

From the DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

ON BIOMARKERS IN TRAUMATIC BRAIN INJURY

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**Karolinska
Institutet**

Stockholm 2015

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Cover Art: Immunohistochemistry of a coronar section of injured rat brain. The blue color represents neurons (NeuN) and the red complement system activation (C5b9). The molecule is a S100B homodimer (©Wikimedia Commons).

Published by Karolinska Institutet.

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ISBN 978-91-7549-864-5

Printed by E-print AB

On Biomarkers in Traumatic Brain Injury

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my grandfather Hugo Thelin

Who despite coming from very simple conditions always worked unfailingly and enthusiastically to improve medical research in Sweden and worldwide. You are a true inspiration.

To our dog Link

You lived a very brief and stormy life yet you were able to give us so much love. You will be missed.

“So come up to the lab and see what's on the slab.

I see you shiver with antici... pation!”

Dr. Frank-N-Furter (Tim Curry), *The Rocky Horror Picture Show*, 1975

ABSTRACT

Traumatic brain injury (TBI) is a common cause of death and disability. Unfortunately, TBI patients will be affected by secondary insults, such as hypoxia and increased intracranial pressure, which may lead to secondary brain injuries. Because of this, these patients are treated in specialized neuro-intensive care units (NICU) where the brain is monitored in order to prevent secondary lesion development. Cerebral monitoring is limited by its locality and more generalized markers to monitor the injured brain are warranted. Biomarkers have been introduced in the field of TBI, where they may be evaluated to examine potential pathophysiological processes. S100B, a primarily astrocytic protein, is the most studied serum biomarker in TBI, but other candidates exist. The aims of this thesis were to validate biomarkers toward long-term functional outcome, to evaluate the effect of biomarkers and a new global method of microdialysis in multimodal monitoring of NICU patients and in a translational methodology assess how biomarkers may facilitate in the damage analysis in a hypoxic-TBI animal model.

In **Paper I**, a retrospective study including 265 NICU TBI patients, where S100B samples were acquired at admission and every 12 hours the first 48 hours after injury, we detected a significant, and independent, correlation between S100B levels and long-term functional outcome. The predictive capabilities increased sharply after 12 hours and remained high up to 36 hours after injury. S100B levels were only significantly correlated to pathology detected on computerized tomography (CT) and not to extracranial trauma.

In **Paper II**, a retrospective study including 250 NICU TBI patients, we analyzed S100B samples acquired later than 48 hours after injury. We noted that secondary increases of S100B even as low as 0.05µg/L is sensitive and specific enough to detect radiological verified cerebral deteriorations, undetected by conventional monitoring.

In **Paper III**, a prospective study including 14 NICU TBI patients, we monitored patients using microdialysis (MD) in flowing cerebrospinal fluid (CSF) for a more “global” overview of cerebral metabolism. We validated the method using conventional CSF samples, and found that the MD-CSF method yielded adequate results. Also, albeit a small sample size, we noted that lactate and pyruvate levels were significantly elevated in patients with an unfavorable outcome.

In **Paper IV**, a retrospective study including 182 NICU TBI patients, we analyzed serum and CSF levels of Neurofilament light, a protein of axonal origin thus different from S100B. We showed that NFL levels significantly correlated independently to outcome, even in the presence of S100B. However, we could not correlate NFL levels to injuries visible on CT and magnetic resonance imaging (MRI).

In **Paper V**, a preclinical study including 73 Sprague-Dawley rats, we analyzed how hypoxia exacerbates TBI. We detected increased neuronal death using immunohistochemistry and increased lesion size on MRI in the hypoxic animals compared to normoxic animals. A trend was found towards higher S100B levels in serum after 24 hours in the hypoxic group. Vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1-alpha (HIF1α) expressions were significantly increased in the normoxic group.

In summary, the biomarker S100B provides important information towards long-term outcome, even more so than other known predictors of long-term outcome. Outcome prediction models including both S100B and NFL presents the highest explanatory variance, presumably by monitoring different pathophysiological processes. S100B is a valuable asset in the multimodal monitoring in order to detect secondary cerebral injuries and together with the MD-CSF technique; it could improve conventional NICU care with a more global approach. Hypoxic insults following TBI aggravate injury development and this pathophysiological process could presumably be monitored using S100B as an indicator of injury severity.

LIST OF SCIENTIFIC PAPERS

- I. **S100B is an important outcome predictor in traumatic brain injury.** Thelin EP, Johannesson L, Nelson D, Bellander BM. J Neurotrauma. 2013 Apr 1;30(7):519-28
- II. **Secondary peaks of S100B in serum relate to subsequent radiological pathology in traumatic brain injury.** Thelin EP, Nelson DW, Bellander BM. Neurocrit Care. 2014 Apr;20(2):217-29
- III. **Microdialysis Monitoring of CSF Parameters in Severe Traumatic Brain Injury Patients: A Novel Approach.** Thelin EP, Nelson DW, Ghatan PH, Bellander BM. Front Neurol. 2014 Sep 2;5:159
- IV. **Comparative assessment of the prognostic value of biomarkers in traumatic brain injury reveals an independent role for serum levels of neurofilament light.** Al-Nimer F*,Thelin EP*, Nyström H, Dring AM, Svenningsson A, Piehl F, Nelson DW*, Bellander BM*
* = Authors contributed equally
Submitted manuscript
- V. **Hypoxia following traumatic brain injury in rats exacerbates lesion size whereas hypoxia-inducible factor 1 alpha and vascular endothelial growth factor were increased in normoxic rats.** Thelin EP, Frostell A, Mulder J, Mitsios N, Damberg P, Nikkhou-Aski S, Risling M, Svensson M, Morganti-Kossmann MC, Bellander BM.

Manuscript

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LIST OF ABBREVIATIONS

TBI	Traumatic Brain Injury
NICU	Neuro-intensive Care Unit
CT	Computerized Tomography
MRI	Magnetic Resonance Imaging
MD	Microdialysis
NFL	Neurofilament Light
GFAP	Glial Fibrillary Acidic Protein
HIF1 α	Hypoxia-inducible Factor 1-Alpha
VEGF	Vascular Endothelial Growth Factor
MAC	Membrane Attack Complex (C5b9)
NSE	Neuron-Specific Enolase
CSF	Cerebrospinal Fluid
GCS	Glasgow Coma Scale
GOS	Glasgow Outcome Score/Scale
EUSIG	Edinburgh University Secondary Insult Grade
EDH	Epidural Hematoma
SDH	Subdural hematoma
DAI	Diffuse Axonal Injury
trSAH	Traumatic subarachnoid hemorrhage
RLS-85	Reaction Level Scale-85
AIS	Abbreviated Injury Scale
ISS	Injury Severity Scale
PBtO ₂	Brain Tissue Oxygen Pressure
ICP	Intracranial Pressure
CPP	Cerebral Perfusion Pressure
PTCI	Post-Traumatic Cerebral Infarction
AUC	Area Under Curve

1 INTRODUCTION

1.1 EPIDEMIOLOGY OF TBI

Globally, traumatic brain injury (TBI) is a major health problem (Ghajar, 2000, Corrigan et al., 2010), and the highest contributor to in-hospital trauma related mortality (Acosta et al., 1998). Being a common problem in high income countries today, a vast majority of TBI related morbidity and mortality (90%) affects low and middle income countries (Hofman et al., 2005). Historically, TBI has been the disease of the young population, being the most common cause of mortality up to 44 years of age (Jennett, 1996, Tagliaferri et al., 2006). The effects of TBI are extensive, not only for the affected patient but also for the next of kin, furthermore it results in a huge economic burden for society, resulting in increasing indirect costs for rehabilitation and social welfare (Corrigan et al., 2010, Gustavsson et al., 2011, Leibson et al., 2012). Moreover, demographics are changing with a subsequent increase in frequency of TBI among the elderly, increasing the already high morbidity in that affected patient group (Roozenbeek et al., 2013).

In Europe, studies have shown that the hospital admittance of TBI is 235 per 100.000 (Tagliaferri et al., 2006), even if differences exist throughout nations. In Sweden the prevalence is relatively high, with an admittance of about 450 per 100.000 (Andersson et al., 2003, Styrke et al., 2007). Even if the incidence of TBI is high in Sweden, the vast majority of cases have been shown to comprise of mild TBI (97%) (Styrke et al., 2007). Thus, the mortality rate is quite low, Sweden has a median mortality rate of 9.5 per 100.000 being the lowest in the Nordic countries, lower than the European average of about 20 per 100.000 and the US of about 18 per 100.000 (Tagliaferri et al., 2006, Sundstrom et al., 2007, Corrigan et al., 2010, Coronado et al., 2011). The global mortality rate for severe TBI has drastically decreased during the 20th century, but has remained unchanged since the 1990's, and is presently around 20-35 % (Stein et al., 2010, Flynn-O'Brien et al., 2015). While traffic accidents still remains the leading cause globally for TBI (Hofman et al., 2005), the most common cause in Sweden are falls (55%), predominantly among the elderly, while traffic accidents being the most common cause for the young, in total contributing to 30% of all TBI cases (Jacobsson et al., 2007, Styrke et al., 2007).

1.2 THE PRIMARY BRAIN INJURY

As external forces affect the brain, including meninges, parenchyma and surrounding vessels, structures obtain energy, resulting in injuries of different natures. These forces include acceleration/deceleration, blast-waves or objects impacting, or even penetrating, the cranium and distorting the brain tissue. The severity of the injury, and extent of the primary damage, is determined by the duration and intensity of these forces. These injuries will result in either focal injuries, including hematomas, lacerations and contusions, or diffuse injuries leading to cerebral swelling or axonal injury (Nortje and Menon, 2004). Intracranial mass lesions increase the intracranial pressure, hence increasing the risk for subsequent secondary brain damage, and ultimately brain death due to brain stem herniation. Usually, several different types of injuries are present at the same time in TBI patients, presenting a very heterogeneous challenge for the physician.

1.2.1 Focal traumatic injuries

1.2.1.1 Cerebral contusions and traumatic cerebral hemorrhages

Traumatic parenchymal lesions, or contusions (Figure 1), are common after severe TBI; a pooled incidence of 13-35% in severe TBI has been reported (Bullock et al., 2006a). Primarily, contusions are a result of mechanical forces damaging parenchymal blood vessels, leading to micro- and macroscopic hemorrhages. Contusions are usually present in the frontal and temporal lobes, due to movements of the affected brain tissue over irregularities of the skull base (Adams et al., 1980), but depending on the type of impact, they might be present in several areas of the brain. Traumatic parenchymal lesions often evolve, leading to a more extensive mass-effect (Servadei et al., 1995), and subsequently lead to secondary brain injuries, neurological deterioration (Bullock et al., 1989) and a worse outcome (Mathiesen et al., 1995).

1.2.1.2 Subdural hematomas

Subdural hematomas (SDH) are mass lesions located in the subdural compartment between the arachnoidea- and the dura mater (Figure 1), often as a result from tearing of bridging veins and other dural vessels and frequently in combination with other cerebral injuries. Pooled incidence has been shown to be 21% in severe TBI patients (Bullock et al., 2006c). SDH is more common in the elderly compared to the young population (Hanif et al., 2009), presumably as a result of subdural veins in the aged atrophic brain are more vulnerable to straining, even in low energy trauma (Hanif et al., 2009, Evans et al., 2015). In older cohorts, the correlation between presence of subdural hematoma in unconscious patients and mortality has been shown to be as high as 57 - 90% (Seelig et al., 1981), with a marked increase if midline shift is large and the hematoma is not rapidly evacuated.

1.2.1.3 Epidural hematomas

An epidural hematoma (EDH) is located outside the neuro-axis (Figure 1), and is often the cause of the disruption of meningeal arteries (36%) (Servadei et al., 1989), predominantly the middle meningeal artery, but may also be caused by hemorrhage from the middle meningeal vein or other venous sinuses. Present in up to 9% of unconscious TBI patients, EDHs are most common among patients in their twenties, and are rarely seen in patients above 60 years of age (Bullock et al., 2006b). In unconscious patients, with severe TBI and anisocoria, or an EDH with a volume bigger than 30 ml, regardless of consciousness level, the EDH should be evacuated promptly in order to minimize further damage (Bullock et al., 2006b). EDHs have been correlated to better outcome when larger

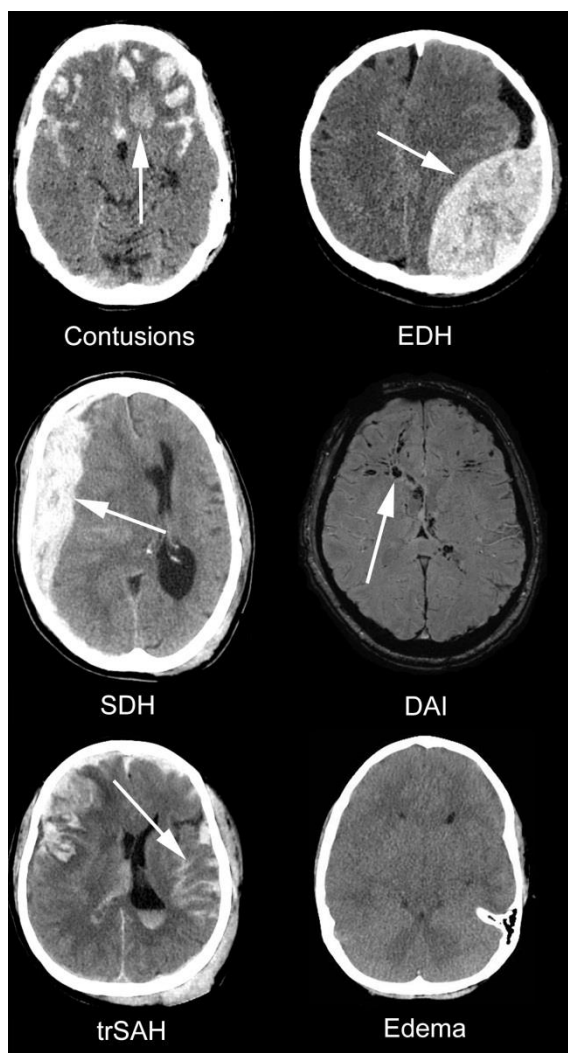


Figure 1 - Computerized tomography illustrating contusions, epidural hematoma (EDH), subdural hematoma (SDH), traumatic subarachnoid hemorrhage (trSAH) and cerebral edema. Magnetic resonance imaging (MRI) presenting diffuse axonal injury (DAI). Pathology is highlighted by white arrows.

retrospective cohorts have been analyzed (Maas et al., 2005, Nelson et al., 2010), possibly since if adequately treated, there is limited damage to the cerebral parenchyma.

1.2.2 Diffuse traumatic injuries

1.2.2.1 Traumatic subarachnoid hemorrhages

Traumatic rupture of subarachnoid arteries and veins are among the most frequent pathologies found among TBI patients subjected to autopsy (Figure 1) (Freytag, 1963). In larger human materials, radiological evidence of traumatic subarachnoid hemorrhage (trSAH) is found in 39% – 52% (Eisenberg et al., 1990, Maas et al., 2005). trSAH has been described, by several studies, as one of the most important factors for an unfavorable outcome following TBI (Maas et al., 2005, Nelson et al., 2010). The presence of trSAH is correlated to the development of secondary ischemic injuries (Harders et al., 1996). This is primarily due to arterial vasospasm, which is present in up to 30% of cases with trSAH, and is more common 4-15 days post TBI (Martin et al., 1997). As a result, inadequate cerebral perfusion subsequently develops, as has also been reported in several cases of spontaneous subarachnoid hemorrhage (Kassell et al., 1985). Different pathophysiological mechanisms could explain the connection between trSAH and vasospasm, including depolarization of smooth muscle cells (Sobey, 2001), endothelin release (Zuccarello et al., 1998), catecholamine surge (Ley et al., 2009) and prostaglandin-induced vasoconstriction (Armstead, 2006). While the calcium inhibitor nimodipine has been shown to decrease the risk of vasospasm following trSAH (Kakarieka, 1997), more recent reviews have failed to show any beneficial treatment effects (Vergouwen et al., 2006).

1.2.2.2 Diffuse axonal injury

First described in the 1940s (Rand and Courville, 1946), traumatic tearing of axons, also called diffuse axonal injury (DAI) (Figure 1), is a common pathology present in up to 70% in closed head injury cases (Skandsen et al., 2010). DAI has been suggested to be the result of mass effect differences between gray and white matter that in rotational injury will slither, and tear, during accelerations (Margulies et al., 1990), resulting in ripped axons. DAI has been graded, dependent on histological presentation: Grade 1, injury to subcortical areas and corpus callosum, Grade 2, with more severe hemorrhagic axonal damage in the corpus callosum, and Grade 3, additional hemorrhages in the rostral brain stem (Adams et al., 1989). When the neuron is damaged, the transport along the axons is altered with subsequent swelling, disrupting which results in a histologically characteristic “bulb” found at the site of disconnection (Christman et al., 1994, Pettus et al., 1994). Moreover, this axonal disconnection has partly been shown to be the result of secondary processes, primarily involving calcium influx, protease activity and subsequent degradation (Buki et al., 1999). Axonal swelling has been shown to commence a few hours after injury (Povlishock and Christman, 1995) but evidence of axonal pathology may be present as late as several months following injury (Blumbergs et al., 1994). Several studies have shown that the presence of DAI on CT, and MRI, is correlated to an unfavorable long-term outcome (Paterakis et al., 2000, Nelson et al., 2010).

1.2.2.3 Cerebral edema

Frequent after TBI, edema formation is caused by parenchymal vascular disruption and osmotic imbalance, increasing the amount of cerebral fluid content (Figure 1) (Unterberg et al., 2004). There are mainly two types of edema affecting the injured brain: Vasogenic and cytotoxic. The vasogenic edema is primarily caused by a vascular collapse affecting the endothelial cellular layer, thereby disrupting the integrity of the blood-brain barrier. Subsequently, an influx of osmotically active ions will accumulate water in the extravascular space (DeWitt and Prough, 2003). Cytotoxic edema is the result of cellular (neuronal, astrocytic, microglial) fluid influx in the CNS, caused by energy depletion, and

thereby ionic pump failure leading to an increased membrane permeability of osmotic substances (Stiefel et al., 2005). The latter type of edema is thought to be more common in TBI, even if both contribute to ischemic events and secondary injury development (Marmarou et al., 2006).

1.2.3 TBI severity classification

The brain injury will affect the patient's clinical abilities. A more severe TBI may result in focal symptoms and loss of consciousness depending on the cerebral structures affected and the extent of the injury, while milder injuries may only lead to nausea, vomiting and headache. At the moment, several classification systems for describing the extent of the TBI exist.

1.2.3.1 Glasgow Coma Scale

The most widely used TBI classification is the Glasgow Coma Scale (GCS, 3-15), assessing the best verbal- (1-5), motor- (1-6) and eye (1-4) response of the patient during physical examination (Table 1) (Teasdale and Jennett, 1974, 1976). The motor response, including pathological extension and flexion motoric patterns in the unconscious patients, reflecting extensive injury, is the component that best correlates to outcome (Ross et al., 1998, Brain Trauma, 2000a). By using these clinical symptoms as surrogate markers of the brain injury severity, it is possible to grade a patient with a positive CT scan into severe (GCS 3-8), moderate (9-13) and mild (14-15) TBI.

Glasgow Coma Scale		
Behavior	Response	Score
Eye opening	Spontaneously	4
	To speech	3
	To pain	2
	No response	1
Verbal response	Oriented to time, place and person	5
	Converses, may be confused	4
	Inappropriate words	3
	Incomprehensible sounds	2
	No response	1
Motor response	Obey commands	6
	Moves to localized pain	5
	Flexion withdrawal from pain	4
	Abnormal flexion	3
	Abnormal extension	2
	No response	1
Total Score		3 - 15
Reaction Level Scale 85		
Consciousness	Response	Score
Conscious	No delay in response	1
	Drowsy or confused	2
	Very drowsy, response to strong stimuli	3
Unconscious	Localizes and ward off pain	4
	Withdrawal from pain	5
	Abnormal flexion	6
	Abnormal extension	7
	No response	8

Table 1 – Glasgow Coma Scale (GCS) and Reaction Level Scale-85

There are however inherent limitations to the GCS classification (Zuercher et al., 2009). A significant difference between pre-resuscitation GCS and in hospital GCS have been detected (Winkler et al., 1984, Majdan et al., 2015), which is probably due to administration of sedatives, alcohol, drugs or other non-cerebral conscious altering events (Stocchetti et al., 2004), even if injury progression cannot be ruled out in individual cases. In some cases, intubation and craniofacial injuries make it impossible to adequately assess GCS. Also, the assessment of GCS has been shown to have a high intra-individual difference among physicians, with many examiners providing GCS scores with low accuracy (Bledsoe et al., 2014).

1.2.3.2 Reaction Level Scale-85

Another clinical assessment score is the Reaction Level Scale 85 (RLS-85, 1-8), based on the GCS (Starmark et al., 1988a) (Table 1), is used nationally in Sweden. This system is better for assessing

intubated patients, as well as patients with swollen eye-lids, not specifically accounting for the verbal or eye command, but grading the score from 1 (alert) to 3 (very drowsy or confused) and then 4 (localizes pain) to 8 (no response to pain) for different levels of unconsciousness (Stalhammar et al., 1988). However, this scoring system shares many similarities with the GCS, especially the motor score evaluation, even if RLS-85 has been shown to have better inter-observer agreement than the GCS score (Starmark et al., 1988b).

1.2.3.3 Abbreviated Injury Scale

Abbreviated Injury Scale (AIS) is another system, ranging from 1 (minimum) – 6 (maximum, fatal) for each injured organ system, including the cerebral injury (Greenspan et al., 1985). It was developed in 1971 in order to aid motor vehicle accident investigators, and the score represents a relative risk of “threat to life”, for a standardized person. The system is based on radiological and clinical criteria which have been shown to correlate with outcome. A head AIS ≥ 3 is considered a severe head injury (Greenspan et al., 1985).

1.3 SECONDARY BRAIN INJURY

Following the initial injury, different pathophysiological processes will commence. These will develop over minutes up to days or weeks (and in some instances years) after the primary brain damage. The severity of the secondary brain injury will be determined by the intensity of these secondary insults during the treatment period (Masel and DeWitt, 2010). Some of the detrimental secondary injuries that may occur in the damaged brain include activation of potentially harmful genes, free-radical generation, calcium-related damage, release of excitatory amino acids, mitochondrial dysfunction, and neuro-inflammatory processes. Often, these processes interact in a synergizing manner, further impairing cerebral tissue, which may lead to secondary injuries, and subsequent permanent cellular death.

1.3.1 The Monro-Kellie doctrine

In general, the central nervous system (CNS) consists of three compartments; blood (venous and arterial) (10%), parenchyma and meninges, (80-85%) and cerebrospinal fluid (CSF) (5-10%). Since the cranium is an enclosed and incompressible space, the relationship between (CSF), blood and brain tissue must all remain in volume equilibrium and the sum of the compartments be constant. This means that if any of the contents within the cranial compartment should increase, as in hydrocephalus and development of hematomas and tumors, it will have a direct effect on the other constituents, which then have to compensate by a decrease in volume. This phenomenon was first described by Alexander Monro, and later confirmed by George Kellie, and is today referred to as the Monro-Kellie doctrine (Mokri, 2001). Using these compensatory mechanisms, primarily by decreasing venous blood flow and CSF, intracranial mass effects may increase in size to quite a degree before the reserves are exhausted, and the intracranial pressure (ICP) will increase. As the ICP increases, the cerebral perfusion pressure (CPP, being calculated as mean arterial pressure (MAP) minus ICP), essential for brain tissue oxygenation will decrease. To adjust for this, expanding mass lesions following TBI could be surgically evacuated. However, in the neuro-intensive care unit (NICU) there are other non-surgical treatments for increasing ICP. On the other hand, if the ICP is allowed to increase, the patient will eventually suffer from Cushing’s triad; an increased pulse pressure with a concomitant raised systolic blood pressure, irregular respirations and bradycardia, as the cerebrum herniates towards vital centers in the medulla and brain stem, subsequently causing circulatory and respiratory arrest (Fodstad et al., 2006).

1.3.2 Cellular pathophysiology

Following the direct tissue damage, the immediate effect of the primary injury, will lead to a devastating environment for the perilesional tissue, not being able to meet blood flow- or metabolic demand. The substrate delivery collapses, creating a harmful acidic cellular environment with increased lactate concentration due to the anaerobic conditions, not adequate to sustain cerebral tissue. This failure leads to membrane depolarization and the release of neurotransmitters which will activate sodium- and calcium channels, henceforth causing an intracellular catabolic process leading to apoptosis or necrosis, of the CNS cells (Werner and Engelhard, 2007).

1.3.3 Secondary insults

During pre-hospital care and in the NICU, secondary events may occur that may exacerbate the primary injury (Miller et al., 1978, Jones et al., 1994) (Table 2). Primarily, these insults may lead to subsequent hypoxic or ischemic damage to the already injured brain (Miller et al., 1978). Increased intracranial pressure is considered the most severe secondary insult and has been extensively correlated to unfavorable

Systemic insults	Intracranial insults
Hypoxia	Cerebral hypoxia
Hypotension	Increased ICP
Anemia	Progression of hematoma
Pyrexia	Seizure
Hyponatremia	Vasospasm
Hypoglycemia	Derranged cerebral metabolism

Table 2 – Some of the secondary insults that TBI patient might suffer from in the NICU.

outcome, however specific limits are a question of debate (Jones et al., 1994, Signorini et al., 1999).

