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Bridging Preclinical and Clinical Perspectives: New Insights on Melatonin's Neuroprotective Effects in Neonatal Rats with Hypoxic-Ischemic Brain Injury and Discovering Prognostic Biomarkers in Patients with Traumatic Brain Injury

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Thesis Overview

Perinatal hypoxic-ischemic (HI) brain injury and traumatic brain injury (TBI) represent two important causes of lifelong disabilities. HI brain injury arises when the brain experiences an inadequate supply of oxygen and blood flow often due to circumstances such as cardiac arrest, respiratory failure, or asphyxiation, leading to neuronal death and cognitive impairment (Allen & Brandon, 2011). Similarly, TBI is a consequence of an external mechanical force leading to brain dysfunction, which can also lead to cell death and cognitive impairment (McAllister, 2011). HI brain injury and TBI share common pathophysiological mechanisms, including excitotoxicity, inflammation, and oxidative stress. The progression of brain damage following both injuries is a dynamic process. Initially, the primary injury transpires, directly caused by the trauma or HI event. Subsequently, secondary injury processes occur, encompassing excitotoxicity, inflammation, oxidative stress, apoptosis, mitochondrial dysfunction, and disruptions to the blood-brain barrier (McAllister, 2011; McLean & Ferriero, 2004; Ng & Lee, 2019). TBI also induces microvascular injury and HI is common following TBI. Virtually 100% of patients who die of TBI have evidence of HI on neuropathological examination of their brains (Vespa, 2016).

The aim of this thesis is two-fold: 1) to gain more insight into the mechanisms underlying the neuroprotective effect of melatonin following HI brain injury in the early postnatal brain in neonatal rats, and 2) to identify prognostic biomarkers in patients with TBI.

This thesis comprises two chapters detailing preclinical and clinical approaches to brain injury. The first chapter, entitled “Neonatal Hypoxic-Ischemic Brain Injury: Involvement of Notch1 Signaling Pathway and SIRT3 in the Neuroprotective Effect of Melatonin” reports data from experiments that I conducted at the Department of Biomolecular Sciences, University of Urbino Carlo Bo in Italy. This research utilized a neonatal rat model of HI to investigate the neuroprotective mechanisms of melatonin, a natural neurohormone secreted by the pineal gland with antioxidant, anti-apoptotic, and anti-inflammatory properties. The study elucidates how melatonin administration immediately following neonatal HI can attenuate brain damage by modulating the Notch1 signaling pathway and SIRT3.

The second chapter, “Traumatic Brain Injury: Identification of Prognostic Biomarkers”, was conducted during a research period that I spent at The Hospital for Sick Children

(SickKids) affiliated with the Faculty of Medicine of the University of Toronto in Canada and is a result of a collaborative study in which I was involved. This chapter deals with a clinical approach to TBI and includes two projects.

The first project, “A Canadian Biobank and Database for TBI (CanTBI)” involves establishing a sustainable platform linking existing regional biobanks to a national database of children and adults with TBI. By creating a centralized, state-of-the-art neuroscience database, this platform enables the translation of molecular biomarker research from the laboratory to clinical settings, ultimately improving the quality of care and outcomes for TBI patients.

The second project, “Prognostic Serum Protein Biomarkers in Children with Severe TBI”, was conducted in a collaborative project between The Hospital For Sick Children and the National Research Council. This study has discovered eight proteins with differential concentrations in three experimental groups including favorable outcome within 6-month post-TBI, unfavorable outcome within 6-month post-TBI, and trauma controls without TBI, offering potential for clinical neuromonitoring, prognosis, and risk stratification in TBI patients, and may lead to the development of new therapies for TBI.

Chapter 1: Neonatal Hypoxic-Ischemic Brain Injury: Involvement of Notch1 Signaling Pathway and SIRT3 in the Neuroprotective Effect of Melatonin

1. Introduction

1.1 Hypoxic Ischemic Encephalopathy

1.1.1 Definition and Overview

Neonatal Hypoxic-ischemic encephalopathy (HIE) is a brain injury in neonates resulting from partial or complete anoxia and decreased or suspended cerebral blood flow due to asphyxia during the perinatal period (Huang et al., 2018; Yıldız et al., 2017). HIE is widely recognized as a leading contributor to neonatal mortality and disability (Huang et al., 2018; Yıldız et al., 2017). It frequently leads to lifelong limiting sequelae, such as cerebral palsy, seizures, or mental retardation (Guo et al., 2021).

Despite significant advancements in neonatal care that have led to improved survival rates for extremely premature infants and severely hypoxic neonates, the risk of brain damage during the perinatal period remains high. The incidence of neonatal HIE is 1.5 to 2.5 per 1000 live births in developed countries. Typically, by the age of 2 years, about 60% of infants with HIE will either die or experience severe disabilities (Allen & Brandon, 2011; Long & Brandon, 2007). Furthermore, in developing countries, the prevalence of this condition has increased to about 26 in every 1000 live births. Approximately 24% of these infants typically do not survive their stay in the neonatal intensive care units. Among the survivors, a significant number with cerebral palsy (10-20%), visual and hearing issues (around 40%), and a range of motor and behavioral disorders, including epilepsy, global developmental delays, and autism are diagnosed (Yıldız et al., 2017).

Therefore, it is of high priority to develop effective interventions for those at risk of cerebral HI. This underscores the critical need to understand the complex pathophysiology of HI brain injury in perinatal healthcare (Carloni & Balduini, 2008).

1.1.2 Pathophysiology

The pathophysiology of HI is multifaceted, involving a range of biological processes such as energy failure, excitotoxicity, oxidative stress, inflammation, cell apoptosis, and autophagy (Fatemi et al., 2009; Huang et al., 2018). Understanding the complex cellular and molecular mechanisms is crucial for the development of effective therapeutic interventions.

- Energy Failure and Adenosine Triphosphate (ATP) Depletion:

HIE is initiated by a decrease in cerebral blood flow and oxygen delivery, leading to inadequate oxygen supply to the brain (Vannucci & Hagberg, 2004). This results in a shift from aerobic to anaerobic metabolism, with a subsequent decrease in ATP production and accumulation of lactate (Yıldız et al., 2017). ATP depletion disrupts essential cellular functions, including ion homeostasis, membrane potential maintenance, and neurotransmitter reuptake, contributing to the onset of excitotoxicity (Johnston et al., 2011).

- Excitotoxicity:

During HIE, the excessive release of excitatory amino acids, particularly glutamate, occurs due to impaired neurotransmitter reuptake and increased presynaptic release (Martin et al., 2000). This leads to overactivation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, causing a massive influx of calcium ions (Ca2+) into neurons (Volpe, 2001). The increased intracellular Ca2+ triggers a series of destructive enzymatic cascades, resulting in mitochondrial dysfunction, free radical production, and ultimately, neuronal death (Barks & Silverstein, 1992).

- Oxidative Stress:

Oxidative stress plays a crucial role in HIE pathogenesis, as the reperfusion of ischemic tissue leads to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Puka-Sundvall et al., 2000). These reactive species interact with cellular macromolecules such as lipids, proteins, and Deoxyribonucleic Acid (DNA), causing oxidative damage and further exacerbating cellular dysfunction (Drury et al., 2014).

- Inflammation and Blood-Brain Barrier Disruption:

Inflammation is a significant component of HI pathophysiology, with pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) contributing to blood-brain barrier (BBB) disruption, edema, and leukocyte infiltration (Liu & Mccullough, 2013). BBB disruption facilitates the entry of inflammatory cells and toxic molecules into the brain, worsening the injury (Rocha-Ferreira & Hristova, 2016).

- Apoptosis:

Apoptosis is a key player in brain injury in newborns and is crucial in delayed neural death.

Cells undergo apoptosis when energy levels drop significantly; however, if energy is entirely depleted, they succumb to necrosis. So, apoptosis has a vital role in brain damage caused by HI. This process starts in the early stages of the injury and can last for several days or even weeks. Gaining insight into when and how cells die after HI is extremely important to develop treatments and preventative measures for brain injury. A group of proteins called caspases, specifically, Caspase-3 plays a key role in the process of apoptosis. Caspase-3 is released in large amounts when HI is triggered and is mainly responsible for the typical changes seen in cell death by apoptosis (Huang et al., 2018; Yıldız et al., 2017).

1.2 Melatonin

1.2.1 Overview and Biological Function

Melatonin (n-acetyl-5-methoxytryptamine) is a lipophilic neurohormone primarily synthesized in the pineal gland. This hormone possesses various physiological roles, including the regulation of circadian rhythms, mitochondrial protection, and exhibiting antioxidant, anti-inflammatory, and antiapoptotic properties. It is particularly a free radical scavenger, neutralizing singlet oxygen, superoxide anion radical, hydroperoxide, hydroxyl radical, and lipid peroxide radical (Tan et al., 2007). Furthermore, it indirectly exhibits antioxidant properties by enhancing mitochondrial electron transport efficiency and activating key antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase (Rodriguez et al., 2004) without showing any pro-oxidant effects (Cuzzocrea & Reiter, 2001).

1.2.2 Role in Neuroprotection

Melatonin's neuroprotective effect, especially concerning neurodegenerative disorders, has been extensively explored (Zhang et al., 2016). This protection stems mainly from its capability to neutralize free radicals and its ability to penetrate morphophysiological barriers, allowing widespread antioxidative effects (Reiter et al., 2002). Its ubiquitous distribution in tissues, cells, and subcellular compartments facilitates widespread antioxidative effects. It has a crucial role in controlling the apoptotic cell death cascade through increasing ATP. It showcases its strong antioxidant prowess by neutralizing free

radicals and activating antioxidant enzymes and pathways (Reiter et al., 2002). Additionally, melatonin exerts anti-inflammatory properties by inhibiting nitric oxide synthase, cyclooxygenase-2, and neutrophil infiltration, adding to its neuroprotective potential (Deng et al., 2006).

The neuroprotective properties of melatonin are documented in various diseases, including stroke (Cheung et al., 2003; Pei et al., 2003). Melatonin exhibits numerous advantageous impacts on Alzheimer's Disease (AD). These include functioning as an antioxidant, a neuroprotective agent, a modulator of mitochondrial operation, and an anti-neuroinflammatory agent (Dragicevic et al., 2011; Hardeland et al., 2015; Zhang et al., 2016). Recent research suggests that melatonin also mitigates memory deficits, accumulation of amyloid-beta (Aβ), and neuroplasticity disruptions observed in a sporadic rat model of AD. Consequently, melatonin emerges as a promising candidate for preventing and treating AD and other age-related neurodegenerative diseases (Furio et al., 2007; Stefanova et al., 2015; Zhang et al., 2016).

Considering melatonin's potential effectiveness and its low toxicity, it has emerged as a promising neuroprotective candidate for perinatal HI brain injury. Despite its current lack of approval for neonatal therapeutic use, preliminary applications in neonatal sepsis, bronchopulmonary dysplasia, and neonatal asphyxia have yielded promising results (Carloni et al., 2008; Fulia et al., 2001; Gitto et al., 2001). Beneficial effects of melatonin have been reported in a variety of diseases, especially with regard to cardiovascular protection (Jiki et al., 2018; Pandi-Perumal et al., 2017) and neuroprotection (Alghamdi, 2018; Hsu et al., 2019; Sanchez-Barcelo et al., 2017) in cases such as TBI (Ansari et al., 2008) and HI brain injury (Zhao et al., 2016). Melatonin holds great promise in diminishing brain injury and its long-term repercussions, either as a stand-alone or as an additional therapy. Despite its promise, a comprehensive understanding of its benefits requires further clinical research (Yıldız et al., 2017).

Our previous research offers valuable insights into this potential. We demonstrated that melatonin effectively protects against brain injury in a neonatal rat model of HI brain injury. We observed a positive effect when: 1) a single 15 mg/kg dose of melatonin was given 5 minutes before HI or 2) the same dosage was administered after HI and repeated 24 and 48 hours later. The latter treatment schedule also significantly improved long-term behavioral outcomes, reducing learning deficits caused by HI brain injury (Carloni et al., 2008).

1.3 Notch Signaling

1.3.1 Overview and Biological Function

Notch signaling pathway is an evolutionary conserved pathway that has a crucial role in the central nervous system (CNS) across a mammal's life span. Its roles range from embryonic development stages of neurogenesis and specification (Pompa et al., 1997), to synaptic plasticity and learning/memory functions during adulthood (Wang et al., 2004). In addition, during brain development, Notch signaling is crucial in maintaining neural progenitors in an undifferentiated state, which is accomplished in part by inhibiting neurogenesis (Ables et al., 2011).

Notch signaling pathway belongs to a newly recognized signaling model known as Regulated Intramembrane Proteolysis (RIP). RIP is a highly regulated proteolytic process involving two sequential proteolysis steps that instigate the activation of a transmembrane receptor and subsequent expression of downstream genes: first an extracytosolic shedding and then an intramembrane cleavage (Lal & Caplan, 2011). RIP operates across two distinct subcellular locales. The initial location is the transmembrane where, in reaction to a stimulus or ligand binding, the full-length receptor undergoes a two-step proteolysis process that releases an intracellular domain. This domain then moves to the second location of action, typically the nucleus - a scenario exemplified by the Notch pathway (Brown et al., 2000).

Four analogous Notch genes including Notch1, Notch2, Notch3, and Notch4 have been identified in mammals (Fortini et al., 1993). Within the nervous system, Notch1 is significantly present in neural stem/progenitor cells, neurons, and astrocytes. Its role is crucial in managing neuronal differentiation and promoting neurogenesis in adults (Xiao et al., 2009). Notch2 expression is temporarily observed in granule neuron precursors in the developing cerebellar cortex (Solecki et al., 2001) and is primarily exhibited by glial cells in the brain after birth (Tanaka et al., 1999). In adult tissues, Notch3 is specifically expressed in vascular smooth muscle cells and plays a role in the pathology of ischemic cerebral small-vessel disease (Louvi et al., 2006). Notch4, on the other hand, is particularly found in endothelial cells, and its activation contributes to the development of brain arteriovenous malformations in mice (Murphy et al., 2008; Uyttendaele et al., 2000). Among four Notch homologs, Notch1 is the most prevalently expressed in neurons and its function extensively explored in both invertebrates and mammals. Consequently, this thesis

will primarily focus on the role of Notch1.

Notch1 signaling pathway is highly conserved cell-cell communication system that has a fundamental role in cellular processes, including regulating neurogenesis, neural networks, synaptic plasticity (Zhang et al., 2016), proliferation, differentiation, death and cell fate decisions during development in a variety of tissues (Ables et al., 2011). It is unique in that most of its ligands are also transmembrane proteins, which limits signaling to adjacent cells. Although the intracellular transduction of the Notch signal is simple and lacks secondary messengers, it plays a vital role in a wide range of developmental processes, and its malfunction is implicated in numerous diseases (Bray, 2006).

1.3.2 Mechanism

The central component of Notch1 signaling is the Notch1 receptor, which is a transmembrane protein (Zhang et al., 2016). The Notch1 signaling is initiated by ligand binding to the Notch1 receptors (Stoeck et al., 2014). Following activation by ligand binding, the Notch intracellular domain (NICD), which is directly involved in transcriptional control, is released into the cytosol (Fortini et al., 2009). Subsequently, NICD translocates to the nucleus and initiates transcription of Notch1 target genes, including those related to cell-fate determination such as Hairy-Enhancer of Split 1 (HES1) (Jarriault et al., 1998), and proliferation such as MYC. HES1 is a transcription factor that belongs to the basic helix-loop-helix (bHLH) family and plays regulatory roles in neuronal function and morphology. HES1 and other proneural genes are crucial in inducing neurogenesis and regulating neuronal differentiation (Zhang et al., 2016). MYC, a transcription factor with a basic helix-loop-helix leucine zipper structure, controls a vast array of target genes linked to cell proliferation and metabolism. Given its pivotal role in cell growth regulation, it is considered one of the most critical oncogenes (Pozzo et al., 2022; Stoeck et al., 2014). The DNA-binding protein RBPJ is the principal effector of this pathway in mammals and together with the transcription factor moiety of Notch, NICD, regulates the expression of Notch1 target genes (Castel et al., 2013).

1.3.3 Notch Signaling in Pathological Conditions

Dysregulation of the Notch1 signaling pathway has been implicated in the pathogenesis of

a wide range of diseases. Some prominent examples include:

Cancer: Abnormal Notch1 signaling has been linked to the development and progression of several types of cancer, including leukemia, breast cancer, and lung cancer (Aster et al., 2017; Ranganathan et al., 2011). Notch1 can act as either an oncogene or a tumor suppressor, depending on the cellular context (Radtke & Raj, 2003).

Cardiovascular diseases: Dysregulation of Notch1 signaling pathway has been implicated in various cardiovascular disorders, such as congenital heart defects, atherosclerosis, and cardiac hypertrophy (Borggrefe & Oswald, 2009; High & Epstein, 2008; MacGrogan et al., 2010).

Multiple sclerosis: Notch1 signaling has been shown to play a role in the pathogenesis of multiple sclerosis by modulating the inflammatory response and contributing to the loss of oligodendrocytes, leading to demyelination (Jurynczyk et al., 2008).

Neurodegenerative diseases: Notch1 signaling has been implicated in the pathogenesis of AD, as it regulates the production of amyloid-beta peptides, which contribute to the formation of amyloid plaques in the brain (Berezovska et al., 1998; Kopan & Ilagan, 2009; S. Zhang et al., 2016).

Brain ischemia: Notch1 signaling contributes to the pathophysiology of brain ischemia, notably by regulating the response of NSCs and endothelial cells to ischemic injury (Androutsellis-Theotokis et al., 2006; Arumugam et al., 2006).

1.4 Sirtuin3 (SIRT3)

1.4.1 Overview and Biological Function

Sirtuins (SIRTs) are a family of seven Nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases and ADP ribosylases, renowned for their cell-protective and lifespan-extending properties. SIRTs undertake diverse functions across different tissues and organs. In mammals, seven SIRTs (SIRT1-7) have been identified. Despite their ubiquitous expression in the body, each SIRT subtype is characterized by a specific cellular localization: SIRTs 1, 6, and 7 are localized in the nucleus; SIRT2 is found in the cytosol; and SIRTs 3, 4, and 5 reside in mitochondria (Carloni et al., 2017; Gleave et al., 2017).

SIRT3, a mitochondrial deacetylase, is prevalent in all forms of human tissues and controls energy metabolism, the respiratory chain, the generation of reactive oxygen species (ROS),

and autophagy through post-translational deacetylation of the substrate. It has a crucial role in maintaining mitochondrial homeostasis by deacetylating substrates in an NAD+-dependent way (Yang et al., 2023). This thesis will primarily focus on the role of SIRT3.

1.4.2 SIRT3 in Pathological Conditions

Recent research suggests that SIRT3 could play a crucial role in various human disorders, such as metabolic disorders, age-related diseases, and various types of cancer (Zhang et al., 2020). SIRT3 expression is significantly down-regulated in the cerebral cortex of AD rats (Yang et al., 2023). SIRT3 gene knockout amplifies neuronal death due to H2O2-induced oxidative stress and exacerbates the degeneration of striatonigral dopaminergic neurons in rats with Parkinson's disease (PD). SIRT3 is implicated in the genesis and progression of nervous disorders, inflammatory damage from microglia activation, and induced cell apoptosis and mitochondrial damage in NSCs (Yang et al., 2023).

Moreover, a large number of studies are pointing to a protective effect of SIRT3 on the brain against diverse types of damage, including cerebral ischemia/reperfusion (I/R) injury, TBI, subarachnoid hemorrhage (SAH), and intracerebral hemorrhage (ICH). However, the specific protective processes involved are still unclear. These might be connected to the maintenance of a state in the mitochondria homeostasis or other roles (He et al., 2020), like combatting oxidative stress and inflammation in the nervous system (Gao et al., 2020), and offering protection against neuronal excitotoxicity (Cheng et al., 2016; H. Yang et al., 2023). The progression of brain damage typically involves a dysfunction of the mitochondria, leading to a variety of pathophysiological events, including inflammation, oxidative stress, and mitophagy (Yang et al., 2023; Zhang et al., 2022). Given the role of SIRT3 in maintaining mitochondrial homeostasis and metabolism, as well as reducing mitochondrial damage, research into how SIRT3 operates could be key in gaining a better understanding of brain injuries (Yang et al., 2023).

