to the brain (Shultz, Bao et al. 2013).

The nature of adrenergic receptor dysregulation in TBI remains poorly understood, and traditional radioligand binding assays have proven inadequate to clearly identify cell-specific receptor variations. Notably, the application of immunofluorescence flow and imaging cytometry represent important analytical techniques for the determination of candidate injury mechanisms that may be therapeutically targeted by various β-blockers in TBI. Numerous investigations examining ligand-receptor interactions on leukocytes have uncovered a multitude of potential pro- and anti-inflammatory signaling mechanisms, differentially regulated by both NE and Epi through various α- and β-AR subtypes (Uotila 1996, Ballard-Croft and Horton...
For example, catecholamines may promote anti-inflammatory actions by stimulating monocytic IL-10 production via β2-ARs; a process mediated by cyclic-AMP (Woiciechowsky, Asadullah et al. 1998). Conversely, when signaling through α2-ARs, catecholamines may inhibit cyclic-AMP activity (Uotila 1996). In addition, NE may act on either α- or β-ARs to induce the potentially pro-inflammatory transcription factor NF-κB (Bierhaus, Wolf et al. 2003). Indeed, adrenergic-leukocyte signaling is complex and currently ill-defined. However, elucidating SNS-immune biology will be essential in determining how and when β-blockers can be effectively implemented after TBI, specifically regarding their potential effects on preventing neuroinflammatory-related parenchymal damage in the CNS, peripheral recruitment of leukocytes to the brain, and in extracranial sequelae such as the inflammatory-related mediation of hemostasis, susceptibility to sepsis and infection, and organ dysfunction. In view of this, our group has begun evaluating the applicability of multiparameter immunofluorescence-based imaging cytometry as a tool to probe adrenergic-immune cell biology within specific human circulating leukocyte subpopulations (Di Battista, Rhind et al. 2013). Such novel analytical techniques offer an attractive alternative to current methods to assess receptor expression and function, and may facilitate the application of clinically useful biomarkers as sensitive and reliable, diagnostic, prognostic, and treatment monitoring adjuncts in TBI. While the application of flow cytometry to human clinical studies can be limited by the time sensitive nature of sample acquisition and analysis, its potential to add significantly to our knowledge of immunopathophysiology far out-weighs the logistical concerns.

The use of multiple analytical techniques with the ability to corroborate mechanisms encompassing both the brain and periphery carries great potential. In view of this,
neuroimaging has the ability to strengthen biomarker findings in TBI. With this approach, it may be possible to investigate potential relationships between brain injury and consequent peripheral perturbations. For example, combinatorial imaging and biomarker analyses could be used to investigate the effects of peripheral leukocyte migration on neuronal injury and cerebral edema, or may be used to assess the influence of ICP on SNS activity and subsequent peripheral inflammation. However, neuroimaging often encompasses substantial costs and feasibility issues, particularly concerning severely injured patients who may not be stable enough for assessment. Nonetheless, moving forward, this technique represents a viable adjunct to peripheral blood biomarker analysis in TBI.
References


