From DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

INTERPLAY BETWEEN BLOOD-BRAIN BARRIER DISRUPTION AND NEUROINFLAMMATION FOLLOWING SEVERE TRAUMATIC BRAIN INJURY

Caroline Lindblad

Karolinska Institutet

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Cover illustration: Subventricular zone-astrocytes, stained following differentiation with GFAP, GLT-1, c-Jun, and DAPI. Color channels were modified for aesthetic purposes.

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Caroline Lindblad

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Principal Supervisor:
Professor Mikael Svensson
Karolinska Institutet
Department of Clinical Neuroscience

Co-supervisors:
Dr Eric P Thelin
Karolinska Institutet
Department of Clinical Neuroscience

Dr Sebastian Thams
Karolinska Institutet
Department of Clinical Neuroscience

Associate Professor Per Mattsson
Karolinska Institutet
Department of Clinical Neuroscience

Opponent:
Professor Iver Arne Langmoen
University of Oslo, Oslo University Hospital
Department of Neurosurgery

Examination Board:
Professor Eddie Weitzberg
Karolinska Institutet
Department of Physiology and Pharmacology

Associate Professor Anders Hånell
Uppsala University
Department of Neuroscience

Associate Professor Per Almqvist
Karolinska Institutet
Department of Clinical Neuroscience

Till min mormor Ingrid

POPULAR SCIENCE SUMMARY OF THE THESIS

A severe traumatic brain injury (TBI) is elicited when an external physical force affects the head and causes structural brain injuries that render the patient unconscious. Despite scientific progress, ~35% of afflicted patients die, and survivors often face disabilities. One explanation for this is that the trauma triggers secondary injury mechanisms within the brain. Although these may clear the tissue from dying cells and heal damaged structures, they also promote additional brain injury. Two particularly interesting mechanisms of such character are blood-brain barrier (BBB) disruption and neuroinflammation. BBB disruption causes the brain's finer blood vessels to break, which provokes an inflammatory reaction within the brain. In addition, the inflammatory reaction is parallelly driven by the brain's inherent immune cells. This thesis aimed to characterize BBB disruption and neuroinflammation following severe TBI.

In **paper I** and **II**, we used cerebrospinal fluid and blood from TBI patients. In **paper I**, 17 patients were included, whereas **paper II** included 190 patients. We found that BBB disruption occurs among numerous patients at the time of trauma, whereafter it is sustained for at least a week (**paper I**). We also found that BBB disruption is of importance for clearance of brain-specific substances from brain to blood. We elaborated on this in **paper II**, where we found that BBB disruption constitutes a novel prognostic marker, strongly related to the neuroinflammatory response within the injured brain. Notably, a specific part of the neuroinflammatory response, the complement system, seemed to be involved. In order to characterize the locally injured tissue in even higher detail we conducted two laboratory studies (**paper III, IV**). In **paper III** we assessed how BBB disruption, neuroinflammation and brain swelling interplay following experimental TBI in rodents. We found that neuroinflammatory responses relating to the brain's inherent immune system cells were active in regions with cellular swelling. Interestingly, this also seemed to be related to one of the cells that make up the BBB, the astrocyte. Astrocytes have processes which cover the BBB surface. At the end of these processes lie a water channel called aquaporin-4. Following experimental TBI, aquaporin-4 had been withdrawn from the BBB, which could be related to local inflammatory reactions (**paper III**). In order to further elucidate how astrocytes are affected by inflammation, we conducted **paper IV**, in which we cultured stem cells that we matured into astrocytes and nerve cells. We found that astrocytes reacted promptly to inflammatory stimuli. Interestingly, an astrocyte stimulated with inflammatory substances seen among TBI patients, could also adopt a behavior that killed nerve cells.

Taken together, we characterized BBB disruption and neuroinflammation following severe TBI. Neuroinflammation is strongly related to BBB disruption and therefore entails an eligible treatment target. Locally in the damaged tissue, additional injury mechanisms are at play, some of which are mediated by astrocytes. These latter findings need further characterization in the laboratory environment preceding pursuit of therapeutical strategies in humans.