1.4.3 Connection between Notch1 Signaling Pathway and SIRT3

Recently reports highlighted a connection between the Notch1 signaling pathway and Sirt3 in several stress conditions. It was found that Gastrodin regulated both the Notch1 signaling pathway and Sirt3 in activated microglia in neonatal rats subjected to cerebral

hypoxic-ischemia (HI), as well as in activated BV-2 microglia (Guo et al., 2021). In human gastric cancer cells, Sirt3 inhibited cell proliferation via the down-regulation of Notch1 (Wang et al., 2015). When the Notch1 signaling pathway was interfered with, it resulted in the inhibition of Sirt3 expression, suggesting that Sirt3 is a downstream gene of the Notch1 signaling pathway. Furthermore, Sirt3 has been found to promote autophagy in HK-2 human proximal tubular epithelial cells via the inhibition of Notch1/Hes1 signaling (Wang et al., 2021).

Melatonin (N-acetyl-5methoxytryptamine), a versatile and ubiquitous molecule well known as a potent indirect antioxidant and direct free radical scavenger (Manchester et al., 2015), significantly improved the perinatal brain damage caused by HI through activation of several protective mechanisms such as antioxidant, anti-inflammatory and antiapoptotic effect (Pluta et al., 2023). We recently found that the protective effects of melatonin are strictly interconnected to the restoration of mitochondria status, which is significantly altered by HI, as well as to the modulation of cell cycle dynamics of newborn hippocampal cells and increased cell proliferation (Nasoni et al., 2021).

1.5 Objectives and Hypothesis

Increasing evidence highlights the beneficial role of melatonin in neurodegenerative diseases, including perinatal brain damage. However, knowledge regarding the cellular mechanisms underpinning the neuroprotective effect of melatonin in HI is not well understood. We hypothesized that melatonin may mitigate HI brain injury via modulation of the Notch1 signaling pathway and SIRT3. The objective of this study was to investigate the modulation of the Notch1 signaling pathway and SIRT3 in the melatonin's neuroprotective effect in the early stage (1 hour) after HI brain injury in neonatal rats. In this study, we investigated the regulatory role of melatonin on Notch1, NICD, Hes1, c-Myc, and SIRT3, which could deepen our understanding of its potential therapeutic application in neurodegenerative diseases.

The rationale for investigating melatonin's protective effects on HI brain injury via modulation of Notch1 signaling and SIRT3 is outlined below:

Prevalence of HI brain injury: HI brain injury is a major cause of neonatal morbidity, mortality, and long-term neurological disabilities in survivors (Johnston et al., 2011). Gaining insight into the mechanisms involved and potential therapeutic interventions is

essential to address this public health issue.

Neuroprotective properties of melatonin: Melatonin possesses antioxidant, anti-inflammatory, and anti-apoptotic properties (Reiter et al., 2016). These neuroprotective effects make melatonin a promising candidate for HI brain injury treatment.

Safety and tolerability of melatonin: Melatonin exhibits a favorable safety profile and is generally well-tolerated, even at high doses (Andersen et al., 2016). This makes it an ideal candidate for further investigation as a potential treatment for neonatal HI brain injury.

Role of Notch1 Signaling in neural development and repair: Notch1 signaling is critical for the development, maintenance, and repair of the CNS, participating in NSC proliferation, differentiation, and survival (Ables et al., 2011), and its dysregulation has been associated with various neurological disorders, including HI brain injury (Zhang et al., 2016)

Potential therapeutic target: Investigating melatonin's impact on neonatal HI brain injury through Notch1 signaling may reveal a possible target for therapeutic intervention. If melatonin effectively modulates Notch1 signaling, it could provide new insights for the development of targeted therapies (Beker et al., 2019).

Role of SIRT3 in neuroprotection: SIRT3 plays a significant role in cellular stress resistance, metabolic regulation, and longevity. SIRT3 has been demonstrated to exert neuroprotective effects including, reducing oxidative stress and maintaining mitochondrial function, which are vital for neuronal survival in HI (Yang et al., 2023; Zhang et al., 2022). Therefore, understanding the role of SIRT3 in the neuroprotective effects of melatonin could offer new insights into the molecular mechanisms of neuroprotection.

2. Materials and Methods

2.1 Cerebral Hypoxia-ischemia (HI)

All surgical and experimental procedures were carried out in accordance with the Italian regulation for the care and use of laboratory animals (according to EU Directive 63/2010; Italian D.L. 26/14; research protocol authorization 582/2020-PR), and were approved by the Animal Care Committee of the University of Urbino Carlo Bo. Pregnant Sprague-Dawley (Charles River) rats were housed in individual cages and the day of delivery was considered day 0. Neonate rats from different litters were randomized, normalized to ten pups per litter, and kept in regular light/dark cycles (lights on 8 am–8 pm).

On postnatal day 7, pup rats were anesthetized with 5% isoflurane in O2 mixture, and underwent unilateral ligation of the right common carotid artery via a midline neck incision. After artery ligation, the wound was sutured and the animals were allowed to recover for 3 hours (h) under a heating lamp. Pups were then placed in an airtight jar and exposed for 2.5 h to a humidified nitrogen–oxygen mixture (92% and 8%, respectively) delivered at 5–6 L/min (HI). Once the HI procedure was finished (and melatonin treatment or vehicle received), pups were returned to their dams until the experimental procedures were performed 1 h, 24 h, or 48 h after HI.

2.2 Drugs Administration

Melatonin (Sigma-Aldrich, Milan, Italy, M5250) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Milan, Italy, D5879) and diluted in normal saline solution to a final concentration of 5% DMSO (vehicle). The melatonin solution was intraperitoneally injected to pup rats 5 minutes (min) after HI at the dose of 15 mg/kg and repeated after 24 h and 48 h. Control animals received the same volume of the vehicle. This dose and schedule of melatonin drug administration was chosen on the basis of previous experiments showing the protective effects of melatonin in ischemic neonatal pup rats (Carloni et al., 2008).

2.3 Western Blot Analysis

Pups were anesthetized and euthanized by decapitation 1, 24 or 48 hours after HI. Brains were rapidly removed, and hippocampal homogenates prepared as previously described

(Carloni et al., 2016). The cytosolic, mitochondrial, and nuclear fractions were prepared from the hippocampus according to Nijboer et al. (Nijboer et al., 2007). Samples were stored at -80°C until use. After mixing with sodium dodecyl sulfate gel-loading buffer and heating for 4 min at 95°C, samples (50 μg protein) were electrophoresed onto sodium dodecyl sulfate-polyacrylamide gel and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. ColorBurstTM electrophoresis marker (3 μL/gel, Sigma, C1992) was used for qualitative molecular mass determinations and visual confirmation of blot transfer efficiency. Blots were then blocked with non-fat dry milk in TBS-T (10 mM Tris, 150 mM NaCl, pH 7.6, plus 0.1% Tween-20) and probed with the following primary antibodies: anti-Notch1 (1:1000, polyclonal; Cell Signaling Technology, #3608), anti-Cleaved Notch1 (NICD) (1:500, polyclonal; Cell Signaling Technology, #4147), anti-Hes 1 (1:1000, polyclonal; Cell Signaling Technology, #11988), anti-c-Myc (1:1000, polyclonal; Cell Signaling Technology, #5605), anti-SIRT3 (1:1000, polyclonal; Cell Signaling Technology, #2627), anti-Hif-1a (1:1000, polyclonal; Cell Signaling Technology, #3716). A monoclonal antibody against β-actin (1:4000, Santa Cruz Biotechnology, sc-8432) was used as a control for protein gel loading. Blots were analyzed using the NIH-Image J 1.45 software (https://imagej.nih.gov/ij/), National Institutes of Health, Bethesda, MD, USA. Data were normalized to β-actin and expressed as % of control.

2.4 Immunohistochemistry

Pups were deeply anesthetized with 5% isoflurane in O2 mixture and perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L PBS. Brains were rapidly removed on ice and processed for antigen retrieval by immersing overnight in 10 mmol/L sodium citrate buffer (pH 6.0, 4°C) and boiling in the same buffer for 3 min (Ino, 2003). After boiling, brains were cryoprotected with 30% sucrose/PBS (72 h, 4°C). Brain sections (thickness 12 μm) were incubated with 1.5% normal blocking serum for 1 h at room temperature, and then overnight at 4°C with anti-Notch1 and anti-NICD (1:1000 and 1:40 respectively, polyclonal; Cell Signaling Technology, #4147), anti-glial fibrillary acidic protein (GFAP, 1:300, monoclonal; Boehringer Mannheim GmbH, 813 369) or anti-neuron-specific nuclear protein (NeuN, 1:500; monoclonal; Millipore, MAB377), anti-microtubule associated protein 2 (MAP2, 1:500; rabbit, polyclonal; Millipore, AB5622). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, or Texas Red goat anti-mouse IgG, or

Alexa Fluor goat anti-rabbit IgG (1:200; Santa Cruz Biotechnology, sc-2359, sc-3797, sc-516248, respectively) were used to demonstrate immunofluorescence reactivity. The specificity of the reactions was evaluated in some slices by omitting the primary antibody from the incubation medium. Peroxidase activity was amplified by 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H2O2 at the appropriate stage. Images were acquired on a Leica TCS SP5 II confocal microscope (Leica Microsystem).

2.5 Data Analysis

Image J 1.45 software (https://imagej.nih.gov/ij/) was used for all the quantitative image analysis. Statistical analyses were performed by one-way ANOVA using the GraphPad Prism 9.0 Computer program (GraphPad Software, San Diego, CA, USA). Bartlett's test was used to determine data homogeneity. The Newman-Keuls multiple-comparison test was used to determine differences between single treatment groups. Results were considered to be significant when $p \leq 0.05$.

3. Results

3.1 Melatonin increases Notch1 expression in ischemic neonatal hippocampus

To examine whether neonatal HI affects Notch1, we studied the hippocampal protein expression at different time points after injury. Western blot analysis revealed that the expression of Notch1, compared to control condition, was significantly increased 1 hour after HI ($p \leq 0.01$ vs control), further increased after 24 hours ($p \leq 0.001$ vs control) and returned to control levels at 48 hours (Fig. 1A). Double labelling immunohistochemical experiments, performed to investigate the cell types in which Notch1 was expressed, showed that Notch1 was expressed in both neuronal and glial cells of ischemic hippocampus, as revealed by a strong co-localization of Notch1 with both the neuronal marker MAP2 and the astrocyte-specific marker GFAP (Fig. 1B).

The effect of melatonin on Notch1 expression was tested 1 hour after HI. Western blot analyses confirmed that Notch1 hippocampal expression significantly increased in the early phase of ischemic brain damage development, i.e. 1 hour ($p \leq 0.01$ vs control), and showed that melatonin further increased Notch1 expression ($p \leq 0.001$ vs control) (Fig. 1C).

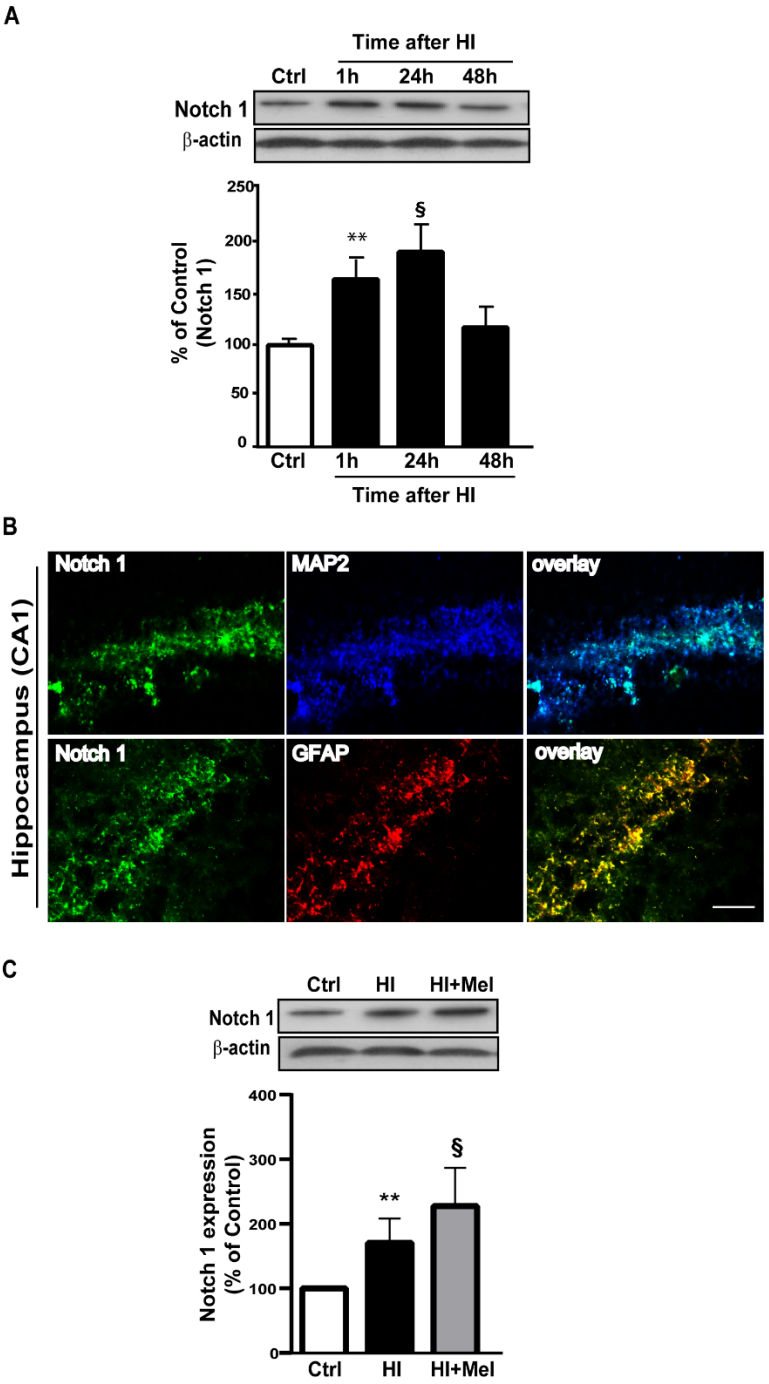

Figure 1. Effect of neonatal hypoxia-ischemia and melatonin on Notch1 expression.

(A) Representative Western blots and quantitative evaluation of Notch1 expression in the hippocampus of Control (Ctrl) and hypoxic-ischemic animals (HI) sacrificed 1 hour, 24 hours, and 48 hours after HI. (B) Representative photomicrographs showing cells labeled with Notch (green), the glial marker GFAP (red), and the neuronal marker MAP2 (blue) in the injured hippocampus of HI animals sacrificed 1 hour after HI. Merge images show the co-localization of Notch/MAP2 (light blue) and Neun/GFAP (yellow). Scale bar, 100µm. (C) Representative Western blots and quantitative evaluation of Notch1 expression in the CA1 region of the hippocampus of Ctrl, HI, and melatonin-treated hypoxic-ischemic (HI+Mel) animals sacrificed 1h after HI. Data are expressed as % of Control (mean±SE, n=5). β-actin was run as an internal standard. **$p < 0.05$ vs Ctrl; § $p < 0.001$ vs Control; One-way ANOVA followed by Newman–Keuls multiple comparison tests.

3.2 Melatonin modulates Notch1 signaling pathway in ischemic neonatal hippocampus

To better analyze the effects of HI and melatonin on Notch1 signaling pathway, we studied the expression of several proteins involved in the pathway, including, NICD, a key protein of the pathway, HES1, a downstream effector of Notch1, and c-Myc, a protein expressed since its gene is a target of Notch (H. Guo et al., 2014).

Western blot studies showed that NICD expression was significantly increased 1 hour after HI ($p \leq 0.001$ vs control) while melatonin administration decreased its expression to control values (Fig. 2A). Immunofluorescence results demonstrated a significant co-localization between NICD and NeuN in the CA1 region of the ischemic hippocampus 1 hour after HI, indicating that the protein is significantly expressed in neurons (Fig. 2B).

Hes1 expression was significantly increased 1 hour after HI ($p \leq 0.05$ vs control) while melatonin administration reduced its expression to control levels (Fig. 3A). c-Myc expression was significantly reduced 1 hour after HI ($p \leq 0.05$ vs control) while melatonin restored its expression to values higher than the control group (Fig. 3B).

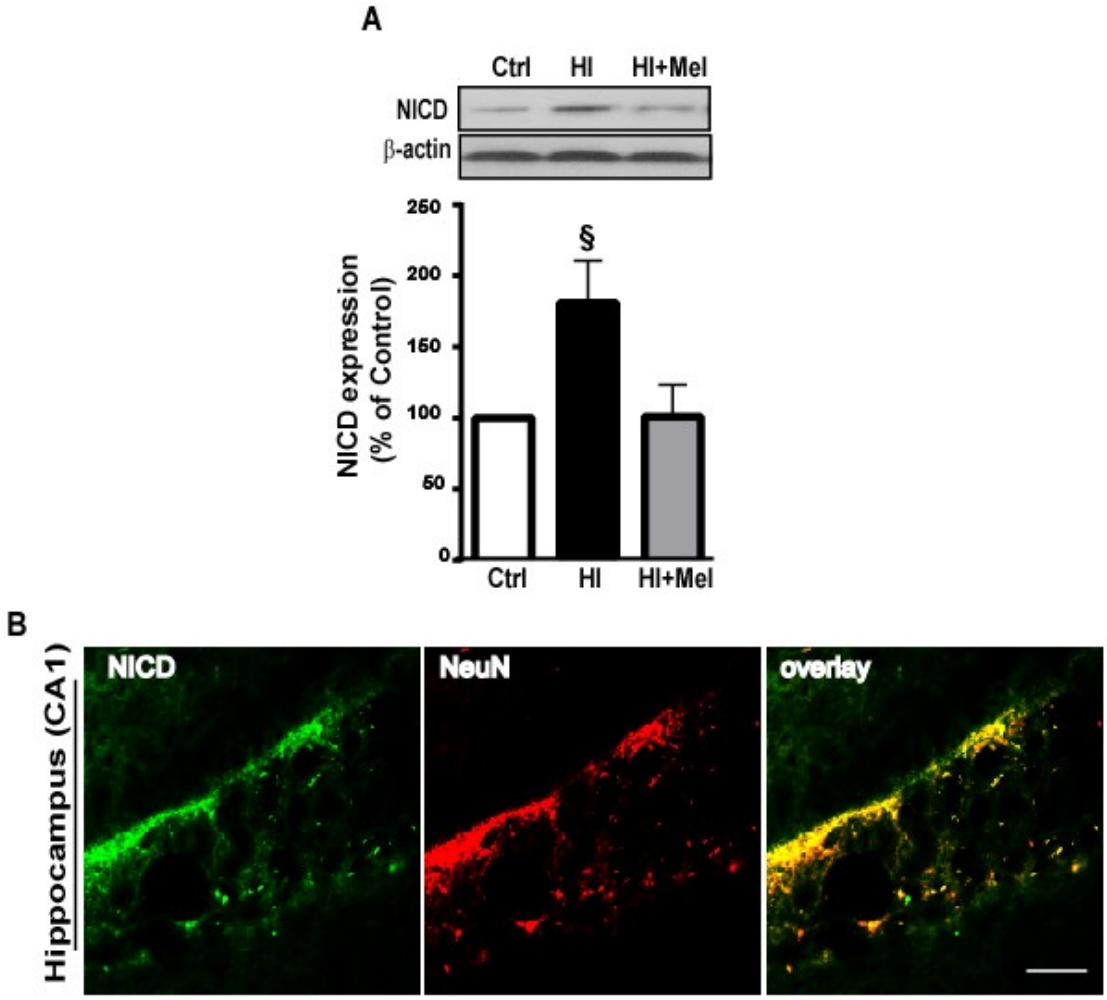

Figure 2. Effect of neonatal hypoxia-ischemia and melatonin on NICD expression.