Appendix 2.1

Percentage of samples within detection limit for all circulating cytokines and chemokines analyzed.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Healthy (n = 21)</th>
<th>Admission (n = 157)</th>
<th>Hours After Admission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 (n = 148)</td>
</tr>
<tr>
<td>Cytokines (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>9 (42.9)</td>
<td>54 (34.4)</td>
<td>48 (32.4)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>7 (33.3)</td>
<td>86 (54.8)</td>
<td>87 (58.8)</td>
</tr>
<tr>
<td>IL-2</td>
<td>9 (42.9)</td>
<td>57 (36.3)</td>
<td>55 (37.2)</td>
</tr>
<tr>
<td>IL-4</td>
<td>0 (0.0)</td>
<td>20 (12.7)</td>
<td>18 (12.2)</td>
</tr>
<tr>
<td>IL-5</td>
<td>17 (80.9)</td>
<td>101 (64.3)</td>
<td>106 (71.6)</td>
</tr>
<tr>
<td>IL-10</td>
<td>19 (90.5)</td>
<td>156 (99.4)</td>
<td>147 (99.3)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>15 (71.4)</td>
<td>50 (31.8)</td>
<td>38 (25.7)</td>
</tr>
<tr>
<td>IL13</td>
<td>9 (42.9)</td>
<td>36 (22.9)</td>
<td>35 (23.6)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>20 (95.2)</td>
<td>154 (98.1)</td>
<td>147 (99.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemokines (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>21 (100)</td>
<td>133 (84.7)</td>
<td>130 (87.8)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>11 (52.4)</td>
<td>111 (70.7)</td>
<td>95 (64.2)</td>
</tr>
<tr>
<td>IL-8</td>
<td>20 (95.2)</td>
<td>148 (94.3)</td>
<td>147 (99.3)</td>
</tr>
<tr>
<td>IP-10</td>
<td>21 (100)</td>
<td>148 (94.3)</td>
<td>148 (100)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>21 (100)</td>
<td>149 (94.9)</td>
<td>148 (100)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>21 (100)</td>
<td>149 (94.9)</td>
<td>147 (99.3)</td>
</tr>
<tr>
<td>MDC</td>
<td>20 (95.2)</td>
<td>146 (93.0)</td>
<td>147 (99.3)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>21 (100)</td>
<td>149 (94.9)</td>
<td>148 (100)</td>
</tr>
<tr>
<td>TARC</td>
<td>21 (100)</td>
<td>149 (94.9)</td>
<td>146 (98.6)</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, traumatic brain injury; GCS, Glasgow coma scale; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor - alpha; IP-10, interferon-gamma induced protein - 10; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP-1β, macrophage inflammatory protein – 1 beta; TARC, thymus and activation regulated chemokine.

Data are presented as the number and percent, n (%), of total available blood samples at each time point within the detection range for the assay, and with a coefficient of variance (CV) less than 25%.
Appendix 2.2

Circulating concentrations of cytokines and chemokines in all TBI patients (GCS 3-13) within 24 hours of hospital admission.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Healthy</th>
<th>Admission</th>
<th>Hours After Admission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Cytokines (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ND</td>
<td>0.4 (0.0 – 2.7)</td>
<td>0.5 (0.1 – 4.9)</td>
</tr>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.4 (0.1 – 2.2)</td>
<td>0.5 (0.1 – 6.7)</td>
<td>0.6 (0.1 – 6.5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.7 (1.4 – 3.9)</td>
<td>14.4 (0.4 – 617.6)</td>
<td>8.2 (0.7 – 136.9)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.9 (0.1 – 4.3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.9 (2.8 – 5.6)</td>
<td>4.9 (1.3 – 19.2)</td>
<td>5.0 (1.6 – 67.8)</td>
</tr>
</tbody>
</table>

Chemokines (pg/mL)

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>590.2 (335.6 – 949.4)</td>
<td>628.7 (234.2 – 2273.0)</td>
<td>446.2 (151.8 – 1448.0)</td>
<td>388.2 (78.4 – 1243.0)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>5.1 (3.6 – 9.4)</td>
<td>9.5 (2.5 – 28.0)</td>
<td>8.7 (2.6 – 34.0)</td>
<td>8.3 (2.2 – 49.6)</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.5 (1.6 – 12.4)</td>
<td>10.0 (2.2 – 182.9)</td>
<td>10.7 (3.2 – 168.3)</td>
<td>9.3 (1.7 – 137.9)</td>
</tr>
<tr>
<td>IP-10</td>
<td>175.6 (102.1 – 283.9)</td>
<td>137.6 (37.4 – 779.3)</td>
<td>119.5 (38.7 – 994.3)</td>
<td>129.5 (41.0 – 1641.0)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>235.4 (143.5 – 335.4)</td>
<td>378.7 (101.1 – 2215.0)</td>
<td>283.5 (87.3 – 3838.0)</td>
<td>267.8 (86.9 – 1588.0)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>295.6 (107.3 – 587.8)</td>
<td>386.7 (71.0 – 1727.0)</td>
<td>252.5 (53.3 – 1423.0)</td>
<td>200.3 (24.3 – 1345.0)</td>
</tr>
<tr>
<td>MDC</td>
<td>321.9 (201.8 – 593.4)</td>
<td>259.9 (41.2 – 791.8)</td>
<td>253.5 (39.3 – 547.3)</td>
<td>237.4 (67.3 – 664.8)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>62.0 (37.0 – 97.9)</td>
<td>110.8 (27.1 – 519.6)</td>
<td>102.7 (26.4 – 555.7)</td>
<td>96.5 (31.3 – 304.7)</td>
</tr>
<tr>
<td>TARC</td>
<td>100.7 (34.8 – 314.0)</td>
<td>171.8 (36.3 – 1584.0)</td>
<td>172.8 (32.6 – 1295.0)</td>
<td>167.0 (42.0 – 978.7)</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, traumatic brain injury; GCS, Glasgow coma scale; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor - alpha; IP-10, interferon-gamma induced protein - 10; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP-1β, macrophage inflammatory protein – 1 beta; TARC, thymus and activation regulated chemokine.