POPULÄRVETENSKAPLIG SAMMANFATTNING

När en människas huvud utsätts för våld kan detta leda till att man förvärvar en svår traumatisk hjärnskada (TBI), vid vilken man blir medvetslös på olycksplatsen till följd av skador inuti hjärnan. Trots forskningsframsteg dör cirka 35% av drabbade patienter och bland överlevande har många funktionsnedsättningar. En delförklaring till detta är att traumat triggar en mängd reaktioner inuti hjärnan. Även om dessa har visats kunna rensa vävnaden från döda celler och laga skadade strukturer kan de också förvärra hjärnskadan. Två särskilt intressanta sådana reaktioner är blod-hjärnbarriärskada (BBB-skada) och inflammation i hjärnan (neuroinflammation). BBB-skada leder till att blodkärl som skyddar hjärnan från resten av kroppens blodflöde och förser hjärnan med näring går sönder. Detta bidrar till att provocera en inflammatorisk reaktion, vilken parallellt också drivs av hjärnans egna immunceller. Den här avhandlingen avsåg att undersöka kopplingen mellan BBB-skada och neuroinflammation vid TBI.

I **studie I** och **II** användes ryggmärgsvätska och blod från TBI-patienter. **Studie I** inkluderade 17 patienter och **studie II** inkluderade 190 patienter. I **studie I** fann vi att en BBB-skada uppstår hos många patienter vid skadeögonblicket och kvarstår i åtminstone en vecka. Vi upptäckte också att en BBB-skada är av betydelse för hur hjärnspecifika ämnen forslas från hjärnan till blodbanan, där några sådana ämnen fraktas över en trasig BBB. I **studie II** fann vi att BBB-skada är en ny prognostisk markör, som är starkt relaterad till den skadade hjärnans neuroinflammatoriska svar, och specifikt en del av detta som kallas för komplementsystemet. För att kartlägga skadeområdet i högre detalj genomförde vi därefter de experimentella **studierna III** och **IV**. I **studie III** undersökte vi hur en BBB-skada, neuroinflammation och hjärnsvullnad samspelar efter att råttor exponerats för en skada liknande svår traumatisk hjärnskada hos människa. Vi fann att i områden där hjärnans celler var svullna fanns en inflammatorisk aktivitet från hjärnans egna immunceller, och intressant nog så verkade detta också vara relaterat till att en av BBB:s celler – astrocyten – var påverkad. Astrocyter har utskott, som de placerar utefter BBB och på dessa sitter en särskild vattenkanal som kallas aquaporin-4. Denna hade dragits bort från BBB i **studie III**, vilket var associerat med lokala inflammatoriska reaktioner. För att vidare kartlägga hur astrocyter påverkas av inflammation så genomförde vi **studie IV**, där vi odlade stamceller som genom olika kemiska substanser utmognade till astrocyter och nervceller. Vi fann att astrocyterna reagerade prompt på inflammatoriska stimuli. Intressant nog så kunde en astrocyt stimulerad med inflammatoriska ämnen, som förekommer hos människor efter traumatisk hjärnskada, också döda nervceller.

Sammantaget kartlade vi BBB-skada och neuroinflammation efter en svår TBI. Neuroinflammation är starkt relaterat till BBB-skada och är därför någonting vi bör sträva att utveckla behandlingar mot. Lokalt i skadeområdet sker ytterligare specifika skademekanismer, som delvis medieras av astrocyter. Dessa fynd måste fortsatt utvärderas i laboratoriemiljö före de kan bli föremål för riktade behandlingar hos människor.

ABSTRACT

A severe traumatic brain injury (TBI) holds deleterious consequences for the afflicted, its next-of-kin and society. Still today, prognosis is semi-desolate. One explanation for this might be pathophysiological processes ensuing the primary trauma that are but indirectly targeted for treatment. Among such processes, blood-brain barrier (BBB) disruption and neuroinflammation constitute two astrocyte-dependent mechanisms that interplay in the aftermath of a severe TBI. The overall aim of this thesis was to characterize both BBB disruption and neuroinflammation translationally.