(A) Representative Western blots and quantitative evaluation of NICD expression in the hippocampus of Control (Ctrl), hypoxic-ischemic (HI), and melatonin-treated hypoxic-ischemic (HI+Mel) animals sacrificed 1 hour after HI. Data are expressed as % of Control (mean±SE, n=5). β-actin was run as internal standard. § $p < 0.001$ vs Control, One-way ANOVA followed by Newman–Keuls multiple comparison test. (B) Photomicrographs showing cells labeled with NICD (green) and NeuN (red) in the injured hippocampus of HI animals sacrificed 1 hour after HI. Overlay images show the co-localization of NICD/NeuN (yellow). Scale bars, 100μm.

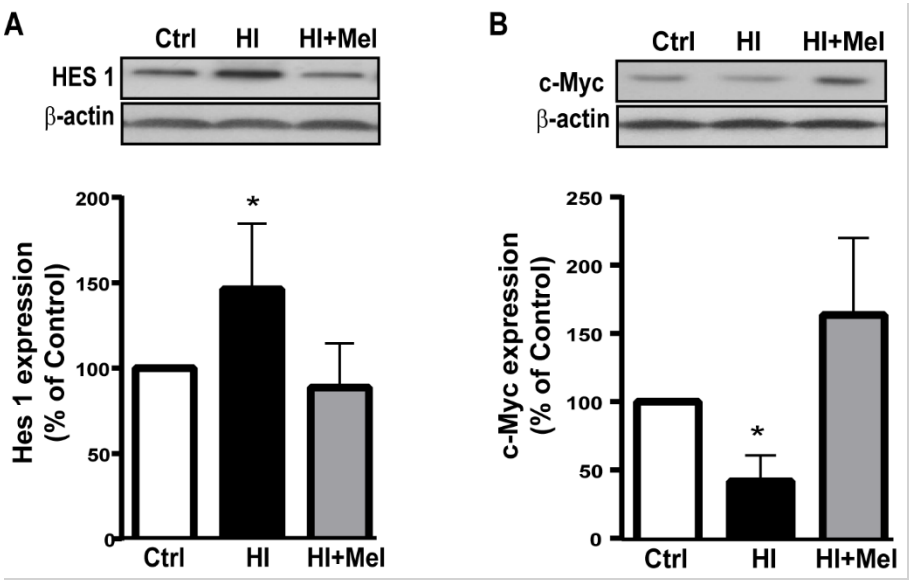

Figure 3. Effect of neonatal hypoxia-ischemia and melatonin on Hes1 and c-Myc expression.

(A) Representative Western blots and quantitative evaluation of Hes1 and c-Myc (B) expression in the hippocampus of Control (Ctrl), hypoxic-ischemic (HI) and melatonin-treated hypoxic-ischemic (HI+Mel) animals sacrificed 1 hour after HI. Data are expressed as % of Control (mean±SE, n=5). β-actin was run as an internal standard. * $p < 0.05$ vs Ctrl, One-way ANOVA followed by Newman–Keuls multiple comparison tests.

3.3 Melatonin preserves SIRT3 expression in neuronal and glial cells of neonatal hippocampus affected by HI

SIRT3 resided in mitochondria, and as reported in several studies (Carloni et al., 2017; Gleave et al., 2017; H. Yang et al., 2023), we confirmed the deacetylase mitochondrial localization also in our experimental conditions. Indeed, immunoblot analysis performed in cytosolic, nuclear and mitochondrial fractions obtained from hippocampus of neonatal rats demonstrated that SIRT3 expression was not detected in the nucleus and cytosol (Fig. 4A and B), whereas was highly expressed in the mitochondria (Fig. 4C). Compared to controls, 1 hour after HI, the SIRT3 expression was significantly reduced in the hippocampus of ischemic animals (Fig. 4C; $p \leq 0.001$). Melatonin did not affect SIRT3 expression in control animals nor in the contralateral hippocampus of ischemic animals, but completely blocked its reduced expression in the lesioned hippocampus (Fig. 4C).

Double-staining of SIRT3 with different antibodies against cell-type-specific antigens revealed a strong co-localization of SIRT3 and NeuN within the CA1 region of the hippocampus (Fig. 5A, panel f) and, in a lower amount, within the deep layers of the cerebral cortex of HI+Mel

animals (Fig. 5A, panel c). Co-labeling with GFAP, instead, showed few SIRT3/GFAP-positive cells in the cerebral cortex (Fig. 5B, panel i), as well as in the hippocampus of ischemic animals treated with melatonin (Fig. 5B, panel n), indicating that melatonin mainly preserved SIRT3 expression in hippocampal neurons (Fig. 5A, panels d, e, f).

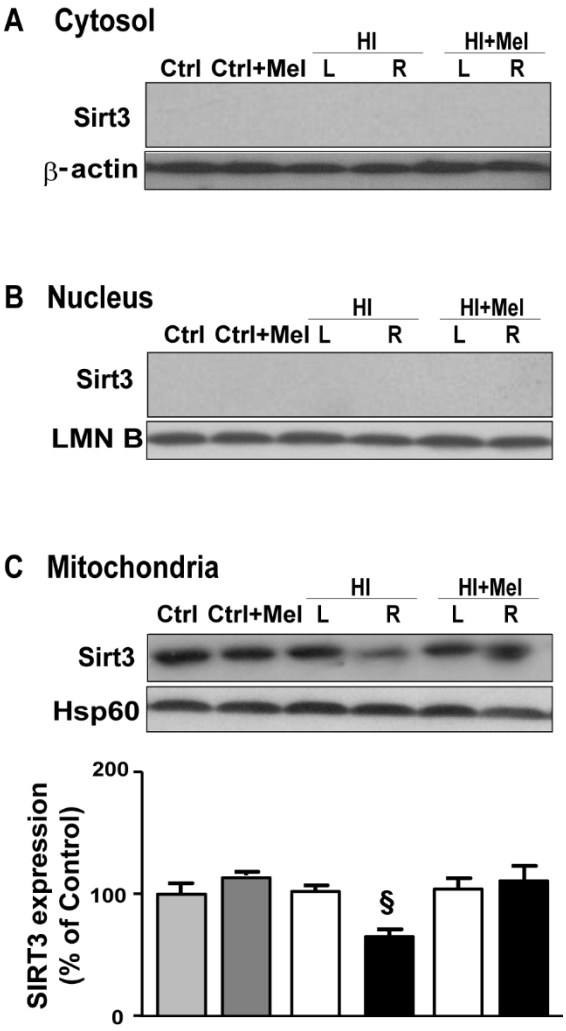

Figure 4. Effect of neonatal hypoxia-ischemia and melatonin on SIRT3 expression.

Representative Western blot and quantitative evaluation of SIRT3 expression in the cytosolic (A), nuclear (B), and mitochondrial (C) fractions of the hippocampus of vehicle-treated controls (C) or melatonin-treated (C+Mel) control animals and vehicle-treated (HI) or melatonin-treated (HI+Mel) ischemic animals sacrificed 1 hour after HI. β-actin, Lamin B (LMN B), and Hsp60 were run as loading controls for the cytosolic, nuclear, and mitochondrial fractions, respectively. Data are expressed as % of control and are the mean ± SEM (N=5/group). L, left side, contralateral; R, right side, ipsilateral to the occluded carotid artery. § $p < 0.001$ vs Control (C) group, one-way ANOVA followed by Newman-Keuls multiple comparison test.

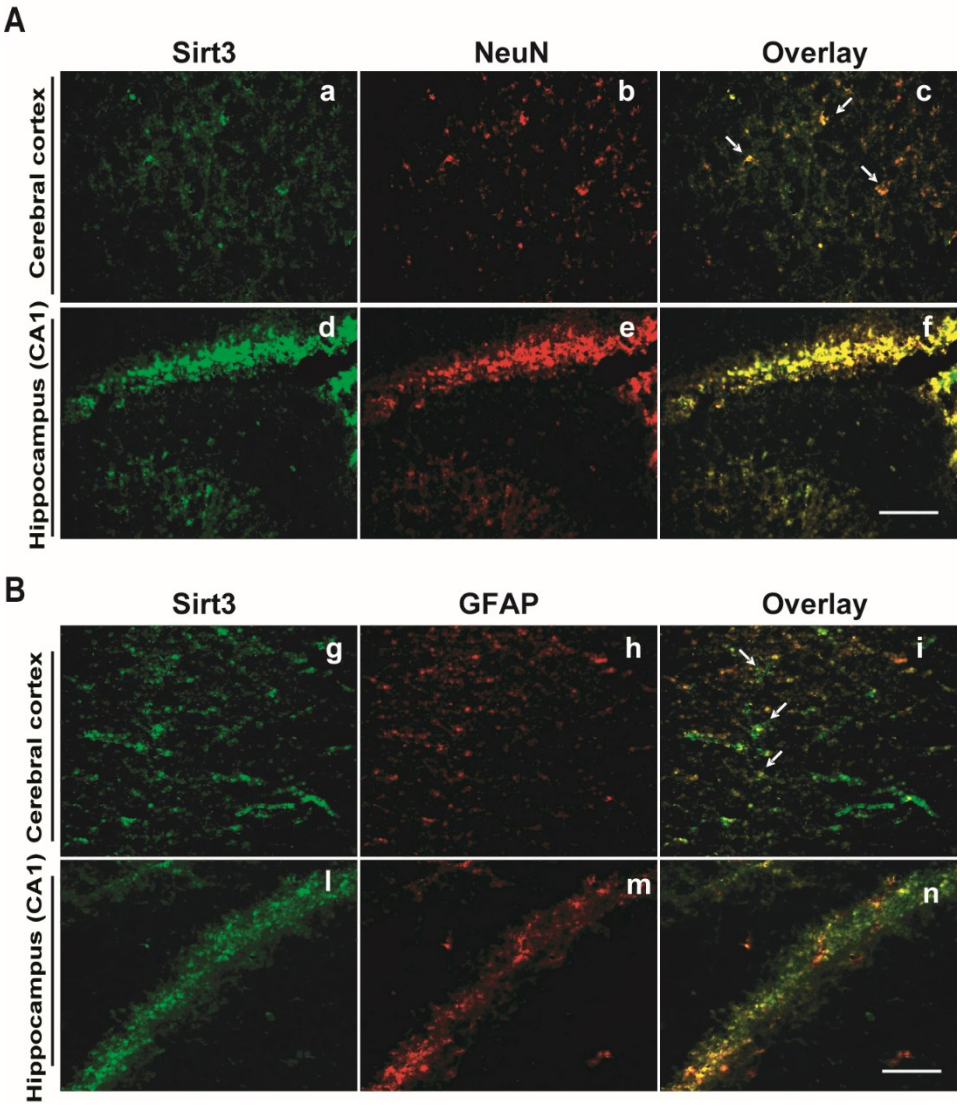

Figure 5. SIRT3 expression in neuronal and glial cells.

(A) Representative photomicrographs of experiments performed 1 hour after HI showing cells labeled with SIRT3 (a and d, green) and NeuN (b and e, red) in the injured cerebral cortex (a, b and c) and hippocampus (CA1 region) (d, e and f) of HI animals treated with melatonin (HI+Mel). Panels c (arrows) and f show the overlay of the images. (B) Representative photomicrographs of experiments performed 1 hour after HI showing cells labeled with SIRT3 (g and l, green) and GFAP (h and m, red) in the injured cerebral cortex (g, h and i) and hippocampus (CA1 region) (l, m and n) of HI+Mel animals. Panels i (arrows) and n show the overlay of the images. N = 5/group. Scale bars, 100 μm.

4. Discussion

The primary focus of our research was to investigate the neuroprotective mechanisms of melatonin through modulating the Notch1 signaling pathway and SIRT3 in neonatal rats after HI brain injury. Our results showed a significant increase in Notch1 expression in the hippocampus of neonatal rats following HI, with a significant expression in both neurons and glial cells. We observed that Notch1 expression slightly increased in the hippocampus of neonatal rats 1 hour after HI and melatonin administered 5 minutes after HI, further increased this expression. At the same time point, HI also significantly increased NICD expression, the key protein of the Notch1 signaling pathway, while melatonin administration maintained its expression to control values. Furthermore, the expression of c-Myc and Hes1, which represent target genes of Notch1 signaling pathway, were significantly modulated after HI while melatonin reversed these HI effects. These results clearly show the modulation of the Notch1 signaling pathway after neonatal HI and treatment with melatonin.

We also observed a significant decrease in SIRT3 expression in the hippocampal mitochondria 1 hour after HI, which was effectively restored to control values following melatonin administration, indicating a protective role of melatonin in preserving mitochondrial function in vivo as we previously observed after ischemic-like injury in hippocampal HT22 cells and in organotypic hippocampal cultures (Luchetti et al., 2022). Our results also demonstrated that melatonin significantly increased SIRT3 expression in neurons. Taken together, these results demonstrate that melatonin administration immediately after HI injury preserved the expression of Notch1 signaling pathway and SIRT3 that are significantly affected by neonatal HI.

Our findings are consistent with the current understanding of the role of the Notch1 signaling pathway in neuronal damage and neurodevelopmental disorders. Our study provides novel insights into the role of Notch1 signaling in neonatal HI brain injury which leads to excitotoxicity, oxidative stress, inflammation, and eventually, cell death (Hagberg et al., 2015). Notch1 signaling pathway has been shown to play an important role in cell-fate determination during embryonic development and in the regulation of diverse processes in adulthood (Artavanis-Tsakonas et al., 1999; Fortini, 2009). Its significance in the context of neuronal damage has been gaining interest; indeed, Notch1 signaling was found to be activated in various brain injuries including ischemia by regulating the response of NSC and endothelial cells to ischemic injury (Androutsellis-Theotokis et al., 2006; Arumugam et al., 2006). Our results

showed an increase in Notch1 expression 1 hour after HI, further supporting this relationship. The early upregulation of Notch1 expression after HI confirms previous research showing its role in neuroprotective mechanisms (Androutsellis-Theotokis et al., 2006). Moreover, the observed expression of Notch1 in both neurons and glial cells points to the pathway's contribution to neurogenesis and glial cell function following neonatal brain injury (Ables et al., 2011; Alberi et al., 2013).

In the context of neuroprotective responses to HI brain injury, our findings highlight the involvement of the Notch1 signaling pathway. Melatonin is a neurohormone known for its potent antioxidant and anti-inflammatory properties, which are conferred through several cellular mechanisms, such as the activation of antioxidant enzymes and the inhibition of pro-inflammatory cytokines (Reiter et al., 2016). Our study demonstrated the impact of melatonin on Notch1 signaling pathway in neonatal HI. Interestingly, while Notch1 expression was increased after melatonin administration, NICD expression, in contrast, was maintained to control values. This seemingly paradoxical effect could be related to the multi-faceted nature of Notch1 signaling, which may exert different effects depending on the cellular context and the activation of other signaling cascades (Bolós et al., 2007).

Furthermore, the role of SIRT3 in maintaining mitochondrial function is crucial, as mitochondria are fundamental for the survival of cells, especially neurons, due to their high-energy demand. A decrease in SIRT3 expression, as seen in our study, could imply mitochondrial dysfunction, contributing to neuronal damage in HI brain injury. The fact that melatonin administration led to an increase in SIRT3 expression suggests that melatonin's neuroprotective effects may be partly through preserving mitochondrial integrity, a finding that we previously observed also in in vitro and ex vivo studies (Luchetti et al., 2022).

SIRT3 in brain injuries, especially in the context of HI, is a subject of recent studies (Yang et al., 2023). Recognized as one of the critical members of the Sirtuin family of NAD+-dependent protein deacetylases, SIRT3 is primarily localized within the mitochondria and holds a crucial role in metabolic regulation and stress responses (Lombard et al., 2007). SIRT3 is implicated in the genesis and progression of nervous disorders, inflammatory damage from microglia activation, and induced cell apoptosis and mitochondrial damage in NSCs (Yang et al., 2023). Several studies have highlighted the potential neuroprotective effects of SIRT3 in different types of brain injuries such as HI, TBI, stroke, and neurodegenerative diseases (Ahn et al., 2008; Yang et al., 2023; Yang et al., 2021). For instance, overexpression of SIRT3 in cerebral ischemia models has demonstrated promising outcomes, including reduced oxidative stress,

improved mitochondrial function, and decreased apoptosis, thereby underscoring its potential therapeutic relevance (Ahn et al., 2008; Yang et al., 2018). Moreover, an increasing number of studies are pointing to a neuroprotective effect of SIRT3 against different types of damage, including cerebral ischemia/reperfusion (I/R) injury, TBI, subarachnoid hemorrhage (SAH), and intracerebral hemorrhage (ICH). These might be connected to the maintenance of a state in the mitochondria homeostasis or other roles (He et al., 2020), like combatting oxidative stress and inflammation in the nervous system (Gao et al., 2020), and offering protection against neuronal excitotoxicity (Cheng et al., 2016; Yang et al., 2023). The progression of brain damage typically involves a dysfunction of the mitochondria, leading to a variety of pathophysiological events, including inflammation, oxidative stress, and mitophagy (Yang et al., 2023; Zhang et al., 2022). Given the role of SIRT3 in maintaining mitochondrial homeostasis and metabolism, as well as reducing mitochondrial damage, research into how SIRT3 operates could be key in gaining a better understanding of brain injuries (Yang et al., 2023).

The relationship between SIRT3 and melatonin was another important finding of our study. Our results showed that SIRT3 was significantly decreased in the hippocampal mitochondria 1 hour after HI, and that melatonin administration preserves its expression affected by neonatal brain ischemia. These findings align with previous studies that underscored the neuroprotective effects of melatonin against mitochondrial damage through the regulation of SIRT3 (Yang et al., 2018). Hence, it seems plausible that the preservation of SIRT3 by melatonin might support its neuroprotective role in HI brain injury. These findings were supported by our experimental findings, which suggested an important role for SIRT3 in HI brain injury. We observed a significant reduction in SIRT3 expression following cerebral ischemia, suggesting that SIRT3 is associated with deregulation and the pathogenesis of brain injury (Fan et al., 2021). Interestingly, our research also demonstrates that melatonin treatment could restore SIRT3 expression in the early phase of ischemic brain damage, suggesting melatonin's neuroprotective effect might operate via modulating the mitochondrial deacetylase. Such findings align with previous research that points to SIRT3 as a possible downstream target of melatonin's neuroprotective effects (Yang et al., 2018).

The existing scientific literature provides strong support for melatonin's neuroprotective effects in the context of acute HI perinatal brain injury, driven by its antioxidative, antiapoptotic, and anti-inflammatory properties (Hassell et al., 2015). The application of pharmacological concentrations of melatonin immediately after HI led to modulation of the Notch1 signaling

pathway and SIRT3 expression in our neonatal rat model of HI. This observation further confirms the growing consensus on melatonin's neuroprotective effect.

The neuroprotective effect of melatonin against various kinds of brain injuries has been confirmed by various studies. Melatonin's impact in decreasing endoplasmic reticulum stress following ischemia/reperfusion has been demonstrated in neurons exposed to oxygen-glucose deprivation. In addition, post-ischemic melatonin administration in models of transient focal cerebral artery occlusion and reperfusion led to a significant reduction in both infarction volumes and individual cortical lesion dimensions, as well as increased numbers of surviving neurons (Lin et al., 2018). Moreover, melatonin administration was shown to mitigate neuronal apoptosis induced by oxygen-glucose deprivation in vitro (Lin et al., 2018). In addition, post-ischemic melatonin administration exhibited a substantial positive impact on survival rates and neurological outcomes in a mouse model of transient middle cerebral ischemic/reperfusion injury (Chern et al., 2012). This was achieved by preserving the integrity of the BBB through mitigating stroke-induced free radical production. Furthermore, post-stroke melatonin treatment significantly augmented endogenous neurogenesis and cellular proliferation within the peri-infarct region, emphasizing its therapeutic potential in neuroprotective strategies (Chern et al., 2012).