Data are presented as median (range).

* = adjusted P < 0.05 vs. healthy control subjects.

ND = >50% of the samples below the assay detection level.
### Appendix 3.1

Plasma concentrations of biomarkers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>Admission</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endotheliopathy (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-Sel</td>
<td>11.6 (3.1 – 20.8)</td>
<td>7.7 (0.5 – 32.9)*</td>
<td>10.3 (0.5 – 51.1)</td>
</tr>
<tr>
<td>P-Sel</td>
<td>51.0 (18.4 – 116.1)</td>
<td>85.1 (24.8 – 312.7)*</td>
<td>84.5 (28.4 – 213.4)*</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>299.3 (185.6 – 607.7)</td>
<td>226.1 (51.9 – 1092.5)</td>
<td>287.2 (131.8 – 1317.8)</td>
</tr>
<tr>
<td>ICAM-3</td>
<td>0.4 (0.3 – 0.7)</td>
<td>0.6 (0.1 – 50.5)</td>
<td>0.4 (0.1 – 36.7)*</td>
</tr>
<tr>
<td>VCA-M-1</td>
<td>455.3 (255.3 – 823.8)</td>
<td>359.3 (136.5 – 1595.1)*</td>
<td>614.8 (228.2 – 1746.3)*</td>
</tr>
<tr>
<td>VAP-1</td>
<td>223.6 (77.4 – 316.6)</td>
<td>365.1 (32.7 – 688.0)*</td>
<td>267.7 (104.9 – 560.5)*</td>
</tr>
<tr>
<td>SDC-1</td>
<td>22.9 (7.5 – 41.1)</td>
<td>58.8 (16.7 – 295.0)*</td>
<td>41.9 (10.4 – 264.0)*</td>
</tr>
<tr>
<td><strong>Coagulopathy (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT</td>
<td>2.7 (1.0 – 5.5)</td>
<td>56.74 (8.4 – 187.5)</td>
<td>13.0 (2.1 – 92.2)*</td>
</tr>
<tr>
<td>TF</td>
<td>185.3 (107.4 – 229.6)</td>
<td>430.2 (128.7 – 1302.0)*</td>
<td>208.8 (113.1 – 1276.6)*</td>
</tr>
<tr>
<td>TFPI</td>
<td>30.2 (10.6 – 47.2)</td>
<td>59.0 (32.3 – 97.6)*</td>
<td>47.8 (24.0 – 84.8)*</td>
</tr>
<tr>
<td>TM</td>
<td>3.8 (2.3 – 5.1)</td>
<td>3.2 (1.7 – 8.0)*</td>
<td>4.0 (1.7 – 13.1)</td>
</tr>
<tr>
<td>tPA</td>
<td>1.6 (0.3 – 5.9)</td>
<td>17.2 (2.3 – 47.6)*</td>
<td>11.0 (2.0 – 45.0)*</td>
</tr>
<tr>
<td>DD</td>
<td>115.2 (21.3 – 251.3)</td>
<td>4911.2 (122.5 – 13456.2)*</td>
<td>1656.9 (144.2 – 9962.7)*</td>
</tr>
<tr>
<td>PAI1</td>
<td>17.2 (5.1 – 26.9)</td>
<td>43.9 (9.8 – 208.1)*</td>
<td>47.4 (16.9 – 155.3)*</td>
</tr>
<tr>
<td><strong>Inflammatory (ng/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>--</td>
<td>21.1 (0.6 – 529.4)</td>
<td>28.8 (2.6 – 2953.5)</td>
</tr>
<tr>
<td>CRP (µg/mL)</td>
<td>8.2 (1.4 – 29.2)</td>
<td>1.6 (0.0 – 141.3)</td>
<td>115.0 (4.9 – 195.2)</td>
</tr>
<tr>
<td>SAA (µg/mL)</td>
<td>5.3 (1.1 – 21.7)</td>
<td>2.3 (0.2 – 146.9)*</td>
<td>113.9 (15.5 – 195.4)*</td>
</tr>
</tbody>
</table>