1.3.4 Altered cerebral perfusion

Under normal conditions, the cerebral blood flow (CBF) has been shown to be about 50mL per 100g/min, providing that the CPP is adequate (Phillips and Whisnant, 1992). Studies have shown that both global and focal ischemic events occur following hypoperfusion in TBI, which effect long-term outcome negatively (Inoue et al., 2005). Similar to ischemic stroke, a CBF <15 ml 100g/min will lead to irreversible tissue damage in TBI (Cunningham et al., 2005a). Unfortunately, post-traumatic hypoperfusion is further facilitated by vessel distortion due to mechanical injury and auto-regulatory failure (Rodriguez-Baeza et al., 2003).

On the other hand, cerebral hyperperfusion (CBF >55 ml 100g/min), resulting in hyperemia, is also common following TBI, resulting in unfavorable outcome (Kelly et al., 1996). This phenomenon, which is more common 1-3 days following trauma (Martin et al., 1997) may be as harmful as hypoperfusion, since an auto-regulatory mismatch may relate to vasoparalysis, hence an inability for the brain to control for the cerebral blood volume, which may lead to a life-threatening increase in ICP (Kelly et al., 1997).

1.3.5 Autonomic dysregulation and CO₂-reactivity

Cerebral vessels are dependent on chemo- and mechanical receptors to regulate blood flow due to metabolic demand (Rossanda and Vecchi, 1979). Thus, indicating a higher metabolic rate and subsequent blood flow if the pCO₂ is high and a low blood flow if the pCO₂ is low, something that may be manipulated using hyperventilation in order to manage ICP (Raichle and Plum, 1972). To provide an adequate cerebral perfusion, cerebrovascular auto-regulation and vascular CO₂-reactivity, are important mechanisms to avoid the development of secondary injuries. Unfortunately, blood flow auto-regulation, vascular variability to flow volume, is many times impaired in the injured brain, and may be so up to several days after injury (Lee et al., 2001, Hlatky et al., 2002). This supports the current therapy regimes in providing an adequate perfusion pressure to the injured brain in order to prevent the development of ischemia or hyperemia. However, the cerebrovascular CO₂-reactivity remains

more robust, only affected in the severe TBI, hence being possible to treat patients using hyperventilation (McLaughlin and Marion, 1996), especially in cases of hyperemia since hyperventilation has a more profound effect on the CBF than the ICP (Obrist et al., 1984).

1.3.6 Cerebral metabolic dysfunction

As an effect of the hypoperfusion and hypoxia in TBI, the cerebral metabolism and energy state will be altered in damaged tissue (Cunningham et al., 2005a), reflected by an inadequate substrate delivery, disturbed lactate:pyruvate ratio and affected glucose metabolism (Glenn et al., 2003), with a subsequent effect on outcome (Wu et al., 2004). This energy disturbance might lead to a mitochondrial dysfunction (Cheng et al., 2012), hence a decreased production of adenosine triphosphate (ATP), and subsequent calcium overload in the mitochondria (Verweij et al., 2000). This will lead to further cellular damage, apoptosis and an impaired intracranial status. Also, increased concentrations of glucose (hyperglucolysis) in the injured brain might occur, exceeding the metabolic demand (Bergsneider et al., 1997), perhaps indicating seizure activity or a disturbed metabolic control of the injured brain.

1.3.7 Cerebral Oxygenation

The cerebral oxygen consumption during normal conditions is approximately 3.5 mL per 100 g/min, which is about 20% of the total oxygen consumption of the body, despite the brain accounting for only about 2% of the body weight in an adult. In the injured brain, there is a miss-match between oxygen delivery (decreased) and oxygen consumption (increased), leading to brain tissue hypoxia. A brain tissue oxygen pressure (PBtO₂) >20 mmHg is considered sufficient, and levels below 10 mmHg are considered harmful (Rose et al., 2006, Brain Trauma et al., 2007g). Low levels of PBtO₂ result in ischemic lesions and consequently a worse outcome, even in patients that are hemodynamically stable (Stiefel et al., 2006).

1.3.7.1 Hypoxia-inducible factor-1 alpha

Hypoxia-inducible factor-1 alpha (HIF-1 α), is a transcription factor that is involved in oxygen homeostasis (Wang and Semenza, 1995) and provides an adaptive response to the unfavorable conditions present during hypoxia (Singh et al., 2012). When oxygen saturation drops, HIF-1 α triggers the up-regulation of several genes, which in severe hypoxia may result in activation of detrimental cellular pathways leading to apoptosis (Chen et al., 2007). However, in milder hypoxic states (Fan et al., 2009, Singh et al., 2012), it may promote neuro-protective effects including angiogenesis via the production of vascular endothelial growth factor (VEGF) (Liu et al., 1995), erythropoiesis via induction of erythropoietin (Wenger, 2002, Shein et al., 2005), mitochondrial function (Ebert et al., 1995) and cell survival (Lawrence et al., 1996).

1.3.8 Blood-brain barrier disintegration in TBI

Surrounding the vessels of the brain are tightly connected endothelial cells and astrocytes connected by tight junctions, making up the blood-brain barrier (BBB). The BBB is responsible for creating a highly restricted environment in the CNS as it regulates the entry of blood-borne metabolites and immune cells, regulating the cerebral environment with an influx of vital substrates and secretion of waste products. The astrocytic podocytes, covering the BBB, as well as microglial cells and the basal cell membrane of endothelial cells are an essential part of the BBB transportation, connecting to parenchymal microvessels (Lassmann et al., 1991, Abbott et al., 2006).

Following injury, there is a disruption of the vascular integrity, functional changes in the pericontusional area and an increased permeability of the BBB to high molecular weight proteins, such as albumin due primary to functional changes (Chodobski et al., 2011). Rat models of TBI

have revealed an increased permeability 4-6 hours after injury, with a secondary peak after 3 days (Shapira et al., 1993, Baskaya et al., 1997, Hicks et al., 1997), while in humans, elevated albumin quota (Q_A) between CSF:serum, is detected up to a week following TBI (Bellander et al., 2011). The disruption of BBB also results in edema development (Unterberg et al., 2004).

1.3.9 The glymphatic system following TBI

A route between the interstitial fluid of the brain, cerebrospinal fluid and venous outflow has recently been discovered, entitled the glymphatic system because of the connection between glial cells and aquaporin-4(AQP4)-dependent paravascular pathways (mimicking a lymphatic drainage from the brain) (Iliff et al., 2012). This para-arterial influx of CSF through the brain extracellular fluid (ECF) to a para-venous outflow has been suggested to be the main efflux of cerebral protein debris (Nedergaard, 2013), and is driven by arterial pulsations (Iliff et al., 2013).

TBI has shown to result in a lost perivascular polarization of AQP4 up to 28 days after injury (Ren et al., 2013), yielding a decreased outflow of tau proteins after TBI (Iliff et al., 2014). A recent study shows that the glymphatic system works independently from BBB integrity following brain injury and that proteins of cerebral origin predominantly drain through the glymphatic system from the injured brain (Plog et al., 2015).

1.3.10 Excitatory amino acids and oxidative stress

Glutamate is the most prominent excitatory neurotransmitter substance in the human brain (Faden et al., 1989), which, together with aspartate, is excessively released as a result of injury in the perilesional area (Bullock et al., 1998). The rapid increase affects cerebral cells, over-stimulating glutamate receptors, leading to an influx of osmotically active ions, primarily sodium and calcium (Floyd et al., 2005), triggering successive catabolic breakdown. Reactive oxygen species (ROS) (oxygen radicals including hydrogen peroxide, superoxides, nitric oxide and peroxynitrite) have been shown to generate oxidative stress in the pericontusional area following TBI, leading to vascular-, membrane-, protein- and genomic injury due to peroxidation (Lewen and Hillered, 1998, Bayir et al., 2005, Chong et al., 2005, Shao et al., 2006).

1.3.11 Coagulopathy in TBI

The effect on the systemic coagulation following TBI has been previously reviewed (Harhangi et al., 2008, Kurland et al., 2012, Zhang et al., 2012), and is a common issue in operating theatres and intensive care units worldwide.

In healthy humans, clot formation and fibrinolysis are in balance as not to develop excessive hemorrhage or thrombo-embolic events (Bennett and Ratnoff, 1972). The pathophysiology of TBI might lead to both a hyper- and a hypocoagulative state (Touho et al., 1986). To add insult to injury, activated endothelial factors (Sulfonylurea receptor 1, SUR-1) in border zones surrounding the

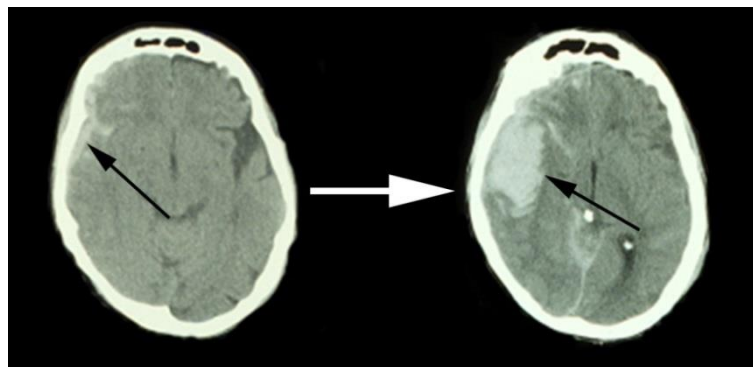


Figure 2 - The left picture reveals a small contusion and SDH, while the right shows a substantial progression of the intracranial hemorrhages (black arrow) in a patient suffering from TBI.

contusions, may lead to cell death through hemorrhagic necrosis and structural failure of microvessels (Simard et al., 2009, Patel et al., 2010). Also, the aggregative capabilities of thrombocytes are affected following TBI, with loss of the thromboxane A_2 -receptor function and/or an impairment of

platelet cyclooxygenase (Nekludov et al., 2007). Even though this process is not fully understood, its effect has been suggested to come from circulating micro particles which affect the coagulative capabilities following TBI (Nekludov et al., 2014).

1.3.11.1 Progressive intracranial hemorrhage

After impact, the inflicted injuries will lead to hematomas and lesions that may progress in size over time (Alahmadi et al., 2010), which has been shown in up to 80% of TBI patients (Figure 2) (Chieragato et al., 2005). Because of this, many early CT-scans may provide a result that does not fully reflect the true size of the fully developed hematoma (Oertel et al., 2002), which is why a limit of 90-120 minutes after injury has been suggested to perform radiological examinations to more accurately illustrate the extent of intracranial lesions (Oertel et al., 2002, Velmahos et al., 2006). However, while the evolution of an intracranial hemorrhage is usually an ongoing process early after injury, it may occur as late as 4 days following TBI (Kurland et al., 2012). If coagulopathy is detected (increased prothrombin time (INR), increased activated partial thromboplastin time (APT-T) and low platelet count), the risk of the patient to develop a progressive intracranial hemorrhage is increased, with up to 31% (Stein et al., 1992), and is correlated to an unfavorable outcome (Greuters et al., 2011). In the IMPACT-material, where available, increased INR was found in 26% while low platelet was found count in 7% (Van Beek et al., 2007), both correlated with an unfavorable outcome.

1.3.11.2 Post-traumatic cerebral infarctions

Ischemic injuries, due to vascular complications such as mechanical compression, thromboembolic events, venous stasis, or vasospasm in cerebral vessels are common in autopsy material from TBI patients (up to 90%) (Graham et al., 1978), and are seen in 10-20% during and after treatment in patients suffering from TBI (Figure 3) (Mirvis et al., 1990, Marino et al., 2006, Tawil et al., 2008, Tian et al., 2008, Chen et al., 2013). Thrombocytopenia, elevated

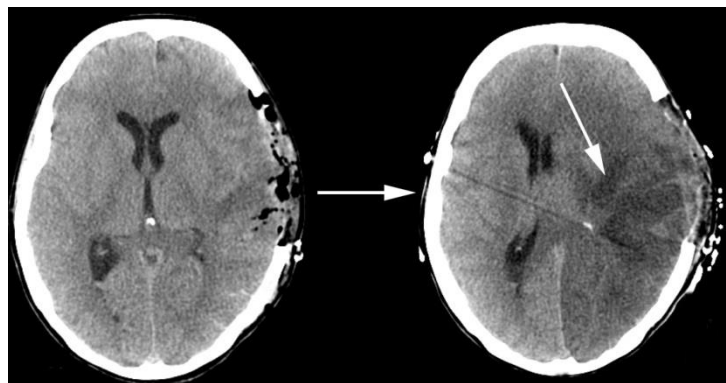


Figure 3 - The left picture shows an injured brain following surgery. The right picture shows the same patient developing a post-traumatic cerebral infarction of the left hemisphere, as indicated by the white arrow.

APT-T and increased D-dimer are seen in patients demonstrating post-traumatic cerebral infarction (PTCI), suggesting disseminated intravascular coagulation (DIC) as a conceivable cause in some cases (Chen et al., 2013). PTCI have also been shown to affect major cerebral vascular territories, such as the middle cerebral artery or smaller cerebral vascular systems (Server et al., 2001), detrimentally altered by increased pressure and subsequent herniation by lobes against the rigid tentorium (Rothfus et al., 1987), common for the posterior cerebral artery vascular territory (Ham et al., 2011). Even with aggressive decompressive treatment, the outcome for these patients are generally unfavorable (Ham et al., 2011). PTCI may also occur due to vasospasm as a consequence of trSAH (Kakarieka, 1997).

1.4 CEREBRAL INFLAMMATION FOLLOWING TBI

The brain has long been considered a privileged organ in concern to systemic inflammatory reactions, being surrounded by a shielding blood-brain barrier regulating the metabolism and flow of substrates, and being protected by its own immune system, consisting primarily of microglial cells (Benarroch, 2013). However, the last decades have shed new light on the complex mechanism of neuro-inflammation, being involved in a number of diseases, including, among others, multiple sclerosis

(MS) (Xiao and Link, 1999). The inflammatory response to TBI has been reviewed extensively (Morganti-Kossmann et al., 2001, Morganti-Kossmann et al., 2002, Ziebell and Morganti-Kossmann, 2010, Helmy et al., 2011b, Woodcock and Morganti-Kossmann, 2013), highlighting both beneficial and detrimental effects of the neuro-inflammatory response following TBI.

1.4.1 The innate immune system in TBI

Being unspecific to pathogens, the innate immune system is comprised of physical barriers, inflammatory cells, anti-bacterial peptides, complement proteins and cytokines, protecting the human body. A disruption of the BBB will lead to a facilitated transport of cells and inflammatory proteins to the brain parenchyma. It is important to note that the inflammatory cascade is complex, with probably several unknown interactions between involved immunological agents that have yet to be identified.

1.4.1.1 Cytokines

Cytokines are short-lived, small signaling proteins that form a multifaceted network of inflammatory processes, being both pro- and anti-inflammatory. A recent review have shown that Interleukin (IL)-1 beta, IL-6, IL-8, IL-10, tumor necrosis factor alpha (TNF-A), all potent mediators of inflammation, are increased in response to the severity of the traumatic brain injury, suggesting a major role of the innate immune system in TBI (Helmy et al., 2011b, Woodcock and Morganti-Kossmann, 2013). Measurements of intracranial cytokine levels using the microdialysis (MD) technique reveal a temporal patterns in humans (Helmy et al., 2011a), presumably highlighting an ongoing role in the pathophysiology of TBI.

1.4.1.2 Neutrophil granulocytes

Immediately following TBI and disruption of the BBB, neutrophil granulocytes, the most abundant type of white blood cell, will infiltrate the cerebral tissue (Holmin et al., 1995, Holmin et al., 1998), a transport facilitated by chemotaxins (CXCL1) (Szmydynger-Chodobska et al., 2009) and activated vascular adhesion molecules (ICAM-1) (Carlos et al., 1997). A reduction of neutrophils in the brain parenchyma is possible to achieve using a soluble human recombinant complement receptor SCR1, and has been shown to correlate to less edema formation and tissue loss, probably due to the cytotoxic nature of granulocytes (Kenne et al., 2012). The concentration of neutrophils start to increase adjacent to the injury immediately to 24 hours after trauma (Al Nimer et al., 2013, Schwarzmaier et al., 2013), and has been shown to increase up to 7 days following injury, and thereafter steadily decline (Holmin et al., 1995, Bellander et al., 2010).

1.4.1.3 Macrophages

Macrophages have been shown to swiftly (within 12 hours) migrate to the injured area in the CNS following injury (Zhang et al., 2006), and to be able to phagocyte tissue debris and promote regeneration (David et al., 1990), thus having beneficial effects in the injured CNS. However, macrophages have also been shown to release different cytokines in the perilesional tissue (Turtzo et al., 2014), promoting inflammation and increase tissue loss (Lehrmann et al., 1997). The peak migration of macrophages have been seen in the lesion area 7 to 14 days after injury (Bellander et al., 2010), exacerbated by diffuse traumatic hypoxic injury (Hellewell et al., 2010).

1.4.1.4 Complement system activation

The complement system, a highly regulated cascade of the innate immune system, has also been shown to play an active role in TBI (Stahel et al., 1998, Helmy et al., 2011b, Brennan et al., 2012). These immunological active molecules are predominantly synthesized in the liver, and are involved in agglutination of pathogens, chemotaxis, opsonization and direct lysis of cells, having an active role in

both the innate and adaptive immunological response to TBI. The complement system is triggered, and activated, by three distinct pathways; the classical pathway (by the factor C1 and C4, because of immunoglobulin (Ig) M or G binding), the alternative pathway (by the factor C3) and the lectin pathway. The following cascade of chemotaxis, and opsonization, substances is tightly regulated, ending up with C5b9, the Membrane Attack Complex (MAC), which will cause a lysis of cells (for review, see (Ricklin et al., 2010)). C1q, C3, C4, C3b, C3d and C5b9 all have been noted to be upregulated in the border zone surrounding contusions in human TBI (Bellander et al., 2001), indicating it to have an important role in the evolving injury process. Moreover, in the CSF, C5b9 is elevated following TBI (Stahel et al., 2001) and has been correlated to increased intracranial pressure following severe TBI (Bellander et al., 2011). In an in vitro model, C1q and C5b9 have been shown to be upregulated in the perilesional area for up to 7 days after experimental injury in the absence of circulatory blood (Bellander et al., 2004a). The MAC is more prominent in the secondary injury development in focal injury, compared to diffuse axonal injury (Rostami et al., 2013). Also, by pharmaceutically inhibiting MAC, using a C6 antisense oligonucleotide, an increased neuronal and axonal survival has been seen following TBI (Fluiter et al., 2014).

1.4.1.5 Microglia

Being the primary immune cell in the CNS in mediating response to infection and injury, microglia plays an important role in TBI migrating to the damaged area, forming a line of defense to protect surviving cerebral tissue (Cunningham et al., 2005b, Davalos et al., 2005). The up-regulation of microglia is 1-3 days after TBI, but has been shown to continue up to 28 days (Bellander et al., 2010). Moreover, studies in humans have shown an increased microglia and macrophage activity up to 1 year after injury, indicating an ongoing immunological process (Smith et al., 2013), a pro-inflammatory condition that might be chronic (Ramackhansingh et al., 2011). Also, hypoxia has been seen to aggravate the microglial response to injury in neonatal rats (Leonardo et al., 2008).

1.4.2 Adaptive immune response in TBI

While originally being considered immune-privileged, we today know that several diseases in the CNS are dependent on the adaptive immune system (Xiao and Link, 1999) and the severity has been connected to the amount of infiltrating T-cells (McGavern and Truong, 2004). It has been suggested that the brain injury itself creates an early low-grade MS lesion, albeit the extent, and the role of the adaptive immune system in TBI, are not fully understood (Ling et al., 2006). CD4-positive and CD8-positive, regulatory and cytotoxic T-cells, have been shown upregulated in the proximity to traumatic contusions 3 to 5 days following TBI and are thought to be involved in the ongoing, inflammatory cerebral response (Holmin et al., 1998).

1.4.3 Cellular death in TBI

The primary brain injury will lead to an immediate loss of viable cerebral tissue. However, the ongoing secondary injury cascade may lead to a subsequent development of cellular death in the CNS (Zhang et al., 2005, Stoica and Faden, 2010), up to a year following TBI (Williams et al., 2001). The dominant forms of cellular death described in TBI are mainly apoptosis and necrosis, while autophagic cell death and necroptosis also have been identified.

1.4.3.1 Necrosis and necroptosis

Immediately following trauma, necrosis occurs, as cell dies due to ischemic and mechanical tissue damages resulting in karyolysis and cell swelling (Fink and Cookson, 2005). The cell dissolves and different cytotoxic proteases and peroxidases will be released and thus further aggravate the cytotoxic environment in the injured tissue (Trump et al., 1997). A special kind of controlled necrosis has been

described, labeled necroptosis, characterized by a morphologic death similar to necrosis (energy independent) yet regulated by Necrostatin-1 (Degterev et al., 2005, Li et al., 2008).

1.4.3.2 Autophagy

Autophagic death, a degradation of cellular organelles and proteins in cells during intense stress, have been detected up to 24 hours after TBI (Liu et al., 2008), and contributes to outcome in animal TBI models (Luo et al., 2011). Autophagic cell death may be present at the same time as apoptosis (Uchiyama et al., 2008), but has been suggested more neuro-protective since it maintains a cellular hemostasis yet is energy dependent unlike necrosis. However, if the energy is depleted during the autophagic process, necroptosis will occur instead (Amaravadi and Thompson, 2007).

1.4.3.3 Apoptosis

Apoptosis, was first described 1972 as a programmed cell death presenting morphological changes, nuclear condensation, vesicle formation and cell shrinkage, and eventually phagocytosis by macrophages (Kerr et al., 1972). Its role in TBI has been thoroughly reviewed (Raghupathi et al., 2000, Wong et al., 2005). Apoptosis may be activated through several different pathways, the consecutive activation of caspases (primarily caspase 3), through either the activation of “death receptor” ligation (extrinsic pathway) or mitochondrial disruption (intrinsic pathway) (Eldadah and Faden, 2000). While perilesional cortical neurons are susceptible to secondary injury mechanism, with an increased apoptotic frequency days after trauma, thalamic and hippocampal neurons are also sensitive to apoptosis, which have been shown in several animal- (Nawashiro et al., 1995, Clark et al., 1997a, Bramlett et al., 1999b) and human studies (Kotapka et al., 1992, Ross et al., 1993), probably due to their high metabolic rate and substrate demand. Several drugs, primarily caspase inhibitors, have been proposed as potential therapeutic targets following TBI in order to limit the extent of apoptosis in experimental models (Eldadah and Faden, 2000), none has yet shown an improved clinical outcome in human TBI.

1.5 ANIMAL MODELS OF TBI

Patients being admitted for TBI present with various injury severities, different injury types, different ages and sex, genetic backgrounds and often with multiple trauma of extracranial origin. This heterogeneity makes it difficult to generalize findings and to perform clinical trials. To better reproduce the different conditions present at impact, animal models (using primarily rodents) have been created that mimic the affecting forces and settings, hence being able to monitor pathophysiological processes in TBI. However, the transition from animal to human models and trials has been shown to be difficult, presumably due to inherent discrepancies between species (Bullock et al., 1999), partly due to metabolic and age differences (Sengupta, 2013).

1.5.1 Focal TBI models

1.5.1.1 Weight drop device

By dropping a free falling weight on to the exposed brain, a contusion type injury is formed (Feeney et al., 1981). The severity can be adjusted, using different weights and heights, however it has seen to cause “rebound” injuries from the falling weight which is a limitation and makes it difficult to control the reproducibility. Nevertheless, it is easily accessible and is fairly cheap, making it a common rodent brain injury model.

1.5.1.2 Controlled cortical impact

A controlled cortical impact (CCI) uses a device to impact the cortex of brain during a controlled fashion, and might be considered an upgrade from the drop-device and fluid percussion injury models (Dixon et al., 1991). Usually, the CCI impact is performed on an area adjacent to the midline. A computer controlled piston will impact the exposed dura, with velocity, severity and depth of injury determined by the user (Gilmer et al., 2009). In comparison to the fluid percussion injury, the CCI injury is more focal.

1.5.1.3 Penetrating brain injury

Missiles, gunshots and other sharp objects may result in penetrating brain injuries (2001). Historically, large animal models have been used to mimic these conditions (Crockard et al., 1977). Today, small animal models of penetrating brain injury, where a sharp metal object penetrates the brain, have been well described regarding lesion size, edema and neuro-degeneration (Plantman et al., 2012, Cernak et al., 2014). Like the CCI, the penetrating brain injury is considered a focal injury.

1.5.2 Diffuse TBI models

1.5.2.1 Fluid percussion injury

In order to create an experimental brain concussion, a state frequently observed in the more common mild TBI, the fluid percussion device has been suggested (Sullivan et al., 1976). After having performed a craniectomy around the midline (or more laterally) and exposed the dura, a cylindrical reservoir saline reservoir is attached. A strike at the other end of the cylindrical reservoir creates a pressure pulse which will travel to the intact dura and result in cerebral deformation. The injury has been shown to mimic a contusion injury as well as to have a diffuse injury pathophysiology (Thompson et al., 2005).

1.5.2.2 DAI-models of injury

DAI is the result of accelerations of the brain parenchyma, due to mass effect discrepancies which will tear and damage axons. In order to mimic these circumstances in a rodent, without hemorrhages or focal injuries, several models have been developed (Marmarou et al., 1994, Cernak et al., 2004, Davidsson and Risling, 2011). The most common models used are variations of the model described by Marmarou et al where a disc is being attached to the head of an animal, where later on a weight is dropped, hence resulting in an impact that will yield axonal injuries in the brain stem, corpus callosum, basal ganglia and subcortical tracts (Marmarou et al., 1994).

1.5.2.3 Blast injury

Improvised explosive devices, and conventional weapons, create blast injuries which through a shock-wave will injure the brain (Langlois et al., 2006). These blast injuries create diffuse injuries, similar to the diffuse axonal injuries, and have been reproduced in detail in animal models following shock-wave injuries (Risling et al., 2011, Gunther et al., 2014).