Our study provides significant insights into the pathophysiological mechanisms of HI brain injury in neonates. Our findings suggest a dual neuroprotective role of melatonin, involving both modulation of the Notch1 signaling pathway and the preservation of mitochondrial function via SIRT3. It also points to potential therapeutic avenues. Considering melatonin's unique properties and safety profile, it emerges as a promising candidate for treatment in neonatal brain injuries. Melatonin demonstrates an exceptional tolerance, even when administered in high doses. Its safety record extends to special circumstances like pregnancy and immediate postnatal periods, showing no recorded side effects (Andersen et al., 2016; Reiter et al., 2016; Sanchez-Barcelo et al., 2017). Furthermore, its lipophilic characteristics facilitate easy traversal through biological barriers. Notably, it can cross biological barriers including the placenta as well as the BBB, an important feature for any potential brain injury treatment. Hence, clinical trials need to be performed to explore melatonin's therapeutic potential, aiming to determine its efficacy in treating neonatal HI brain injuries. These trials could potentially be designed either to administer melatonin to pregnant mothers during deliveries, where there is a high risk of HIE in the fetus, or in the immediate post-natal period in newborns with HIE.

Despite these significant findings, many questions remain unanswered. A deeper understanding of the mechanisms underlying the modulatory effects of melatonin on the Notch1 signaling pathway and SIRT3 could provide critical insights into the neuroprotective effects of melatonin. Also, the potential crosstalk between Notch1 and SIRT3 and the role of this interaction in HI brain injury could be a compelling area for future research. Moreover, exploration of other Notch receptors, their downstream targets, and their response to melatonin would provide a broader perspective on the role of Notch1 signaling in HI brain injury. Further, future research could focus on more targeted approaches, such as gene knockdown or overexpression experiments, which could provide more detailed insights into the role of Notch1 and SIRT3 in HI brain injury.

In conclusion, our study adds new dimensions to our understanding of the complex molecular pathways involved in HI brain injury and highlights the potential therapeutic applications of melatonin in neonates. Our study provides valuable insights into the potential roles of Notch1 and SIRT3 in the pathophysiology of neonatal HI and suggests that melatonin might exert its neuroprotective effects through the modulation of these proteins. By shedding light on the molecular responses to HI, our research contributes to a broader understanding of this complex pathological process and paves the way for further investigations into potential therapeutic interventions for neonatal HI brain injury.

Chapter 2: Traumatic Brain Injury: Identification of Prognostic Biomarkers

- **Project 1: A Canadian Biobank and Database for Traumatic Brain Injury (CanTBI)**
- **Project 2: Prognostic Serum Protein Biomarkers in Children with Severe Traumatic Brain Injury**

Project 1: A Canadian Biobank and Database for Traumatic Brain Injury (CanTBI)

1. Introduction

1.1 Traumatic Brain Injury: Definition and Overview

Traumatic brain injury (TBI) is a leading cause of death and acquired disability worldwide affecting individuals of all ages (Abu Hamdeh et al., 2021; Hutchison et al., 2018; Nguyen et al., 2016). Survivors often suffer from chronic physical, psychological, cognitive, behavioral, and emotional disabilities that pose a significant burden on both patients and their communities (Borgen et al., 2020; Rosenfeld et al., 2012).

TBI is defined as a traumatically induced structural injury or physiological disruption of brain function as a result of an external force (Nishimura et al., 2022; Returning Home from Iraq and Afghanistan, 2013). A clinical assessment of TBI could indicate: (i) periods of diminished or lost consciousness; (ii) amnesia for events immediately preceding or following the injury; (iii) focal neurological deficits, which might include muscle weakness, visual impairment, or altered speech; and (iv) changes in mental state like confusion, slowed cognition, and disorientation (Nishimura et al., 2022).

1.2 Global Incidence and Prevalence of TBI

The most comprehensive study to date on global TBI rates, conducted in 2016, recorded over 27 million new TBI cases that received medical treatment. This means 369 out of every 100,000 people worldwide had a medically treated TBI that year (James et al., 2019). According to this research, more than 55 million people, or 0.7% of the world's population, live with a brain injury. This data is provided by the Global Burden of Disease study and other research (Feigin et al., 2013; Theadom et al., 2012). Other focused studies have given even broader TBI estimates. For instance, the BIONIC study in New Zealand reported a rate of 790 TBIs per 100,000 people, indicating that over 50 million TBIs occur annually worldwide, with nearly a third of them not receiving immediate medical care (Feigin et al., 2013).

Another study by Dewan and colleagues in 2019 estimated the worldwide occurrence of TBIs across all severity levels, irrespective of medical intervention. Their estimation drew from the percentage of TBIs resulting from road traffic incidents (like car accidents). Their analysis, encompassing over 240 research papers, estimated an alarming rate of 939 TBIs per 100,000

people each year, including 55.9 million mild and 5.48 million severe cases (Dewan et al., 2019). This higher estimate suggests that many TBIs may go undetected.

Comparing TBI rates between countries or over time is challenging because of differences in reporting and data collection methods. Some reports indicate that Central and Eastern Europe and Central Asia have the highest TBI rates (James et al., 2019). Interestingly, even though wealthier countries reported higher per-person TBI rates, poorer countries had almost triple the total number of TBIs. According to the Global Burden of Disease Consortium, central and eastern Europe and Central Asia showed the highest TBI rates (James et al., 2019). This disparity highlights a potential 2 million lives that could be saved annually if trauma care from high-income nations was accessible in lower-income regions (Mock et al., 2012).

Globally, the incidence and prevalence of medically treated TBI have risen by 3.6% and 8.4%, respectively, from 1990 to 2016 (James et al., 2019). This increase is primarily attributed to a rise in fall-related TBI in the elderly population of developed countries and higher rates of road accidents and violence in developing countries. The Global Burden of Disease TBI consortium estimated that TBI accounts for 8.1 million disability per year, with a global disability rate of 111 per 100,000 individuals (James et al., 2019). These statistics underscore the growing global health challenge posed by TBI, necessitating heightened awareness, improved prevention strategies, and more accessible treatment options worldwide.

1.3 TBI Pathophysiology

The pathophysiology of TBI is multifaceted, with the underlying molecular and cellular mechanisms remaining largely unknown. This pathology involves a progression over time, divided into a primary and a secondary injury phase. The link between the primary and secondary phases is inconsistent and weak (Ghaith et al., 2022). The primary phase results directly from mechanical forces during the head impact, potentially disrupting the brain parenchyma and compromising the blood-brain barrier (BBB) integrity. This phase is followed by a systemic and neuroinflammatory response or the secondary phase, mediated by peripheral immune cells and the activation of immune-competent neural cells. The release of molecular mediators, such as cytokines, growth factors, and adhesion molecules, activates a complicated network of pathways that may continue to evolve for months after the primary injury. While some of these pathways aim for restoration, others exacerbate injury, leading to metabolic dysregulation, hypoxic-ischemic events, brain swelling, and edema (Dadas et al., 2018).

Moreover, activation of nitric oxide pathways and calcium influx into injured cells significantly induce apoptosis (Ghaith et al., 2022). These molecular and cellular events can trigger further neuroinflammation, oxidative stress, excitotoxicity, and edema leading to neurodegeneration, cerebral atrophy, and long-lasting changes that affect a patient's quality of life (Bramlett & Dietrich, 2015).

1.4 TBI Classification

Classification is central to understanding the progression and nature of TBI, aiding in diagnostic, prognostic, and therapeutic decisions (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022). TBI can be classified by using trauma mechanism, clinical severity, presence of structural damage on neuroimaging, and prognostic risk (Maas et al., 2008).

1.4.1 Glasgow Coma Scale (GCS)

For nearly half a century, the Glasgow Coma Scale (GCS) has been an instrumental tool in TBI assessment and classification for both clinical care and research. It is used to evaluate patients using a three-component scale, which includes assessments of eye-opening (E, 1-4), vocal response (V, 1-5), and motor response (M, 1-6), resulting in a structured numerical score (e.g., E4V4M6) (Teasdale & Jennett, 1974). The combined sum score (3-15) of E, V, and M is used to categorize patients into three severity groups: mild TBI (GCS 13-15), moderate TBI (GCS 9-12), and severe TBI (GCS 3-8) (Dewan et al., 2019). Although this was designed to simplify the classification of TBI, its use may have negatively impacted the clinical care and research in this field. Use of the GCS for classification of the severity of TBI may have led to less precise individual patient information, particularly when certain components were untestable or missing, like the verbal response for a patient that has had endotracheal intubation to protect their airway or control their ventilation. Considering TBI's heterogeneity and the diverse pathoanatomical variations, a single GCS score is likely insufficient for its characterization. It is hard to imagine that TBI could be simply classified as mild, moderate, or severe, using the GCS, when determining diagnosis, prognosis, and treatment likely requires more detailed information (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022).

The current method of classification can also lead to biases affecting patient care. Often, those with mild TBI are overlooked for subsequent care under the presumption that they will naturally recover fully. This is despite emerging evidence suggesting that some of these patients face long-term symptoms and impairments post-injury (Nelson et al., 2019). On the other hand, those classified with a severe TBI sometimes face a defeatist approach, which includes early withdrawal of life-sustaining treatments, even when there is data indicating that a portion of these individuals can make notable recoveries in their global functional neurological outcome (McCrea et al., 2021).

1.4.2 Imaging

Recent extensive studies over the past ten years advocate for the incorporation of imaging in conjunction with GCS, to develop a more accurate and insightful TBI classification system (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022). Computed Tomography (CT) scans continue to be the primary tool for TBI evaluation in emergency departments due to their ability to objectively detect immediate injuries and pinpoint specific anatomical features demanding varied clinical interventions. Indeed, CT scan results play a pivotal role in deciding hospital triage and surgical decision-making for TBI patients (Ratcliff et al., 2014). CT scans are recognized as well-known prognostic biomarkers, especially in patients with more severe injuries (GCS 3–12, moderate to severe TBI). Recent research has even highlighted their prognostic significance for patients classified as having mild TBI (GCS 13–15) (Yuh et al., 2021). This diagnostic and prognostic capability of CT scans partly explains their extensive use, even amidst concerns about radiation risks (Kirsch et al., 2011).

While CT scans are quick and commonly accessible, MRI usage is gradually on the rise due to its high sensitivity and reduced risks. MRI has proven effective in detecting TBI-related anomalies missed by CT scans, and there is growing data attesting to its prognostic value (Yue et al., 2019). However, MRI is not as widely available as CT, might necessitate patient movement within the hospital, and generally takes more time. Yet, when both imaging techniques are accessible, they collectively offer insights that can make the TBI classification process more accurate (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022). Although we acknowledge the potential utility of MRI in TBI this is beyond the scope of this thesis proposal.

1.4.3 Blood-derived Biomarkers

Growing evidence advocates for incorporating blood-derived biomarkers to improve the TBI classification system. Recent research indicates that such biomarkers can reliably predict intracranial hemorrhages on head CT scans, potentially diminishing the number of unnecessary CT scans in emergency care settings (Wang et al., 2007). Additionally, these biomarkers correlate with specific TBI-related pathologies (like contusions or diffuse axonal injuries) in patients with neurotrauma, marking a pivotal move towards a tailored approach for TBI treatment (Okonkwo et al., 2020). A growing body of research indicates that these biomarkers offer both diagnostic and predictive insights across the entirety of TBI severities, ranging from minor concussions to deep comas. Importantly, these blood biomarkers provide prognostic information that surpasses those from conventional clinical assessments (Frankel et al., 2019).

1.4.4 Comprehensive TBI Classification

While GCS scores, neuroimaging results, and blood-based biomarkers are all strongly correlated, they each offer distinct and supplementary insights, making the optimal classification system one that integrates all three indicators of brain injury severity. Blood-based biomarkers of glial and neuronal damage give a quantitative measure of brain cell injury, without being confined to the specific location or resulting functional impact (Okonkwo et al., 2020). On the other hand, neuroimaging not only provides insight into the severity of brain cell and vascular injury but also pinpoints the precise location and anatomical implications of the injury, such as a midline shift. GCS score, meanwhile, provides information about the level of coma immediately following the injury and is associated with the long-term functional consequences of the injury (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022).

A reduced GCS score tends to be associated with a greater chance of pathology on neuroimaging scans (Amyot et al., 2015). There is also evidence linking lower GCS scores with increased levels of certain blood markers indicating injury to brain cells of glial (such as glial fibrillary acidic protein [GFAP]) and neuronal (such as ubiquitin carboxy-terminal hydrolase L1 [UCH-L1]) injury (Okonkwo et al., 2013). This connection is present even in milder TBI cases with a GCS score of 15 (M. McCrea et al., 2020). Nonetheless, there are factors, such as intoxication, sedation, endotracheal intubation, dementia, or language barriers, that can lead to challenges in obtaining accurate GCS scores. These factors help provide a strong rationale for not relying

solely on the GCS for TBI classification (Zuercher et al., 2009). These confounders can lead to excessive use of CT scans for TBI diagnosis, even when many CT results come back normal and it is not as accurate as MRI (Yue et al., 2013, 2019).

Therefore, a comprehensive classification system that amalgamates clinical assessments like the GCS, neuroimaging, and blood biomarkers would facilitate a multifaceted classification and characterization of the injury. Further enriching our understanding of TBI with data from sources like blood biomarkers can also refine clinical decision-making processes regarding the use of CT and MRI scans. A more comprehensive classification system would also help reorganize this complex condition into more specific subcategories, paving the way for tailored therapeutic interventions (Committee on Accelerating Progress in Traumatic Brain Injury Research and Care et al., 2022).

1.5 Categories of TBI

TBI is categorized into three types, based on the specific physical trauma mechanisms involved: (1) Closed (non-penetrating); (2) Open (penetrating); and (3) Explosive blast injury (Andriessen et al., 2010).

- Closed (also known as non-penetrating): The primary cause of closed TBI is blunt force trauma, most frequently seen in falls, motor vehicle collisions, and sports-related injuries. This type of TBI has the highest occurrence in the civilian population. The forceful blunt impact affects the brain, particularly at the impact site, leading to instant damage to the brain vasculature and neural cells. Additionally, biophysical rotational and reverberative forces leading to impact and re-impact between the brain and skull can compress the brain parenchyma, the blood vessels, and the microcirculation and diminish cerebral blood flow. These forces can result in either focal localized contusions or injury to other brain areas or diffuse shear injuries. Laceration of brain tissues mainly causes focal damage, cerebral edema, intracranial hemorrhage, and ischemia (Ng & Lee, 2019).
- Open (also known as penetrating): Open TBI arises when an object pierces the skull (e.g., a bullet, shrapnel, bone fragment, or by a weapon such as a hammer or knife) and enters the brain tissue. The impact and subsequent injury can be magnified if the penetrating object moves swiftly, causing cavitation in the brain tissue. The extent and nature of the neurological damage can vary based on the characteristics of the penetrating object, such as its size, velocity, and trajectory. Given the exposure of the brain to such injuries, the risk of infection

is elevated. Furthermore, this injury type is linked to more immediate medical complications compared to closed TBI. Like closed TBI, laceration of brain tissues mainly causes focal damage, cerebral edema, intracranial hemorrhage, and ischemia (Black et al., 2002).

- Explosive blast injury: Explosive blast TBI became more prominent due to the high number of war-related injuries in the 20th century (Warden, 2006). This injury type differs from the previous two as it is caused by the rapid pressure changes from an explosion. These pressure waves can transmit a significant amount of energy from the skull into the brain parenchyma or even from forces transmitted through the body to the brain (Ling & Ecklund, 2011). Blast injuries can have varying damage patterns, such as primary (internal damage from the shock wave), secondary (penetrating), tertiary (physical injuries from the blast wave), and quaternary (other associated injuries) (Risdall & Menon, 2011). The energy from the explosion can distort the brain's structure, causing extensive damage across brain tissue, leading to a range of outcomes including compromised BBB, neuronal death, axonal injury, vasospasm, pseudoaneurysm formation, hyperemia, contusion and cerebral edema and widespread diffuse injury in both the gray and the white matter (Cernak & Noble-Haeusslein, 2010). Notably, many individuals with blast-related TBI also experience post-traumatic stress disorder, a condition found to be prevalent among survivors (Risdall & Menon, 2011).

In our studies, when we refer to TBI, we are focusing on closed TBI. This specific focus allows us to delve deeper into the characteristics, mechanisms, and outcomes associated with this type of injury.

1.6 TBI Outcome Assessments

TBI outcome assessments are essential tools used in clinical and research settings to evaluate the consequences and quality of life for individuals affected by TBI. Patients enrolled in the study participate in a battery of questionnaires and performance-based cognitive and behavioral assessments at three or four time points, depending on the severity of the injury. The battery of questionnaires and assessment tools we used at each time point in our studies focus on four key outcome domains: global neurological functional outcome and death; TBI-related symptoms; mood and behavior problems and quality of life. We collected these outcomes over the telephone. For quality of life outcomes, in certain age groups, the assessment should involve both the patient and their parents in the case of

children or proxy in the case of severely disabled adults who could not be interviewed. It is crucial that, if attainable, the same individual carries out the assessment for each time point. For instance, if the patient's father initiated the first evaluation, he and the patient should ideally engage in subsequent evaluations.

Our choice of outcome measures adhered to these criteria: (i) endorsed by the inter-agency TBI Common Data Elements workgroup (McCauley et al., 2012), (ii) suitable for different ages (including age-specific versions when necessary), (iii) accessible in both English and French, (iv) designed for distant evaluations from a central site, and (v) aiming to reduce the strain on participants.

To better understand and evaluate TBI-related outcomes, several assessment tools have been developed. In this summary, we highlight key TBI outcome assessments:

- Glasgow Outcome Scale-Extended (GOS-E): This scale was extended from the Glasgow Outcome Scale (GOS) which is a global scale for neurological functional outcome and death that rates patient status into one of five categories: dead, vegetative state, severe disability, moderate disability or good recovery. The GOSE provides more detailed categorization by subdividing the categories of severe disability, moderate disability, and good recovery into a lower and upper category. It assigns a singular score across eight levels, ranging from death to full recovery without any impairments. It is assessed using a structured interview designed to classify the global functional outcomes for those recovering from TBI (Wilson et al., 1998). Among TBI research tools, the GOS-E stands out as the most prevalent (Lu et al., 2012). Versions designed specifically for children aged 1-17 and for infants less than 1 year of age were introduced and authenticated (Beers et al., 2012). To holistically represent the potential consequences of biopsychosocial aspects, it is advisable to pair the GOS-E with tools that evaluate TBI-induced symptoms, cognitive functions, and overall life quality (Bagiella et al., 2010).

- Health and Behavior Inventory (HBI): HBI is utilized to evaluate post-concussive symptoms in children (McCauley et al., 2012). This 20-item scale is based on the Likert system, where symptoms are rated from 1 (Never) to 3 (Often). Both child and parent versions exist, containing the same content but with different phrasing to accommodate either a first versus third-person viewpoint. Recognized as a foundational tool in the Common Data Elements for Pediatric TBI (Hicks et al., 2013), the HBI is also featured in the child sport concussion assessment tool. The inventory produces distinct results for

cognitive and somatic symptom categories, as well as total scores, all determined through factor analysis.

- Rivermead Postconcussion Symptom Questionnaire (RPQ): The RPQ was created to measure frequently observed symptoms post mild-to-moderate TBI in adults (King et al., 1995). It allows the participants to compare the frequency of 16 specific symptoms to their state before TBI. A score of 0 suggests the absence of a symptom, 1 means the symptom is present but not intensified post-TBI, while ratings from 2 to 4 describe mild, moderate, or significant symptom severity, respectively. Even though many factor analytic evaluations advocate for a three-fold structure, incorporating somatic, emotional, and cognitive facets (Potter et al., 2006) the total score offers an overview of the overall symptom impact.

- Pediatric Quality of Life Inventory (PedsQL): The PedsQL (Wilson et al., 1998) is employed to delve into the broader dimensions of life quality. It evaluates life quality in four domains: physical, emotional, social, and school or work function aspects. It provides a total score, as well as physical health and psychosocial health summary scores. Different versions of this tool include diverse age groups from children to adults. It was first designed as a "Pediatric" tool and later versions were developed for young and old adults.