**Abbreviations:** TBI, traumatic brain injury; E-sel, E-selectin; P-Sel, P-Selectin; ICAM, intercellular adhesion molecule; VCA-M, vascular cell adhesion molecule; VAP, vascular activation protein; SDC, syndecan; TAT, thrombin anti-thrombin complex III; TF, tissue factor; TFPI, tissue factor platelet inhibitor; TM, thrombomodulin; tPA, tissue plasminogen activator; DD, D-dimer; PAI, plasminogen activation inhibitor; IL, interleukin; CRP, c-reactive protein; SAA, serum amyloid a.

Data presented as the median (range).

* = p <0.05 by Kruskal Wallis with Dunns post hoc.

-- = <50% of samples were within the assay detection range.
Appendix 3.2

Biomarker covariance with injury severity

A

Fraction of Variance Explained = 0.67

B

Fraction of Variance Explained = 0.68

Partial Least Squares analysis of covariance between injury severity and markers of endotheliopathy, coagulopathy, and inflammation. Injury severity indices – Glasgow coma
scale (GCS), abbreviated injury score (AIS) head, injury severity score (ISS); SNS biomarkers – epinephrine (Epi) and norepinephrine (NE); endotheliopathy biomarkers - E-selectin (E-sel), P-Selectin (P-Sel), intercellular adhesion molecule (ICAM)-1, -3, vascular cell adhesion molecule (VCAM)-1, vascular activation protein (VAP)-1, syndecan (SDC)-1; coagulopathy biomarkers - thrombin anti-thrombin complex (TAT), tissue factor (TF), tissue factor platelet inhibitor (TFPI), thrombomodulin (TM), tissue plasminogen activator (tPA), d-dimer (DD), plasminogen activation inhibitor (PAI)-1; inflammation biomarkers – interleukin (IL)-6, tumor necrosis factor (TNF)-α, c-reactive protein (CRP), serum amyloid a (SAA), IL-10, -8, interferon producing protein (IP) -10, monocyte chemoattractant protein (MCP)-1, -4, macrophage derived chemokine (MDC), macrophage inflammatory protein (MIP)-1β, thymus and activation regulated chemokine (TARC), eotaxin, and eotaxin-3 (ET-3). Blood biomarker contributions are displayed at admission (A) and 24 h (B). Bars represent z-scores derived from individual bootstrapped loadings divided by the standard error of the mean. *Represents significance at FDR = 0.05.
### Appendix 5.1