1.6 TBI MANAGEMENT

1.6.1 Pre-hospital management

In order to provide an optimal treatment immediately after injury to TBI patients, evidence based management, resuscitation and central nervous system protection, should be initiated at the scene of accident (Badjatia et al., 2008). Secondary insults, if present at the scene of accident, have been

shown to lead to the development of secondary injuries and negatively affect patient outcome (Cooke et al., 1995, Stocchetti et al., 1996).

1.6.1.1 Hypotension

A low blood pressure, hypotension, due to hypovolemia, decreased cardiac output or other systemic dysfunctions, might lead to an inadequate cerebral perfusion. If the brain does not sustain a satisfactory blood flow, it may lead to insufficient substrate and oxygen delivery, which in turn may result in tissue ischemia.

Studies have shown that a cut-off of <90 mmHg systolic blood pressure, at the scene of accident on patients suffering from TBI, is independently correlated to an unfavorable outcome (Chesnut et al., 1993, McHugh et al., 2007b). Between 18%, up to 35%, of TBI patients suffer from pre-hospital hypotension, presumably as a result of concomitant injuries (Chesnut et al., 1993, McHugh et al., 2007b).

1.6.1.2 Hypoxia

A decreased level of oxygen saturation in the brain parenchyma, cerebral hypoxia, may be the result of an obstructed airway, respiratory failure or any other damages to the lungs or associated vessels. Also, a lower surrounding oxygen pressure, used in models of hypoxic TBI, will mimic the conditions present during pre-hospital hypoxic situations.

Studies have shown that 20% - 45% of TBI patients suffer from pre-hospital hypoxia (Jeremitsky et al., 2003, McHugh et al., 2007b), a condition which subsequently has been shown to correlate with worse outcome in humans (Jones et al., 1994, Chi et al., 2006, McHugh et al., 2007b, Yan et al., 2014). A cut-off of 90% oxygen saturation, and airway obstruction, are often defined as significant pre-hospital hypoxia. Hypoxia may lead to cerebral ischemia, an irreversible secondary brain injury frequently seen in autopsy materials of TBI patients (Graham et al., 1989). In experimental conditions, hypoxic TBI seems to lead to an exacerbated cerebral inflammation (Hellewell et al., 2010, Yan et al., 2011), an aggravated neuronal death and lesion size (Yamamoto et al., 1999, Matsushita et al., 2001, Gao et al., 2010, Hellewell et al., 2010), and having a detrimental effect on the BBB and edema formation (Ishige et al., 1987, Tanno et al., 1992, Van Putten et al., 2005, Yan et al., 2011), as well as leading to worse functional outcomes (Ishige et al., 1987, Clark et al., 1997b, Bramlett et al., 1999a, Hallam et al., 2004, Hellewell et al., 2010, Yan et al., 2011).

1.6.1.3 Combination of hypoxia and hypotension

Patients that suffer from both low blood pressure and oxygen saturation at the scene of accident are in greater risk of ending up with an unfavorable outcome (Miller et al., 1978, Chesnut et al., 1993, McHugh et al., 2007b). Of the two, hypotension has been shown to be better correlated with poor outcome (Manley et al., 2001), perhaps as an aggregate for more extensive and severe intracranial- and extracranial injury. In animal models, a general cerebral hypoxia has been seen to generate a systemic hypotension (Clark et al., 1997b, Bramlett et al., 1999a) perhaps indicating a greater co-existence of the two secondary insults than what is commonly described.

1.6.1.4 Hypothermia

Patients suffering from hypothermia at the scene of accident, present in about 10% of cases, more common during winter, have been seen to correlate to an unfavorable outcome to the same extent as hypoxia and hypotension (McHugh et al., 2007b, Tohme et al., 2014). This finding could partially be a result of extended time between the traumatic insult and the initial/or definitive care, potentially worsening secondary injuries.

1.6.2 Radiological examinations

1.6.2.1 Computerized tomography

The computerized tomography (CT) scan of the head was first introduced in the early 1970s (Ambrose, 1973, Ambrose and Hounsfield, 1973) but has since then been extensively modified and updated to become the important tool it is today for diagnosing neurological pathology. Due to its availability, speed and accuracy to detect acute cerebral conditions, it's the modality of choice in the emergency setting (Wilson, 2009). Using a scanning X-ray tube circulating around the head, two detectors on the opposite side will absorb the radiation, as described by Hounsfield (Hounsfield, 1973). The absorption will be dependent on the intensity of X-rays at the source and the intensity of X-rays hit by the detector, air will not absorb any X-rays while tissue will absorb more. The amount of absorption is called Hounsfield units, where air has -1000, water 0, bone 500 to 3000, and brain parenchyma 20-45 (Ambrose, 1973, Hounsfield, 1973). Therefore, acute hemorrhages, and the bone, appear hyperdense while edema and ischemia hypodense as they contain more water (Ambrose, 1973). Today, multi-detector CT (MDCT) scanners provide a rapid acquisition of several submillimeter axial-section data from a single gantry rotation, hence being able to reconstruct the injury into different 2D and 3D pictures (Kubal, 2012), substantially improving the diagnostics of TBI patients.

1.6.2.2 Magnetic resonance imaging

The magnetic resonance imaging technique (MRI), or nuclear magnetic resonance as it was originally called, was developed by Lauterbur in 1973 (Lauterbur, 1973). MRI was later on introduced clinically in the 1980s and the different techniques associated with MRI have developed and improved tremendously since then.

The principle behind MRI is to use radiofrequency pulses in a magnetic field which will excite hydrogen nuclei (protons) residing in water molecules in the body. The magnetic field and pulses may be modulated to acquire a spatially recorded image. Depending on the density of protons, there will be different intensity on images. Two different visualization protocols are often used, T1 and T2, depending on the time taken for proton relaxation. For instance, T2 is bright in areas with high water content (as in the CSF). Different techniques have developed over the years, including diffused weighted imaging (DWI), useful to detect edema and stroke (Lansberg et al., 2000, Unterberg et al., 2004, Le and Gean, 2009), susceptible weighted imaging (SWI), to detect micro-hemorrhages in diffuse axonal injury (Haacke et al., 2009) and diffuse tensor imaging (DTI) to measure white matter integrity, hence axonal disruption (Basser et al., 2000, Shin et al., 2012). In DWI, the water diffusion rate is measured for every image element (referred to as voxels), making it interesting in pathologies where cellular environments are affected, such as edema and ischemia (Lansberg et al., 2000, Unterberg et al., 2004). Diffusion is then calculated in apparent diffusion coefficients (ADC) which measures differences of water content within the analyzed tissue (Sener, 2001). DTI is similar to DWI, but is capable to detect and visualize homologous structures within the brain, such as axons, since the water content will diffuse more rapidly in the direction of the axonal structure. SWI detects susceptibility differences in the brain, following different image post-processes, making it very sensitive in the detection of iron deposits and hemorrhages. Another technique, called fluid attenuated inversion recovery (FLAIR), uses a protocol that nullifies fluid, thus making it possible to remove the effect of CSF, and may thus better visualize periventricular lesions present in e.g. multiple sclerosis (De Coene et al., 1992).

MRI has better resolution than CT scans, and also a better contrast between gray and white matter, making MRI superior to CT scans in detecting edema (Unterberg et al., 2004), even differentiating them between vasogenic and cytotoxic, and ischemic injuries (Lansberg et al., 2000). However, the imaging technique is slower and more expensive, and not as accurate as CT to detect traumatic

subarachnoid hemorrhages (Snow et al., 1986) and fractures (Roguski et al., 2015), thus not readily an option in the emergency setting.

1.6.3 Radiological grading of injury

By examining large cohort of TBI patients, different protocols have been formed which may facilitate interpretation and prognostication of TBI.

1.6.3.1 Marshall CT-score

Today, the dominant scoring system used is the Marshall CT-score (Table 3) (Marshall et al., 1991). Generally, it is divided into no visible injury, diffuse- (3 steps, depending on severity) and focal injury (any lesion $>25 \text{ mm}^3$) related to mortality and is based on patients in the extensive Traumatic Coma Data Bank (TCDB) study (Marshall et al., 1983). Moreover, “evacuated mass lesion” is also noted in the scale. They authors detected an increase in unfavorable outcome as the diffuse swelling exacerbated, suggesting it an indirect sign of increased ICP (Marshall et al., 1991). However, later studies have revealed a limited capacity in outcome prediction using the Marshall CT-score compared to the Rotterdam- and Stockholm CT-scoring systems (Maas et al., 2005, Nelson et al., 2010).

1.6.3.2 Rotterdam CT-score

By using the Marshall CT-score variables, Maas et al pioneered the Rotterdam CT-score (Table 3) (Maas et al., 2005). This systems re-weights the parameters into a graded scale from 1 (best) to 6 (worse) where trSAH, basal cistern compression and midline shift ($>5\text{mm}$) are dichotomized as unfavorable parameters while the presence of EDH to have a favorable correlation towards outcome. EDH has in bigger patient materials been shown to be a positive predictor of outcome (Maas et al., 2007), presumably since in these patients, if treatment is rapidly provided, the brain parenchyma will remain relatively uninjured.

1.6.3.3 Stockholm CT-score

The Stockholm CT-score was introduced in 2010 and, in comparison to the other systems, uses a continuous scale to grade severity (Table 3) (Nelson et al., 2010). The scoring system uses the magnitude of midline shift, as an aggregate of all focal and diffuse lesions, although the presence of EDH is favorable, as well as its own grading of subarachnoid hemorrhage and is the only CT-scoring system to include DAI visible on CT scans as a negative predictor (Nelson et al., 2010).

Marshall CT classification	
Diffuse injury I	No visible intracranial pathology detected on CT scan
Diffuse injury II	Cisterns are present with midline shift of 0-5 mm and/or lesions densities present; no high or mixed density lesion $>25 \text{ cm}^3$ may include bone fragments and foreign bodies
Diffuse injury III	Cisterns compressed or absent with midline shift of 0-5 mm; no high or mixed density lesion $>25 \text{ cm}^3$
Diffuse injury IV	Midline shift $>5 \text{ mm}$; no high or mixed density lesion $>25 \text{ cm}^3$
Evacuated mass lesion (V)	Any lesion surgically evacuated
Non-evacuated mass lesion (VI)	High or mixed density lesion $>25 \text{ cm}^3$; not surgically evacuated
Rotterdam CT score	
Basal cisterns	0 = normal, 1 = compressed, 2 = absent
Midline shift	0 = $\leq 5 \text{ mm}$ shift, 1 = $>5 \text{ mm}$
Epidural mass lesion	0 = present, 1 = absent
Intraventricular/subarachnoid blood	0 = basent, 1 = present
Final sum + 1 = Rotterdam CT score	
Stockholm CT score (tally)	
Increasing sum results in an increased risk of unfavorable outcome	
(Midline shift (in mm))/10 + (extent of subarachnoid hemorrhage)/2 - 1 (if EDH + 1 (if DAI) + 1 (if dual-sided SDH)	
Final sum + 1 = Stockholm CT score (tally)	

Table 3 – Describing the Marshall-, Rotterdam- and Stockholm CT scoring classification systems.

1.6.4 The neuro-intensive care unit – Multimodal monitoring

The main goal with neuro-intensive care is to minimize the burden of secondary insults in order to prevent the development of permanent secondary injuries (Helmy et al., 2007). To improve the neuro-intensive care, evidence based guidelines have been introduced by the Brain Trauma Foundation (BTF) (Brain Trauma, 2000a, Brain Trauma et al., 2007a), where departments that have adhered to these regimes have shown an improved outcome (Hesdorffer and Ghajar, 2007). These therapies aim to improve conditions by emphasizing intracranial monitoring, and the necessity to optimize cerebral perfusion and regulate intracranial pressure. By setting up clinically important threshold levels for blood pressure (Brain Trauma et al., 2007b), intracranial pressure (Brain Trauma et al., 2007f), cerebral perfusion pressure (Brain Trauma et al., 2007d), cerebral oxygenation (Brain Trauma et al., 2007g) as well as evaluating current therapies, including hyperventilation (Brain Trauma et al., 2007j), hyperosmolar therapies (Brain Trauma et al., 2007c), anti-seizure prophylaxis (Brain Trauma et al., 2007i) and nutrition (Brain Trauma et al., 2007h), it provides physicians with a robust tool to treat patients suffering from moderate and severe TBI.

Other therapies exist, including the Swedish “Lund-concept”, which emphasizes, in contrast to the BTF-guidelines, that ICP reducing therapy should start immediately following admission, even during normal intracranial conditions, in order to maintain normal physiology in the injured brain, minimize future cerebral swelling, and hence prevent a potentially low CPP (Asgeirsson et al., 1994).

1.6.4.1 Measuring Intracranial pressure

The ICP, and thereby also the CPP, is commonly measured using a ventricular catheter (extra ventricular drain, EVD), being introduced in the 1960s (Lundberg, 1960). In cases where performing a ventriculostomy is not feasible, an intraparenchymal pressure device may be used, though they have been shown to have higher internal variation of ICP levels compared to EVDs (Brain Trauma et al., 2007e). Many therapies are today focused on decreasing ICP, since increased levels, >20-25 mmHg, have been shown to negatively affect outcome (Brain Trauma et al., 2007f). Surgical methods, removing space occupying mass lesions, are obvious in the acute care of TBI patients in order to decrease potentially fatal intracranial pressure. However, sedation using certain pharmaceuticals, mainly propofol, midazolam and barbiturates, have shown to decrease metabolic demand (Stewart et al., 1994), and to lower ICP (Eisenberg et al., 1988) (Nugent et al., 1982, Herregods et al., 1988). Moreover, hyperosmolar therapy, using mannitol or hypertonic saline (HTS) to osmotically extract fluid from the intracranial space, is used to decrease ICP, with HTS being suggested to be the new golden-standard therapy (Marko, 2012).

1.6.4.2 Measuring Intracranial blood flow

It is possible to monitor the cerebral blood flow continuously using a surgically implanted cerebral thermal diffusion probe (TDP; Hemedex® Cambridge, Massachusetts, USA) (Vajkoczy et al., 2000), albeit only monitoring a very regional area. Transcranial Doppler (TCD), a non-invasive way to measure flow velocities in major cerebral vessels, is also used to detect intracranial vasospasm and to assess cerebral auto-regulation (Bouzat et al., 2014).

1.6.4.3 Measuring brain oxygenation

Approximately 25% of the patients suffering from TBI, and presenting normal ICP, still suffer from cerebral hypoxia (Stiefel et al., 2006). In order to monitor the $PBtO_2$, it's possible to surgically implant catheters in affected brain areas (LICOX®, IntegraLifeSciences, Plainsboro, NJ, USA or Neurotrend, Diametrics Medical, St Paul, MN) to make sure that the damaged brain does not suffer from hypoxia. Levels of $PBtO_2$ below 15(-10) mmHg (Dings et al., 1998, Valadka et al., 1998) have been correlated to hypoxia, and an unfavorable long term outcome (Maloney-Wilensky et al., 2009), and should thus

be avoided. If PbO_2 decreases, it could be treated by decreasing ICP (or increasing CPP), or to improve systemic respiratory conditions. Hyperoxia is not recommended as it does not lead to an improved cerebral metabolism (Diringer et al., 2007), but instead impaired cerebral blood flow (Johnston et al., 2003) and worse functional outcomes in animal models (Liu et al., 1998), suggested to be a result of an increased oxidative stress and lipid peroxidation in the injured brain.

By inserting a catheter into the internal jugular vein just below the skull base, it's possible to measure the oxygenation of the blood leaving the brain hence providing an indirect measurement of cerebral oxygen consumption ($CMRO_2$) (Schell and Cole, 2000). The technique is referred to as jugular bulb oximetry and is used in the NICU, especially during active hyperventilation. Excessive hyperventilation in order to treat intracranial hypertension has been seen to cause a significant decrease in PbO_2 (Sarrafzadeh et al., 2003), thus prompting hyperventilated patients to be extensively monitored.

While hyperbaric oxygen treatment has been seen to improve conditions following TBI (Rockswold et al., 2013), it's still unclear which TBI patients that will best benefit from this type of treatment. Monitoring of cerebral oxygen pressure is important to continuously monitor for potential deterioration in the injured brain and should be used together with ICP-monitoring to improve patient outcome (Nangunoori et al., 2012).

1.6.4.4 Measuring cerebral metabolism - Microdialysis

In order to analyze focal brain biochemistry in patients suffering from TBI, the microdialysis (MD) technique has been used since the 1990s (Persson and Hillered, 1992). Using a semipermeable membrane, usually with pores the size of 20 kDa, substrates of the cerebral metabolism, including pyruvate, glutamate, glucose, glycerol, and lactate, may be measured in the extracellular fluid (ECF) of the brain parenchyma. The recovery of substrates, using the MD technique in ECF, has been shown to be around 70% (Hutchinson et al., 2000). High levels of lactate:pyruvate ratio, or isolated high lactate and low glucose (Hlatky et al., 2004), are presumably associated with cerebral ischemia, and correlated to an unfavorable outcome (Timofeev et al., 2011, Sanchez et al., 2013). Moreover, increased glycerol has been shown to correlate to phospholipid membrane degradation (Hillered et al., 1998), hence could be used as a surrogate marker of cellular damage. Guidelines have been introduced to better determine the use of cerebral microdialysis in the treatment of TBI patients (Bellander et al., 2004b).

Presumably, the catheter should be placed in perilesional tissue, since this area is more susceptible to secondary injury, usually presenting higher lactate:pyruvate ratio and glycerol levels, thus is more crucial to monitor (Timofeev et al., 2011). Even if focal monitoring might be a strength, it is also a major weakness of the MD method since a lot is depending on the exact placement and location of the catheter (Hillered et al., 2005), resulting in highly individualistic patterns among different patients (Nelson et al., 2004), making the method difficult to generalize in treatment regimes.

In an attempt to monitor the cerebral metabolism on a more global scale, including the entire brain, a "metabolic crisis" has been suggested, with simultaneous low glucose and elevated LPR, when the MD catheter is placed in uninjured brain tissue (Stein et al., 2012), which has been shown to correlate to an unfavorable outcome. Additional ways to monitor global cerebral metabolism are necessary to further improve detection of brain tissue at risk of secondary deterioration.

1.7 OUTCOME MEASUREMENTS FOLLOWING TBI

Good outcome measurements and predictions are essential for care providers to distribute resources more effectively, conduct better randomized trials in TBI, to balance benefits and risks of early treatment and to evaluate the effect of new therapeutic regimes. TBI poses a challenge when it comes to outcome prediction due to the inherent heterogeneity among its patient population, making it difficult

to detect independent factors associated with long-term functional outcome. In order to accumulate enough patients to find significantly independent parameters, multicenter studies are a necessity. Some of the multicenter trials in TBI are the IMPACT (International Mission for Prognosis and analysis of Clinical Trials in TBI) study (Marmarou et al., 2007b) and the CRASH (Corticosteroid Randomization After Significant Head Injury) study (Roberts et al., 2004, Perel et al., 2008).

Several parameters have been found to correlate to poor outcome. The most important are increasing age (probably due to an increased morbidity, as indicated by Charlson co-morbidity index (Charlson et al., 1987)) (Miller et al., 1981, Hukkelhoven CW et al., 2003, Mushkudiani et al., 2007, Vos et al., 2010), low Glasgow Coma Scale score (GCS) (Stocchetti et al., 2004, Husson et al., 2010) and unresponsive pupil reaction (Marmarou et al., 2007a, Martins et al., 2009). Multivariate models also indicate that age, GCS motor response and pupil responsiveness, all explain outcome with a partial- R^2 of 3.5-6%, are the most important factors in predicting outcome after TBI (Murray et al., 2007).

Glasgow Outcome Scale/Score (GOS)	
5	Good recovery, resumption of normal life even if minor disabilities may persist.
4	Independent, able to work in a sheltered environment due to disabilities but may independently use public transportation and manage personal hygiene
3	Dependent, in need of daily support due to mental or physical disabilities
2	Vegetative state, unaware of self and environment
1	Death
Glasgow Outcome Score Extended (GOSe)	
8	Upper good recovery
7	Lower good recovery
6	Upper moderate disability
5	Lower moderate disability
4	Upper severe disability
3	Lower severe disability
2	Vegetative state
1	Death

Table 4 – Describing the two most frequently used assessments of long term functional outcome; Glasgow Coma Scale and the Extended Glasgow Coma Scale.

Today, the most common tool for functional outcome assessment following TBI is the Glasgow Outcome Score (GOS), pioneered in 1975 (Jennett and Bond, 1975) (Table 4). This grading consists of five levels where GOS1 = death and GOS5 = Good recovery. Usually, the GOS is assessed at 6 months, but further functional outcome improvement has been seen up to >12 months (Corral et al., 2007). GOS has been criticized for its broad use of “dependent” state (GOS3) and to improve this, the extended GOS (GOSe) was introduced in 1981 using 8 levels to better describe disabilities following TBI (Jennett et al., 1981). Other functional scales for assessment of outcome and disabilities after brain injury exist, including the Disability Rating Scale (DRS) (Rappaport et al., 1982), the Functional Independence Measure (FIM) and supplement Functional Assessment Measure (FAM) (Ottenbacher et al., 1996), all with more in depth interviews and questions corresponding to the patients quality of life.

1.8 BIOMARKERS IN TBI

A biological marker (biomarker) of injury is defined as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working, 2001). It may be proteins, or other substances, functioning as surrogate markers of injury. In several areas of medicine biomarkers play an important role, for instance the protein Troponin-T is used to detect myocardial infarction (Hamm et al., 1997) or D-Dimer to detect deep vein thrombosis (Wells et al., 2006) and pulmonary embolism (Corwin et al., 2009). Ideally, a biomarker of brain injury should fulfill the following criteria (Kleindienst et al., 2007, Papa et al., 2008);

1. Demonstrate a high sensitivity and specificity for brain injury.
2. A passive release from the CNS without any stimulated active release.
3. Lack of specific effects on CNS cells interfering with the initial injury.
4. Stratify patients by severity of injury.
5. Have a rapid appearance in accessible biological fluids.
6. An unlimited passage through the BBB.
7. Provide information about injury mechanisms.
8. Have well defined bio-kinetic properties.
9. Monitor progress of disease and response to treatment.
10. Predict functional outcome.

Also, biomarkers might aid in the stratification of treatment, where it may serve as a companion diagnostics in order to guide treatment decisions in the dynamic clinical situation of neuro-intensive care. Today, no TBI biomarker candidate fulfills the criteria stated above yet several exist and aid physicians. The most studied biomarker of brain injury, with almost 800 publications on the subject (Pubmed®), is S100B.

1.8.1 S100B

The protein S100 was originally isolated from bovine brain almost 50 years ago and got the name from its 100% solubility in a saturated ammonium sulfate solution (Moore, 1965). S100 is a small protein, 9-14 kDa, present as different monomers but mainly as homodimers (Donato, 2001). It belongs to a family of intracellular, calcium-binding proteins predominantly present in astrocytes in the central nervous system (CNS), but several cells in the CNS and peripheral nervous system (PNS) express S100 (Steiner et al., 2007, Donato et al., 2009). It exists mainly as the homodimer S100BB (Figure 4), or heterodimer S100AB in nervous tissue (Isobe et al., 1983, Haimoto et al., 1987). Together, these proteins make up the levels of what is usually referred to clinically as “S100B”.

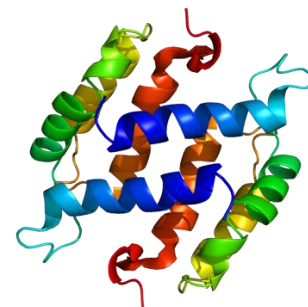


Figure 4 - Homodimer structure of the S100B protein. ©Wikimedia Commons.

1.8.1.1 Different proteins of the S100 family

The function of the proteins in the S100 family has been described in several review articles (Donato, 2003, Kleindienst et al., 2007, Donato et al., 2013). Some notable functions include induction of pro-inflammatory chemotaxis (S100A4, S100A8, S100A9 and S100A12) (Hofmann et al., 1999, Donato, 2003, Meijer et al., 2012, Tong et al., 2014), increased malignancy risk (S100A4, S100A8 and S100A9) (Saleem et al., 2006, Srikrishna, 2012), regulation of allergy (S100A6) (Lesniak et al., 2009), neurite proliferation and neuronal survival (S100B, S100A1 and S100A4) (Huttunen et al., 2000, Novitskaya et al., 2000).

1.8.1.2 Functions of S100B

The biochemical properties of S100B have previously been thoroughly reviewed (Donato, 2001, 2003, Van Eldik and Wainwright, 2003, Goncalves et al., 2008, Michetti et al., 2012, Donato et al., 2013).

Intracellularly, S100B is a part of the calcium hemostasis, thereby transferring signals from second messengers (Heizmann et al., 2002) but also involved in cell differentiation and cell cycle progression (Schafer and Heizmann, 1996). It has been shown to be able to inhibit apoptosis if applied in experimental conditions (Brewton et al., 2001). Extracellularly, S100B has been shown to have a neuro-protective properties as well as to promote neurogenesis (Haglid KG et al., 1997, Ahlemeyer et al., 2000), to be neuro-modulating and to promote neuronal plasticity (McAdory et al., 1998,

Nishiyama H et al., 2002), as well as to enhance processes involved in memory and learning (Fazeli et al., 1990, Kleindienst et al., 2013) if S100B is provided in both traumatic, and normal, conditions.

The effect of S100B has been shown to be concentration dependent, where lower concentrations (nanomolar) are beneficial and higher concentrations (micromolar levels) are correlated to harmful effects (Rothermundt et al., 2003, Van Eldik and Wainwright, 2003). Increased extracellular levels of S100B may lead to neuronal dysfunction or cell death due to an inflammatory response that stimulate astrocytes and microglia to recruit and produce pro-inflammatory cytokines with a subsequent increase of the extracellular levels of calcium and activation of nitric oxide, with harmful effects (Hu et al., 1997, Koppal et al., 2001). The different effects of S100B has been suggested to depend on the receptor for advanced glycation endproducts (RAGE) which is upregulated by S100B levels and may cause pro-inflammatory gene activation (Donato et al., 2013).