- Quality of Life after Brain Injury-Overall Scale (QOLIBRI-OS): The QOLIBRI-OS Originally conceptualized as a detailed 36-item tool for measuring the health-related quality of life in TBI-affected adults (Von Steinbuechel et al., 2016). This 6-item version has demonstrated significant correlations with its detailed counterpart and other TBI metrics, affirming its validity (A brief 6-item short form, the QOLIBRI-OS, has been shown to correlate strongly with the full QOLIBRI and other TBI outcome measures, supporting its construct validity). Participants rate their satisfaction in various domains, such as physical and emotional health, daily activities, and social connections, on a scale ranging from 1 (Not at all) to 5 (Very). These scores are then mathematically transformed into a percentage, where 100 denotes the best life quality.

1.7 TBI Knowledge Gaps

Despite significant progress in basic neuroscience, no new therapies and few new diagnostic or prognostic tests have been introduced into clinical care. The complexity and heterogeneities (injury type, severity and mechanism, and patient characteristics) of TBI

present challenges for clinicians and researchers to accurately diagnose, prognosticate, and predict long-term outcomes with existing tools, and tailor care, rehabilitation interventions, and support services accordingly (Dadas et al., 2018; Ghaith et al., 2022; Saatman et al., 2008). The current TBI classification system based on GCS fails to capture the disease's complexity and heterogeneity (Dadas et al., 2018), minimizes the real burden of mild TBI, and makes it difficult to distinguish between minor head injuries and mild TBI/concussion (Posti & Tenovuo, 2022). Current treatment monitoring is largely based on gross physiology and clinical symptoms, and treatment focuses on addressing the consequences and symptoms of TBI rather than predicting and preventing it or controlling ongoing pathophysiology (Posti & Tenovuo, 2022). A comprehensive understanding of the specific subcellular mechanisms is necessary to bridge the knowledge gaps and develop effective treatment options (Park et al., 2008).

1.8 Future Perspective

The discovery and validation of molecular biomarkers in humans will help to understand the pathophysiological mechanisms of TBI, revealing molecular pathways involved in inflammation, defense response, cellular edema, cell death, and neuronal recovery (Dadas et al., 2018; Mendoza et al., 2020) leading to the discovery of novel therapies. Biomarkers also have potential utility for multiple contexts of clinical use for TBI patients, including classification (Gravesteijn et al., 2020; Tosetti et al., 2013), risk stratification (Nishimura et al., 2022b; Posti & Tenovuo, 2022), diagnosis (Nishimura et al., 2022b; Posti & Tenovuo, 2022) assessment of susceptibility to secondary brain injury (Dadas et al., 2018), monitoring (Posti & Tenovuo, 2022), prognosis and prediction (Mendoza et al., 2020; Posti & Tenovuo, 2022), pharmacodynamic (Nishimura et al., 2022) and neuroprotective response to therapies (Dadas et al., 2018), and patient safety (Nishimura et al., 2022), addressing TBI heterogeneity and paving the way for a precision medicine approach (Huie et al., 2021; Tosetti et al., 2013). These insights may be used to help surrogate decision-makers and clinicians make decisions regarding intensive therapy versus end-of-life care, and to help plan follow-up and rehabilitation for survivors (Dadas et al., 2018).

The process of biomarker translational research necessitates the continual discovery of biomarkers and the development of standardized protocols for sample collection, processing, and analysis. These biomarkers must also be under analytical validation to

ensure quality control of test assay performance before clinical application, and a clear association between biomarkers and clinical endpoints must be validated (Tan et al., 2014). Future applications of biomarkers may incorporate multi-analyte assays, which are likely to better capture the heterogeneity of TBI disease processes. A robust research infrastructure, such as the establishment of biobanks and databases complying with best-practice guidelines, could be instrumental in fostering international partnerships to further advance this field of TBI research (Ketharanathan et al., 2019).

1.9 Objectives

To address these gaps, Canadian scientists developed a platform to be used as a resource for biomarker research. We conducted a prospective multi-center study, the Canadian biobank and database for patients with TBI (CanTBI), in which we banked fluid samples and collected data on children and adults with mild, moderate, and severe TBI on behalf of the Canadian TBI Research Consortium (Hutchison et al., 2018). In this chapter of the thesis, I aimed to 1. report the methods and standard operating procedures for this CanTBI platform. 2. report the baseline patient data and compliance to follow-up, and 3. report the compliance to study outcome follow-up for patients enrolled in the study.

2. Materials and Methods

2.1 Study Design, Setting and Participants

All participating sites received local research ethics board (REB) approval, including approval of deferred consent for early blood sampling and informed consent for the collection of demographics, personal health information, and clinical and follow-up data. Participants, or their surrogate decision-makers, were initially given a study information sheet and later approached for informed consent.

We prospectively screened potentially eligible patients in hospital emergency departments, hospital wards, and intensive care units from Monday to Friday between 7:00 and 17:00 hours and enrolled eligible children and adults with TBI. This study was conducted at four children's hospitals (British Columbia Children's Hospital, Vancouver, British Colombia, Alberta Children's Hospital, Calgary, Alberta, The Hospital for Sick Children, Toronto, Ontario and Sainte-Justine Hospital, Montreal, Quebec) and three adult hospitals (Vancouver General Hospital, Vancouver, British Columbia, Foothills Hospital, Calgary, Alberta and Halifax Infirmary Hospital, Halifax, Nova Scotia. We included patients with 1. TBI of any severity who were admitted to pediatric and adult emergency departments, hospital wards, or critical care units. We used the Centre for Disease Control's definition of TBI; a disruption in the normal function of the brain that can be caused by a bump, blow, or jolt to the head, or penetrating head injury (Capizzi et al., 2020); 2. ability to provide a minimum of one blood sample within 30 hours following injury; 3. ability of patient or the surrogate decision maker to speak and read in English and/or French; and 4. a fixed address. We excluded patients with 1. pre-existing severe neurodevelopmental disorders, ongoing neurologic deficits from previous TBI or other acquired injury (e.g. stroke); 2. cardiac arrest leading to hypoxic-ischemic brain injury in combination with TBI; 3. confirmed or suspected brain death; 4. terminal illness where the patient was expected to live less than 12 months following TBI; 5. unwillingness to participate in follow-up assessments; 6. injury occurring before 38 weeks corrected age in premature infants; 7. weight less than 3 kg (6.6 lbs); and 8. neonates with a diagnosis of birth trauma as the cause of the TBI. More detailed information to assess eligibility was included in the study procedures manual used by the research coordinators at each study site. We classified the severity of the TBI based on the maximum GCS recorded on the initial assessment at the study site. Our rationale for this early classification was that the follow-up and outcome measurement protocol was different for those with mild compared to moderate to severe TBI and the first

outcome time point for mild TBI was at 7 to 10 days post-injury (see the 'Outcome data collection' section below).

2.2 Biobanking Protocol

Study research coordinators enrolled eligible patients using deferred consent. Trained research and laboratory personnel collected and processed biological samples, including serum, plasma, and buffy coat for DNA extraction and, when possible, in cases of severe TBI, cerebrospinal fluid (CSF) according to the CanTBI biobanking standard operating procedure. The first blood sample was collected as early as possible following TBI. Up to three blood samples could be collected before informed consent. In cases where consent was refused, these samples were destroyed. Following informed consent, a total of up to six blood samples could be collected at specific time intervals: two samples on day 1 and one sample on each of days 4, 7, 14, and 28 post-injury. If possible, these research samples were collected simultaneously with clinical blood samples to minimize the burden on patients.

The volumes of blood collected at each time were based on weight, banking larger volumes for older children and adults. Blood was collected into two tubes: 1. for separation of serum and buffy coat (no anti-coagulant), and 2. for plasma [Ethylenediaminetetraacetic acid (EDTA) anti-coagulant]. Within two hours of blood sampling, tubes of serum, plasma, buffy coat, and CSF were frozen and stored at –80 ° Celsius. CSF was collected from patients with severe TBI if an external ventricular drain (EVD) was placed by the neurosurgeons as part of clinical care. In these cases, CSF was collected and banked daily until the removal of the EVD. To help monitor the processing and storage of biological samples, three dates and times were recorded: 1. sample collection; 2. arrival in the laboratory for sample processing; and 3. sample placement in the freezer. These data were used for quality improvement during the conduct of the study with the goal of consistent rapid processing and storage of biological samples across the study sites.

Batches of biological samples were later shipped on dry ice to four regional biobanks in Vancouver, Calgary, Toronto, and Montreal. At these biobanks, the samples were thawed and divided into aliquots and then labeled with bar codes, cataloged, frozen, and stored in boxes at –80 ° Celsius. A database of biological sample aliquot numbers and locations was created and entered in the central CanTBI study database for tracking the use and availability of biological samples for studies of biomarkers of TBI.

2.3 Demographic and Clinical Data Collection

We selected core National Institute of Health common data elements for TBI (Yue et al., 2013) and combined them in an electronic case report form (CRF) linked to a detailed procedures manual. We collected demographic, pre-hospital, and clinical data including age at the time of the TBI, biological sex, mechanism of injury, pre-hospital events and interventions, highest GCS score on admission to the hospital, clinical monitoring data, medications, and medical and surgical treatments and procedures for TBI from the patient's medical record. We also calculated the Abbreviated Injury Scale (AIS) score (Loftis et al., 2018) and Injury Severity Score (ISS) (Brown et al., 2017) in adults and the Pediatric Trauma Score in children (Asuquo et al., 2022).

2.4 Laboratory and Neuroimaging

Data on clinical laboratory tests of organ function were collected and entered into the study database. Trained physicians collected brain injury characteristics from the radiologist's report from the first CT scan of the head done at the study site and entered these into the CanTBI database. Dates and times of MRI and neurophysiologic tests, done as part of routine clinical care, were collected for future use if further funding permitted, but analyses of these data were beyond the scope of the current thesis.

2.5 Outcome Data Collection

Trained research coordinators interviewed patients or their surrogate decision-makers to collect questionnaires and performance-based global functional neurological outcome and quality of life across all age groups and severities of TBI and post-concussion symptoms in mild TBI only. These assessments were conducted either by phone or during face-to-face interviews if the research coordinators were able to schedule an interview at the same time as a clinical follow-up at the study site.

The importance of follow-up was explained in the study consent forms and during the informed consent process. To help ensure optimal compliance to follow-up the research coordinators established a relationship with the patient and/or surrogate decision-makers as soon as possible after the injury. We collected addresses, phone numbers, and email addresses and the preferred method of communication to schedule follow-up assessments for each patient or their surrogate

decision maker. The research coordinators attempted to contact as many times as was necessary, and within reason, to schedule these follow-up appointments. In situations where the research coordinators found it difficult to schedule follow-up appointments, they were trained to prioritize assessment of the GOS-E at 6 months following the injury, which was the primary outcome measure for the study.

Compliance to follow-up for primary and secondary outcomes was evaluated at four time points: 7 to 10 days and at 3-, 6- and 12-months post-injury. Only patients with mild TBI were approached for the outcome measurements at the first 7 to 10 days post-injury time point. The primary outcome for the study, the GOS-E, infant, pediatrics, and adult versions, was used to assess global neurological function and death (Jennett, 1975; Lu et al., 2012; McCrea et al., 2021; Wilson et al., 1998) at 6 months following injury. This was the only outcome measure collected at 6 months following injury. The secondary outcomes were the 1. GOS-E collected at the 7-10 days (mild TBI only) and at the 3- and 12-months' time points following injury, 2. Pediatric Quality of Life scale (PedsQL, infant, child, adolescent, and adult versions) (McCauley et al., 2012; Varni et al., 2001) at 7 to 10 days (mild TBI only) and at 3- and 12-months following injury, 3. Quality of Life after Brain Injury Outcome Scale (QOLIBRI-OS, adults only) (Von Steinbuechel et al., 2012, 2016) at 7 to 10 days, in mild TBI only, and at 3- and 12-months following injury in all patients. 4. Health and Behaviour Inventory (HBI) in children and adolescents with mild TBI only (Ayr et al., 2009; McCauley et al., 2012) at 7 to 10 days and at 3- and 12-months following injury and 5. Rivermead Postconcussion Symptom Questionnaire (RPSQ) in adults with mild TBI only (King et al., 1995; Potter et al., 2006) at 7 to 10 days and at 3- and 12-months following injury. The outcome assessors were also trained to assess the risk of suicidality and psychiatric illness sequelae (e.g. depression), and to make an emergent referral or appropriate clinical follow-up referral in high-risk patients, using a battery of validated risk assessment tools. These data were kept confidential and will not be reported in this thesis.

2.6 Personal Health Information (PHI)

A component of the CanTBI platform was designed to collect PHI for future healthcare utilization research. Patients enrolled in our study consented to the storage of PHI which was needed to link our study database to federal or provincial health care utilization databases. This collection of PHI was approved by all study site REBs and was explicitly outlined in the

informed consent forms with an opt out option. The PHI was stored securely at each study site and linked to the unique study identification number for each subject. This PHI was not entered into the central CanTBI study database.

2.7 National Database

We developed an Oracle database at The Hospital for Sick Children (Toronto, ON). We positioned this web portal database behind the hospital's firewall and protected it with a password. Study personnel had role-based access to enter, view, and edit data from their site. The coordinating site had access to the full study cohort. Enrolling sites were responsible for the accuracy of study data entered into the database. The coordinating site conducted frequent data audits to ensure rigorous data quality and integrity.

2.8 Data Storage

We stored physical study documents in a secure location at each enrolling site, ensuring access only for study personnel. We stored documents containing study-specific data separate from the consent form and source documents.

2.9 Data Sharing and Access to Biosamples

We will share data and biosamples from this platform with scientists from research networks, including the International Initiative for TBI Research (InTBIR), the Canadian Critical Care Translational Biology Group (CCCTBG), and the Canadian Traumatic Brain Injury Research Consortium (CTRC) and potentially with industry. The plans to potentially share biosamples and data with other scientists and for-profit companies were explicitly outlined in the study consent forms. We created a standard operating procedure for CanTBI sub studies which outlined how to develop a study proposal and budget. These sub-study proposals require approval by the CanTBI study Steering Committee before initiation.

2.10 Data Analysis

We performed exploratory data analysis using box plots, histograms, and dot plots. We used standard parametric and non-parametric methods to summarize data, as appropriate. The primary focus was to understand the distribution and patterns of the data visually and

descriptively across the study times. We performed analyses using IBM SPSS version 24, SAS, and R statistical software.

3. Results

3.1 Patient Recruitment

The flow diagram of patient screening and recruitment is shown in Figure 1. 8,182 patients with suspected TBI were screened across the seven study sites. Of these, 7,595 patients were excluded as eligibility criteria were not met. Some patients often had more than one exclusion criteria, so we only listed the first exclusion criteria, to add up to 7,595. Of the remaining 587 patients meeting eligibility criteria, 132 were not enrolled because the patient or surrogate decision-maker declined consent, an opportunity to obtain informed consent was missed, or consent was obtained but later withdrawn. Screening and recruitment occurred between September 2016 and January 2022. The long-term outcomes of the final patient recruited to the study were collected in January 2023. Although most recruiting sites concluded their recruitment by June 2019, when grant funding ended, one site continued recruitment, until January 2022, enabling us to reach the final number of 455 participants (Figure 2).

3.2 Demographics and Injury Characteristics

Table 1 shows the demographic and injury characteristics of TBI patients: pediatric mild (n=114, 25.1%) and moderate to severe TBI (n=47, 10.3%) and adult mild (n=202, 44.4%) and moderate to severe TBI (n=92, 20.2%). The severity of TBI was divided into 2 groups according to the highest GCS on admission: Mild TBI, GCS of 13 to 15, and moderate to severe TBI, GCS of 3 to 12. The median age for children with mild TBI was 10.2 years [interquartile range (IQR) 5.3-13.8] and for those with moderate/severe TBI, was 9.0 years (IQR 3.5-13.8). For adults with mild TBI, the median age was 44 years (IQR 30.0-61.0) and for those with moderate to severe TBI, it was 48.2 years (IQR 29.0-65.3). Most of the patients were male with a male: female ratio of 1.8:1. The median age for all 455 patients was 29.0 years (IQR 13.3-52.0), and most were mild TBI (n=322, 71%). Most injuries were sustained via falls (37.8%) or motor vehicle collisions (32.7%). Those with moderate to severe TBI were more likely to have exhibited abnormal clinical CT scans compared to those with mild TBI, with subarachnoid hemorrhage (28.1%) and subdural hemorrhage (24.4%) being the most common pathology diagnosed among all patients.

In Table 2, we show the incidence of physiologic conditions and diagnoses known to be associated with secondary brain injury. These included hypotension, hypoxia, cardiac arrest, intracranial hypertension, and fixed dilated pupils, which often indicate trans tentorial

cerebral herniation. We observed that the most common conditions associated with secondary brain injuries were intracranial hypertension (23.1%) and hypotension (14.5%) in our patient population.

3.3 Compliance to Follow-up for Outcome Measures

Compliance for each outcome measured across four time points is shown in Table 4. The primary outcome, the GOS-E, was the only outcome assessed at 6 months following injury. The GOS-E was also assessed at the three other time points: 7-10 days (mild TBI only) and at 3- and 12-months post-injury. We were able to collect GOS-E for 71-78% of patients at each of these time points. The other secondary outcome assessments were conducted three times: 1 week and at 3- and 12-months following injury. Compliance with follow-up for secondary outcomes varied across outcome measures and the 3 time points where they were collected. For PedsQL, which was evaluated across all age groups and injury severities, compliance rates ranged from 36% to 55%. For QOLIBRI-OS, solely assessed in adults across all severities of TBI, compliance rates ranged between 36% and 55%. RPSQ was exclusively evaluated in adults with mild TBI, and compliance rates ranged from 67% to 73%, and for HBI, assessed only in pediatric patients with mild TBI, the compliance rates ranged from 50% to 60%.

4. Discussion

We effectively addressed our primary objectives to develop a biobank and database as a resource for scientists to test the most relevant hypotheses for biomarker research in patients with TBI. We prospectively enrolled 455 patients from four pediatric and three adult hospitals in Canada, banked biological samples, and collected and stored demographic and clinical data and outcome measures into a central database over 6 ½ years. The biobank and database are serving as an essential resource for Canadian and international scientists conducting biomarker research in TBI. Most of our hospitalized patients (71%) were classified as mild TBI. The most common mechanisms of injury were falls and motor vehicle collisions. A significant proportion of patients with moderate to severe TBI displayed abnormalities on clinical brain CT scans, most commonly subarachnoid and subdural hemorrhages. Furthermore, physiologic abnormalities associated with secondary brain injuries, especially intracranial hypertension, emerged as a significant concern among our cohort. The compliance to follow-up and collection of the outcome measures varied across our four follow-up time points but we achieved a reasonably high collection of the primary outcome, the GOS-E of 76% at 6 months following injury and 78% of patients at 12 months post-TBI. For secondary outcomes, compliance rates ranged from 36% to 73 % across different measures and time points.

Addressing the pressing gaps in TBI research, especially about the identification and validation of biomarkers, requires an integrative, standardized, and collaborative approach. The CanTBI platform stands as a testament to this need, aiming to facilitate advanced biomarker research. The platform's design and deployment resonate with global calls to centralize and standardize TBI data for research, and we aligned our study with other initiatives including the Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) in the United States (Yue et al., 2013) and the Collaborative European Neuro Trauma Effectiveness Research in TBI (CENTRE-TBI) study in Europe (Volovici et al., 2022). At the beginning of our 3 studies, as part of the InTBIR, we aligned and collected demographic, clinical, and outcomes National Institutes of Health TBI common data elements (Meeuws et al., 2020). For biobanking we also aligned our biobanking standard operating procedures including the types of biological samples collected [serum, plasma, buffy coat (for DNA extraction), and CSF], the days post-TBI that we collected biological samples, and high-quality standards for processing and banking of biological samples with these 2 studies in the United States and Europe.