Biomarker values according to concussion history

<table>
<thead>
<tr>
<th>Markers (pg/mL)</th>
<th>Males (n = 60)</th>
<th>Females (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No concussion history (n = 38)</td>
<td>Concussion history (n = 22)</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-1β</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-7</td>
<td>2.1 (1.8 – 3.5)</td>
<td>3.7 (2.9 – 5.0)</td>
</tr>
<tr>
<td>IL-10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>120.6 (93.7 – 140.9)</td>
<td>113.5 (77.9 – 142.5)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-13</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-15</td>
<td>2.3 (2.0 – 2.8)</td>
<td>2.2 (2.0 – 2.7)</td>
</tr>
<tr>
<td>IL-16</td>
<td>304.9 (191.4 – 374.6)</td>
<td>305.9 (224.9 – 395.2)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.8 (1.5 – 2.2)</td>
<td>1.9 (1.7 – 2.1)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VEGF</td>
<td>34.6 (27.5 – 51.1)</td>
<td>37.4 (29.8 – 55.6)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>78.1 (60.2 – 101.2)</td>
<td>83.6 (68.5 – 101.7)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>21.7 (18.5 – 28.1)</td>
<td>23.4 (21.5 – 43.3)</td>
</tr>
<tr>
<td>IP-10</td>
<td>207.1 (162.9 – 257.6)</td>
<td>207.0 (156.4 – 264.2)</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.7 (1.5 – 2.6)</td>
<td>2.5 (1.6 – 4.1)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>89.9 (76.5 – 117.4)</td>
<td>98.6 (84.7 – 116.9)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>26.5 (18.7 – 34.0)</td>
<td>31.9 (23.9 – 54.7)</td>
</tr>
<tr>
<td>MDC</td>
<td>809.4 (706.1 – 924.3)</td>
<td>824.9 (711.9 – 1063.2)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>37.8 (32.7 – 49.4)</td>
<td>42.4 (32.9 – 54.5)</td>
</tr>
<tr>
<td>TARC</td>
<td>39.2 (29.2 – 68.4)</td>
<td>43.2 (23.8 – 58.5)</td>
</tr>
<tr>
<td><strong>Brain injury</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s100B</td>
<td>698.4 (591.2 – 858.0)</td>
<td>738.8 (669.4 – 946.9)</td>
</tr>
<tr>
<td>GFAP</td>
<td>75.9 (63.9 – 83.2)</td>
<td>69.9 (64.4 – 91.1)</td>
</tr>
<tr>
<td>NSE (ng/mL)</td>
<td>1.6 (1.2 – 2.1)</td>
<td>1.7 (1.4 – 2.4)</td>
</tr>
<tr>
<td>NRGN (ng/mL)</td>
<td>7.3 (4.5 – 11.8)</td>
<td>8.0 (6.1 – 11.0)</td>
</tr>
<tr>
<td>CKBB</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VILIP-1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tau</td>
<td>25.9 (21.5 – 45.5)</td>
<td>25.6 (20.7 – 33.9)</td>
</tr>
<tr>
<td>vWF (µg/mL)</td>
<td>34.6 (24.1 – 43.8)</td>
<td>41.7 (18.3 – 54.9)</td>
</tr>
<tr>
<td>BDNF (ng/mL)</td>
<td>851.6 (507.5 – 1910.7)</td>
<td>902.1 (570.9 – 1758.8)</td>
</tr>
<tr>
<td>PRDX-6 (ng/mL)</td>
<td>27.5 (18.2 – 34.0)</td>
<td>29.0 (26.3 – 39.2)</td>
</tr>
</tbody>
</table>
Interleukin (IL) -1α, -1β, -2, -4, -5, -6, -7, -10, -12p40, -12p70, -13, -15, -16, -17A, tumor necrosis factor (TNF) -α, -β, granulocyte macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ), eotaxin, eotaxin-3, interferon gamma-induced protein (IP) -10, IL-8, monocyte chemoattractant protein (MCP) -1, -4, macrophage derived chemokine, (MDC), thymocyte- and activation-regulated chemokine (TARC), s100 calcium binding protein beta (s100B), glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), creatine kinase-BB isoenzyme (CKBB), visinin-like protein (VILIP-1), von Willebran factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX) -6.

* = all markers reported as pg/mL unless otherwise stated

“--” = below assay quantitation in ≥50% of samples analyzed.
## Appendix 5.2

Biomarker values in athletes stratified by collision sport participation.