1.8.1.3 Release and elimination of S100B

In vitro studies reveal that astrocytes, when affected by trauma or metabolic distress (Gerlach et al., 2006), will release stored S100B, which has been measured extracellularly as fast as 15 seconds after a lesion is established (Willoughby et al., 2004). S100B mRNA concentration, as a sign of ongoing intracellular synthesis of the protein, will increase shortly after injury (Hinkle et al., 1997), thus the concentrations that is measured in blood is presumably both from secreted, and newly synthesized, origin.

It is known that the concentration of S100B in the CSF could be up to 100x higher than in serum (Petzold et al., 2003). When the patient suffers from a TBI, the BBB will disrupt causing a leakage of proteins from the CSF with subsequent cerebral deterioration and edema formation (Marmarou, 2003). The ratio of albumin between CSF:Serum (Q_A) is often used to quantify the degree of BBB disruption (Tibbling et al., 1977). Some authors claim that S100B is released into the serum through the disrupted BBB (Kapural et al., 2002, Kanner et al., 2003, Marchi et al., 2003), thus is a good marker of BBB permeability (Blyth et al., 2011, Lopez et al., 2012). However, the studies supporting S100B as a marker for BBB integrity are limited in sample size, use osmotic- or chemo therapy in non-TBI patients to disrupt the BBB and are not focused on the complex BBB disruption present following traumatic conditions. Studies that do focus on TBI patients do not show any correlation between a disrupted BBB, using Q_A , and the peak serum levels of S100B (Bellander et al., 2011) or by using a ratio between CSF and serum S100B compared to Q_A (Kleindienst et al., 2010b), hence indicating a better correlation between actual injury and S100B levels, and not to the degree of BBB disruption. Furthermore, it has recently been shown that the glymphatic system, the drainage of CSF and ECF into the venous system, plays a very important role in the outflow of S100B from the CNS, independently from the BBB integrity, presumably explaining the findings in the TBI studies (Plog et al., 2015).

Systemically, S100B has been shown to be excreted and eliminated 100% through the kidneys (Usui et al., 1989). Patients with renal failure, or renal impairment, have been shown to have increased S100B levels (Molina et al., 2002). Kidney transplanted patients have higher S100B levels, which also correlated to creatinine clearance (Gross et al., 2010), while another study could not detect any correlation between S100B levels and the glomeruli filtration rate (Jonsson et al., 2000).

1.8.1.4 Half-life of S100B

It is difficult to assess the half-life of S100B in serum after TBI since there will be a continuous release of the protein from the damaged brain, and perhaps a contribution from the CSF due to a disruption of the BBB (Kapural et al., 2002), outflow from through the glymphatic system (Plog et al., 2015), ongoing cerebral synthesis and/or active secretion (Hinkle et al., 1997, Gerlach et al., 2006). The half-

life of S100B has shown to be everything from 132 minutes (Blomquist et al., 1997), 120 minutes (Ingebrigtsen and Romner, 1996, Jackson et al., 2000), 90 minutes (in melanoma patients) (Ghanem et al., 2001), 60 minutes (Ingebrigtsen and Romner, 2002) while another, more controlled method, puts it as short as 25 minutes (Jonsson et al., 2000), calculated from patients undergoing coronary artery bypass grafting not suffering from TBI. Nevertheless, compared to the other brain biomarkers NSE (20-48 hours) (Schmechel et al., 1978, Johnsson et al., 2000, Ingebrigtsen and Romner, 2002) and GFAP (in vitro 16-144 hours) (Chiu and Goldman, 1984, Smith et al., 1984, Rolland et al., 1990) and UCH-L1 (11 hours) (Brophy et al., 2011) the half-life of S100B is significantly shorter, indicating that an elevation of S100B better designates a more imminent injury, and that the duration of the increase might correspond to the extent of the damage sustained.

1.8.1.5 Extracranial sources of S100B

There are known extracerebral sources of protein S100B, such as Langerhan's cells, adipocytes, epithelial cells, cardiac and skeletal muscle cells and chondrocytes (Haimoto et al., 1987) and patients that suffer from trauma, presenting multiple injuries to the thorax, extremities and abdominal organs without any identified damages to the central nervous system have been shown to exhibit elevated S100B concentrations in serum (Anderson et al., 2001, Unden et al., 2005b, Routsis et al., 2006). However, S100B release of extracerebral origin appears to have a faster clearance than S100B released from the CNS (Pelinka et al., 2003, Savola et al., 2004, da Rocha et al., 2006) or even not to significantly influence the total serum concentration of S100B at all (Pham et al., 2010). Furthermore, probably due to its short half-life, S100B elevations seen in extra-cranial trauma are generally lower (median levels ranging 0.18 µg/l – 0.57µg/l) (Korfias et al., 2006b, Stalnacke et al., 2006) than those seen in TBI (mean levels ranging 1.7 µg/l – 4.01 µg/l) (Herrmann et al., 2001, Savola et al., 2004). While possible extracranial sources should be taken into consideration when assessing TBI patients (Persson et al., 2012), repeated sampling could perhaps exclude several of these confounding factors.

1.8.1.6 Assays to analyze S100B

There are several available assay kits to measure S100B used in the clinics and laboratories. An advantage of S100B is that it is stable, unaffected by storing and changes in temperature (Raabe et al., 2003a), which greatly facilitates the handling of the protein and reliability of the analyses. ELISA has become the golden standard in measuring S100B in laboratories, and are available from different manufacturers today (Diasorin, Sangtec, Italy; CanAG Diagnostics, Sweden; Roche Diagnostics, Switzerland; Syn X Pharma Inc. Nanogen, USA) and usually measures “S100AB” and “S100BB” dimers as “S100B”.

In clinical studies, the two most frequently used systems are the quantitative automated luminometric immunoassay, LIASON-mat S100 system (Diasorin, Sangtec, Italy) and the electrochemiluminescence immunoassay (Elecsys S100B®; Roche Diagnostics, Penzberg, Germany). The LIASON-system is primarily used to detect and screen for S100B in malignant melanoma and other tumors, thus is not manufactured for quick analyses (Mussack et al., 2006). The Elecsys system is increasing in popularity since it may analyze a sample faster and at a cheaper price (Smit et al., 2005).

1.8.1.7 Clinical use of S100B

The clinical properties of S100B in TBI have been thoroughly reviewed. (Korfias et al., 2006a, Bloomfield et al., 2007, Kleindienst et al., 2007, Kochanek et al., 2008, Filippidis et al., 2010, Schiavi et al., 2012, Astrand et al., 2013, Mercier et al., 2013).

Extracranially, S100B has long been used to monitor the advancement, and evaluate the efficacy of therapy, of melanocytic tumors (Kindblom et al., 1984, Smit et al., 2008, Egberts et al., 2009). Though lately, a clinical role has also been suggested in different cerebral conditions, including stroke (Foerch et al., 2004, Brea et al., 2009), global ischemia (Usui et al., 1989, Mussack et al., 2002), neurodegenerative disorders such as MS and Alzheimer's disease (Rothermundt et al., 2003), as well as in cerebral herniation (Unden et al., 2004), spontaneous subarachnoid hemorrhage (Oertel et al., 2006, Stranjalis et al., 2007, Sanchez-Pena et al., 2008, Moritz et al., 2010), and cerebral vasospasm (Oertel et al., 2006). However, it is in the field of traumatic brain injury where it has been mostly studied. The first human TBI study of S100B in serum was published by Ingebrigtsen et al. in 1995 (Ingebrigtsen et al., 1995), though increased S100B levels in CSF following TBI had been previously described (Sindic et al., 1982). Later on, S100B was shown to be sensitive to detect a plethora of different intracranial lesions, including cerebral contusions (Raabe et al., 1998), subdural hematomas and traumatic subarachnoid hemorrhages (Romner et al., 2000), as well as epidural hematomas (Unden et al., 2005a).

1.8.1.8 S100B in mild TBI in adults

A vast majority (95%) of all TBI cases present as mild (Cassidy et al., 2004). In order to determine which patients that are at risk of developing, or are suffering from, intracranial hemorrhage, specific guidelines have been set-up to better select patients for CT investigations, such as the Scandinavian CT guidelines (Ingebrigtsen et al., 2000). Using the Scandinavian CT-guidelines, a sensitivity to detect an intracranial lesion is 96%, but the specificity is only 53% (Stein et al., 2009), resulting in unnecessary performed CT scans with excess, potentially harmful (Brenner and Hall, 2007), radiation and costs. The introduction of S100B testing in the emergency rooms, have introduced a way to potentially screen which patients that are in need of a CT scan. A recent meta-analysis on mild TBI and S100B, including 2466 patients from 12 scientific articles, states that the pooled negative predictive value (NPV) was more than 99% (CI 98%-100%) with a sensitivity of 97% in S100B detecting CT-visual brain pathology, using 0.10 µg/l as cut-off (Unden and Romner, 2010). With that sensitivity and NPV it makes S100B superior to, for example, D-dimer in detecting pulmonary embolism and deep vein thrombosis (NPV: 92%) (Wells et al., 2006) and Troponin-T (NPV: 96%) (Hamm et al., 1997) in detecting myocardial infarction. The authors, however, do stress the importance of acquiring the sample early after trauma and not to use it in patients with extracranial injuries or focal neurological deficits, since in these patients S100B is not enough to select patients for CT scans (Unden and Romner, 2010). This meta-analysis formed the foundation for the new Scandinavian guidelines for mild and moderate TBI (Unden et al., 2013). These guidelines assist the physicians and personnel in the emergency rooms to better determine which patients are in need of a head CT scan by acquiring a serum sample of S100B. If the concentration is less than 0.10 µg/L, within 6 hours after trauma, and the patient is suffering from a mild TBI without extracranial trauma and other risk factors, performing a head CT-scan could be reconsidered (Unden et al., 2013). It is estimated that this could reduce the number of unnecessary CT investigations by 32% in the emergency departments with more effective resource allocation.

1.8.1.9 S100B in moderate-to-severe TBI

As previously mentioned, several reviews have analyzed how S100B may be utilized in moderate and severe TBI patients. The latest review from 2013 includes a meta-analysis of 39 studies including a total of 1862 patients (Mercier et al., 2013). In this analysis, serum levels between 2.16µg/L to 14.0µg/L predicted an unfavorable outcome (GOS1-3), while some included studies showed that similar levels are associated with mortality (Mercier et al., 2013). Comparable findings have led some authors to the conclusion that S100B could be used as a marker for facilitating brain death diagnosis (Egea-Guerrero et al., 2013, Shakeri et al., 2013).

However, the optimal time for collecting S100B to predict outcome after trauma in moderate-to-severe TBI has been a matter of intense discussions. In several studies, the initial sample of serum S100B after trauma is considered the most important for outcome prediction (Jackson et al., 2000, Romner et al., 2000, Petzold et al., 2002, Raabe et al., 2003b). Later time frames have also been suggested to have clinical significance, ranging from within 6 hours (Woertgen et al., 2002), 6 to 12 hours (Raabe et al., 2003b), within 12 hours (Muller et al., 2007), after 12 hours (Raabe et al., 1999), 24 hours (Gonzalez-Mao et al., 2011), within 42 to 79 hours (Herrmann et al., 2000), within 48 hours (Pelinka et al., 2003, Watt et al., 2006), 72 hours (Murillo-Cabezas et al., 2010) and finally up to >84 hours after trauma (Pelinka et al., 2003). It is becoming more and more obvious that the serum concentration of S100B itself is of limited use to predict outcome if the time that have lapsed since the trauma is unknown. Repeated sampling of S100B shows improved correlation towards outcome (Herrmann et al., 2000), even better than repeated radiological examinations (Gradisek et al., 2012).

A study including pediatric TBI patients highlights the fact that the temporal profile, or trajectory analysis, of serum S100B concentration is of great importance since patients with increasing serum levels have a higher risk of unfavorable outcome compared to patients with subsequent serum concentrations in steady decline (Berger et al., 2010). These patterns have been seen in adult TBI as well, using trajectory analysis of repeated S100B CSF sampling, patients with worse outcome have high concentrations over a prolonged period time (Niyonkuru et al., 2013).

1.8.1.10 S100B as monitoring marker of ongoing injury

As studies have described scenarios where the levels of S100B continue to increase following TBI (Pelinka et al., 2003, Kleindienst et al., 2010a), there are some studies that have monitored patients in the NICU using subsequent samples of S100B. Raabe and co-workers monitored their patients, constituting mainly of those suffering from subarachnoid hemorrhages and post-operative intracranial tumor surgery, with daily S100B samples and found that an increase of S100B >0.5µg/L significantly correlated to the development of a severe secondary injury, such as a cerebral infarction or hematoma progression (Raabe et al., 2004). Moreover, they noted that a secondary increase of S100B influenced treatment in 21% of the cases. Another study by Undén et al revealed that increased levels of S100B were correlated to secondary neurological complications, in a material consisting primarily of spontaneous SAH patients (Undén et al., 2007), but the authors were not able to demonstrate that the secondary peak of S100B could predict these complications, nor were these complications associated with the long-term outcome. Other studies have shown a correlation between neurological deterioration with an increase of S100B during the NICU stay, suggesting it to be a useful therapeutic tool being closely related to pathophysiological mechanisms in TBI (Dimopoulou et al., 2003, Korfiatis et al., 2007, Hendoui et al., 2013). Several researchers have investigated the concentration of S100B as a surrogate marker for increased ICP and have found a correlation (Pelinka et al., 2003, Hayakata et al., 2004, Olivecrona et al., 2014). These findings strengthen the role of S100B as a laboratory test for monitoring patients admitted to NICU.

1.8.2 Other biomarkers of brain injury

There are other proteins of cerebral origin that have been elevated in serum following TBI (Yokobori et al., 2013, Chou et al., 2014), including myelin based protein (MBP) (Baker et al., 2009), microtubule-associated tau proteins (C-tau) (Zemlan et al., 2002), spectrin-break down products (Farkas et al., 2005, Mondello et al., 2010) and apolipoprotein-E (Kay et al., 2003). There are studies that propose that a combination of biomarkers following TBI, such as GFAP and S100B or UCHL-1 and GFAP, to further increase diagnostic capabilities and outcome predictions (Vos et al., 2010, Mondello et al., 2012). NSE, NFL, UCHL-1 and GFAP are presented in short.

1.8.2.1 Neuron-Specific Enolase

Neuron-specific Enolase (NSE) is the second most studied biomarker in TBI. NSE is an isoenzyme of enolase, a glycolytic protein that is present primarily in the cytoplasm of neurons and neuroendocrine cells (Marangos and Schmechel, 1987). Intracranially, it has been shown to be a biomarker of cerebral injury (Pleines et al., 2001, Cheng et al., 2014). Moreover, it may detect and monitor differentiation of specific cancers of neuronal origin (Herman et al., 1989). In axonal injury, NSE has been shown to upregulate in order to preserve cerebral homeostasis (Wu et al., 2004). The half-life in serum is over 20 hours (Ingebrigtsen and Romner, 2002). Unfortunately, NSE is present in erythrocytes, which limits its usefulness as a biomarker of brain damage as serum levels will be elevated if hemolysis occurs in the acquired blood sample (Johnsson et al., 2000).

1.8.2.2 Neurofilament-Light

Neurofilaments are the main components of the intermediate filaments that make up the axonal cytoskeleton. Neurofilaments consist of three chains, a heavy subunit (190-210kDa), an intermediate subunit (150 kDa) and a light subunit (68 kDa). The neurofilament-light (NFL) has, in situations of axonal damage, been seen to increase in biological fluids (Teunissen and Khalil, 2012). In neuroinflammatory diseases, such as amyotrophic lateral sclerosis and multiple sclerosis, NFL has emerged as a promising biomarker in CSF, where it may work as a surrogate therapeutic target and outcome of neurological disability and axonal damage (Tortelli et al., 2012, Gaiottino et al., 2013, Khademi et al., 2013). It is also increased in CSF following concussions in mild TBI, in the ECF of pericontusional areas in TBI and in the serum of patients with spinal cord injury (SCI). (Magnoni et al., 2012, Neselius et al., 2012, Kuhle et al., 2014). The in-vivo half-life of NFL has been shown to be about 3 weeks (Barry et al., 2007).

1.8.2.3 Glial fibrillary acidic protein

Being a cytoskeletal filament protein present in astrocytes, glial fibrillary acidic protein (GFAP) was first identified in 1971 (Eng et al., 1971). It has been measured in serum and shown to be elevated in patients with head injury (Missler et al., 1999). Like S100, it's an astrocytic protein, but since extracranial sources are limited, it has been suggested as more brain specific than S100B (Papa et al., 2014). Being considered a better marker for focal than diffuse brain injury (Pelinka et al., 2004), it has been used in ratios with neuronal markers (Ubiquitin C-Terminal Hydrolase-L1 (UCHL1)) in order to detect injury type in TBI patients (Mondello et al., 2012).

1.8.2.4 Ubiquitin C-Terminal Hydrolase-L1

Known as neuronal-specific protein gene product (PGP 9.3), ubiquitin C-terminal hydrolase L-1 (UCHL1) is present in the soma of neurons (Jackson and Thompson, 1981) and is involved in turnover of ubiquitinated proteins set for internal metabolism in cells (Tongaonkar et al., 2000). In comparison to GFAP, it is more prominent in serum following diffuse brain injury than after focal injury (Mondello et al., 2011), providing additional information to clinical outcome, also when combined with other biomarkers of TBI (Mondello et al., 2012).

2 AIMS

The general aims were to validate biomarkers collected in NICU TBI patients as predictors of long-term functional outcome. Furthermore, in addition to conventional multimodal monitoring, analyze how biomarkers and CSF microdialysis may improve the detection of cerebral deterioration in TBI patients. Finally, through a translational approach, we aimed to analyze how biomarkers may assist in stratification of injury if hypoxia is added to the TBI. The aims of the individual papers were to;

2.1.1.1 *Paper I*

investigate S100B as an outcome predictor compared to, and adjusted for, other known parameters known to influence TBI outcome. Secondary aim was to examine which parameters that best correlated to serum concentrations of S100B. In addition, the optimal sampling time of S100B was investigated in relation to outcome prediction.

2.1.1.2 *Paper II*

study the occurrence of secondary increases in serum concentration of S100B and its association to possible pathological findings seen on neuro-radiological investigations. Additional aims were to identify what levels of secondary increases of S100B are optimal when monitoring TBI patients, and to relate secondary events and S100B fluctuation for monitoring secondary insults and outcome.

2.1.1.3 *Paper III*

validate a method of global MD monitoring by using a MD catheter placed in CSF. As a secondary aim, conventional CSF samples were used to calculate the recovery of the MD catheter and to correlate MD-CSF and MD-Brain parameters to patient outcome.

2.1.1.4 *Paper IV*

analyze a possible predictive value of CSF and/or serum NFL, as compared and adjusted to other known predictive factors including the two biomarkers S100B and NSE. Secondary objectives included to explore the dynamics of NFL levels after TBI in serum and CSF and to investigate if NFL and S100B reflect different pathophysiologic phenomena as analyzed by neuro-radiological examinations.

2.1.1.5 *Paper V*

to develop a hypoxic focal TBI rat model using controlled cortical impact. With the model, we aimed to investigate the effect of post-traumatic hypoxia on brain morphological changes including lesion progression and serum changes of the biomarker S100B, but also to vascular/cytotoxic edema, metabolic parameters (lactate), brain histopathological features such as neuronal survival, leukocyte and macrophage infiltration, all of which were compared to normoxic rats over 4 weeks.

3 MATERIAL AND METHODS

3.1 ETHICAL CONSIDERATIONS

The studies were approved by the Regional ethical review board in Stockholm (**Paper I, II, III and IV**) or the Swedish department of agriculture (**Paper V**). The identification numbers are: 2009/1668-31/2 (**Paper I and Paper II**), 2009/1112-31/3 (**Paper III**), 2014/2025-31 (**Paper IV**), N369/12 and N126/13 (**Paper V**).

At our department, every patient and next of kin receive information that they may be included in studies and quality follow-ups, and may voluntarily opt-out. For **Paper I, II and IV** no consent for participation was acquired due to the retrospective nature of the study. Furthermore, all data was anonymized and presented on group level making it impossible to identify unique patients. **Paper III** was a prospective study where informed consent was gathered from the next of kin, since the patients were unconscious when they were admitted the NICU. In case a patient decided later on that they did not want to participate in the study, they were withdrawn. The animal study, **Paper V**, was approved by the Department of Agriculture, and was performed in such a manner that animal suffering and morbidity were minimized.

3.2 STUDY POPULATION

The patients in the retrospective studies (**Paper I, II and IV**) had all been treated for severe-to-mild (GCS 3-15) TBI between 2005-2009 (**I and II**), and 2007-2013 (**IV**). All patients older than 15 years admitted to NICU were included in the studies. All admissions needed NICU care, approved by the attending neurosurgeon in charge. Following NICU admission, the patients were included in the Karolinska TBI database, a database administered by physicians at the NICU department and regularly updated by a NICU specialized nurse with extensive experience of TBI patients. Pre-hospital-, admission-, and hospital parameters were gathered in conjunction with discharge data.

Patients in **Paper III**, were also in need of NICU care and included in the TBI database, but the data and samples were prospectively collected during the NICU stay from 2010-2012. In contrast to **Paper I, II and IV**, only patients suffering from severe TBI (GCS 3-8 at admission) were included in this study.

The animals in **Paper V** were all female Sprague-Dawley rats, being about 15 weeks old, weighing approximately 250 grams, on the day of surgery.

3.3 OUTCOME ASSESSMENT

For **Paper I, II and IV** patient outcome was assessed systematically at discharge from the hospital, at a follow-up visit at the department of neurosurgery and/or the department of neuro-rehabilitation 3-6 months after discharge, and through a questionnaire regarding quality of life at about 12 months after discharge. Therefore, a combination of assessed and self-reported outcome was used. The final GOS score available for each patient, between 6 to 12 months after discharge, was used.

For **Paper III**, a board-certified specialist in neuro-rehabilitation examined the patient 6 months after trauma during a follow-up visit assessing extended GOS. The extended GOS was dichotomized (Unfavorable GOS 1-5 vs Favorable GOS 6-8), as this correlated to current GOS scores for the patients (Unfavorable GOS 1-3 vs Favorable GOS 4-5).

3.4 TREATMENT

3.4.1 Patient treatment

For **Paper I, II, III and IV**, our department used the guidelines set forth by the Brain Trauma Foundation for treatment of patients suffering from TBI (Brain Trauma, 2000b, Brain Trauma et al., 2007a), albeit with small modifications.

All unconscious TBI patients were intubated, mechanically ventilated and sedated using morphine, propofol and/or midazolam. Mass lesions, if detected, were evacuated as deemed appropriate by the attending neurosurgeon. ICP was measured predominantly using extraventricular drains (Medtronic, Fridley, MN, USA), or in cases where drain insertion was not possible, by intraparenchymal pressure monitors (Codman Microsensor, DePuy Synthes, Johnson&Johnson, New Brunswick, NJ, USA or Rehau AG+CO, Rehau, Germany). ICP was targeted at <20 mmHg and guided further treatment. MAP was continuously measured invasively, usually in the radial artery, and with transducers located at midlateral ventricular level. The head was slightly elevated at a 30 degree angle. CPP, calculated as MAP-ICP, was usually targeted at 50-70 mmHg. These targets were attained using intravascular infusions (primarily ringer's acetate and albumin), vasopressors (norepinephrine) and osmotic therapy (hypertonic NaCl and, occasionally, mannitol). Intermittently, dynamic CSF drainage from ventricular catheters to reduce ICP was performed in **Paper I, II and IV**, but not in **Paper III** as they instead had a constant flow of CSF out through a CSF-pump.

Mild hyperventilation and/or decompressive craniectomy were performed, if deemed necessary to improve intracranial conditions. If mild hyperventilation was deemed necessary for ICP control, it was guided using jugular bulbar saturation and venous-arterial-jugular lactate difference. Temperature was kept at 37°C, using paracetamol or external wrapping cooling systems. Mild hypothermia (35–36°C), was occasionally used for high refractory ICP. If ICP remained elevated despite all other measures, sodium thiopental was infused, limited by burst suppression and monitored with continuous electroencephalography.

If clinically relevant traumatic subarachnoid hemorrhage (trSAH) was detected, nimodipine treatment, and monitoring with transcranial Doppler (TCD), was provided to reduce the risk of potential vasospasm and subsequent tissue ischemia.

3.4.2 Animal treatment

In **Paper V**, female Sprague-Dawley rats were used, housed in a 12-hour light/dark cycle, with food and water ad libitum. A room temperature of 21°C +/- 1°C and normal air humidity were kept throughout the experiment. In the experimental procedure, the rats were anaesthetized using 5% Isoflurane, intubated, and mechanically ventilated using 2-3% Isoflurane as maintenance. Marcain® was provided as local anesthetic and Temgesic® and Paracetamol® for analgesia. Following a mid-line incision, a craniectomy, 8x5 mm, was performed revealing the dura. A 3 mm deep, 3 mm Ø wide, controlled cortical impact was made (Figure 5) (TBI 0310, Precision Systems and Instrumentation LLC, Lexington, KY, USA), followed by a 30 minute period where the rat inhaled either a hypoxic- (11%O₂) or a normoxic (22%O₂) air mixture. During this time period, the animals were monitored using a pulsoxymeter device (MouseSTAT™, Kent Scientific, Torrington, CT, USA) following by arterial blood gas sample from the tail artery (ABL800 FLEX analyzer, Radiometer Medical, Brønshøj,

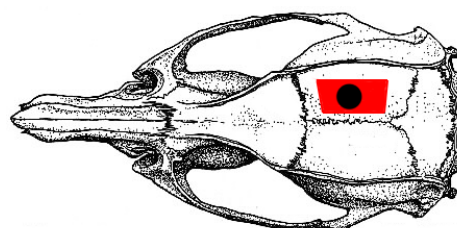


Figure 5 - Rat skull illustrating the performed craniectomy over the right hemisphere (box). The 3 mm in diameter wide impact hit in the middle of the craniectomy (black circle) (image in the public domain).