When comparing our current study to previous studies we also found a male predominance for TBI likely due to risk-taking behavior, emphasizing a gender disparity in TBI occurrence (Dadas et al., 2018; Nguyen et al., 2016; Vasudevan et al., 2022). In previous studies by our group and others, where patients were recruited from hospital emergency departments, hospital wards, and intensive care units, the proportion of patients with the different TBI severities was similar, with about 70–80% of patients classified as mild based on GCS (Tagliaferri et al., 2006; Voormolen et al., 2019; Yuh et al., 2021). Similar proportions of moderate (5-10%) and severe TBI (15-20%) were also reported (Tagliaferri et al., 2006). Notably, our observation of a varied age at injury for those with mild TBI compared to moderate to severe TBI resonates with other studies and may have been influenced by where patients were recruited and/or by age-specific susceptibilities to the different severities of TBI (Narayan et al., 1981; Vasudevan et al., 2022; Wiles, 2022). The injury mechanisms, with falls and motor vehicle collisions predominating, also align with global trends as reported in various studies (Gravesteijn et al., 2020; Langlois & Sattin, 2005; Roozenbeek et al., 2013; Tagliaferri et al., 2006). The most common causes of injury were falls and road traffic incidents in the 4,509 patients in the CENTER-TBI study (Dadas et al., 2018) and 1,935 TRACK-TBI study (Dadas et al., 2018; Yuh et al., 2021).

Our study has several limitations which we will summarize here. This study includes only patients admitted to seven tertiary or quaternary referral hospitals in Canada, which raises questions about the generalizability of our findings to other populations or regions. We must consider potential bias from our samples, which were limited to patients who could provide a blood sample within 24-30 hours following injury, speak and read English or French, and had a fixed address. Additionally, the study's reliance on data collected from medical records and self-reports may have introduced some degrees of bias and error. Despite being a multi-center study, our sample size remains relatively modest, especially when dissecting specific subgroups of TBI. Additionally, our challenges with follow-up still pose questions regarding the overall generalizability of our results. Loss of patients to follow-up may introduce bias into biomarker research studies, especially for prognostication (Helmrich et al., 2021). The challenges faced, especially in terms of follow-up, underline the need for enhanced patient engagement strategies. Despite our rigorous methodology, the challenges we encountered in follow-up compliance, especially for the secondary outcomes, are notable. Our encounter with challenges in follow-up compliance reflects a recurrent issue in previous longitudinal TBI studies (Yue et al., 2013). The reasons for the loss of follow-up data for the secondary

outcomes are beyond the scope of the current thesis but need to be examined in more detail to help ensure improved compliance rates for follow-up in future studies. Our follow-up rates were however comparable to both the TRACK-TBI and CENTRE-TBI studies. It is also important to note that our mild TBI patient population was skewed towards a more severe group of patients with mild TBI compared to patients with concussion, who have a less severe mild TBI and are predominantly caused by sport-related injury and/or are described in outpatient settings.

Despite these challenges, the CanTBI platform offers a beacon for future TBI research. In our prospective study, we used a strong integrative approach, bringing together clinical assessments, high-quality biobanking procedures, and patient-centric metrics to help ensure our future research goals of a personalized medicine approach for hospitalized patients with TBI. Our platform has great potential to help scientists identify and validate biomarkers and revolutionize TBI diagnostics, prognostication, and monitoring. Earlier studies, like those from TRACK-TBI, have hinted at the potential of composite biomarker scores in predicting long-term recovery post-TBI (Huie et al., 2021). Most studies have shown that many biomarkers can distinguish mild TBI from moderate to severe TBI, including the CENTER-TBI study, which is the largest biomarker study cohort to date (Posti & Tenovuo, 2022). In recent research on childhood TBI, there has been a focus on using biomarkers as predictors of outcome (Wilkinson et al., 2017). Several promising biomarker candidates have been found, but the translation of TBI biomarkers into clinical applications has been minimal. The only blood-based biomarker application that is officially approved for clinical practice is to assess the need for head CT imaging (Posti & Tenovuo, 2022). We have recently used mass spectrometry and other technologies to analyze biosamples from the CanTBI platform and discovered blood metabolite biomarkers with potential prognostic utility in adults with severe TBI (Banoei et al., 2023).

Scientists from the biomarker working group of the InTBIR consortium are combining biobanks and databases from CanTBI, TRACK-TBI, and CENTRE TBI to provide a pooled resource for analytical validation studies for TBI biomarkers (Wang et al., 2021). Another potential aim of this future research project is to complete proof of principal studies with adequate power to validate blood biomarkers for several contexts of clinical use (e.g. prognosis) using samples and data from these three studies. The results of these studies will be used to help design and implement prospective clinical validation studies for bedside use of blood biomarkers in patients with TBI.

5. Figures and Tables

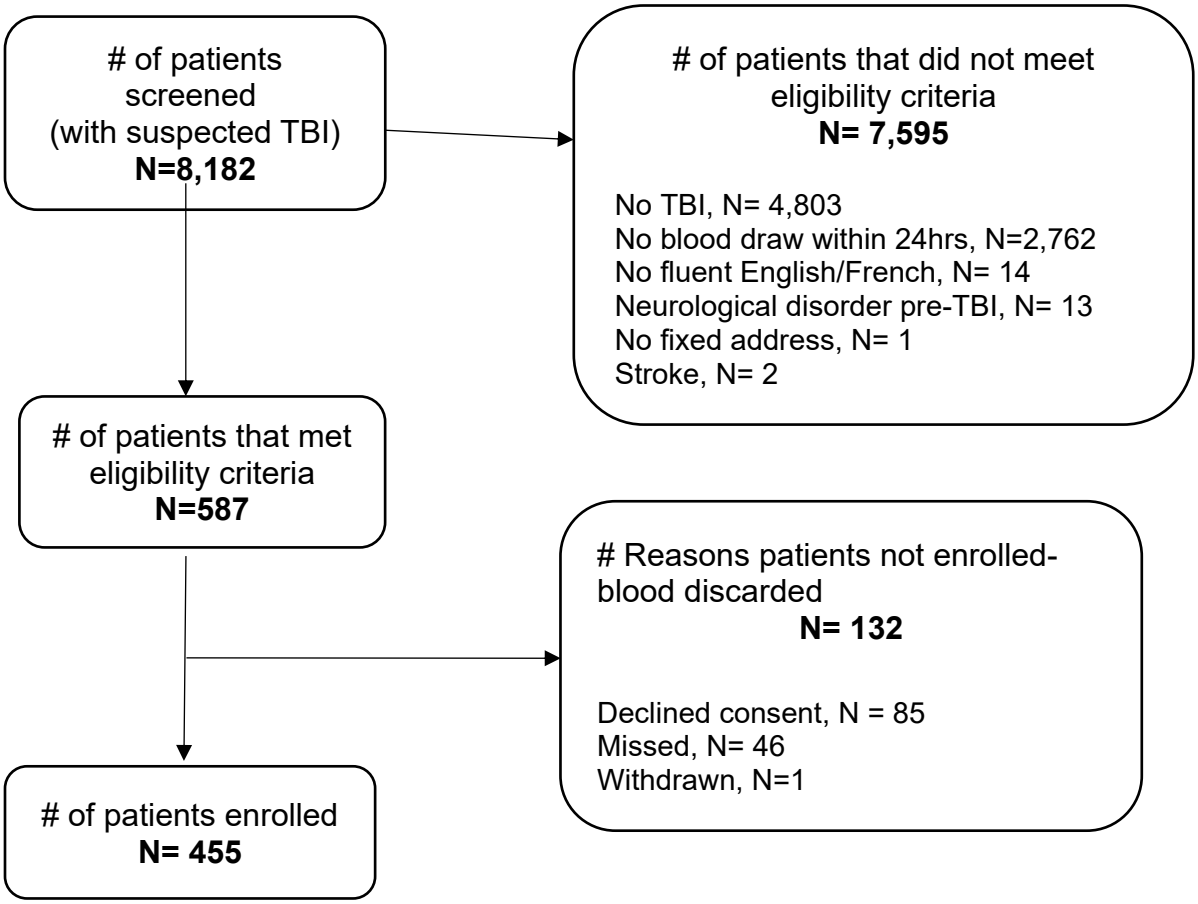

Figure 1. Flow diagram of screening and recruitment of patients with suspected traumatic brain injury.

TBI, Traumatic brain injury.

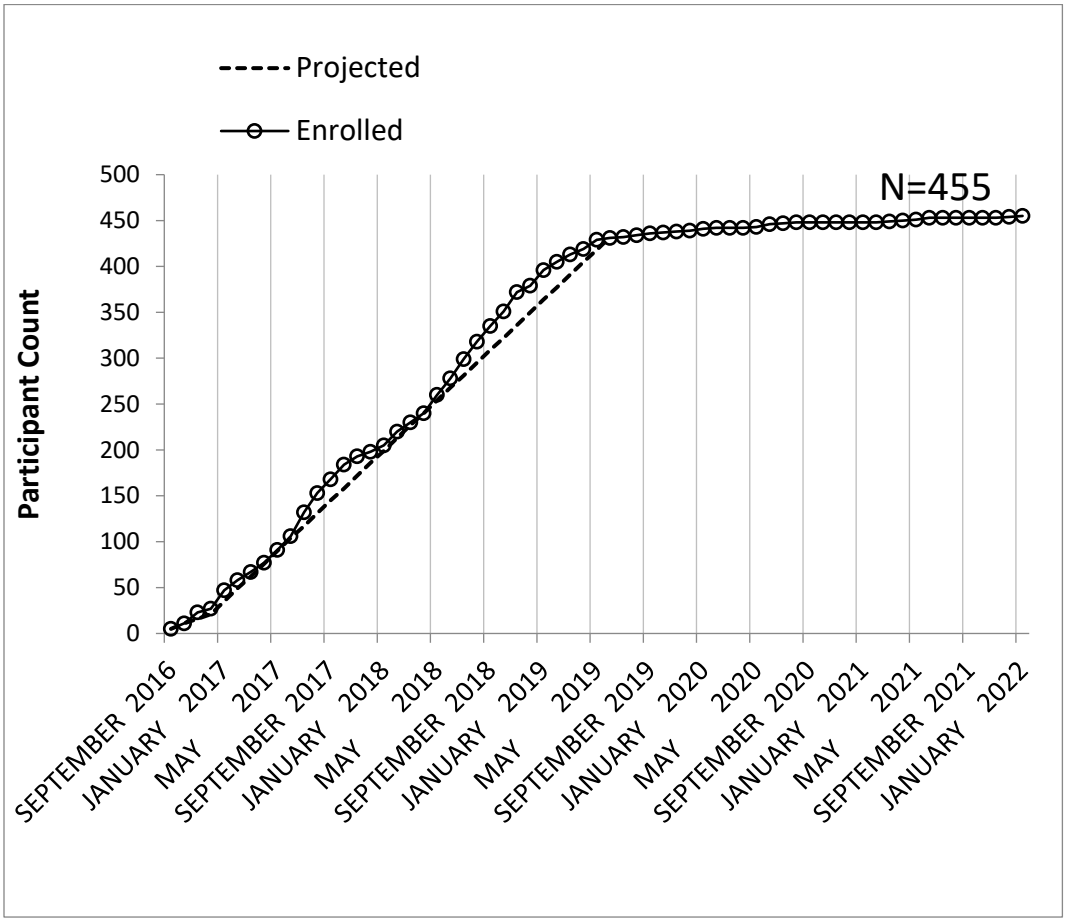

Figure 2. Recruitment of patients with traumatic brain injury over time.

The line graph illustrates the recruitment trajectory from September 2016 to January 2022. The Projected line indicates the initially expected recruitment, which was planned to conclude in May 2019. The Enrolled line shows that the actual recruitment period was extended and continued until January 2022, ultimately enrolling 455 participants.

Characteristics	Pediatric TBI		Adult TBI		Total combined age and severity
	Mild	Moderate to Severe	Mild	Moderate to severe	
Number of patients	114 (25.1)	47 (10.3)	202 (44.4)	92 (20.2)	455 (100)
Age (years)	10.2 (5.3-13.8)	8.95 (3.5-13.8)	44 (30-61)	48.15 (29-65.3)	29 (13.3-52)
Sex (Male: Female ratio)	2.08 : 1	1.6 : 1	1.4 : 1	3.6 : 1	1.8 : 1
Weight (kilograms)	31 (19.8-52)	26 (16-58)	75 (68.3 - 83.5)	78 (70-88)	59 (28-75)
Glasgow Coma Scale	15 (15-15)	4 (3-7)	15 (15-15)	6 (3-8)	15 (8-15)
Abbreviated Injury Scale	NA	NA	3 (2-6)	10 (7-14)	4 (2-8)
Injury Severity Score	NA	NA	5 (4-13)	38 (25.5-51.5)	12 (4-27)
Pediatric Trauma Score	9 (7-10)	2 (0.5-4)	NA	NA	8 (4-10)
Motor vehicle collision	40 (35.1)	24 (51.1)	44 (21.8)	41 (44.6)	149 (32.7)
Passenger	17 (14.9)	16 (34)	3 (1.5)	6 (6.5)	42 (9.2)
Pedestrian	16 (14)	6 (12.8)	13 (6.4)	15 (16.3)	50 (11)
Bicycle	16 (14)	1 (2.1)	31 (15.3)	6 (6.5)	54 (11.9)
Fall	39 (34.2)	8 (17)	92 (45.5)	33 (35.9)	172 (37.8)
Sport	14 (12.3)	2 (4.3)	28 (13.9)	6 (6.5)	50 (11)
Other	9 (7.9)	12 (25.5)	26 (12.9)	13 (14.1)	60 (13.2)
Number (% total) with CT scans	92 (80.7)	46 (97.9)	153 (75.7)	90 (97.8)	381 (83.7)
Cerebral contusion	7 (7.6)	13 (28.3)	13 (8.5)	27 (30)	60 (15.7)
Intracerebral hemorrhage	7 (7.6)	14 (30.4)	13 (8.5)	37 (41.1)	71 (18.6)

Subdural hemorrhage	9 (9.8)	20 (43.5)	16 (10.5)	48 (53.3)	93 (24.4)
Epidural hemorrhage	7 (7.6)	9 (19.6)	4 (2.6)	9 (10)	29 (7.6)
Subarachnoid hemorrhage	12 (13)	16 (34.8)	27 (17.6)	52 (57.8)	107 (28.1)
Cerebral edema*	6 (6.5)	10 (21.7)	2 (1.3)	17 (18.9)	35 (9.2)
Decrease in size of lateral ventricle(s)	4 (4.3)	10 (21.7)	1 (0.7)	12 (13.3)	27 (7.1)
Decrease in size of basal cisterns	6 (6.5)	12 (26.1)	0 (0)	10 (11.1)	28 (7.3)
Midline shift	3 (3.3)	10 (21.7)	2 (1.3)	22 (24.4)	37 (9.7)
Isolated TBI - N (%)	34 (29.8)	4 (8.5)	110 (54.5)	17 (18.5)	165 (36.3)
TBI + injury to other organ/system	79 (69.3)	43 (91.5)	91 (45)	74 (80.4)	287 (63.1)

Table 1. Injury characteristics of patients with traumatic brain injury.

Data are number (%) or median (interquartile range, IQR), unless otherwise indicated. TBI, traumatic brain injury; CT, computed tomography; NA, not applicable.

Characteristic	**Pediatric**		**Adult**		**Total combined age and severity**
	Mild	**Moderate to Severe**	**Mild**	**Moderate to Severe**	
Hypotension - pre-adm	5 (4.4)	10 (21.3)	5 (2.5)	16 (17.4)	36 (7.9)
Hypotension - monitoring	10 (8.8)	15 (31.9)	7 (3.5)	18 (19.6)	50 (11)
Hypotension - combined	13 (11.4)	17 (36.2)	9 (4.5)	27 (29.3)	66 (14.5)
Hypoxia - pre-adm	2 (1.8)	6 (12.8)	2 (1)	17 (18.5)	27 (5.9)
Hypoxia - monitoring	4 (3.5)	9 (19.1)	5 (2.5)	11 (12)	29 (6.4)
Hypoxia - combined	5 (4.4)	14 (29.8)	6 (3)	24 (26.1)	49 (10.8)

Cardiac arrest - pre-adm	1 (0.9)	5 (10.6)	0	1 (1.1)	7 (1.5)
Cardiac arrest - monitoring	1 (0.9)	1 (2.1)	0	3 (3.3)	5 (1.1)
Cardiac arrest - combined	2 (1.8)	6 (12.8)	0	4 (4.3)	12 (2.6)
Intracranial hypertension	13 (11.4)	35 (74.5)	2 (1)	55 (59.8)	105 (23.1)
Unilateral pupil fix+dil - admission	0	1 (2.1)	0	0	1 (0.2)
Unilateral pupil fix+dil - monitoring	1 (0.9)	1 (2.1)	0	1 (1.1)	3 (0.7)
Unilateral pupil fix+dil - combined	1 (0.9)	1 (2.1)	0	1 (1.1)	3 (0.7)
Bilateral pupil fix+dil - admission	0	2 (4.3)	0	0	2 (0.4)
Bilateral pupil fix+dil - monitoring	0	1 (2.1)	0	1 (1.1)	2 (0.4)
Bilateral pupil fix+dil - combined	0	2 (4.3)	0	1 (1.1)	3 (0.7)

Table 2. Physiology and diagnoses associated with secondary brain injury.

Data are number (%). TBI, traumatic brain injury; CT, computed tomography; fix+dil, fixed and dilated; pre-adm, pre-admission.

Age group	TBI severity group	Assessment time point post-TBI			
		7 days # (%)	3 months # (%)	6 months # (%)	12 months # (%)
GOSE					
ADULT	Mild	141 (70)	137 (68)	144 (71)	151 (75)
	Moderate to severe	NA	59 (64)	61 (66)	66 (72)
PEDIATRIC	Mild	88 (77)	92 (81)	92 (81)	96 (84)
	Moderate to severe	NA	35 (74)	41 (87)	40 (85)
Total		229 (71)	323 (71)	338 (74)	353 (78)
PedsQL					
ADULT	Mild	95(47)	98 (49)	NA	146 (72)
	Moderate to severe	NA	40 (43)	NA	53 (58)
PEDIATRIC	Mild	57 (50)	68 (60)	NA	99 (87)
	Moderate to severe	NA	26 (55)	NA	35 (74)
Total		152 (47)	232 (51)	NA	333 (73)
QOLIBRI-OS					
ADULT	Mild	80 (40)	86 (43)	NA	130 (64)
	Moderate to severe	NA	19 (21)	NA	33 (36)
Total		89 (40)	105 (36)	NA	163 (55)
RPSQ					
ADULT	Mild	139 (69)	136 (67)	NA	148 (73)

					HBI
PEDIATRIC	Mild	57 (50)	63 (55)	NA	68 (60)

Table 4. Compliance to follow-up for outcome measures in patients with traumatic brain injury.

TBI, traumatic brain injury; GOSE, Glasgow outcome scale extended; PedsQL, Pediatric Quality of Life Score; QOLIBRI-OS, Quality of Life after Brain Injury Outcome Scale; RPSQ, Rivermead Postconcussion Symptom Questionnaire; HBI, Health Behaviour Inventory; NA, not applicable.

Project 2: Prognostic Serum Protein Biomarkers in Children with Severe Traumatic Brain Injury

1. Introduction

1.1 Overview of Traumatic Brain Injury

Traumatic brain injury (TBI) is a significant public health challenge, particularly in children. It is a leading cause of death and long-term disability among children and adolescents worldwide, posing substantial burdens on healthcare systems and society (Dewan et al., 2016; Fraser et al., 2011; Marzano et al., 2022). The Centers for Disease Control and Prevention (CDC) reports that about 7% of children between 3 and 17 years old have had a head injury (Tabor, 2015). While less than 10% of TBI cases are severe, they account for over 80% of the total related global cost (Nishimura et al., 2022).

1.2 Challenges in Pediatric TBI

Pediatric TBI presents many challenges that set it apart from adult TBI, making its management and prognosis particularly complex (Kennedy et al., 2022; Zurek & Fedora, 2012). Despite extensive research efforts, advances in prognosticating the long-term outcomes after severe TBI in children have been limited and challenging (Kennedy et al., 2022; Zurek & Fedora, 2012) due to several factors:

1. Developmental Stage: The developing brain undergoes significant changes, including myelination, particularly in the first three years of life, which affects the impact and recovery from TBI (Kennedy et al., 2022; Serpa et al., 2021).