<table>
<thead>
<tr>
<th>Markers (pg/mL)*</th>
<th>Non-collision sport (n = 46)</th>
<th>Collision sport (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female (n = 25)</td>
<td>Male (n = 21)</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-1β</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-2</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-6</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-7</td>
<td>2.4 (2.1 – 3.6)</td>
<td>2.7 (1.9 – 4.5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>132.2 (104.1 – 160.2)</td>
<td>124.0 (102.0 – 138.4)</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-13</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>IL-15</td>
<td>2.5 (2.1 – 2.7)</td>
<td>2.2 (2.0 – 2.6)</td>
</tr>
<tr>
<td>IL-16</td>
<td>214.5 (176.9 – 290.0)</td>
<td>250.6 (207.2 – 349.7)</td>
</tr>
<tr>
<td>IL-17A</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.8 (1.5 – 2.2)</td>
<td>1.9 (1.4 – 2.4)</td>
</tr>
<tr>
<td>TNF-β</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VEGF</td>
<td>36.5 (23.5 – 48.5)</td>
<td>38.7 (30.8 – 59.9)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eotaxin</td>
<td>73.6 (62.2 – 86.2)</td>
<td>83.9 (64.0 – 101.2)</td>
</tr>
<tr>
<td>Eotaxin-3</td>
<td>--</td>
<td>22.2 (20.4 – 32.0)</td>
</tr>
<tr>
<td>IP-10</td>
<td>183.4 (148.4 – 297.7)</td>
<td>218.4 (189.1 – 277.2)</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.1 (1.5 – 2.6)</td>
<td>2.0 (1.5 – 2.7)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>79.3 (65.1 – 95.4)</td>
<td>84.1 (65.3 – 92.8)</td>
</tr>
<tr>
<td>MCP-4</td>
<td>23.0 (16.8 – 38.3)</td>
<td>28.1 (24.1 – 41.1)</td>
</tr>
<tr>
<td>MDC</td>
<td>773.1 (701.5 – 1043.6)</td>
<td>790.8 (658.3 – 930.0)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>39.6 (27.4 – 48.9)</td>
<td>43.1 (32.7 – 54.2)</td>
</tr>
<tr>
<td>TARC</td>
<td>42.2 (25.2 – 54.5)</td>
<td>42.8 (27.3 – 88.2)</td>
</tr>
<tr>
<td><strong>Brain injury</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s100B</td>
<td>651.6 (578.9 – 764.6)</td>
<td>654.1 (585.8 – 746.6)</td>
</tr>
<tr>
<td>GFAP</td>
<td>87.0 (62.3 – 110.9)</td>
<td>68.2 (58.1 – 99.4)</td>
</tr>
<tr>
<td>NSE (ng/mL)</td>
<td>1.3 (1.0 – 1.6)</td>
<td>1.6 (1.2 – 2.1)</td>
</tr>
<tr>
<td>Neurogranin (ng/mL)</td>
<td>8.1 (3.5 – 12.0)</td>
<td>7.0 (3.6 – 12.5)</td>
</tr>
<tr>
<td>CKBB</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>VILIP-1</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Tau</td>
<td>20.1 (13.6 – 26.9)</td>
<td>21.0 (15.5 – 22.3)</td>
</tr>
<tr>
<td>vWF (μg/mL)</td>
<td>44.3 (24.8 – 63.6)</td>
<td>24.6 (17.3 – 37.7)</td>
</tr>
<tr>
<td>BDNF</td>
<td>969.4 (631.2 – 2031.8)</td>
<td>1219.2 (688.7 – 2480.1)</td>
</tr>
<tr>
<td>PRDX-6 (ng/mL)</td>
<td>44.3 (24.8 – 63.6)</td>
<td>27.5 (18.6 – 33.3)</td>
</tr>
</tbody>
</table>

Interleukin (IL) -1α, -1β, -2, -4, -5, -6, -7, -10, -12p40, -12p70, -13, -15, -16, -17A, tumor necrosis factor (TNF) -α, -β, granulocyte macrophage colony-stimulating factor (GM-CSF),
vascular endothelial growth factor (VEGF), interferon-gamma (IFN-γ), eotaxin, eotaxin-3, interferon gamma-induced protein (IP) -10, IL-8. monocyte chemoattractant protein (MCP) -1, -4, macrophage derived chemokine, (MDC), thymocyte- and activation-regulated chemokine (TARC), s100 calcium binding protein beta (s100B), glial fibrillary acidic protein (GFAP), neuron specific enolase (NSE), creatine kinase- BB isoenzyme (CKBB), visin-in-like protein (VILIP-1), von Willebran factor (vWF), brain derived neurotrophic factor (BDNF), peroxiredoxin (PRDX) -6.

* = all markers reported as pg/mL unless otherwise stated

“--” = below assay quantitation in ≥50% of samples analyzed.