Denmark). Afterwards, the animals were allowed to regain consciousness on a heating pad, and then returned to their home cages. The procedure is explained in greater detail in **Paper V**.

3.5 BIOMARKER ANALYSIS

All human serum S100B samples were analyzed at Karolinska University Hospital Solna, Department of Clinical Chemistry. As per clinical routine in our NICU, since around 2006, serum samples of S100B are acquired at admission, and every 12 hours (06:00 and 18:00), as long as the patient remains unconscious. Before 2006, samples were acquired more sporadically, yet there were often samples during the first days of NICU admission. Except for venous serum samples acquired in the emergency room, all S100B samples were arterial. Arterial and venous samples have been shown to present similar results (Astrand et al., 2012). For **Paper I, II, III and IV** S100B, and NSE (**Paper III and IV**), in serum were acquired using the medical files from the hospital electronic charts system Take Care® (CompuGroup Medical Sweden AB, Farsta, Sweden) for each patient.

In **Paper I**, the patients had at least one serum sample of S100B within the first 48 hours after reported trauma, and at least three samples within the first 72 hours. The S100B Area Under Curve (AUC) was calculated using ICU-Pilot® (mDialysis AB, Stockholm, Sweden). For sake of standardization, all S100B levels were set to 0 µg/L at time of trauma in the AUC calculations. The highest level of S100B, as well as AUC in the first 48 hours after the traumatic insult, were used.

In **Paper II**, the patients had at least three serum samples of S100B acquired, where at least one sampled had to be obtained 48 hours following the trauma (Figure 6). Patient journals were scanned to detect potential “secondary peaks” of S100B. This peak was defined as an increase of serum S100B that developed more than 48 hours after the initial trauma. This time cut-off was arbitrary in an attempt to separate the primary S100B increase from the secondary peak. Three cut-off levels

defining secondary peaks were investigated; $\geq 0.05\mu\text{g/L}$, $\geq 0.1\mu\text{g/L}$ and $\geq 0.5\mu\text{g/L}$, persisting for at least 24 hours, thus not falling below the baseline serum concentration that existed prior to the increase.

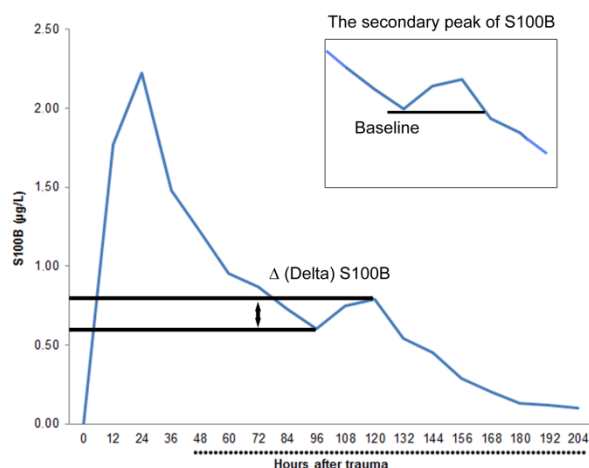


Figure 6 - Illustrative demonstration of a secondary peak of S100B in a patient suffering from TBI. The delta (difference) from the “baseline” is highlighted. Only peaks detected 48 hours after trauma were used (dotted line) (**Paper II**).

In **Paper III**, peak S100B concentrations in arterial serum, acquired between 12 to 36 hours after trauma were used. NSE peak levels in the first 48 hours were included.

In **Paper IV**, serum S100B concentrations at 12-36 hours after injury as well as serum and CSF levels acquired concomitantly with NFL-levels were used. For NSE, peak levels and levels acquired with NFL were used.

In **Paper V**, after the animal had received a lethal dose pentobarbital, the heart was punctured using an angiocatheter (18G, KD Medical, Berlin, Germany) and blood was collected in two test tubes (2 x 1.5 mL test tube, 3810X, Eppendorf, Hamburg, Germany). The tubes were placed vertically for about 2 hours to allow separation. Afterwards, the blood was centrifuged (Spectrafuge16M®, Labnet International, Edison, USA) for 10 minutes at 10.000g (about 11.000 rotations per minute (RPM)). The serum was collected and immediately frozen at -80 °C.

3.5.1 S100B analyses

In **Paper I, II and IV** up until September 2008, a quantitative automated immunoassay was used (LIASON, DiaSorin, Italy). It is a two-site chemiluminescence immunoassay (sandwich principle) based on paramagnetic particles coated with two monoclonal antibodies and a monoclonal conjugate antibody labelled with an isoluminol derivative. During a first incubation, S-100 present in samples binds to the solid phase monoclonal antibodies, and subsequently after a washing step in a second incubation the antibody conjugate reacts with S-100 already bound to the solid phase. After incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents (alkaline peroxidase and a catalytic solution, with protohematin) are added and a flash chemiluminescence reaction is thus induced. The light signal (425 nm), and hence the amount of isoluminol-antibody conjugate, is measured by a photomultiplier as relative light units (RLU) and is directly proportional to S-100 concentration present in calibrators, samples or controls.

In September 2008, an automatic electrochemoluminescence immunoassay assay was introduced (Modular E170, Roche Diagnostics, Germany). The assay uses a sandwich complex, comprising of a biotinylated monoclonal S100B specific antibody and a monoclonal S100B specific antibody labeled with ruthenium. For incubation, streptavidin-coated microparticles were added and attached the complex to the solid phase through an interaction between biotin and streptavidin. Magnetic particles were captured on the electrode surface, and unbound particles were washed off. An electrode voltage was applied which induced the chemiluminiscent reaction detected using a photomultiplier. Some samples in **Paper I, II and IV**, as well as all samples in **Paper III**, were analyzed using the Roche Modular E170.

A commercially available S100B ELISA kit was used in **Paper V** (CanAg EIA S100, Fujirebio Diagnostics AB, Göteborg, Sweden). The samples were thawed overnight at 4°C and were added (50 µl per well) to a pre-coated 96 well plate and run in duplicates, according to the protocol. The results were analyzed using a Multiskan EX and Ascent Software V2.6 (ThermoLabSystems, Thermo Scientific, Waltham, USA). Absorbance was measured at 620 nm and a cubic spline curve fit method was used to fit the absorbance as recommended by the manufacturer. Extrapolation was used for sample levels outside calibration reference levels. Outliers (deviating more than 3 standard deviations) were excluded (n=3) (two normoxic, day 7 and one sham, day 1), as well as animals with an inadequate sample volume (n=1) (hypoxic, day 7).

All methods analyze the BB and AB dimers of S100B, and have been used in several other studies. The LIASON assay was developed for diagnosis of malignant melanoma, while the Roche Modular E170 is more tailored for TBI and emergency use, hence being faster (36 hours vs 18 minutes). The two assays have been shown to yield slightly dissimilar results, with a mean difference of 0.14 µg/L, with a variance being higher in more elevated concentrations >0.6 µg/L (Alber et al., 2005, Muller et al., 2006). A general pattern has emerged, revealing that the Roche Modular E170 presenting significantly lower levels. However, the two methods usually show a good correlation, up to $r=0.932$ (Smit et al., 2005, Mussack et al., 2006). In order to validate the two assays, the Department of Clinical Chemistry, Karolinska University Hospital ran both methods simultaneously for 6 months and no clinical significant difference between the two assays could be found, as supported by an internal publication of verification (Bergman, 2009).

3.5.2 NSE analysis

In **Paper III and IV** NSE samples were analyzed using the same method as for S100B prior to September 2008, a quantitative automated immunoassay (LIASON, DiaSorin, Italy). It is a two-site chemiluminescence immunoassay (sandwich principle). The method is very sensitive to hemolysis, so a sample was not analyzed if hemolysis (defined by haptoglobin concentration >0.5 g/L) was present.

3.5.3 NFL analysis

In **Paper IV**, determination of serum and CSF levels of NFL were analyzed using a commercially available ELISA kit (Uman Diagnostics, Umeå, Sweden) in line with the manufacturer's instructions. Measurements were performed in duplicates, using 50 µl undiluted cell-free serum, or 10µl CSF, per well. If levels were not within detection limits, that specific samples were re-run at a lower or higher dilution. NFL has been shown to be relatively stable, not affected by repetitive thawing (up to 4 thaws) (Kuhle et al., 2013).

3.6 MICRODIALYSIS MONITORING

In **Paper I, II, III and IV** intra-parenchymal microdialysis probes (CMA70, 10mm membrane, mDialysis AB, Stockholm, Sweden) were inserted when deemed appropriate by the attending neurosurgeon on call. In focal injuries, the probe was aimed for the peri-contusional locale (Nelson et al., 2011), while in diffuse injuries, the probe was placed in the right frontal lobe, according to international guidelines (Bellander et al., 2004b). Following the surgical procedure, the MD-catheter was connected to a MD-pump device (CMA 106, mDialysis AB, Stockholm, Sweden). A commercially available perfusion fluid ("Perfusion Fluid CNS", mDialysis AB, Stockholm, Sweden), was used as a carrier, pumped at 0.3µL/min. The MD-pumps acquired samples were stored in microvials (holding 200µL). The microvials were analyzed simultaneously every hour with a CMA600 enzyme-photometric analyzer (mDialysis AB, Stockholm, Sweden). Glucose, lactate, pyruvate and glycerol were analyzed regularly every hour. MD monitoring, as well as all other invasive intracranial monitoring, were withdrawn when the patient regained consciousness. While MD monitoring was used sporadically for patients in **Paper I, II and IV**, only **Paper III** presents analyzed MD data.

In **Paper III**, all patients had a CMA70 intra-parenchymal catheter, as previously described, and a CMA64 IView catheter (10mm membrane, mDialysis AB, Stockholm, Sweden) inserted in an external ventricular drain device. The CMA64 catheter was connected to a CMA106 MD pump, with the same perfusion fluid and perfusion velocity as the intra-parenchymal catheter. The CMA64 was placed inside a four-way stopcock (Multiflo3, BDConnecta, Franklin Lakes, NJ, USA), connected to both an extraventricular drain and a LiquoGuard® (Möller-Medical, Fulda, Germany) CSF-pump set to pump CSF with 2 mL/h, hence constantly being located in flowing CSF. The microvials were sampled with the same method and frequency as the CMA70 microvials. Data was presented as median and interquartile range.

3.7 NEURORADIOLOGICAL EXAMINATIONS

In **Paper I**, the presence of traumatic subarachnoid hemorrhage, subdural hematoma, hypodense lesions, epidural hematoma and cerebral contusions, on admission CT scan, were noted. Any midline shift was measured in millimeters and reported. Further, potential progression of the intracranial hematoma between the first and second CT-scan, was documented (Narayan et al., 2008).

For **Paper II**, all specific pathology noted on initial CT-scans included in **Paper I** were used. Also, all subsequent CT and MRI readings were reviewed by a neuroradiologist and if a secondary pathological development was detected, it was documented. Infarctions were discriminated from edema using either MRI, or subsequent CT-scans to determine if the initial hypodense region had diminished, and if so it was defined as edema.

In **Paper III and IV**, the admission CT scans were reviewed using different classification protocols, including Marshall CT classification (Marshall et al., 1991), Rotterdam CT score (Maas et al., 2005) and Stockholm CT score (Nelson et al., 2010).

Paper V used an ex-vivo (decapitated heads) MRI examination, utilizing a 9.4 Tesla machine with the following protocols; T2, fractional anisotropy (FA) and diffusion weighted imaging (DWI). T2 protocols were used to determine lesion size using the “free-hand tool” in ImageJ (1.48v, NIH, Bethesda, MD, USA) while ADC was measured for cytotoxic and vasogenic edema. It was performed in two rats per survival day and normoxia/hypoxia group, including sham. In total n=23 were performed (one hypoxic brain day 28 was missing due to procedural errors).

3.7.1 Computerized Tomography (CT)

All patients, in **Paper I, II, III and IV**, had been subjected to a head CT-scan prior to admission either at Karolinska University Hospital or at another hospital. By protocol, subsequent neuroradiological investigations were often performed following neurosurgical interventions, when patient deterioration was present or in absence of clinical improvement.

3.7.2 Magnetic Resonance Imaging (MRI)

MRI was often performed within 10 days after injury to detect potential DAI in **Paper I, II, III and IV**. As can be seen in **Paper II**, it was performed predominantly in the severe TBI patients (79%). The clinical course, and structural damage on CT, determined if a MRI was performed. For the human studies, the MRI used was a 1.5 Tesla machine using the following protocols; Echo Planar (EP) diffusion, Fluid Attenuated Inversion Recovery (FLAIR), Gradient Echo (GRE), T1 and T2. As clinical routine, the neuroradiological scans were all reviewed by two specialists in neuroradiology. In **Paper IV**, a blinded, dedicated specialist in neuroradiology reviewed all scans noting the extent of DAI.

In **Paper V**, the software ImageJ® (1.48v, NIH, Bethesda, MD, USA) was used to map the lesion area using the “Freehand selections”. T2 images from survival times at 7, 14 and 28 days were used since they had well-defined lesion areas. The largest lesion area per brain was chosen for quantification. Also, in **Paper V**, the freehand tool in ImageJ was used to detect the pericontusional area with an increased diffusion by measuring apparent diffusion coefficient (ADC) signaling indicating vasogenic edema and a decreased ADC indicating cytotoxic edema (Unterberg et al., 1997, Maeda et al., 2003, Unterberg et al., 2004). All MRI slides including edema were quantified and displayed as max value per animal.

3.8 CLINICAL PARAMETERS AND SECONDARY INSULTS

Age, sex, pupil responsiveness (graded as 0 = bilateral responsive, 1 = unilateral unresponsive and 2 = bilateral unresponsive) and Glasgow Coma Scale (Teasdale and Jennett, 1974) were documented at admission to the hospital in **Paper I, II, III and IV**.

In **Paper I**, pre-admission hypoxia (oxygen saturation <90% or respiratory obstruction) and hypotension (systolic blood pressure <90mmHg), were retrieved from ambulance charts. Multitrauma was defined according to Advance Trauma and Life Support (ATLS) guidelines, if thoracic, abdominal or skeletal injury were present on initial radiological examinations, or if the cause was a high-energy injury trauma (Committee on Trauma, 2008) (also noted in **Paper II**).

Paper II included several secondary insults monitored in the NICU, graded according to the Edinburgh University Secondary Insult Grade (EUSIG) (Jones et al., 1994). ICU-Pilot® (mDialysis AB, Stockholm, Sweden) was used to monitor patients in the NICU, and record secondary insults. The peak level of ICP during the treatment period, exceeding 5 minutes, was noted and divided into 4 groups; <20 mmHg (no secondary insult), 20-29 mmHg (mild secondary insult), 30-39 mmHg (moderate secondary insult) or ≥40 mmHg (severe secondary insult). Also, during 1.5 days prior to a secondary peak of S100B and 1.5 days after, secondary insults were extracted from the monitoring data. The secondary insults were present for >5 minutes, in order to be recorded. Presence of

increased ICP (>20 mmHg) was noted. The total burden of secondary insults were also recorded, including increased ICP (>20 mmHg), low cerebral perfusion pressure (CPP, <50mmHg), hypertension (systolic blood pressure >160mmHg, mean arterial pressure >110mmHg), hypotension (systolic blood pressure <90mmHg, mean arterial pressure <70mmHg) and pyrexia (>38°C) prior to, and after the secondary peaks of S100B. The lowest concentration of hemoglobin was also noted, as it was thought to possibly affect the development of secondary ischemic lesions.

Additionally, **Paper III** noted Abbreviated Injury Scale Score (AIS) and Injury Severity Score (ISS) at the admission to the NICU. Conventional CSF parameters (glucose, lactate, erythrocytes, leukocytes, and albumin) were acquired as they were sampled in the NICU.

In **Paper V**, per-operative data (pulse, saturation, perfusion) as well as post-operative blood gas samples (pO₂, pCO₂, pH, Lactate, Glucose, Hb, Na, K) were acquired. Data were displayed as median and interquartile range.

3.9 IMMUNOHISTOCHEMISTRY

3.9.1 Sample preparation

In **Paper V**, following euthanasia, the brain was formaldehyde perfused, and transferred to PBS solution in +4°C, waiting sectioning. Later on, the brain were moved to a 30% sucrose solution for three days and where then immediately frozen to -80°C. The brains were then sectioned in coronal in 14 µm thick sections. The sections were frozen in -10°C.

3.9.2 Antibodies used in immunohistochemistry

In **Paper V**, commercially available primary and secondary antibodies were used. For information concerning secondary antibodies, please see **Paper V**. To best visualize the immune cell activation to cerebral injury, we used CD68/ED1 to detect macrophages (and activated microglia) and CD43/W3/13 for granulocytes. NeuN was used to detect viable neurons and measure the lesion size. For detection of the end stage of the complement cascade, we used a specific antibody against the membrane attack complex (C5b9). Due

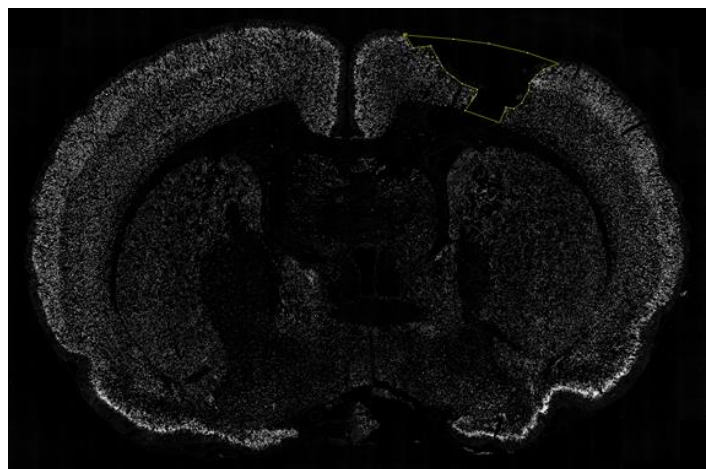


Figure 7 – NeuN (white) expression on a coronal section following cortical controlled impact. The missing cortical area is quantified.

to the lack of signal using the C5b9 immunofluorescence, an alternative tyramide signal amplification (TSA) procedure was employed. Also, expression of HIF-1α was included to monitor its changes following hypoxia/normoxia while vascular endothelial growth factor (VEGF) was used to detect an increased angiogenesis signaling and IgG extravasation to quantify the extent of BBB disintegration. DAPI was chosen as cellular counterstain. As control, all secondary antibodies were tested with omission of the primary antibodies resulting in no significant background noise. Sham surgery animals were also used (n=2 from normoxia and hypoxia, day 1).

3.9.3 Immunohistochemistry protocol

A standard staining protocol was used to primary, and secondary, incubate the sections of formaldehyde perfused, frozen brain tissue (see **Paper V** for a more detailed description). The primary antibodies were diluted in 0.3% TX-100 (X100, Sigma-Aldrich, St.Louis, USA) + 0.1% NaN₃ in PBS

pH7.4 for 16 hours (4°C) while the secondary antibodies were mixed in a Tris-NaCl-blocking buffer (TNB-buffer, FP1020, PerkinElmer, Waltham, USA), incubating in the dark for 90 minutes in room temperature. Sudan black was used to minimize background fluorescence. Sections were mounted using PVA/DABCO (ProLong® Gold antifade with DAPI, P36931, Life Technologies, Thermo Fisher Scientific, Waltham, USA) and stored in -20°C before being scanned. For more information concerning which primary and secondary antibodies that were used, see **Paper V**.

3.9.4 Microscope

In **Paper V**, a scanning microscope (MetaSystems, Alltussheim, Germany), using a 10x objective and different filter sets were used. Whole microscope slides were pre-scanned at 2.5x to generate a tissue-map. All tissue-covered areas were scanned using 10x primary objective. Individual images were stitched together (VSlide) to generate a large fluorescence image of the entire slide.

3.9.5 Image analysis

In **Paper V**, to quantify neuronal survival, the software ImageJ was used to map the cortical layer where NeuN expression was absent, using the “Freehand selections” (Figure 7). All brains were used and at least three sections per brain were quantified. The larger area of neuronal loss in the cortical layer was chosen for further analyses. The NeuN area revealed a more consistent result than the DAPI area since DAPI is more sensitive to debris in the lesion cavity and to the quality of the section (if the tissue is folded, slightly out of focus etc.).

ImageJ was also used to analyze all other antibody expression in the cortical tissue surrounding the lesion (upper right corner). A cut-off threshold was chosen for each antibody. The threshold varied depending on the fluorescence intensity of each antibody in order to eliminate potential background expression. The staining was automatically quantified using macros created in ImageJ similar to methods described previously (Papadopoulos et al., 2007, Gandhi et al., 2012). All available slides were used, with a minimum of three sections per brain for each antibody. The slide with the highest staining intensity per brain was selected and expressed as “Integrated density”, including all pixels above the specified cut-off. One rat (ET012, hypoxia 7 days) presented as an outlier for all antibodies used in the immunofluorescence experiments and was thus excluded from the study.

3.10 STATISTICAL ANALYSIS

The statistical program R (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>) was used throughout **all Papers**. To plot the line graphs in **Paper III**, GraphPad Prism 6.0 (GraphPad Software Inc., 2014) was used. In **Paper I, II and IV**, GCS was used as continuous variable, as has been done previously (Perel et al., 2008). Throughout **all Papers**, a p-value <0.05 was considered statistically significant; however a Bonferroni correction was applied in **Paper III** due to a low sample size. For more detailed analyses, see the specific papers.

3.10.1.1 Paper I

A prediction model was used, using admission parameters known to influence outcome, where S100B was added to establish to which extent the estimated explained variance was increased, using the “rms”-package in R. Accuracy of all models was evaluated using Nagelkerke’s pseudo- R^2 , which provides a partial prediction between 0 and 1, where 1 is a fully explained model (also used in **Paper II, III, IV and V**). Multivariate analyses, with a stepwise reduction based on Akaike Information Criterion, were performed with imputed data to determine which variables had an independent correlation to outcome. Additionally, a linear regression was performed to determine what parameters were correlated to levels of S100B. The temporal relation between sample-time, and the strength of

correlation between S100B and outcome, was investigated using a sliding time window of 100 samples examined using a proportional odds model of GOS prediction.

3.10.1.2 Paper II

Logistical regression was performed, to determine parameters correlated to the development of secondary radiological findings, and proportional odds analyses were performed to analyze parameters correlated to patient outcome. The “rms”-package in R, and Nagelkerke’s pseudo- R^2 , was used in univariate analyses. Multivariate analysis, using a step-wise reduction of parameters, was performed to determine which parameters were independently correlated to the development of secondary radiological pathology, and to patient outcome. Sensitivity, specificity, negative predictive value and positive predictive value for the different cut-off levels, of secondary peaks of S100B, were calculated. A receiver operating characteristic curve (ROC curve) was provided as to best determine which cut-off level to use. A Wilcoxon Rank-Sum test was performed to determine any significant difference between secondary insults (ICP and total secondary insult burden), prior to and after, the secondary increase of S100B.

3.10.1.3 Paper III

The “rms”-package in R was used to perform univariate logistic regression analyses, examining the correlations between levels of conventional CSF- and MD-CSF glucose and lactate, as well as for outcome predictions. Bland-Altman plots were used to illustrate similarity between conventional CSF-samples and MD-CSF samples (Bland and Altman, 1986). A Mann-Whitney-U Test was used to assess association between median MD parameters (in brain and CSF) and favorable- and unfavorable outcome.

3.10.1.4 Paper IV

The “rms” package for proportional odds models was used. Parameters known to be predictive of TBI outcome were included (Age, GCS, Pupil response, CT score, and trauma grade (ISS, AISS)) as adjusting parameters. Since data on NFL was sampled 1-3 times per patient and at different time points, mixed model analyses were not possible. As an alternative median, mean and max values were determined and used in the statistical models. To near a normal distribution, logarithm values for NFL, S100B and NSE data were used. Multivariate proportional odds analyses were performed towards GOS. The explained variance of linear models when identifying parameters related to serum and CSF NFL is given as an adjusted R^2 . A Mann-Whitney U-Test was used to compare NFL levels in patients with and without DAI in Marshall Grade II patients.

3.10.1.5 Paper V

To correlate intra-operative monitored data and post-surgery metabolic data, Mann-Whitney U Tests were employed to allow comparisons between normoxic and hypoxic animals. The MRI lesion size was illustrated by using bar plots and compared using a Mann-Whitney U Test. Linear regression analyses were performed to correlate antibody expression, as well as S100B levels and edema, to hypoxic/normoxic group and to time after injury. A Student-T-Test was performed on S100B levels the first day to compare hypoxic and normoxic animals.

3.10.2 Missing Data

In **Paper I, II and IV** missing data was imputed using multiple imputations (MI). This was done by using the “MICE” package in R. Seven imputations have been suggested by the International Mission for Prognosis and Analysis of Clinical Trials in TBI (IMPACT) investigators, in order to further improve the quality of retrospective analyses (McHugh et al., 2007a). MI exchanges missing values by an

estimate drawn from a distribution of known data, retaining the uncertainty of the imputed data in following analyses.

In **Paper III and V**, missing data were excluded from the analyses.