2. Anatomical Differences: Infants and young children have open fontanelles and split cranial sutures, as well as a larger head-to-body ratio with relatively poor muscle tone and head control. These anatomical differences result in differing biophysics in the event of an injury (Lipsett et al., 2023)

3. Heterogeneity of TBI: The diverse nature of TBI in children, influenced by age, developmental stage, and mechanism of injury, adds to the complexity (Papa et al., 2013; Sandler et al., 2010).

4. Lack of Pediatric-Specific Tools: The absence of tools specifically designed for pediatric

TBI assessment limits the ability to understand and manage these injuries effectively (Papa et al., 2013; Park et al., 2019).

5. Pathophysiological Understanding: Limited knowledge of the specific pathophysiology of TBI in children hinders the development of targeted treatments (Ganeshalingham & Beca, 2021; Serpa et al., 2021).

6. Severity and Outcome Correlation: There is a lack of clear correlation between the severity of the injury and the long-term outcome in pediatric cases (Forde et al., 2014).

Traditional evaluation methods may not provide enough information to predict long-term outcomes accurately, making it difficult to provide appropriate care and support services (Berger et al., 2007; Park et al., 2019). In pediatric intensive care units (PICUs), accurately predicting death and long-term neurological outcomes among comatose children with severe TBI remains a significant challenge for clinicians. The high risk of secondary insults and uncertain patient outcomes necessitates urgent and comprehensive medical attention (Marzano et al., 2022).

1.3 Importance of Early Prognostication

Accurate early prognostication in pediatric TBI is crucial. It informs clinical decision-making, guiding interventions, and rehabilitation strategies tailored to individual needs. Effective early prognostic tools can potentially improve patient outcomes, assist in resource allocation, and support families in understanding the potential long-term implications of the injury. Objective tools to better understand the degree of injury and to aid early prognostication during rehabilitation are pressing (Ganeshalingham & Beca, 2021).

1.4 Serum Biomarkers as a Promising Avenue

The exploration of serum biomarkers has emerged as a promising avenue in the prognostication of TBI. These biomarkers offer a non-invasive and accessible means to determine the severity of brain injury and predict outcomes. While considerable research has been done in adult populations, there remains a gap in understanding the role and efficacy of serum biomarkers in pediatric TBI. Recent research has focused on the use of serum biomarkers as predictors of outcome (Wilkinson et al., 2017). The use of prognostic protein biomarkers in severe TBI management for children has the potential to improve the accuracy

of early prognostication, enhance rehabilitation, and reduce the burden of TBI on children and their families (Kennedy et al., 2022). Serum biomarkers are readily available and non-invasive and have gained significant popularity (Berger et al., 2012; Neher et al., 2014) as an adjunctive measure in the evaluation and prognosis of TBI in the last decade (Fraser et al., 2011; Thelin et al., 2019; Zurek & Fedora, 2012). Elevations in serum biomarkers have the potential to predict injury severity, secondary brain damage, early indication of recovery, mortality, morbidity, and neurological outcomes, thus allowing for early intervention and rehabilitation (Fraser et al., 2011; Park & Hwang, 2018). While no single biomarker has shown the necessary sensitivity and specificity for predicting outcome, the admission concentrations of multiple biomarkers combined have been shown to have better outcome predictive values (Wilkinson et al., 2017) and may distinguish between favorable and unfavorable outcomes, as well as survival and death (Wang et al., 2018). Early levels of some biomarkers have been demonstrated to provide enhanced prognostic capabilities for mortality at 6 months (Bandyopadhyay, 2005). Biomarkers also offer the potential to better characterize the heterogeneity of TBI (Whitehouse et al., 2022). The guideline on the prediction of recovery from coma has accepted that in addition to clinical indices, a serum brain-specific biomarker, can reliably assist in accurately predicting unfavorable outcome or death (Vos et al., 2010).

Although numerous biomarkers have been studied in pediatric TBI patients (Papa et al., 2013; Wang et al., 2018), to date, no ideal biomarker has been validated as a clinical tool in pediatric severe TBI patients (Papa et al., 2013; Zurek & Fedora, 2012). A clinically ideal biomarker for TBI should demonstrate high specificity and sensitivity for brain injury, quick appearance in an accessible biological fluid (preferentially in serum or plasma) (Papa et al., 2013; Sandler et al., 2010), only be released after irreversible brain tissue damage, display low variability based on age and sex (Sandler et al., 2010), and be informative on prognosis (Haqqani et al., 2007; Papa et al., 2013).

1.5 Objectives and Hypotheses

To address these gaps, our prospective multicenter cohort study aimed to identify brain-specific prognostic biomarkers in children and adolescents with severe TBI. We analyzed serum protein levels within 24 hours following injury and compared the levels among three groups: 1) TBI patients with favorable outcome at 6 months post-TBI, 2) those with unfavorable outcome at 6 months post-TBI, and 3) those with orthopedic injuries without

TBI. We hypothesized that certain TBI-specific low abundance serum proteins, measured within the first 24-hour post-injury, could predict six-month global neurological outcome and death, and can be used for early prognostication and as potential therapeutic targets in TBI management.

2. Materials and Methods

2.1 Study Design and Setting

This prospective observational study was conducted in five children's hospitals in Canada, including the Hospital for Sick Children (Toronto, Ontario), Children's Hospital at London Health Sciences Centre (London, Ontario), Children's Hospital of Eastern Ontario (Ottawa, Ontario), Centre Hospitalier Universitaire Ste Justine (Montreal, Quebec), and McMaster Children's Hospital (Hamilton, Ontario) with a follow-up through 6 months after enrollment. In addition, control samples from children with long-bone injuries without TBI were obtained from the University of Alberta and the Hospital for Sick Children. This study was conducted prior to the CanTBI study presented in Chapter 2, Project 1 of this thesis. The Brain Canada Platform support grant, which funded CanTBI, also funded this proteomics study in children with severe TBI as a "demonstration" research project. Approval was obtained from Institutional Review Boards at each participating site, and written informed consent was obtained from the parent or legal guardian of each child enrolled.

2.2 Participant Recruitment

Eligibility criteria were age from 0 to 17 years at the time of injury, presentation to the emergency department requiring observation for a minimum of 4 hours, or admission to the hospital ward or intensive care unit, within 24 hours of a clinically diagnosed TBI of any severity, and consent for blood sampling. Participants were excluded if they had a previous TBI that had required hospital admission, birth trauma (i.e., injuries sustained during labor or delivery), or whose parents or guardians were not fluent in English or French.

2.3 Data Collection and Patient Selection

Demographic data, injury characteristics, clinical Computed Tomography (CT) scan data, physiologic data, and global neurological outcome or death data were prospectively collected by research coordinators using a procedures manual and entered into a central database at the Hospital for Sick Children. The global neurological functional outcome and death were assessed using a scripted phone interview of the parents or surrogate decision makers by trained research coordinators using the 8-point pediatric cerebral performance category (PCPC) score at 6 months following injury (Fiser, 1992). The Pediatric Cerebral Performance Category (PCPC) scores represent normal function (PCPC score of 1), mild disability (PCPC score of 2), moderate disability (PCPC score of 3), severe disability (PCPC score of 4), coma

or persistent vegetative state (PCPC score of 5) and death (PCPC score of 6) (Fiser, 1992).

We recruited 210 hospitalized children with mild, moderate, or severe TBI. 112/210 (53%) of these children had severe TBI, defined as an admission Glasgow coma scale (GCS) score ≤ 8. Of the 112 children with severe TBI, we chose 8 of these patients, with blood samples drawn on the first day following severe TBI and an unfavorable outcome, defined as severe disability or death (severe disability or death, indicated by a PCPC score of 4 or 6) at 6 months, following TBI. We matched each of these 8 children using admission GCS, age, and biological sex to 8 children with a favorable outcome defined as normal or mild disability (normal or mild disability, PCPC score of 1 or 2) at 6 months following severe TBI. 8 patients with orthopedic injury but no TBI (OI controls) from the Hospital for Sick Children (N=3) and the University of Alberta (N=5) were then matched by age and sex to these 16 patients with severe TBI (Fig. 1).

2.4 Blood Sample Collection and Banking

Blood samples were collected on day 1 following the injury. The samples were placed in a tube with no anticoagulant, allowed to clot at room temperature, centrifuged at 5,000 rpm at 4 °C for 10 minutes and serum was separated, divided into 100 µL aliquots, and stored at -80°C as per our biobanking standard operating procedure until analysis. Samples were first stored at study sites, and then shipped on dry ice and stored at –80°C in a biobank at the Hospital for Sick Children.

2.5 Proteomic Analysis

Proteomic analyses involved both untargeted and targeted liquid chromatography and tandem mass spectroscopy (LC-MS/MS). The untargeted LC-MS/MS, conducted at the Hospital for Sick Children's Analytical Facility for Bioactive Molecules aimed to measure low-abundance serum proteins. This untargeted LC-MS/MS protocol was then repeated, followed by a targeted LC-MS assay to measure trends in the most likely prognostic proteins, at the Proteomics facility at the National Research Council (NR), Ottawa, to confirm these findings.

2.5.1 Sample Processing and Protein Digestion for MS Analysis

Clinical human serum samples as well as a commercial human serum (Sigma Aldrich, St. Louis, Missouri, United States) were diluted 1:25 in 25 mM ammonium bicarbonate (Sigma

Aldrich, St. Louis, Missouri, United States). The samples were reduced with 50 mM dithiothreitol at a final concentration of 5 mM (DTT, Sigma Aldrich, St. Louis, Missouri, United States) at 95°C for 10 minutes. Following reduction and cooling to room temperature the samples were alkylated using 100 mM iodoacetamide (Sigma Aldrich, St. Louis, Missouri, United States) at a final concentration of 10 mM at room temperature for 30 minutes in the dark. The proteins were digested by trypsin (Promega, Madison, Wisconsin, United States) (enzyme-to-substrate ratio [w/w] of 1:40) at 37 °C overnight. The digested peptides were acidified with formic acid (Sigma Aldrich, St. Louis, Missouri, United States). Stable heavy isotope-labeled (SIL) synthetic peptides (U–13C6, U–15N2-lysine and U–13C6, U–15N4 arginine-labeled, ≥95% chemical purity, New England Peptide, Gardner, Massachusetts, United States) were spiked at ~6 fmol/µL.

2.5.2 Untargeted Proteomic Analysis

Low abundance serum proteins were measured in serum aliquots using untargeted LC-MS/MS. First, 12 large abundant proteins (Albumin, IgG, IgA, IgM, α1-Acid Glycoprotein, α1-Antitrypsin, α2-Macroglobulin, Apolipoprotein A-I, Apolipoprotein A-II, Fibrinogen, Haptoglobin, and Transferin) were depleted from the serum samples, and then digested with trypsin, and isotope-labeled for untargeted LC-MS/MS analysis using Tandem-Mass Tags (TMT™). TMT labeling was performed using the X tagging kit (Thermo Scientific) according to the manufacturer's instructions. The resulting peptides were separated by LC-MS/MS instrumentation consisting of an EASY-nLC 1000. Spectra were matched to known peptide sequences using Sequest and X!, and raw data was imported into Scaffold software for protein identification. Scaffold was used to match the identified peptides to known proteins from the National Center for Biotechnology Information (NCBI) database to identify proteins and quantify their abundance. We then used specialized proteomic software to compare protein concentrations between these three groups.

2.5.3 Targeted Proteomic Analysis

We developed a new targeted MS method focusing on 8 identified prognostic proteins to validate the results from our untargeted proteomic data. We tested this novel targeted proteomic methods protocol in both serum aliquots that were not depleted, and in serum aliquots that were first depleted of abundant proteins. These proteins were analyzed in serum aliquots from all 24 patients from blood samples drawn at the same dates and times. We also

measured GFAP, which we and others have reported as a prognostic serum protein in pediatric severe TBI (Fraser et al., 2011) as a positive control protein, along with several "negative" control proteins that did not show significant changes across our 3 experimental groups form our untargeted proteomic analysis. To compare the protein expression trends between the untargeted LC-MS/MS data with the targeted MS data, we visually examined trends in peptide /protein fold expression and analyzed statistical differences in these proteins between the 3 experimental groups.

A panel of 13 target proteins (8 novel prognostic proteins, GFAP and 4 negative control proteins that were not expected to change across the 3 experimental groups) were measured in the samples by parallel reaction monitoring (PRM). The samples were loaded onto a reversed-phase UltiMate™ 3000 RSLCnano System with ProFlow Meter (Thermo Fisher Scientific, Waltham, MA) coupled with an Orbitrap Eclipse™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA) for analysis using a nanoelectrospray source operated in positive ion mode. Approximately 0.1 µg of the sample was injected and loaded onto a 300 µm I.D. × 0.5 mm 3 µm PepMaps® C18 trap (Thermo Fisher) followed by separation on a 100 µm I.D. × 10 cm 1.7 µm BEH130C18 nanoLC column (Waters, Milford, MA, USA). The eluted peptides were ionized by electrospray ionization and a full MS scan was acquired in the Orbitrap between 350 and 1800 m/z in profile mode at a 60,000 resolution. This was followed by HCD activation at a normalized collision energy of 28%. Xcalibur was used to build the inclusion list, which incorporated the target precursor mass-to-charge ratio (m/z), and their precursor charge state. The inclusion list contained the 2-3 peptides of the 13 target proteins. The peptides were separated on a 72-minute step-wise gradient from 6% to 85% solvent B (100% ACN and 0.1% formic acid). The MS data were acquired in PRM mode with an isolation width of 1.6 m/z, a resolution of 30,000 on an Orbitrap, a normalized AGC value of 100%, and a maximum injection time of 50 ms. All data was recorded with Xcalibur software (ThermoFisher Scientific, San Jose, CA). Raw data extraction and data analysis were performed using Skyline software (https://skyline.ms). The extracted peptide intensities (peak areas) were normalized against a median intensity value calculated from all peptide intensities in each run.

2.6 Data and Statistical Analysis

We performed exploratory data analysis of blood protein data using box plots, histograms,

and dot plots. The primary focus was to understand the distribution and patterns of the data visually and descriptively across the 3 experimental groups. A Mann-Whitney analysis was performed to compare the mean expression level of each protein between the favorable and unfavorable groups. Using our untargeted proteomic dataset we first identified prognostic proteins that differed statistically in concentrations (fold changes) between the favorable and unfavorable TBI outcome groups. We then used more strict analytical and statistical criteria to identify a smaller group of potential prognostic proteins that also had potential specificity for TBI. These criteria included proteins that had at least 3 peptides from a protein of interest measured in all 24 blood samples, that differed statistically between the favorable and unfavorable outcome groups and also differed statistically between the OI control group and at least 1 of the 2 TBI outcome groups (favorable or unfavorable outcomes) with a conservative p-value of < 0.0002. We performed these statistical analyses using IBM SPSS version 24, SAS, and R statistical softwar

3. Results

One patient was excluded from the initial untargeted proteomics analysis because the 1st blood sample was drawn after 24 hours post-injury but all 24 patients were included in the targeted protein analysis which included a time course of serum proteins. The initial untargeted LC-MS/MS analysis identified over 2000 serum peptides and 253 unique proteins that were expressed in the serum of all 23 samples. 40 low-abundance proteins out of 253 identified proteins were statistically different between favorable and unfavorable groups including metabolic, anti-oxidant and vasoactive proteins (Fig. 2). To select the most promising candidates for further analysis, 8 proteins were selected based on the magnitude of the log-fold difference between favorable and unfavorable outcome groups, statistically significant differences between the OI control group and at least 1 of the TBI outcome groups and the consistency of expression of these peptides/proteins across all samples. Untargeted LC-MS/MS identified 8 prognostic proteins differing in concentration between these 3 experimental groups (Fig. 2).

Figure 3 displays the result of the initial National Research Council (NRC) pathway analysis of the top 40 differentially expressed proteins between three experimental group using the SPARC 2017 TMT datasets. The analysis utilized appropriate statistical methods to identify the differentially expressed proteins and used them as input for the NRC pathway analysis. The software compared these proteins to a database of known pathways and processes to identify those that are overrepresented among the differentially expressed proteins. The resulting list of top pathways and processes was used to generate a hypothesis about the underlying biological mechanisms responsible for the observed differential expression of the proteins.

2 of the 8 prognostic proteins were higher in concentration in the favorable outcome compared to the unfavorable outcome group and 6 of these 8 proteins were higher in concentration in the unfavorable outcome group compared to the favorable outcome group. Patients with favorable and unfavorable outcome had a significantly higher expression of Complement C1r subcomponent (C1R) and Complement C1s subcomponent (C1S) proteins compared to controls ($p < 0.0001$). Furthermore, the expression of these proteins in patients with an unfavorable outcome was significantly higher compared to the favorable group ($p < 0.0001$). Patients with favorable and unfavorable outcome had a significantly

higher expression of Zinc α-2glycoprotein (AZGP1) protein compared to controls ($p < 0.0001$). Furthermore, AZGP1 expression in patients with favorable outcome was significantly higher than patients with unfavorable outcome ($p <0.0001$). Patients with unfavorable outcome had a significantly lower expression of Kininogen-1 (KNG1) protein compared to both patients with favorable outcome ($p < 0.0001$) and controls ($p < 0.0001$). Patients with favorable outcome had a significantly lower expression of Plasma kallikrein (KLKB1) protein compared to both patients with unfavorable outcome ($p < 0.0001$) and controls ($p < 0.0001$). Patients with unfavorable outcome had a significantly higher expression of Lactate dehydrogenase (LDH) protein compared to both favorable groups ($p < 0.0001$) and controls ($p < 0.0001$). Patients with unfavorable outcome had a significantly higher expression of Fructose-bisphosphate aldolase A isoform-2 (ALDOA) protein compared to both patients with favorable outcome ($p < 0.0001$) and controls ($p < 0.05$). Patients with favorable outcome had a significantly higher expression of Peroxiredoxin-2 (PRDX2) protein compared to controls ($p < 0.05$). Its expression in patients with favorable outcome was significantly lower compared to patients with unfavorable outcome ($p < 0.0001$) and controls ($p < 0.0001$) (Fig. 4).

In addition, 3 control proteins were chosen, due to their consistent expression and no log-fold change across all samples. These control proteins were β-ala-His dipeptidase (Log2 fold change = 0, Mann-Whitney Test = 0.99), Hemopexin (Log2 fold change = 0, Mann-Whitney Test = 0.67), and Tetranectin (Log2 fold change = 0, Mann-Whitney Test = 0.74).

Comparing the previous quantities from untargeted LC-MS/MS with depleting abundant proteins to the new targeted MS data without abundant protein depletion developed by NRC, 4 of 8 initial proteins including PRDX2, ALDOA, KLKB1, and KNG1 appear to be showing similar trends as before (Fig. 5).

Based on literature mining results, hypoxia, ischemia, melatonin, Notch1 signaling pathway components, and SIRT3 studied in the first chapter of this thesis are associated with the 8 discovered biomarkers (Table 1). In addition, based on our interatomic result, mapping of the biomarkers to the Notch1 signaling network, only 6 of the 8 discovered biomarkers including, C1S, C1R, KLKB1, KNG1, PRDX2, and LDHB were found to have interaction with Notch1 network. Although the 6 biomarkers either directly or indirectly interact with each other, only one biomarker, PRDX2, was found to interact directly with the Notch1 network via Synaptojanin 2 binding protein (SYNJ2BP) (Fig. 6).

4. Discussion

The findings from our study represent a pivotal step forward in the understanding and management of pediatric severe TBI. By identifying specific serum biomarkers that correlate with six-month post-injury outcomes, this research lays the groundwork for more precise and individualized approaches to TBI care in children and adolescents.

One of the most compelling aspects of this study is the discovery of specific proteins that significantly differ in expression between patients with favorable and unfavorable outcomes. These biomarkers, including Complement C1r and C1s subcomponents, Zinc α-2glycoprotein, Kininogen-1, Plasma kallikrein, Lactate dehydrogenase, Fructose-bisphosphate aldolase A isoform-2, and Peroxiredoxin-2, offer valuable insights into the pathophysiological mechanisms in pediatric TBI. The 8 proteins that differ in concentration between the three experimental groups have the potential to be used by clinicians for neuromonitoring, prognosis, and risk-stratifying patients in future clinical trials. Furthermore, the 2 proteins associated with favorable outcomes may yield insights that lead to novel therapies for patients with TBI, offering hope for improved recovery and treatment outcomes. Interestingly, we found a strong connection between the two chapters of this thesis, the projects in Italy and Canada. Our literature mining results revealed that hypoxia, ischemia, melatonin, the Notch1 signaling pathway, and SIRT3 all studied in the first chapter, are associated with the 8 discovered biomarkers. Furthermore, our interatomic analysis indicated that 6 of the 8 discovered biomarkers interact with the Notch1 network.

The identification of these biomarkers within 24 hours post-injury is particularly noteworthy. This early detection is critical for timely and effective intervention strategies, potentially enabling healthcare providers to tailor treatments based on individual risk profiles and improve outcomes. This study's findings underscore the potential of serum biomarkers in predicting outcomes in pediatric patients with severe TBI.

Elevations in serum biomarkers have the potential to predict injury severity, secondary brain damage, an early indication of recovery, mortality, morbidity, and neurological outcomes, thus improving the prognosis and allowing for early intervention and rehabilitation (Fraser et al., 2011; Ganeshalingham & Beca, 2021; Park & Hwang, 2018; Sandler et al., 2010; Wang et al., 2018; Wilkinson et al., 2017). While no single biomarker has shown the necessary sensitivity and specificity for predicting outcome, the admission concentrations of multiple biomarkers combined have been shown to have better outcome predictive values (Papa et al., 2013; Wilkinson et al., 2016, 2017) and may distinguish between favorable and unfavorable outcomes, as well as survival and death (Wang et al., 2018). Early levels of some biomarkers

have been demonstrated to provide enhanced prognostic capabilities for mortality at 6 months (Bandyopadhyay, 2005). The guideline on the prediction of recovery from coma has accepted that in addition to clinical indices, a serum brain-specific biomarker, can reliably assist in accurately predicting unfavorable outcome or death (Vos et al., 2010).

Although numerous biomarkers have been studied in pediatric TBI patients (Papa et al., 2013; Wang et al., 2018), to date, no ideal biomarker has been validated as a clinical tool in pediatric severe TBI patients (Papa et al., 2013; Sandler et al., 2010; Zurek & Fedora, 2012). Several biomarkers of TBI have been identified, but they carry the disadvantage of either not being sensitive or specific to TBI, which diminishes their clinical utility (Martinez & Stabenfeldt, 2019). Moreover, the predictive value for adverse outcome remains to be determined (Forde et al., 2014; Neher et al., 2014; Thelin et al., 2019).

In conclusion, our research offers a new avenue for enhanced patient care and treatment strategies in pediatric TBI offering hope for better outcomes and recovery paths for this population. While further research is needed to fully realize the clinical potential of these findings, our study provides a promising foundation for improving the prognosis and treatment of children and adolescents with severe TBI.

5. Figures and Tables

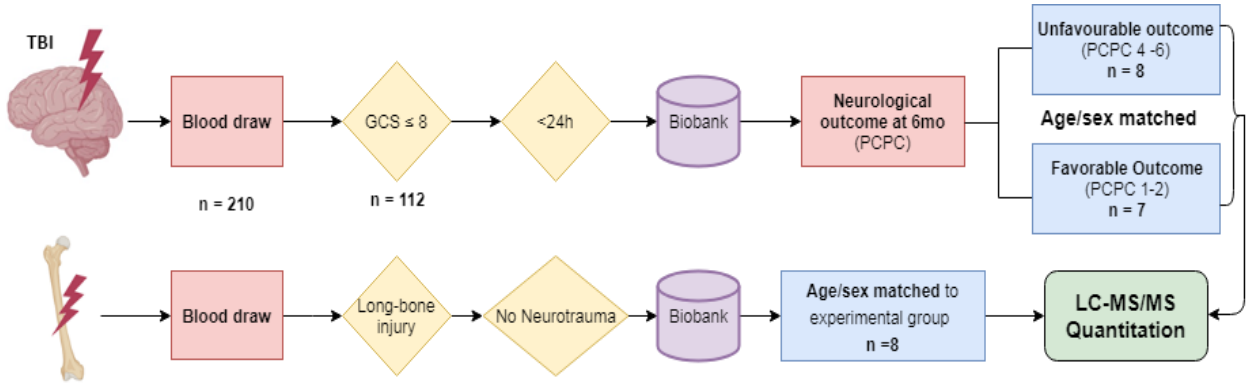

Figure 1. Overview of study design and selection criteria of traumatic brain injury patients with favorable and unfavorable outcome, and trauma controls.

This figure displays the matched case-control study design and inclusion criteria for the proteomic analysis of a biobank of 210 children with mild, moderate, and severe TBI. The study focused on 112 patients with severe TBI, selected based on admission GCS score ≤8. 15 patients met the eligibility criteria of being aged 8-18 and providing blood samples within 24 hours post-injury. We then matched 8 children with unfavorable outcomes to 7 children with favorable outcomes at 6 months post-injury based on age, sex, and admission GCS. In addition, the study included 8 children with orthopedic injury but no TBI as trauma controls, matched by age and biological sex to the 15 TBI outcome patients. The study utilized untargeted LC-MS/MS to measure low-abundance serum proteins.

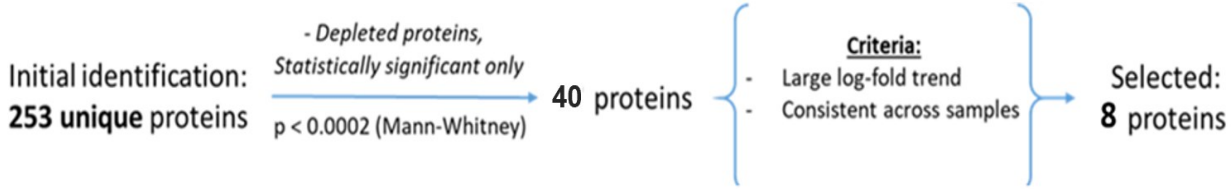

Figure 2. Biomarker selection.

Initial untargeted LC-MS/MS analysis identified 253 unique proteins expressed in the serum of all 23 samples. Following the depletion of abundant proteins, 40 proteins exhibited significant statistical differences between favorable and unfavorable groups relative to orthopedic injury

controls. To identify the most promising candidates for further analysis, these proteins were evaluated based on both the magnitude of the log-fold difference between favorable and unfavorable samples and the consistency of expression across all samples which ended up identifying 8 proteins.

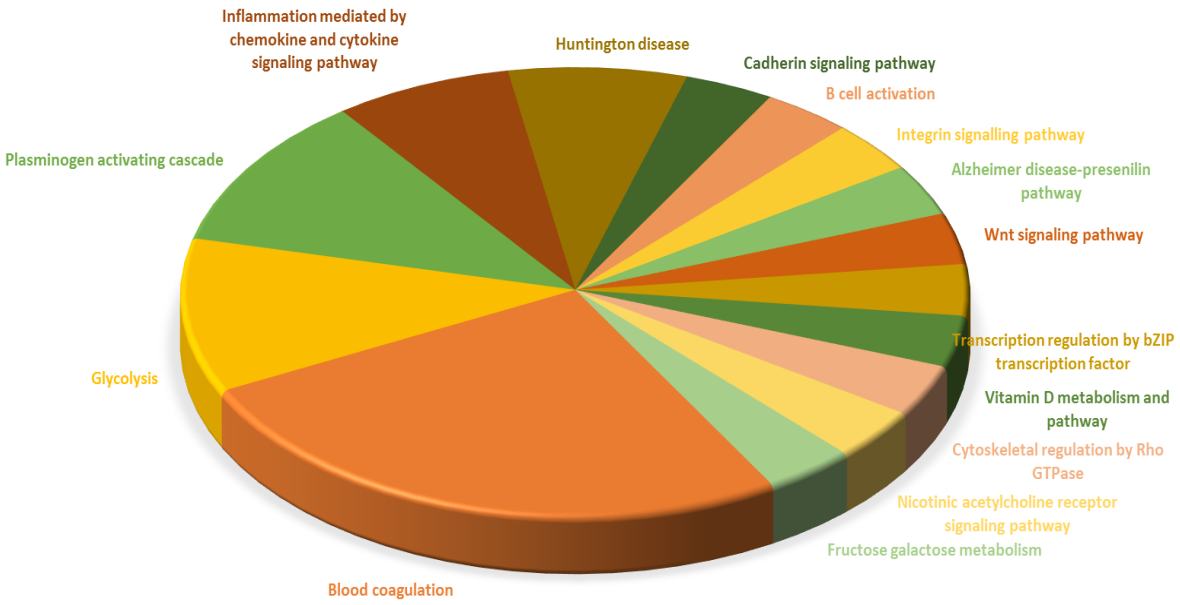

Figure 3. Categories of 40 serum proteins with different concentrations between at least 2 of the experimental groups.

This figure presents the initial pathway analysis conducted by the National Research Council (NRC) on the top 40 serum proteins exhibiting differential expression among at least 2 of the experimental groups, as identified from the SPARC 2017 Tandem Mass Tag (TMT) datasets. The resulting list of top pathways and processes was used to generate a hypothesis about the underlying biological mechanisms responsible for the observed differential expression of the proteins. This visualization serves as a foundation for hypothesizing the biological mechanisms underlying the protein expression differences noted in the study, offering insights into potential therapeutic targets or biomarkers for disease progression and prognosis.

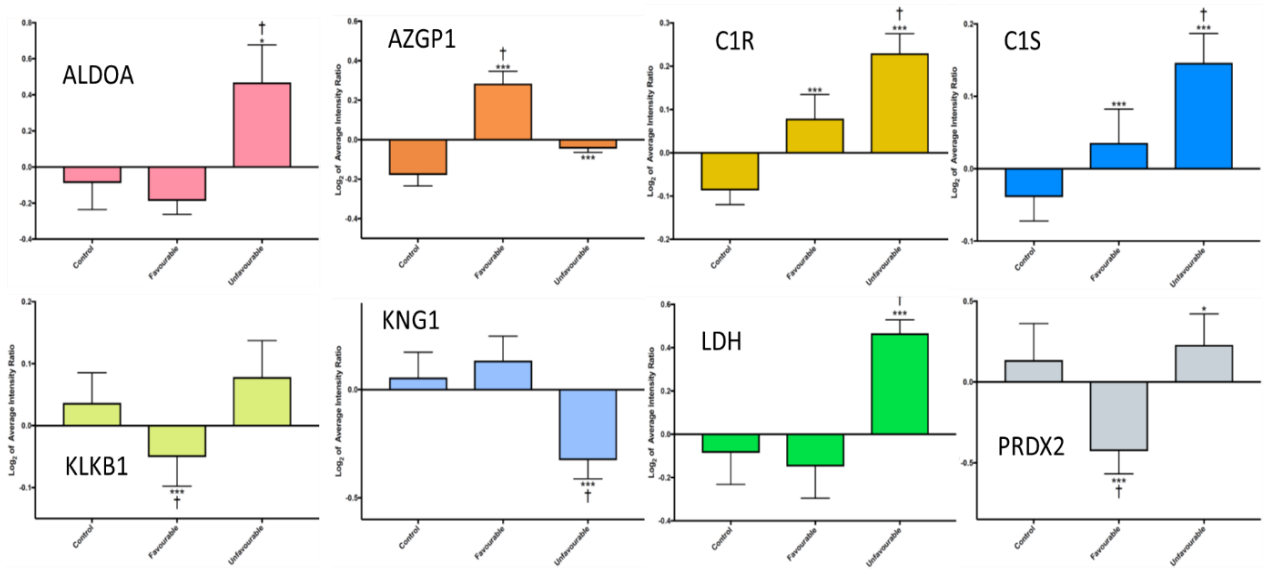

Figure 4. Potential candidate prognostic biomarkers identified by untargeted LC-MS/MS.

The graphs show proteins measured in serum from blood drawn at < 24 hours following injury using untargeted LC-MS/MS. 8 serum proteins, measured using untargeted mass spectrometry, were detected in all serum samples from all 24 patients and had significantly different concentrations between at least one of the TBI outcome groups and the control group. Data are expressed as mean + standard error of the mean. The statistical significance of differences between groups was determined using the Mann-Whitney-U test, with * $p<0.05$ and *** $p<0.0001$ indicating significance compared to orthopedic injury controls, and † $p < 0.0001$ comparing the favorable and unfavorable outcome groups. C1R, Complement C1r subcomponent; C1S, Complement C1s subcomponent; AZGP1, Zinc α-2 glycoprotein; KNG1, Kininogen-1; KLKB1, Plasma kallikrein; LDH, Lactate dehydrogenase; ALDOA, Fructose-bisphosphate aldolase A isoform-2; PRDX2, Peroxiredoxin-2.

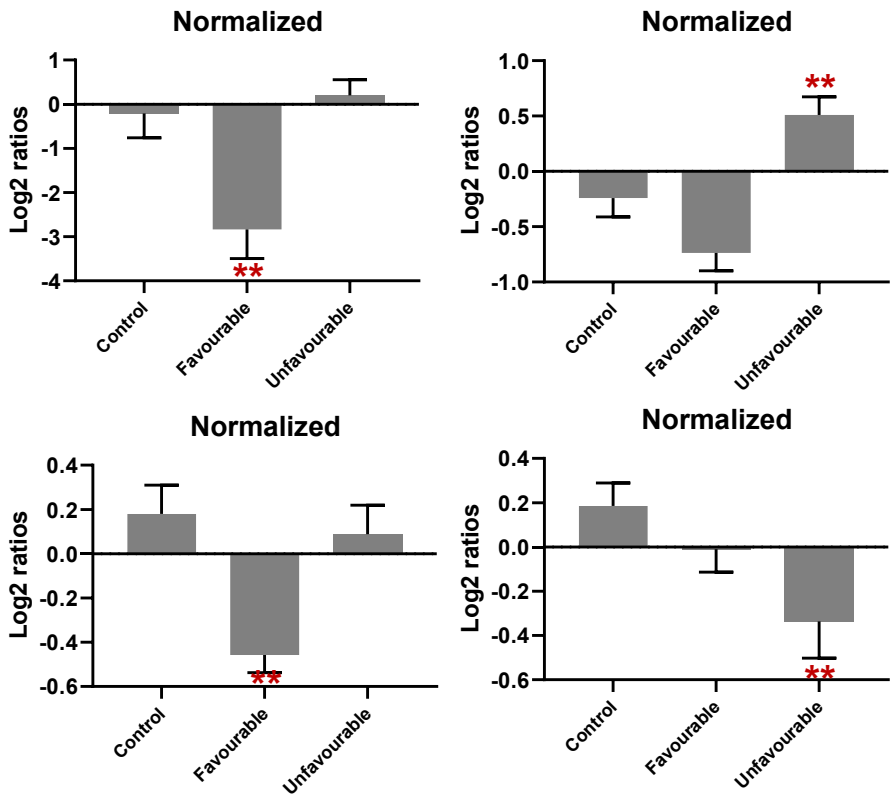

Figure 5. Discovered prognostic biomarkers by targeted LC-MS/MS.

The graphs show proteins measured in serum from blood drawn at < 24 hours following injury using targeted LC-MS/MS. This figure depicts the differential expression of four key proteins across three experimental groups: control, favorable outcome, and unfavorable outcome. Data are expressed as mean + standard error of the mean. The statistical significance of differences between groups was determined using the Mann-Whitney-U test, with ** $p<0.001$. KNG1, Kininogen-1; KLKB1, Plasma kallikrein; ALDOA, Fructose-bisphosphate aldolase A isoform-2; PRDX2, Peroxiredoxin-2.

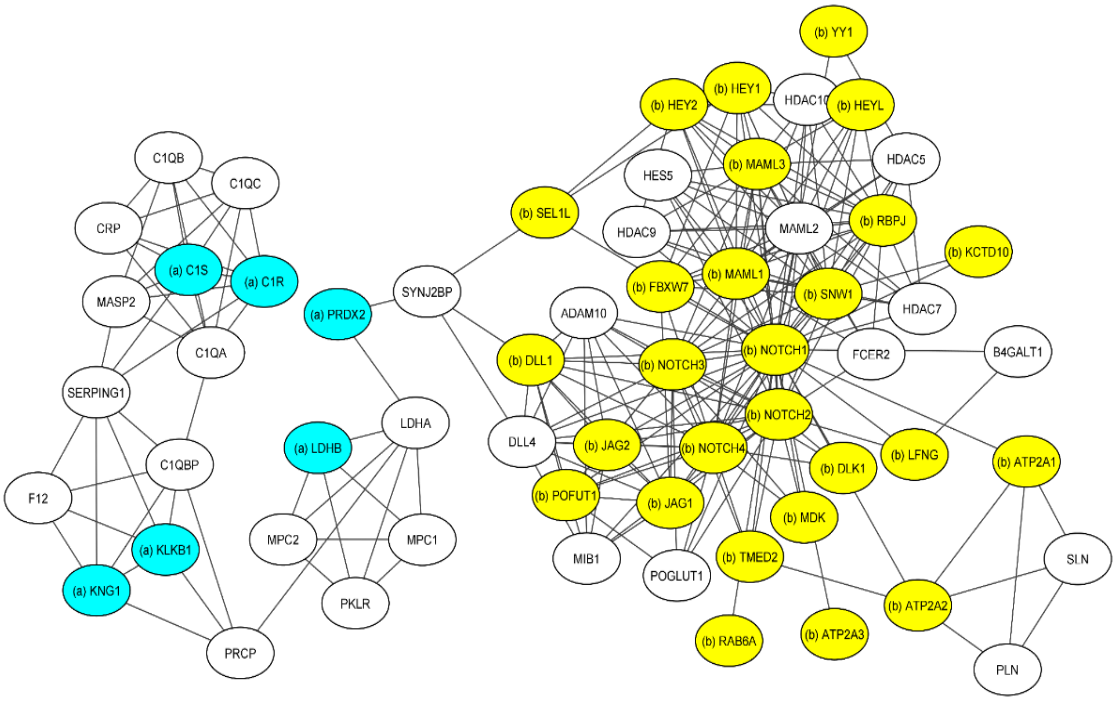

Figure 6. Interactomics of the Notch1 signaling proteins: mapping of the 8 discovered biomarkers to Notch1 signaling network.

Only 6 of the 8 biomarkers were found to have interaction data. Although the 6 biomarkers either directly or indirectly interact with each other, only one biomarker, PRDX2, was found to interact directly with the Notch1 network via SYNJ2BP protein. The 6 biomarkers are shown in blue as (a) and proteins involved in Notch1 signaling are shown in yellow as (b). The network was created using known protein-protein interactions (Carta DB PMID 31364059) compiled at NRC and plotted using Cytoscape 3.9.1. PRDX2, Peroxiredoxin-2; SYNJ2BP, Synaptojanin 2 binding protein

	Notch1	NICD	Notch1 Receptor	HES1	c-MYC	Cyclin D3	p21Waf 1/Cip1	Jagged1 OR JAG1	ADAM10	gamma-Secretase Complex	SIRT3	Melatonin	Hypoxia	Ischemia
C1R										✓			✓	
C1S										✓		✓	✓	
AZGP1	✓													
KNG1								✓	✓				✓	
KLKB1												✓		
LDH	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ALDOA					✓								✓	

PRDX2					✓							✓	✓	

Table 1. Literature mining shows the connection between a pre-clinical study conducted in Italy and a clinical study conducted during a research period in Canada.

Based on our literature mining results, hypoxia, ischemia, melatonin, Notch1 signaling components, and SIRT3 studied in our project in Italy are associated with the 8 discovered biomarkers. C1R, Complement C1r subcomponent; C1S, Complement C1s subcomponent; AZGP1, Zinc α-2 glycoprotein; KNG1, Kininogen-1; KLKB1, Plasma kallikrein; LDH, Lactate dehydrogenase; ALDOA, Fructose-bisphosphate aldolase A isoform-2; PRDX2, Peroxiredoxin-2; NICD, Notch intracellular Domain; HES1, Hairy and Enhancer of Split 1; SIRT3, Sirtuin 3; ADAM10, A Disintegrin And Metalloproteinase 10.

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