4 RESULTS

4.1 BIOMARKERS IN RELATION TO OUTCOME CORRELATION

Paper I showed that later S100B levels sampled later resulted in higher predictive capabilities toward long-term outcome than the initial samples. The peak and area under curve (AUC) levels of S100B both had a higher pseudo- R^2 in explaining outcome prediction than age, pupil responsiveness and GCS at admission (Table 5). In multivariate analysis, age, pupil responsiveness and S100B levels (log AUC) correlated independently to GOS. Unexpectedly, GCS was not independently correlated to outcome. Using a core model of CT-findings, age, pupil responsiveness and GCS, S100B provided an additional explained variance of 6.6%, yielding a total of 35.8%. In **Paper IV**, we found that, in addition to serum levels of S100B, serum levels of NFL provided a significantly ($p=0.006$) increased estimated partial pseudo- R^2 of 2.3% towards functional outcome prediction if used together with other core parameters (Stockholm CT score, pupil responsiveness, age, S100B and GCS at admission).

Also, **Paper I** revealed a discrepancy between AUC and peak levels in some patients, especially if the peak of S100B occurred early (within 12 hours) after trauma (Figure 9). It was concluded that S100B should be sampled 12-36 hours after injury in order to better improve outcome predictions, where it may reach a pseudo- R^2 of 0.20-0.25% towards long-term outcome prediction.

Factor	p-value	pseudo R^2
log S100B AUC first 48 hours	<0.0001	0.119
log S100B Peak level first 48h	<0.0001	0.109
Age	<0.0001	0.102
Pupil responsiveness	<0.0001	0.079
EDH on CT scan	0.0008	0.044
Progression of intracranial	0.0012	0.037
Hypodense lesion on CT scan	0.0016	0.039
GCS at admission	0.0062	0.023
SDH on CT scan	0.0126	0.021
Midline shift on CT scan	0.0305	0.017
Hypotension at SoA	0.0532	NS
trSAH on CT scan	0.055	NS
Contusion on CT scan	0.087	NS
Gender	0.427	NS
Multitrauma	0.525	NS
Hypoxia at SoA	0.880	NS

Table 5 - Ordinal regression analyses of parameters and their correlation to outcome (GOS 1–5)

Paper II showed that the development of secondary pathological findings on CT/MRI was independently correlated to outcome. In univariate ordinal regression analysis, a secondary injury development and a secondary peak of S100B were highly correlated to outcome (pseudo- $R^2 = 0.111$ and 0.100, respectively), only exceeded by age (pseudo- $R^2 = 0.122$). A multivariate analysis revealed that, both secondary peaks of S100B and subsequent radiological findings are both independently correlated to long-term outcome, however they present significant co-linearity, thus not being independently correlated if they are present together in prediction models. Using secondary injury development on CT/MRI, an additional 1.3% is added of explained variance to predict long term outcome.

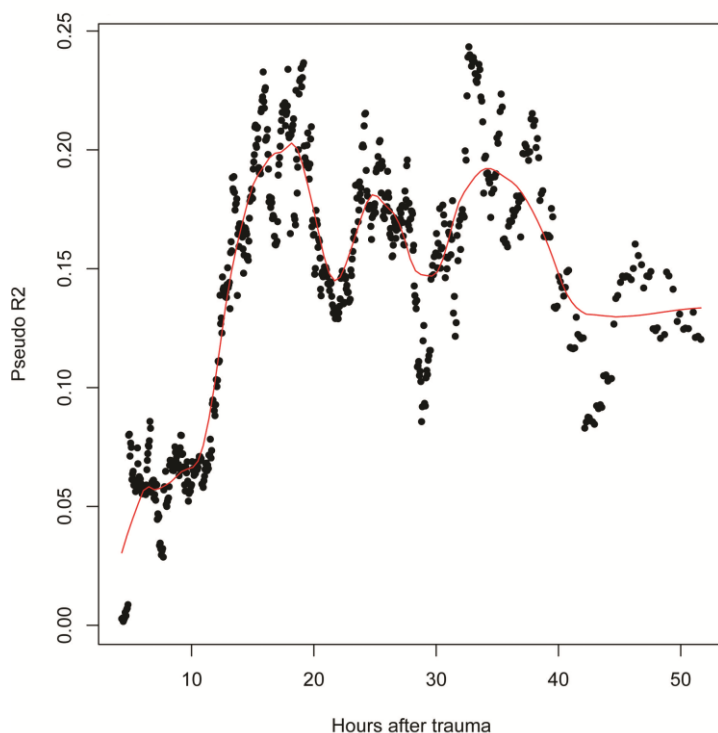


Figure 9 - Precision of S100B to predict outcome in relation to time from injury. The x-axis indicates when S100B was sampled in relation to the time of trauma (hours). The y-axis represents the pseudo R^2 of a proportional odds model predicting outcome as Glasgow Outcome Score (GOS). The smooth line represents a locally weighted scatterplot smoothing (LOWESS), a regression of data points.

findings. Other cut-offs of secondary S100B were used, including $0.10\mu\text{g/L}$ and $0.5\mu\text{g/L}$, which also yielded significant results. Moreover, in a multivariate model, low admission GCS, hypodense lesions on CT-scans and an increased ICP (present sometime during the NICU stay) also independently correlated to the development of subsequent radiological pathology. In total, a pseudo- R^2 of 0.704 towards predicting these secondary findings was found using a core-model of secondary peaks of S100B, age, pupil and GCS-, hemoglobin-, ICP- and admission CT-parameters. Using a lower ($0.05\mu\text{g/L}$) cut off compared to $0.1\mu\text{g/L}$, or $0.5\mu\text{g/L}$, there is an increase of sensitivity ($0.5=16\% - 0.1=62\% - 0.05=80\%$) with only a limited loss of specificity ($0.5=98\% - 0.1=95\% - 0.05=89\%$), to detect secondary radiological findings. A receiver operating characteristic (ROC) curve yielded an AUC of 0.855 using secondary peaks of S100B to detect subsequent radiological pathology (Figure 10).

Paper III showed that increased levels of lactate and pyruvate, extracted using MD-CSF, were correlated to an unfavorable outcome (GOSe 1-5 vs 6-8) ($p=0.0167$ and $p=0.0293$, respectively) (Figure 8). Using conventional (regional) microdialysis analyzing ECF samples, no significant correlation could be found.

4.2 BIOMARKERS IN RELATION TO MONITORING

In **Paper II**, we detected secondary CT/MRI pathology, not present at admission, in 39% TBI of cases, predominantly cerebral infarctions or other ischemic injuries. These secondary radiological events occurred in proximity to secondary increases of S100B. A secondary increase of $0.05\mu\text{g/L}$ of S100B resulted in a pseudo- R^2 of 0.532 in correlation to the development of secondary radiological

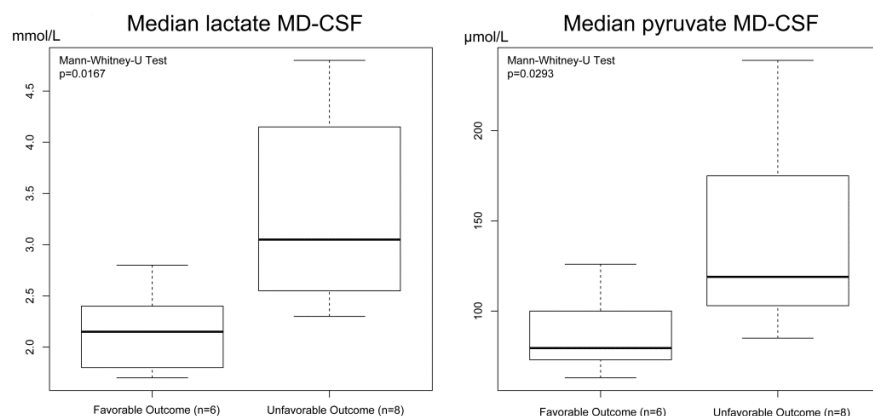


Figure 8 - Median MD-CSF lactate and MD-CSF pyruvate levels versus outcome (GOSe 1-5 vs 6-8). MD-CSF lactate levels and MD-CSF pyruvate levels are significantly higher in patients with an unfavorable outcome ($p=0.0167$ and $p=0.0293$ respectively).

In an attempt to predict the secondary increases of S100B with conventional monitoring and subsequent deterioration in the NICU, increased ICP alone, and increased ICP together with decreased CPP, hypertension, hypotension and pyrexia were monitored 36 hours prior to and after the secondary peak of S100B but no significant difference could be detected.

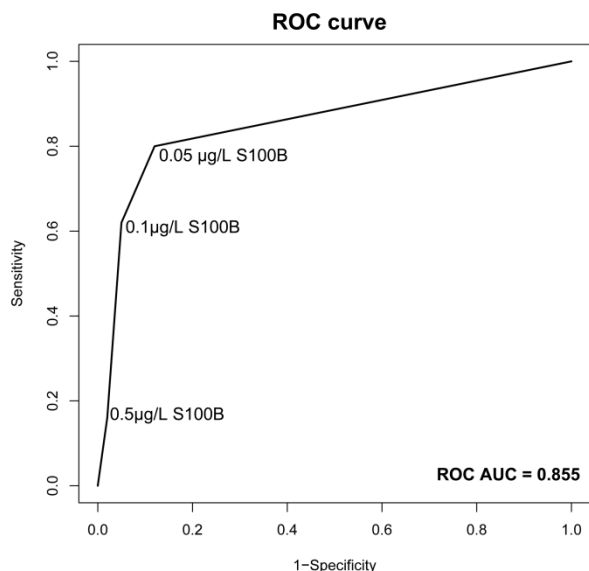


Figure 10 - ROC curve of secondary peak of S100B cut-off levels and the predictability to detect a subsequent pathology on CT or MRI.

In **Paper III**, a new method of monitoring cerebral metabolism using MD catheters in flowing CSF was used. The method revealed a good concordance between conventionally sampled glucose and lactate levels from the CSF. According to us, a clinically accepted variance of 0.51 and 0.13 was detected (Figure 11). The MD-CSF samples were not significantly influenced by CSF erythrocyte-, leukocyte- or albumin concentrations. Using the MD-CSF, metabolites in CSF could be detected with a better temporal resolution than has been previously described. This new monitoring technique detected higher levels of glucose in CSF while lactate, pyruvate, LPR and glycerol were higher in ECF, independent of pericontusional or non-pericontusional location.

Paper IV showed that NFL levels in serum following TBI remain relatively unchanged over time in the individual patient, even if there are concentration differences between patients (Figure 12).

In **Paper V**, per-operative monitoring and post-surgery metabolic data confirmed a hypoxic status. Hypoxic animals had significantly higher pulse and lower peripheral perfusion than normoxic animals ($p=0.0314$ and $p<0.0001$, respectively). The hypoxic animals had, apart from lower pO_2 concentrations, significantly higher lactate, glucose and pCO_2 concentrations following 30 minutes of hypoxia.

4.3 BIOMARKERS IN RELATION TO STRUCTURAL DAMAGE

In **Paper I**, a univariate analysis was performed to determine which factors that correlated with serum concentrations of S100B. The step-wise reduction model retained only CT parameters, including traumatic subarachnoid hemorrhage, absence of epidural hematoma, contusions, midline shift, hypodense lesions and progression of intracranial hematomas as significantly correlated to S100B levels. Noteworthy was that a progression of intracranial hematomas was seen in 59% - 61% of the patients (**Paper I and Paper II**, respectively). Interestingly, multitrauma did not correlate significantly to S100B levels.

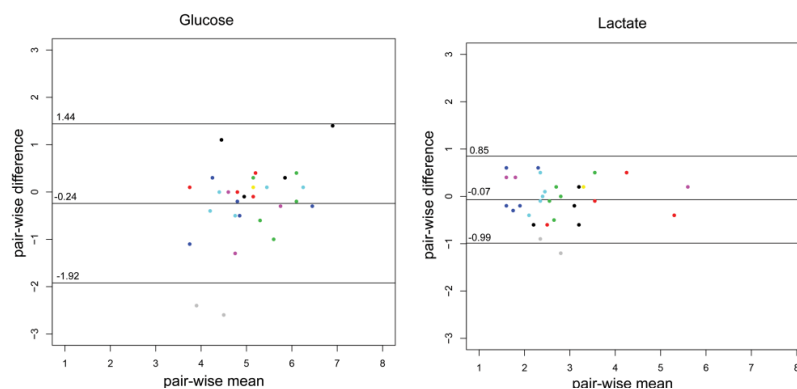


Figure 11 - Bland-Altman plots of the CSF glucose and CSF lactate samples ($n=29$) corresponding with MD-CSF glucose (left) and MD-CSF lactate (right). The points are plotted with the difference between two observations (pair-wise difference) on the y-axis, and the mean of the two observations (mean-wise difference) on the x-axis. The confidence limits and the mean are plotted as black lines. The variance for glucose and lactate are 0.51 and 0.13 respectively. Every patient ($n=14$) is represented by a unique color. Two points lay outside the confidence limits for glucose while one point is outside the confidence limits for lactate.

Serum NFL levels were not significantly correlated to diffuse axonal injury as noted on MRI scans, or to damage assessed using CT scoring protocols, as was shown by **Paper IV**. Levels of NFL were significantly correlated to levels of NSE, but not to S100B.

Using ex-vivo MRT in **Paper V**, a significantly larger lesion size was detected in the hypoxic animals at day 7, 14 and 28 compared to the normoxic animals ($p=0.017$) (Figure 13). Neuronal survival, as defined by NeuN expression, was also significantly increased in the normoxic animals ($p=0.032$).

In **Paper V**, surprisingly, HIF-1 α and VEGF expression were higher in normoxic animals compared to hypoxic animals ($p=0.042$ and $p=0.038$, respectively). HIF-1 α decreased significantly over time while VEGF expression remained unchanged.

In **Paper V**, based on MRI analysis, we noted that cytotoxic edema increased rapidly after TBI and then gradually decreased over time ($p<0.0001$). However, the area of vasogenic edema had a lesser pronounced temporal pattern. When comparing both treatment groups, we found no differences in the extent of brain edema between hypoxic and normoxic animals. C5b9 was upregulated in the perilesional area, primarily expressed in surrounding neurons. The highest expression was seen immediately after trauma and then steadily decreased over time but no significant differences were seen between oxygenation groups.

ED1 expression in **Paper V** was predominantly seen in the peri-lesional subcortical white matter, although also spread out over the cortical areas while CD43 (granulocyte) expression was only detected in the peri-lesional area. ED1 expression revealed a temporal pattern, increasing significantly over time whereas CD43 remained unchanged.

Interestingly, there was a correlation between the amount of macrophage infiltration and the area of vasogenic edema ($p=0.011$, $r^2=0.381$). IgG extravasation, as an indicator of BBB disruption, was expressed in the perilesional zone and peaked early after injury and then steadily decreased without

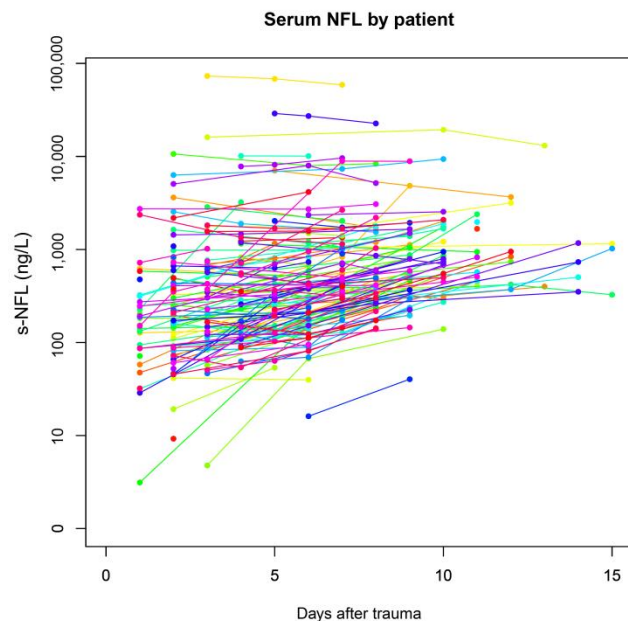


Figure 12 - Serum NFL levels for all patients (**Paper IV**). Every line (separate color) represents an individual patient. Generally, even if there were differences in concentration between patients they were limited over time for the individual patient.

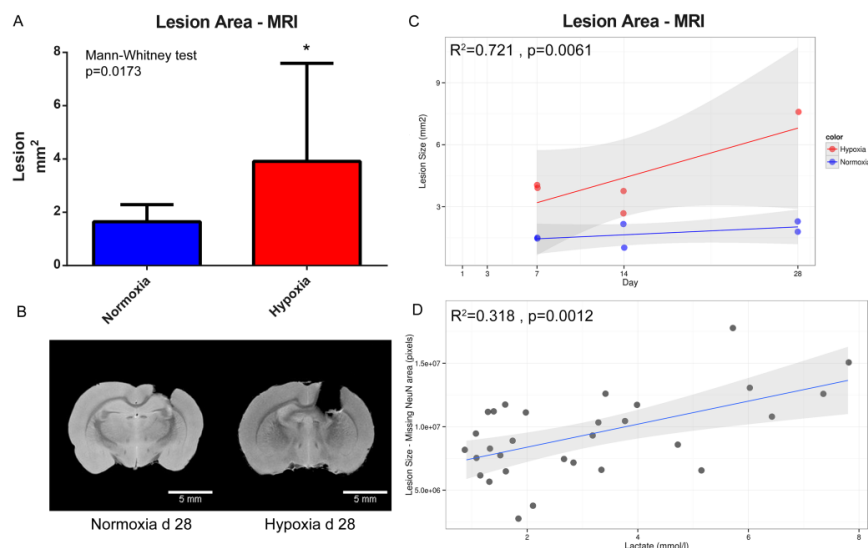


Figure 13 - **A** displays the difference between lesion size for normoxic- ($n=6$) and hypoxic rats ($n=5$) during survival times d7, d14 and d28. In **B**, the lesion size on two d28 animals are visualized; normoxia 28 days following TBI (left) and hypoxia 28 days following TBI (right). **C** illustrates how the lesion size on MRI increased over time in hypoxic (red) and normoxic (blue) ($R^2=0.721$). The gray area represents 95% confidence interval while the line is a regression line. **D** correlates lesion size using missing NeuN area and lactate levels acquired from blood gas post-surgery; as lactate increases, so does the lesion size ($R^2=0.318$).

any significant difference between hypoxic and normoxic animals. Notably, IgG extravasation was highly correlated to C5b9 expression ($p<0.0001$, $r^2=0.759$).

Further, in **Paper V**, S100B levels in serum 24 hours after inflicted injury revealed a trend towards being higher in hypoxic compared to normoxic animals ($p=0.0844$).

5 GENERAL DISCUSSION

5.1 BIOMARKERS IN RELATION TO OUTCOME CORRELATION

In this thesis, we present two of the largest studies to date of TBI patients in neuro-intensive care monitored using serum levels of the biomarker S100B (**Paper I and II**). While several studies have shown a correlation between levels of S100B and outcome, **Paper I** analyzed more frequent S100B sampling in multivariate models towards outcome. Our data indicates that serum levels of S100B have a robust correlation to clinical outcome, adding 6.6% in addition to core parameters (CT findings, age, GCS at admission and pupil responsiveness), which is difficult to compare with other studies but is probably on par or better than parameters such as age, pupil responsiveness and age (Murray et al., 2007). Furthermore, in a univariate analysis, we found S100B to better than age, GCS and pupil responsiveness explain long-term outcome. This could partly be explained by the timing of assessments, since GCS and pupil responsiveness were examined at admission to the hospital and not like S100B which was sampled up to 48 hours after injury. Low GCS two days after injury would probably have had a greater explained variance towards outcome than on admission, which has been previously shown (Lee et al., 2012). However, it is not always possible and could be harmful to wake a sedated patient with a possible intracranial hypertension in the first days following trauma for adequately assess the GCS (Skoglund et al., 2009, Helbok et al., 2012). Moreover, pupil responsiveness is useful in the emergency setting in order to detect intracranial mass lesions, but following hematoma evacuation it may not be as accurately correlated to long-term outcome. In conclusion, future outcome prediction models of TBI will improve their predictive capabilities should S100B levels be incorporated (Lesko et al., 2014), in addition to other core parameters.

Unexpectedly, GCS in our material (in **Paper I, II and IV**) was found not be a strong predictor towards long-term outcome (pseudo- R^2 of 2.3%, 1.8% and 1.6% ($p>0.05$) respectively in each paper). The hospital admission GCS, usually the golden standard, was used in our study, which sometimes is affected by sedative medications (Balestreri et al., 2004) and may thus be inferior to other assessed intervals, such as the post-resuscitation GCS or GCS assessed at the scene of accident. GCS is also complicated to assess due to multitrauma, speech impediments, eye injury and has been seen to present a high intra-personnel variance (Bledsoe et al., 2014). Also, by using only the motor component in GCS, a better prediction to long-term outcome has been seen compared to the other components (Healey et al., 2003). In aggregate, we believe that if the GCS score was assessed at another time interval, or if the three different GCS components had been available for analysis, perhaps it would better reflect the severity of the intracranial injury and thus improve outcome prediction in TBI patients.

In **Paper IV**, we found that by adding serum levels of NFL to an outcome prediction model including S100B (and CT-findings, age, GCS and pupil responsiveness), additional significant explained variance was found (2.3%), presumably indicating that another clinically important pathophysiological processes was monitored by analyzing serum levels of NFL in addition to S100B. Other biomarker combinations have shown to increase outcome prediction, including GFAP+S100B (Vos et al., 2010), NSE+S100B+GFAP (Vos et al., 2004) and UCHL-1+GFAP (Mondello et al., 2012), presumably as surrogate markers for different cellular damages. However, also in **Paper IV**, CSF levels of NFL failed to show any significant correlation to long-term outcome, an analysis that perhaps was relatively underpowered due to a lower sample size. Altogether, we believe that a panel of biomarkers should be used to monitor different clinically important pathophysiological intracranial processes following TBI.

In **Paper I**, a discrepancy between early peak levels and AUC levels of S100B was detected. This phenomenon has been previously described for very early samples of S100B, which will quickly decrease within the first hours (Jackson et al., 2000). It is postulated that these quick falls could be

due to decreased permeability of the BBB, which has been shown in experimental TBI to only be open 30 minutes after injury (Barzo et al., 1997a). Although more novel research has suggested that the glymphatic system drains S100B from the brain to serum, independent of BBB integrity (Plog et al., 2015), and this would release high levels of S100B into serum from CSF and the damaged brain. Either way, systemic S100B levels would decrease swiftly as the in-vivo half-life has been shown to be as short as 20 minutes (Jonsson et al., 2000). Another reason why S100B increases shortly after injury could be a contribution of S100B from extracranial injury, bone/adipose/muscle tissue in multitrauma patients (Pelinka et al., 2003), but has been shown to have a very limited contribution for the total concentration over time (Pham et al., 2010). After 12 hours, we saw that the predictive ability of S100B levels increased dramatically, which is in concordance with other studies that indicate better correlation from later sampling. Altogether, timing of S100B sampling is very important since early high levels seem to less accurately predict long-term outcome perhaps due to extracranial sources of S100B.

The development of secondary cerebral injuries visible on MRI and CT scans was significantly, and independently, correlated to long-term functional outcome in **Paper II**. This finding emphasizes the need for advanced neuro-intensive care after the first days following injury in order to detect and potentially treat or minimize the extent of secondary injuries.

Increased CSF lactate and pyruvate levels were seen to correlate to long-term functional outcome in **Paper III**. Isolated increase in lactate levels have previously been described as a predictor of unfavorable outcome in TBI patients, perhaps as a marker of hypoxic anaerobic metabolism or impaired acid-base status of cerebral cells (Crockard and Taylor, 1972, DeSalles et al., 1986). Increased pyruvate levels, together with increased lactate levels, have previously been seen in TBI (Toczylowska et al., 2006). It has been postulated that this metabolic pattern could be due to be an effect of red blood cell glycolysis in the CSF, or glycolysis in the ischemic affected brain tissue to exceeding the ability to adequately metabolize pyruvate, thus leading to increased levels of both lactate and pyruvate (Hlatky et al., 2004). Also, cerebral hypermetabolism could lead to similar metabolic changes (Foley et al., 2008). However, the erythrocyte levels in CSF were strongly correlated to the lactate concentration thus probably indicating a contribution of lactate from red blood cells. Altogether, the metabolic pattern of increased lactate and pyruvate levels in CSF seen in **Paper III** are probably the result of high levels of erythrocytes in the CSF, yet other detrimental metabolic causes in the affected brain cannot be excluded.

5.2 BIOMARKERS IN RELATION TO MONITORING

In **Paper II**, we detected secondary injuries by radiological tests, predominantly ischemic, in 39% of the patients. These findings are greater than previously reported (10-22%) (Mirvis et al., 1990, Raabe et al., 2004, Marino et al., 2006, Tawil et al., 2008, Tian et al., 2008, Chen et al., 2013), which could be explained by a more liberal use of MRI detecting ischemic injuries not visible on CT scans.

Our choice of a low cut-off limit (0.05 µg/L) for a secondary peak of S100B greatly increased the sensitivity to detect secondary lesions present on CT/MRI scans (n=73 (true positive), a sensitivity of 80%). If the higher cut-off limit described by Raabe et al. (0.5µg/L) would have been used (Raabe et al., 2004), a lot of secondary lesions would have been missed (n=81, a sensitivity of 16%). However, these steeper increases of S100B are perhaps more clinically relevant and should thus prompt a more rapid diagnostic/therapeutic response i.e. invasive vasospasm treatment, evacuating of mass lesion and aggressive ICP management. Transporting ICU patients to perform a radiological examination, or for other logistic purposes, are correlated to an increased risk of secondary insults (Andrews et al., 1990), hence should be done only when they are absolutely necessary, a decision making that perhaps could be facilitated by the use of S100B. In a study from Undén et al., they did not see any significant correlation of an increase of S100B prior to the development of a secondary neurological

deterioration (Uندن et al., 2007). In our study (**Paper II**), it's impossible to say if imminent treatment would have averted the secondary injury that occurred in conjunction with the secondary S100B peak, it is however probable that a swift response would, if not prevent, limit the extent of the secondary injury. Our twice-per-day sampling of S100B is the most frequent that has been described but presumably, several more peaks occurred during the treatment periods since the in-vivo half-life of S100B has been shown to be as low as 25 minutes (Jonsson et al., 2000). Moreover, several lesion developments probably occurred during the first 48 hours and were perhaps concealed by the primary peak of S100B, and could thus not be detected using the current method. In aggregate, repeated sampling of S100B revealed secondary peaks which correlated to subsequent injury development and should thus be implemented in the multi-modal monitoring of patients suffering from TBI.

While increased ICP throughout the whole treatment period was correlated to the development of a secondary injury on MRI/CT, the ICP (and other secondary insults) 36h before versus 36h after secondary peak of S100B did not show any significant difference. Our choice of comparing insults to the timing of secondary peak S100B, instead of when the secondary injury was shown on CT/MRI, was based on the temporal resolution (CT could be up to several days after the peak while S100B was acquired every 12 hours). While some studies have shown that increased S100B levels precedes an increase in ICP, they use different definitions of secondary insults, persisting for different amount of time and are correlated to different increases of S100B, making comparisons between models difficult (Raabe et al., 1999, Olivecrona et al., 2009, Bellander et al., 2011, Olivecrona et al., 2014). However, as increased ICP occurring sometime during the NICU treatment correlated to secondary injury development, we do believe that intracranial hypertension should be treated and prevented, but that the exact timing of subsequent deterioration of intracranial pathology is better assessed if S100B is used in addition to conventional multi-modal monitoring.

In **Paper III**, we developed a new way of “global” cerebral metabolic monitoring by using a MD catheter located in a steady flow of CSF. We found that the recovery of the catheter was higher (98%) than from ECF (65%) (Hutchinson et al., 2000) and blood (80%) (Rooyackers et al., 2010), and similar to what is found when the MD is located in reference solutions (>90%) (Afinowi et al., 2009). Recovery for lactate revealed a lower variance than for glucose, which could be a result due to the small sample size but a similar pattern with a strict metabolic control between cerebral compartments have previously been described (Guerra-Romero et al., 1992). Presumably, the medium analyzed greatly affects the recovery of the MD catheter, increasing in a more water-like solution.

When monitoring metabolic parameters in CSF and ECF with high temporal resolution (**Paper III**), we noted that glucose levels remained higher in the CSF while lactate, pyruvate and glycerol showed higher levels in the ECF. This is probably the result of higher metabolic activity with ongoing cellular stress in the ECF of the injured cerebral tissue, compared to the CSF which is an ultra-filtration of blood and where the different metabolites will become more diluted, or added from the blood (glucose). Anyhow, this further reveals a potential benefit of monitoring both compartments as they tell different stories. Furthermore, with the discovery of the glymphatic system and thus newly suggested movements between different cerebral compartments, MD will become an even more important clinical tool to validate novel findings (Iliff et al., 2012).

In **Paper IV**, the intra-individual levels of NFL only generally had limited changes during the first two weeks after trauma. This could be the effect of logarithmic data, but could also be explained by the long in vivo half-life of NFL which has been shown to be about three weeks (Barry et al., 2007), thus considerably longer than the 20 hours for NSE (Ingebrigtsen and Romner, 2002) and 25 minutes for S100B (Jonsson et al., 2000). If NFL levels were to be elevated initially after trauma, they will probably remain high throughout the first two weeks after TBI, since they will be heavily influenced by the primary injury and early pathophysiological cascades. S100B has been shown to have a more

dynamic range in its temporal profile and thus perhaps works better as a monitoring biomarker to detect secondary injuries in the neuro-intensive care unit (as seen in **Paper II**).

In **Paper V**, we found that the lactate and glucose blood gas levels were increased following 30 minutes of hypoxia, presumable as a response to the anaerobic environment and a subsequent metabolic distress and catecholamine surge in these animals, which has been shown to affect outcome in humans (Woolf et al., 1987). This is further supported by pO_2 levels that were lower in the hypoxic group. Paradoxically, pH was higher in the hypoxic animals than in the normoxic animals, even though lactate was increased creating a presumably acidic environment. Perhaps a compensatory mechanism, with spontaneous hyperventilation and a secondary respiratory alkalosis occurred, as supported by lower levels of pCO_2 .

5.3 BIOMARKERS IN RELATION TO STRUCTURAL DAMAGE

In an attempt to detect which parameters that could explain the levels of S100B in serum, only intracranial parameters remained significant in uni- and multivariate analysis. Progression of intracranial hematoma was the parameter with the greatest pseudo- R^2 in univariate analysis (11.6%), indicating an ongoing cerebral deterioration in these patients with subsequent high levels of S100B. That only intracranial CT parameters were significantly correlated to levels of S100B in serum also demonstrates the biomarker's higher cerebral specificity, compared to potential S100B originating from extracranial sources (**Paper I**). Extracranial sources of S100B have been shown to yield increased S100B levels for a limited amount of time (Pelinka et al., 2003) and lower increases than intracranial trauma (Savola et al., 2004), perhaps explaining our results that are sampled relatively late (up to 48 hours) after trauma.

In **Paper V**, we noted that cortical neuronal loss was more pronounced in hypoxic vs normoxic rats, which is supported by results from previous investigators (Clark et al., 1997a). As time after injury passed, so did the neuronal loss, supported by studies that reveal ongoing apoptosis in the cortical region up to a week following hypoxic-TBI (Clark et al., 1997a, Hallam et al., 2004). Blood lactate levels, as a surrogate marker of hypoxia, were also significantly correlated to a diminished neuronal survival. Lactate levels and survival time (days after trauma), if used together in linear models, provided an explanatory pseudo- R^2 as high as 47% to cortical neuronal death. In summary, we demonstrated that hypoxia following focal TBI has detrimental effects on neuronal survival, being directly correlated to enhanced levels of lactate immediately after injury.

Serum concentrations of S100B in **Paper V** were higher in hypoxic rats 24 hours after impact, though not reaching statistical significance ($p=0.0868$), presumably due to low sample sizes. For the later survival times, no difference between the oxygenation groups was observed which is probably the result of the limited half-life of S100B which is as short as 25 minutes in humans (Jonsson et al., 2000). Moreover, protein turnover in rats have been shown to be 10x that in humans, and 28 rat days would theoretically correspond to about 5 years in humans, presumably making it difficult to compare to human conditions (Quinn, 2005, Sengupta, 2013, Agoston, 2015). In patients we have seen in **Paper I** and **Paper II** that S100B correlates to the extent of hypodense lesions at hospital admission as well as subsequent ischemic development, indicating that if aggravated ischemia is added to the brain injury it would lead to an additional increase of S100B. This is further supported by studies on perinatal asphyxia and S100B where it has been shown to correlate to the degree of ischemic injury (Beharier et al., 2012) as well as in human TBI studies where hypoxia correlated to increased levels of S100B (Yan et al., 2014). Altogether, this supports the validity of S100B measured early after trauma as a potential biomarker to monitoring severe hypoxic injuries following TBI, albeit further investigations including earlier survival times are warranted to verify this assumption.

In **Paper V**, we saw that hypoxia increased the lesion size by using ex-vivo MRI examinations, making this the first to our knowledge to analyze lesion size using MRI over an extended time period. Previously, focal hypoxic-TBI models have revealed an increase in cortical lesion size by using immunohistochemical techniques (Bramlett et al., 1999b, Matsushita et al., 2001). Only 7, 14 and 28 day examinations were chosen since they had well defined lesion cavities. The extent of vasogenic and cytotoxic edema was not significantly different between the hypoxic or normoxic groups, as is supported by a similar study by a collaborating group that also failed to detect any significant increase of brain edema during hypoxic conditions (Yan et al., 2011), although different models were used. Previous studies that do detect increased edema in hypoxic vs normoxic TBI all use fluid percussion models (Ishige et al., 1987, Van Putten et al., 2005, Gabrielian et al., 2011) which perhaps provides a more well defined affected area not influenced by the lesion cavity that might be more suitable for edema formation and quantification. In focal TBI models, cytotoxic edema has been suggested to be the prominent type of edema (Unterberg et al., 1997, Stroop et al., 1998), which was further supported by our ex-vivo MRI examinations, being highest immediately after trauma and then decreasing significantly over time (Barzo et al., 1997b, Marmarou, 2003). Vasogenic edema was seen in close proximity to the lesion cavity, as previously described (Kawamata et al., 2000, Maeda et al., 2003) and increased over time, albeit not significantly, which was perhaps due to the small sample size of the animal groups. Interestingly, there was a correlation between macrophage (ED1) accumulation and the presence of vasogenic edema. Perivascular macrophages in these areas (Rosenberg and Yang, 2007), could perhaps exacerbate edema following TBI (Holmin and Mathiesen, 2000). In conclusion, we noted that rats subjected to hypoxia post TBI presented significantly increased lesion areas on MRI, however no significant difference between cytotoxic and vasogenic edema could be detected between the groups.

Normoxic animals had higher expression of HIF1 α in **Paper V**, which was more obvious at later time points (7 and 14 days). **Paper V** is the first study, to our knowledge that uses HIF1 α in a model of hypoxic TBI, while up-regulation of HIF1 α has been used to validate hypoxia-ischemic brain injury in neonatal rats (Li et al., 2007). In normoxic conditions, HIF1 α correlated to apoptosis, has been analyzed in experimental TBI (diffuse and focal models) where an early increase (1 hour up to 3 days), followed by a later decrease in expression, has been noted (Yu et al., 2001, Ding et al., 2009, Li et al., 2013). In our study, a similar temporal pattern of HIF1 α was noted, even if **Paper V** also included later time points. An up-regulation of HIF1 α does not necessarily mean that the injured tissue will perish. Even if it is recognized that HIF1 α promotes apoptotic neuronal death (Singh et al., 2012), it has also been shown to up-regulate angiogenic factors (VEGF), erythropoiesis and act protectively against mitochondrial and cellular damage (Ebert et al., 1995, Liu et al., 1995, Lawrence et al., 1996, Fan et al., 2009). Perhaps, in hypoxic animals, the perilesional tissue die early after injury as supported by a greater neuronal loss, while the border zone in normoxic animals survive, hence being able to express HIF1 α . This to some extent paradoxical finding is supported by two reviews, suggesting milder hypoxic states, HIF-1 α triggers neuro-protective mechanisms, while more severe hypoxia promotes apoptosis and cellular death (Fan et al., 2009, Singh et al., 2012). In **Paper V**, HIF-1 α strongly correlated with VEGF activation (Liu et al., 1995) and like HIF-1 α , VEGF was significantly upregulated in the normoxic animals, primarily 3, 7 and 14 days following injury, presumably highlighting the neuro-protective capabilities of HIF-1 α . VEGF expression has been seen to peak 4-6 days following TBI (Skold et al., 2005) and act as a promotor of neurogenesis after injury (Skold et al., 2005, Skold et al., 2006), supporting its neuro-protective role (Thau-Zuchman et al., 2010). All in all, we do believe that up-regulation of HIF-1 α and VEGF could reflect the neuro-protective capabilities in surviving cells, which could explain their increase in normoxia, and not in hypoxia, following TBI.

IgG extravasation, C5b9-, ED1- and CD43 expression were not significantly different in the hypoxic compared to the normoxic rats in **Paper V**. This is the first CCI model of hypoxic TBI, to our knowledge, that analyzes tissue expression these proteins. These components of the immune system

all followed previously described temporal profiles; IgG (Beaumont et al., 2000) and C5b9 (Rostami et al., 2013) having an increased expression early after injury decreasing over time, while ED1 expression increased over time (Bellander et al., 2010). CD43 expression was not significantly different to survival time. Studies that show a significant increase of ED1 (Hellewell et al., 2010) and IgG (Tanno et al., 1992) after brain injury in hypoxic conditions use diffuse TBI models, which are perhaps better suited for quantification since they do not present with a lesion cavity.

We used female Sprague Dawley rats in **Paper V**, while previous hypoxic TBI-models have used male rats. It's difficult to assess to what extent this presents as a limitation but it should be taken into consideration as the inflammatory response between male and female rats in experimental TBI is different (Gunther et al., 2015), presumably as females have shown better neuro-protective effects, which is suggested to be due to higher levels of estrogen and progesterone (Roof and Hall, 2000, McCullough and Hurn, 2003).

6 CONCLUDING REMARKS

The general conclusions were that S100B, and NFL, contributed independently and significantly toward long-term functional outcome. Furthermore, repeated S100B sampling monitored patients by identifying secondary cerebral deterioration and the more global MD-CSF detected deranged cerebral metabolism. Finally, through a translational approach, we detected a trend for higher S100B levels if hypoxia was added to an experimental model of TBI.

6.1.1.1 *Paper I*

We concluded that serum levels of S100B add substantial information regarding patient outcome, in excess of that provided by age, GCS, pupil reaction and CT findings. In univariate analysis, late samples and AUC levels of S100B presented with a higher pseudo- R^2 than all other analysed parameters toward long-term functional outcome. Elevated S100B levels are best correlated to CT-findings and were not significantly correlated to extracranial injury. Samples acquired 12-36 hours after trauma have best correlation towards long-term outcome.

6.1.1.2 *Paper II*

Secondary peaks of serum S100B, 48 hours after TBI, are highly correlated with secondary radiological pathology. These secondary pathological findings on CT and MRI were found to be correlated to worse outcome in both uni- and multivariate analysis. We suggest increases of S100B $\geq 0.05\mu\text{g/L}$ to be a monitoring trigger level prompting a review of the patient, displaying a sensitivity of 80% and a specificity of 89% of detecting a secondary pathological progression of existing, or a newly developed, injury.

6.1.1.3 *Paper III*

We developed a new technique of “global” microdialysis monitoring of CSF and MD-CSF monitoring correlates with conventional CSF-levels of glucose and lactate. The MD recovery, using the current MD set-up in CSF, is close to 100% for both lactate and glucose which is higher than previously described. Increases in lactate and pyruvate, without any effect on the LPR, significantly correlate to unfavorable outcome, though perhaps indicating an effect of the presence of erythrocytes in the CSF, or possibly a hypermetabolic state in the injured brain.

6.1.1.4 *Paper IV*

We found that NFL levels in serum are significantly correlated to outcome even when adjusting for known strong predictors of TBI, including S100B. In line with its long half-life, logged NFL levels remained relatively unchanged over time, albeit with an upward trend, exhibiting limited intra-patient differences. No correlation between DAI, or CT findings, and NFL levels could be detected. We hypothesize that NFL might reflect a separate, more long term, pathophysiological process than current TBI biomarkers.

6.1.1.5 *Paper V*

We found that adding a hypoxic insult to a model of TBI using the CCI paradigm, it lead to a decreased neuronal survival, increased lesion size and a trend towards higher levels of the biomarker S100B in serum 1 day after trauma compared to normoxic animals. The levels of lactate and survival time when analyzed together provided a pseudo- R^2 of 47% in determining the area of neuronal death, thereby highlighting the hypoxic effect on brain pathology. HIF-1 α and VEGF were found upregulated following normoxic TBI, primarily at later survival times, perhaps indicating neuro-protective mechanisms.

7 FUTURE PERSPECTIVES

In **Paper I**, we noted that S100B was an independent predictor of long-term functional outcome. We were not able to find a significant increase of registered extracranial trauma. However, as S100B has been detected in several extracranial tissues and have been shown to be elevated after extracranial injury, a more brain specific biomarker might perhaps further increase the explained variance towards long-term outcome (such as we did in **Paper IV**). Even if we monitored a substantial amount of clinical data, perhaps we would be able to better predict long-term outcome than the 35.9% pseudo- R^2 that the core model (age, GCS, pupil responsiveness, CT-findings, hypoxia and hypotension at admission and S100B AUC) provided. In **Paper I**, perhaps if more monitoring data, including microdialysis data, cerebral oxygen saturation, intracranial pressure, perfusion pressure, hypotension and pyrexia, as well as other biomarkers of other cerebral origin was used, a better explained variance could be achieved. Also, pre-hospital morbidity probably represents a substantial part of the explained variance towards outcome and is something that is unfortunately lacking from many studies analyzing TBI and outcome. While GOS is somewhat of a golden standard in the field, its levels are a bit too wide and unspecific. Also, there is no clear cut line when it should be assessed, with studies ranging from hospital discharge to up to two years following trauma. Newer classifications, such as the eight graded scale extended GOS would perhaps better highlight aspects of functional outcome that are now missed. Also, a better and more regular and structured follow-up should be used to better assess outcome. This is important for all papers in this thesis. In **Paper I**, While we included samples up to 48 hours after trauma, being an arbitrary time-line that we thought represented the acute phase after TBI, later samples would perhaps have further improved outcome predictions and is something that has the be explored in future studies.

In **Paper II**, while we correlated secondary peaks of S100B to radiological findings, but we cannot say if the peaks came before the deterioration or vice versa, neither was it possible to determine if the care or diagnostics provided lead to an improved outcome. This is something that has to be further explored in a prospective study, perhaps using even more frequent S100B monitoring than twice per day. In **Paper II**, we used a combination of MRI and CT scans. If a more regular follow up examinations, with repeated MRI scans the first week following injury, a better comparison between S100B dynamics and extent of structural damage could be assessed, such as in the upcoming multicenter Center-TBI study. The EUSIG (Edinburgh University Secondary Insult Grades) was used to grade different secondary insults in the NICU. While this system using different cut-offs (e.g. 20-30mmHg, 30-40mmHg and >40 mmHg for ICP) a more individualized system, using the area under curve of each monitored secondary insult would perhaps provide a better and tailor-made monitoring of secondary burden and perhaps a better correlation towards outcome.

In **Paper III**, we developed a new method of monitoring CSF using the MD technique. While we applied it on TBI patients, it's entirely possible to use it on all patients where draining of CSF improves the clinical situation, such as patients suffering from subarachnoid hemorrhage. Another area where repeated sampling of lactate and glucose, through a closed system, is important is in acute bacterial meningitis where we believe that the published method would provide advantages to current monitoring methods. While we monitored the "conventional" metabolites using a 20 kDa MD probe, it would be possible to use the current system to extract larger molecules. Preferably, it would be able to monitor more brain specific proteins with a high temporal resolution. As mentioned, with the discovery of the glymphatic system, our method of global MD-CSF provides an important research platform in order to further validate the flow of proteins between different cerebral compartments with an increased temporal resolution. The major limitation of **Paper III** is the limited amount of patients and we hope that more patients will be monitored in this fashion in the future as to further validate the method.

In **Paper IV**, we found that NFL added significant and independent outcome information. We hope that future studies in the field of biomarker discovery have samples acquired in a more regular and constructed fashion with samples acquired from the same days, which would improve the discovery process. Also, other studies that have looked at outcome prediction at NFL levels have looked several weeks after the time of trauma, which is probably important if you are analyzing a protein with a long half-life or a biomarker that reflects an ongoing pathophysiological process. When assessing DAI, better methods are needed to quantify the extent of injury and perhaps in the future a more exact volume of disrupted axons would be able to improve the specificity and thus better validate the role of biomarkers of axonal origin.

In **Paper V**, we showed that hypoxia exacerbates the lesion development in TBI. While we found an increased trend for S100B in hypoxic animals if sampled 24 hours after injury, perhaps there are better biomarkers to monitor this process which would provide a significant difference that should be explored in further studies. In vivo MRI would perhaps have increased the detection of injuries, including cerebral blood flow, which perhaps is altered following hypoxic injury. The method developed in **Paper V** could be used to validate the properties of different neuro-protective drugs and the biomarker profiles may work as templates to detect treatment effects, and thus be used as therapeutical targets.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Traumatiska hjärnskador är en mycket vanlig dödsorsak globalt och överlevande drabbas oftast av livslånga handikapp. Tidigare har främst yngre drabbats av svårare traumatiska hjärnskador. Nyare studier visar dock att allt fler äldre drabbas, vilket innebär en allt större belastning för en redan utsatt patientgrupp. Individer med allvarliga traumatiska hjärnskador, de som blir medvetslösa efter skadan, vårdas i respirator på specialiserade neurointensivvårdsavdelningar på landets universitetssjukhus. Vården fokuseras i dessa fall på att minimera risken för ytterligare skada av den redan drabbade hjärnan. För att veta vilka patienter som behöver prioriteras högt där man misstänker potentiellt livshotande hjärnskador, används i dag olika parametrar när man diagnosticerar såsom obefintlig pupillreaktion, dålig medvetandegrad, lågt blodtryck, låg syrgasmättnad i blodet samt hög ålder. Upprioritering av denna patientgrupp sker mot bakgrund av att utfallet för patienter med dessa faktorer är betydligt sämre vid långtidsuppföljning jämfört med andra patienter. Patienter som har drabbats av trauma och fått en blödning i hjärnan genomgår ofta kirurgi med utrymning av blodansamlingar samt inoperation av övervakningsutrustning som kan mäta tryck, syrgasnivåer samt ämnesomsättning i hjärnans vävnad för att motverka utvecklingen av ytterligare skador. Tyvärr är denna övervakning oftast mycket regional och kan därför inte ge en bild av hela den skadade hjärnan.

Hjärnans nervceller och stödjeceller innehåller olika proteiner som man har kunnat påvisa frisätts vid skada. Dessa ämnen brukar kallas för biomarkörer och används inom flera medicinska områden för att fastställa och övervaka olika typer av biologiska processer i kroppen som ligger bakom skador och sjukdomstillstånd. En typ av stödjecell, så kallade astrocyter, innehåller höga nivåer av ett protein som heter S100B. Nervceller i sin tur innehåller höga nivåer av neuronspecifikt enolase (NSE) medan de trådliknande utskott som sammanbinder delar av hjärnan, axoner, till stor del består av neurofilament. Vid skada frisätts dessa ämnen i patientens blod och ryggmärgsvätska och kan då mätas för att bestämma omfattningen av hjärnskadan och ge en bredare bild än tidigare medicinska övervaknings- och diagnostiseringsmetoder.

Genom tre olika databasstudier har vi studerat nivåer av biomarkörer samt koppla dessa till funktionellt utfall och allvarlighetsgrad av skada från 181, 250 respektive 265 patienter som vårdats på neurokirurgens intensivvårdsavdelning Karolinska Universitetssjukhuset i Solna mellan 2005 och 2013.

I den första studien som inkluderade 265 patienter ämnade vi fastställa hur nivåer av S100B vid ankomst till sjukhuset, och de följande 48 timmarna, kunde kopplas till utfallet 6 till 12 månader efter olyckan, samt fastställa tidpunkten för när man bör ta provet efter traumat för att få bäst koppling till utfall. Vi ville också se vilka faktorer som ledde till höga nivåer av S100B. Vi såg att förekomsten av serumnivåer av S100B bidrog signifikant till att förutsäga utfallet för patienten, mer än hög ålder, avsaknad av pupillreaktion samt låg medvetandegrad vid ankomst till sjukhus. De oberoende faktorererna för att förutspå utfall utöver nivåer av S100B var ålder samt pupillreaktion. Vi kunde genom studien fastställa att optimal tidpunkt för provtagning är 12-36 timmar efter trauma för att erhålla bäst koppling till utfall efter 6-12 månader. Enbart skador på hjärnan upptäckta med skiktröntgen kunde signifikant korreleras till förhöjda S100B nivåer. Skador vid olyckan i andra delar av kroppen resulterade inte i signifikant förhöjda nivåer av S100B i blodserum, vilket talar för att S100B är hjärnspecifikt.

I den andra studien, innehållandes 250 patienter, ville vi se hur sekundära ökningarna av S100B i serum 48 timmar efter traumat gick att koppla till försämringar, eller nytillkomna skador, upptäckta vid datortomografi samt magnetkameraundersökningar. Vi ville även se hur dessa skadliga förändringar gick att koppla till utfallet 6-12 månader efter olyckstillfället. Genom studien fann vi en tydlig koppling mellan de patienter som haft sekundära stegringar av S100B i serum och där sekundära skador kunnat fastställas genom röntgen-, och magnetkameraundersökningar. Vi kunde även visa att dessa

sekundära ökningar av S100B, samt skadeutveckling, signifikant bidrar till utfallet för patienter efter 6-12 månader. En sekundär stegringsnivå redan på 0.05 µg/L är tillräcklig för att med god känslighet kunna detektera skadeutveckling på undersökningar, en nivå som är betydligt lägre än vad som redovisats i tidigare studier.

I den tredje databasstudien som inkluderade 181 patienter där analys av neurofilament light (NFL) i blod (samt hos vissa även i ryggmärgsvätska) hade utförts ville vi se hur dessa nivåer enskilt kunde kopplas till långtidsutfallet, även i utfallsmodeller med S100B. Vi ville också se om graden av diffus axonal skada, en typ av hjärnskada som uppstår djupt i hjärnan som bara kan noteras på magnetkameraundersökning, kunde kopplas till nivåerna av NFL. Vi kunde fastställa att NFL signifikant bidrog till att förutspå utfallet, oberoende av nivåerna av S100B. Däremot kunde vi inte se någon koppling mellan diffus axonal skada eller annan hjärnskada noterad på datortomografi och magnetkameraundersökning av hjärnan och nivåer av NFL. Det förefaller vara så att nivåerna av NFL i blodet står för en kliniskt viktig skadeprocess som inte kan åskådliggöras med de metoder som finns tillgängliga idag.

Vi har även försökt att förbättra mikrodialys tekniken på en patientgrupp av 14 patienter. Mikrodialys är en tunn slang med hål där små ämnen kan passera. Genom att använda mikrodialys kan man få en överblick av hjärnans ämnesomsättning och cellsönderfall genom att mäta nivåerna av druvsocker (glukos), mjölksyra (laktat), pyrodruvsyra (pyruvat) samt glycerol (ett vanligt ämne i cellens membran). Genom att placera en mikrodialysslang i flödande ryggmärgsvätska som pumpas ut ur hålrummen i hjärnan, de så kallade sidoventrikulerna, kan man mäta ämnesomsättningen i ryggmärgsvätska och på så sätt få en mer global bild av hjärnans status. Genom vår studie kunde vi konstatera att en god överensstämmelse mellan laktat- och glukosnivåerna mellan prover tagna med mikrodialys teknik jämfört med prover dragna från ventrikeldränaget med sedvanliga metoder, vilket säkerställer att metoden fungerar. Vi fann att patienter med ett sämre utfall 6 månader efter olyckan uppvisade signifikant förhöjda nivåer av laktat och pyruvat, vilket kan vara resultatet av en skadligt förhöjd ämnesomsättning, men även ett indirekt tecken på att det finns många röda blodkroppar i ryggmärgsvätskan.

För att se hur en hjärnskada försämras vid låga syrgasnivåer, något som är relativt vanligt på olycksplatsen där den drabbades andning och luftvägar på olika sätt kan vara påverkade, användes en djurmodell för syrebrist och traumatisk hjärnskada. Råttor sövdes och fick inandas en syrgasblandning innehållande hälften av syrgaskoncentrationen i vanlig rumsluft efter att en traumatisk hjärnskada hade åsamkats över höger hjässlob.

Gruppen av djur som inandades luften med låg syrgaskoncentration uppvisade lägre nivåer av syrgastrick samt högre nivåer av laktat vid blodgasprovtagning vilket bekräftade att djuren led av syrebrist (hypoxi). Dessa djur utvecklade ett större skadeområde vilket man påvisade med magnetkameraundersökning genomförd efter att djuren hade avlivats. Hjärnvävnaden undersöktes och resultaten visade att överlevnaden för nervceller var sämre för de hypoxiska djuren.

Nivåerna av biomarkören S100B visade en trend till att vara högre i hypoxiska djur 24 timmar efter hjärnskadan, jämfört med nivåerna hos djur som inandades normal syrgaskoncentration. Vi kunde även se att nivåerna av proteinerna "Vascular Endothelial Growth Factor, VEGF" som är inblandad i bildningen av nya blodkärl samt "Hypoxia-Inducing Factor 1 Alpha, HIF1α" som kan uppreglera skyddande effekter i vävnad som utsatts för hypoxi, var signifikant förhöjda i djur som inandats normal syrgaskoncentration. Denna uppreglering skulle kunna indikera en skyddande effekt för den överlevande hjärnvävnaden, dock inte under extrema hypoxiska förhållanden vilket är förenligt med tidigare litteratur. I övrigt uppmätte vi delar av det medfödda immunförsvaret som har visat sig vara involverad i sekundära skadeprocesser i hjärnan i anslutning till skadan med hjälp av vävnadsanalys, så kallad immunohistokemisk teknik. Vi såg ingen signifikant skillnad för aktiviteten i det medfödda

immunförsvaret mellan hypoxiska djur och de djur som inandats normal syrgaskoncentration. Med andra ord visar experimentet att hypoxi vid traumatisk hjärnskada leder till en mer omfattande skada för hjärnan som vi inte kan visa är signifikant medierad av immunförsvarets reaktion i hjärnan.

Sammanfattningsvis har vi påvisat att biomarkören S100B är en stark prediktor för utfall vid traumatisk hjärnskada, framförallt om provet tas inom 12-36 timmar efter olyckan. Vi har även visat att nivåerna av S100B inom 48 timmar efter trauma ökar mest vid skada på hjärnan och inte vid skada på övriga kroppen. Vi har vidare funnit att S100B kan användas för att övervaka medvetslösa patienter efter traumatisk hjärnskada för att kunna detektera när det sker en utveckling, eller försämring, av en för utfallet relevant sekundär hjärnskada. Genom att addera mätningar av S100B till mätningar av NFL, en biomarkör med annat ursprung än S100B, har vi påvisat en signifikant ökning av prediktionen av det långtidfunktionella utfallet för patienterna. Vi har även utvecklat och validerat en ny metod för att övervaka ämnesomsättningen med hjälp av mikrodialys teknik och kunskapen om att nivåerna av laktat och pyruvat är signifikant förhöjda hos patienter i ryggmärgsvätska vilket leder till ett sämre funktionellt utfall. Genom att använda en djurmodell för traumatisk hjärnskada har vi också kunnat konstatera att hypoxi leder till en sämre överlevnad för nervceller samt en större skada i hjärnan. Vi har vid försöken noterat en trend till högre S100B-värden hos hypoxiska djur efter 24 timmar från skadetillfället vilket indikerar att biomarkören är användbar för att se om syrebrist har förvärrat en hjärnskada.

9 ACKNOWLEDGEMENTS

I would like express my sincerest and deepest gratitude to;

Karolinska Institutet, Department of Clinical Neuroscience, for having me as a PhD-student. A special thanks to **Jan Hillert**, Prefect, and **Robert "Bob" Harris**, Director of doctoral studies, for supervision and stimulating discussions on how to improve our mutual research areas. I'm looking forward to future collaborations.

My supervisor, Associate Professor **Bo-Michael "Bomme" Bellander**, for acting not only as my supervisor but also neighbor, car-pooler, boss, mentor, co-author and dear friend. Even if I don't always mention it, you know that I consider my PhD years to be the best years of my life so far and that is much thanks to you. Knowing you means that you get close to your loving family (**Kinna, Hampus, Michaela, Susanna** and **Pamina**), which definitely has been a positive addition. I have never met a physician that cares more for the patient to the same extent as you do. Thank you for forcing me outside my comfort zone (the lab) presenting our research to the world. I might plan to release a book to your future PhD-students which will have to include; how to take care of your grandchildren, which Châteauneuf-du-Pape to choose and how to adequately endeavor 500 grams of beef at Grill Ruby, to name a few essential chapters. It is a true honor working with you.

If there is something a research group needs it's someone with cutting edge English skills and a profound knowledge of statistics (any remaining imperfections in the manuscript are solely caused by me). Beyond a doubt, my co-supervisor Dr. **David Nelson's** expertise fulfills these requirements. Thank you for improving the manuscripts and providing us with models that illustrate the data far better than what I have ever seen in published research. Also, you have introduced "R" in my life and, even if I'm not nearly on par with you, I live in a moRe wondeRful woRld (statistically speaking) today than I have eveR done.

My 3rd semester supervisor who later on became my Co-supervisor, Professor **Mårten Risling**. Without your awesome, laid-back picture of you sipping a drink on a conference on your temporary homepage, I wouldn't have walked up those stairs at the Department of Neuroscience 10 years ago and this thesis probably would not have been a reality. You have my sincere gratitude for assisting me in times of need, especially if there is a shortage of antibodies, and to guiding me in my translational path from rat to man, it has been a true privilege.

My Professor, former Head of the neurosurgical department, and Co-supervisor **Mikael Svensson**. While in the neurosurgery clinic, without your focus on science, I probably wouldn't have continued down the path of clinical research. Your surgical skills are matched only by your scientific contribution. Thank you for supporting my work and assisting me in times of need during my PhD period, your aid has been invaluable.

Co-supervisor and Professor **Denes Agoston**, thank you for all your help and interesting discussion on how to improve brain trauma models, analyses of biomarkers and world political issues. Thank you also for initiating fruitful collaborations. I'm looking forward to continuing working in our scientific expeditions to find better and more useful brain biomarkers to treat patients suffering from TBI.

Dr. **Marcus Ohlsson**, my research mentor, hunting buddy and dear friend. Our contact started way before I sat my foot in the clinic and will probably continue until we are both old and wrinkled. Without your enthusiasm for research and clinical work I wouldn't have been able to be where I am today. Thank you for always being there, available 24/7, if I needed help with everything from work, hamburgers, rifles, U.S imports and co-operative video-gaming.

Professor **Lou Brundin**, for always being supportive and believing in my work. Your enthusiasm is impressive and it always fills me with joy and excitement to tackle the scientific hurdles in front of us. I don't know how to thank you enough.

My dear friend and colleague Dr. **Arvid Frostell**. Without your skills and knowledge, ranging from constructing lasers from garbage to ggplots in R, no rat would have been adequately ventilated, this thesis not nearly as Shiny as it is, the lab a little less biased towards Apple products, but most importantly, my stay in the lab and throughout these years a lot more boring. In my life, I have never met someone with that true spirit of an inventor and researcher that you possess. Thank you so much for your help and friendship, from you and your family. And remember, if something doesn't work, rule number one of troubleshooting is to always ask yourself, "is it because it's a mac?".

Britt Meijer, laboratory technician and superior "do-it-all" at the Neurosurgical research laboratory. Thank you so much for all the teaching and help throughout the experiments, but most of all for being there for me during this whole time. I have never met another person who shares your reliability and stability. The administrative process that research actually is would have been impossible without you. I cannot thank you enough for being and bearing with me throughout my PhD period.

Dr. **Nicholas Mitsios** who shed blood, sweat and perhaps some Tween-20 with me during my immunohistochemical adventures at SciLifeLab. I don't think I've ever met someone as patient as you, with an extremely tolerant and enjoyable attitude towards the Sisyphus-like task to mount and scan approximately 1815 rat brain glass slides. Furthermore, thank you for only calling me *μαλάκα* when I really deserved it. You have been a true friend.

I wish to thank everyone in the neurosurgical research laboratory; **Ramil Hakim**, my research mentee whose academic credit by far exceeds mine and who is probably the most well prepared student Karolinska Institutet ever had the pleasure to educate. **Pendar Kalili**, who is one of the most dedicated persons I've ever seen. Even if you do not work with rats, or with traumatic brain injury, I consider you a nice guy (which is saying a lot coming from me). Already, you have produced impressive results and I'm looking forward to future scientific breakthroughs. **Sebastian Tham**, the U.S home-comer, thanks for all our interesting and fruitful discussion on how to combine clinic with research. Your knowledge about stem cells is difficult to match, globally. **Jonas Gripenland**, for your hard work in spinal cord trauma, cross-country skiing and RNA research. **Ann-Christin Von Vogelsang**, who has thought me that adequate follow-up and improved outcome assessment is *teh shit*. **Peter Alpkvist**, who is probably the most well-educated clinician that I have ever met. I know that clinical research can be tedious and tough, but you will enjoy it when you can reap the harvest of your hard labor, trust me. **Christian Glaumann**, the nerve gluer. Former PhD-students; **Mikael Fagerlund**, who taught me how that Swedish-Finns are actually alright, and that rolling tables make perfect storage units for thesis-books. **Jonathan Nordblom**, for our interesting discussions about life, work and everything in between (or perhaps it is all connected?). **Lisa Arvidsson**, for teaching me the essentials on who to go to, who to ask for what and what stem cells are really all about. Our medical students **Theodore Holmlöv**, **Greta Johansson** and **Emma Jeppsson**. I'm glad that you all have survived my co-supervision. **Paula Mannström** and **Pernilla Klyve Busa**, welcome to our lab!

The CMM part of the lab, or should we say "the smarter part". **Ruxandra Covacu**, my surgical partner who prefers rats with glowing cells. Thank you for keeping me company in the laboratory and sharing my hatred towards beeping machines. With your smarts, and my will, we might just have some great research ahead of us. **Sreenivasa "Sreeni" Sankavaram**, my fellow-impact colleague (even if you slightly miss the brain and hit the spinal cord), thanks for all the support and I'm looking forward to further interesting findings. **Cynthia Perez Estrada**, the astronaut of the lab that with a never-ending smile encourages everyone, thanks for all your support. **Maria Bergsland**, for beautiful CLARITY-

images, encouraging fika-pauses and wonderful horses. **Susanne Neumann**, for taking care of my beloved brain sections.

The Risling Laboratory, for commencing my research career approximately 10 years ago. **Maria Angeria**, little did we know that we were to continue to work together after that semester 10 years ago. Thank you for teaching me immunohistochemistry 101, but remember there is more in life than a beautiful Beta-APP staining. **Lizan Kawa**, with an English accent even more terrific than Emma Watson's and with blast-TBI skills that probably are a lot better than hers. **Yuli Cao**, who taught us that brain injury models does not have to harm animals, even if some unlucky cells inevitably will hurt. **Mattias Günther**, an anesthesiologist who turned to the neurosurgical department, whilst also studying the interesting aspect of sex differences in TBI, as well as underground pubs in Budapest. **Mattias Sköld**, the VEGF guy who was the first hard working PhD-student I met back in 2005. Your papers have been a great source of inspiration. **Elham Rostami**, thank you for all the support and for making the scientific meetings delightful experiences.

The tissue profiling group at the Science for Life Laboratory. **Jan Mulder**, thank you for taking me in and accepting to run my (behemoth) project. Also, thank you for everything you have done for me and to really let me feel welcome in the group, I think this is only the beginning of a fruitful collaboration. **Tony Jimenez-Beristain**, for keeping the tissue profiling lab (and Nick) neat and tidy. Your linguistic skills are unmatched by anyone I have ever met and your quick-witted mindset was one of the true joys of the lab. **Agnieszka Limiszewska**, for intense lab discussions about nothing and everything. **Lora Weidner**, the American who is not afraid of Swedish suburbia and whose English skills I gladly put to use. **Chuang Lyu**, who taught us all that life is beautiful and that "tomato" is very different from "tomato". **Kamila Kamuda**, who migrated back to Denmark. Your mounting skills will be dearly missed. Also, thank you **Ellinore Bäcklin Bergh** and **Carl-Fredrik Bowin** for making the spring of 2014 something to remember.

Department of Neurosurgery, my base from 2009 to 2015 that trained me to become the physician that I am today. **Inti Peredo**, head of the department, for your impressive work in keeping the clinic afloat during harsh times. **Lars Kihlström**, for your skillful presence in both the skull base and in questions regarding medical training. **Jiri Bartek** (and **Sofia**), the on-call machine who never sleeps and is always ready for science. **Tiit Mathiesen**, for your academic approach scientific contribution, your papers have been well read, trust me. **Adrian Elmi Terander**, for friendship and interactive discussions on how to improve health-care. **Jenny Pettersson-Segerlind**, for encouraging extra-curricular skiing activities. **Kyrre Pedersen**, for overseeing the spinal surgery section, but also for being so tech-savvy and introducing me to new TV shows. **Halldor Skulasson**, for your impressive surgical skills, your only flaw is that you are too biased towards Apple products. **Petter Förander**, for teaching me how to clinically maneuver R16, especially in my early days. Keep up the good clinical research, and boulder climb with caution. **Margret Jensdottir** and Emma **Svensdotter**, for your valuable contributions to the department. **Per Almqvist**, for helping and assisting young doctors at our clinic, thirsty for education and knowledge. **Göran Lind**, your never ending patience and good mood is contagious at the clinic. Thank you for all the useful input provided throughout the years. **Gaston Schechtmann**, for nice talks in our corridor and nice plants in the office. **Bengt Gustavsson**, for extremely good surgical supervision. Your work as a pediatric neurosurgeon is invaluable. **Ulrika Sandvik**, for bringing some sisu to the clinic. I'm really looking forward to be your fellow research colleague in the future. **Erik Edström**, for sharing my interest in traumatic brain injury and your hard work in the clinic. **Oscar Persson**, for enjoyable moments in the OR. **Simon Skyrman**, thank you for all our interesting discussions and your friendship. Your esthetics skills have provided much joy for the clinic. **Anders Fytagoridis**, the Greek from Gothenburg, thank you for all the laughs and adventures throughout the years (thanks also to **Isa** and **Musa**). **Ernest Dodoo**, thank you so much for always encouraging me in my work and for teaching me neurosurgical procedures at odd hours. **George**

Sinclair and **Bodo Lippitz**, for keeping the gamma knife running. Furthermore, former colleagues **Pontus Jonsson** and **Gustav Burström**, true friends following in my S:t Göran footsteps. **Olivia Kiwanuka**, your clinical expertise in the clinic, lab and in the wild are impressive. **Jennifer Farde**, for showing us that skiing should be a prioritized activity. **Boel Gustavsson** and **Malin Björnsson**, thanks for all the laughs in the department. **Helena Martinelle**, **Amina Guenna Holmgren** and **Biljana Milovac**, for keeping track of everything that is actually important, our work would be impossible without you. Moreover, a big thank you to all physicians (young and old) nurses, secretaries and nurses aids, novice or experienced, that have supported my work throughout the years.

The Neuro-intensive Care Unit (NICU or “NIVA”) at Karolinska University Hospital, which is, and probably will always be my “home” clinic in one way or the other. **Birgitta Ohlgren**, for running the NIVA-ship and 5 kilometers faster than I do. **Gunilla “Nilla” Malmberg Bornhall**, for keeping the TBI database up to date. I hope you understand that without your contribution the NICU research would come to a halt. Your work is invaluable, thank you. **Johanna Hjelm**, for encouragements that keep Bomme working. My friend **Camilla Smedberg**, for being my friend. **Nils-Johan Lindborg**, for our discussions about our mutual religion, The Colbert Nation. And to everyone else at NIVA, especially you have helped me with my clinical projects (I’m looking at you **Vivian “Vivvi” Hammarbäck**) thank you so much for all your support during my PhD period, this thesis would not have been possible without you.

Department of Neurology, for enduring us in the neurosurgical department. **Magnus Andersson**, head of the department. **Fredrik Piehl** and **Faiez Al-Nimer** for all your help in keeping the “genetic” study running and the ELISA analysis of NFL samples, thank you so much. **Michael Mazya**, for a wonderful thesis defense. To all other people in the Neurological department that have supported me; thank you!

Department of Clinical Chemistry, because if you had not analyzed the samples sent to you, our studies would have been impossible. **Mats Estonius**, for your keen interest in biomarkers in traumatic brain injury. Thank you so much for all your help and a synergistic friendship. **Ann-Charlotte Bergman**, chemist responsible for the S100B assay, I hope you understand just how important your work has been in order for us to perform all our studies.

Department of Anesthesiology and Intensive Care, for all the interesting night shifts we’ve had together. **Eddie Weitzberg** and **Anders Oldner**, thank you so much for all your valuable input on neuro-intensive care in my papers. Your interest and extensive work in this area is impressive. **Michael Nekludov**, for keeping the clinical coagulopathy research at our clinic going. Also, thanks for the magnificent work you have done with the hyperbaric oxygen chamber for our TBI patients. **Jonas Blixt**, my research partner who now is in close proximity at the NICU, let me know if I can guide you in whatever limited way I can. There is less space here to thank than there are great ANOPIVA people, thank you all so much!

Department of Neuroradiology, because without you we would not be able to see a thing. **Harriet Nyström**, a neuroradiologist with a keen interest in traumatic brain injury who helped us to establish the Stockholm CT scoring system. **Anders Lilja**, the MRI-guru. **Magnus Kaijser**, for pseudo-intellectual discussion in the neuro-radiological basement. **Jens Kolloch**, for guiding me in life and determining what is, and what is not, Scheiße. And all other neuroradiologists who have helped and guided me through the years in the basement!

The Neuropediatric Department – because you think of the children (or the “small adults” as I refer them to, which I know that you hate). **Eli Gunnarsson**, your interest in pediatric TBI and astrocytes in particular has been an inspiration. **Ronny Wickström**, for your interest and knowledge about neuro-

inflammatory conditions. **Klas Blomgren**, (and I'll squeeze in **Peter Linhard** here as well, even if he doesn't belong in your department, for devoted interest and funding support, but also) for our joint interest in helicopters.

Karolinska Experimental Research and Imaging Centre (KERIC) including Holmin's group. **Peter Damberg**, for your interest in everything-MRI-related and interest in the realms of radiological physics. **Sahar Nikkhoh Aski**, for providing useful input to Paper V taking good care of my ex-vivo brains in the MRI machine. **Philip Little**, for all our discussions about life, universe and everything at Scilifelab. Sometimes, focus is everything. **Fabian Arnberg**, for your input in everything research in general, and about clinical MRI techniques in particular. **Nasren Jeff**, for all our talks while you were at our department, hope to see you at a house-warming party in your new apartment soon. **Staffan Holmin**, for heading one of the coolest pre-clinical research methods that Karolinska University Hospital has ever had the honor to host, your work is truly impressive.

The Trauma Journal Club and Trauma Department – my home for academic research (and valuable KI credits). **Luis Riddez**, for maneuvering the trauma department. I hope that we will have several collaborative ventures in the future. **Shaihn Mohseni**, for your encouragement of beta blocker therapy to our patients and invaluable aid in language checking this manuscript. **Truls Gråberg**, for hosting the journal club sessions together with me. Thank you for intelligent input and invaluable effort to keep the journal club alive. Looking forward to further interesting discussions in the future. **Lovisa Strömmer** and **Rebecka Rubenson Wahlin**, for your enthusiasm in trauma research. **Poya Ghorbani**, for interesting collaborations with Norway and your endeavors to improve trauma care at Karolinska. **Marcus Wannberg**, for your surgical skills, interest in trauma research and for showing valuable conscience in your animal models.

My friends who dwell into the realms of research. The TV-doctor **Viveca Gyberg** (and my GoT-mate **Logan**), for your hard work in metabolic-screening-related-research. **Veronica Siljehav**, my fellow skipper who correlates prostaglandins to breathing (problems). **Johan Holm**, the skiing Skåne guy with pharmaceutical interactions on his mind. **Emilie Kullring**, because we all know that the inner ear is the best part of the ear. **Johan Ejerhed**, for keeping Danderyd's patients healthy. **Agnieszka Popowicz**, from KI to Ibiza. **Emelie Wahlstedt**, for video games- and VNV company. **Ebba Lindqvist**, because it's all in the blood (somewhere). **Mikael Finder**, because we all know that biomarkers are teh shit. **Michael Wilczek**, who shares my love for radiology. **Hedvig Löfdahl**, who I'm hoping will come back to research. **Per Hamid Ghatan**, **Louise Johannesson**, **Maria Cristina Morganti-Kossmann**, co-authors with invaluable contribution to my Papers, thank you. **Carl Johan Sundberg**, for great advices in my research career.

Roche Diagnostics – Owner of the S100B assay at KS since 2008. **Elisabeth Strand**, who joined me in this quest from the start. Thank you so much for all your help and enthusiasm in spreading the word about biomarkers. **Mathias Egermark** and **Palani Kumaresan**, for your hard work in introducing S100B as a companion diagnostics and screening marker of mild TBI.

CMA Microdialysis – With wise input and support, you made Paper III possible. **Katarina Åsberg**, **Olof Nord** and **Magnus Hedberg**, thank you so much for your contributions and laughs in Berlin. **Urban Ungerstedt**, for your never ending energy to further develop the field of microdialysis.

Dear friends; **Adam Bolcsfoldi** (and **Sarah**)(for taking care of me during my German conferences), **Gabriel Dahl** and **Rafael Kasina** (both for our culinary adventures) and **Arnt Röch-Pettersen** (my Norwegian brother), **Sebastian Weil**, **Aron Berg**, **Marcus Visser** (for keeping me alive during the Poseidon attack of 2013), **Georg Marthin** (and **Stella**), **Peter** (and **Lina**) **Andersson**, **Lina Zakrisson**, **Malin Tengsved**, **Josefin Thomson**, **Annika Svanfeldt**, **Karolina** (and **Robert**) **Viberg**,

Ehsan (and **Maria**) **Zand**, **Nisse** (and **Elena**) **Söderström**, **Ola** (and **Therese**) **Rickardsson**, **Peder** (and **Tatiana**) **Wessel** and all who have supported me during these five years, thank you so much.

My Korpen floor-ball hockey mixed-team **Fredhäll Narwahlis**, for a winning attitude and good teamplay. Hail to the whale!

My family, my father **Peter** and mother **Ann-Sofi** for supporting me throughout this time. Thank you for giving me a safe haven which has always been my platform to build my confidence to achieve great things in the past, present and future. Without your help this thesis would not have been possible. My brother **Carl**, that I've had the pleasure of working with since he also stumbled into the field of biomarkers, who has also stood by my side supporting me throughout this voyage. My sister **Charlotte** who throughout my PhD period became a mother of two (**Selma** and **Stella**), and married to **Jesper**, has been an endless source of weekend happiness throughout these years. **Sam**, my four-legged trustworthy companion. **Vincent** and **Zelda**, for their never ending energy. Furthermore, I would like to thank my grandfather **Hugo**, who always has encouraged me to a career in research; finally we are two Dr. Thelins. My grandmothers **Gun** and **Kajsa**, for the love only grandmothers can give. I would also like to thank my uncle **Clas**, and **Angela**, as well as my aunt **Helen**, and **Mats**, for support throughout my PhD period. Moreover, my cousins **Johanna**, **Linda**, **Jonas**, **Artur** and **Saga** deserve a lot of thanks as well. The maternal branch of the family; the **Ecorchevilles** (**Linnéa**, **Astrid** and **Agnes** with families), thank you so much for your hospitality and wonderful times. My extended family, **Carl** (and **Johanna**) **Sundblad**, (and the rest of the Gothenburgian **Lestrups** and **Sundblad** clan) and **Joakim** (and **Åsa**) **Lindberg** (with families) for taking care of me during all these years and our continuous adventures. My new family **Ankarcrona** (**Carl**, **Ann**, **Oscar**, **Regina**, **Victor** and **Mumlan**) and the **Wrede af Elimä** family, for warm encouragements and wonderful food. **Peter** and **Jenny Ståhl** (with families), for introducing me to the medical field (and "pröpper") all these years ago. The **Edman** family, neighbors are for life!

Throughout my life I have been taught to approach all questions and challenges I have encountered with empirical, scientific models to find adequate solutions. This has led me to understand concepts such as the big bang theory, subatomic particles and cerebral biochemistry and pathophysiology. But the way I feel about you, **Sophie**, cannot be explained by anything that I have been taught in academia. The way you make me smile, goes beyond all models that I apply to understand the universe and the love you give me is more genuine than I can ever comprehend. I cannot put everything down in words, but thank you for everything; I love you and you mean the world to me.

(furthermore, your pipetting-, chatting-up-Finnish-Professors-at-conferences and dropping-me-of-next-to-the-lab-and-hospital skills have been highly appreciated).

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