In **paper I**, n = 17 patients with severe TBI were included in a prospective observational longitudinal study. Here, the protein biomarkers S100B and neuron-specific enolase (NSE) were sampled with high temporal resolution from both cerebrospinal fluid (CSF) and blood. We found that BBB disruption occurred among numerous patients and remained throughout the first week following injury. Interestingly, BBB disruption also affected clearance from brain to blood of S100B, but not NSE. This indicates that biomarkers are cleared differently from the injured CNS. We elaborated on this by utilizing a larger cohort size (n = 190 patients), which enabled outcome prediction modelling, in **paper II**. In this prospective, observational, cross-sectional study, we found that BBB disruption comprised a novel, independent outcome predictor that strongly related to levels of neuroinflammatory proteins in CSF and inflammatory processes within the injured brain. Among pathways assessed, particularly the complement system entailed proteins of future interest. We next assessed the relationship between *in situ* neuroinflammatory protein expression, BBB disruption, and brain edema in **paper III**. By utilizing a rodent model of severe TBI, we found that the cytotoxic edema region was associated with an innate neuroinflammatory response, and astrocytic aquaporin-4 retraction from the BBB interface. In fact, the astrocyte itself is an important neuroinflammatory cell, which we showed in **paper IV**, where we constructed a disease-modelling system of stem cell-derived astrocytes that we exposed to neuroinflammatory substances. Following neuroinflammatory stimulus, astrocytes exhibited an important increase in canonical stress-response pathways. Importantly, following stimulation with clinically relevant neuroinflammatory substances seen in human TBI from **paper II**, they also acquired a neurotoxic potential, of plausible importance for local cell survival following a severe TBI.

Taken together, BBB disruption and neuroinflammation ensue a severe TBI. Neuroinflammation, particularly mediated by the complement system, stands out as a future therapeutic target in order to mitigate exacerbated BBB disruption. Locally in the lesion vicinity, additional neuroinflammatory mechanisms are in part mediated by astrocytes, where these cells seem to have an important role in local cell survival. Onwards, our findings suggest that future efforts should be directed at evaluating if neuroinflammatory modulation of complement inhibition yields improved outcome, while elaborating on the promising experimental data of astrocyte-mediated effects in the lesion vicinity.

LIST OF SCIENTIFIC PAPERS

I. **Lindblad C**, Nelson DW, Zeiler FA, Ercole A, Ghatan PH, von Horn H, Risling M, Svensson M, Agoston DV, Bellander B-M, Thelin EP. Influence of Blood–Brain Barrier Integrity on Brain Protein Biomarker Clearance in Severe Traumatic Brain Injury: A Longitudinal Prospective Study. *J Neurotrauma*. 2020;11:1–11.

II. **Lindblad C**, Pin E, Just D, Al Nimer F, Nilsson P, Bellander B-M, Svensson M, Piehl F, Thelin EP. Fluid Proteomics of CSF and Serum Reveal Important Neuroinflammatory Proteins in Blood-Brain Barrier Disruption and Outcome Prediction Following Severe Traumatic Brain Injury: A Prospective, Observational Study. *Crit Care*. 2021;1–28.

III. **Lindblad C**, Mitsios N, Falk-Delgado A, Mattsson P, Morganti-Kossmann MC, Frostell A, Mulder J, Risling M, Damberg P, Bellander BM, Thelin EP, Svensson M. Cytotoxic Brain Edema is Associated with Neuroinflammation and Retraction of Perivascular Aquaporin-4 following Experimental Severe Traumatic Brain Injury – a Multi-Modal Imaging Study. *Manuscript*.

IV. **Lindblad C**, Neumann S, Kolbeinsdóttir S, Zachariadis V, Thelin EP, Enge M, Thams S, Brundin L, Svensson M. Embryonic stem cell-derived brainstem and spinal astrocyte-like cells develop a neurotoxic phenotype *in vitro* following clinically relevant pro-inflammatory stimulation. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ADC	Apparent Diffusion Coefficient
ARMA	Autoregressive Moving Average
AQP4	Aquaporin-4
BBB	Blood-Brain Barrier
C	Complement Component
CBF	Cerebral Blood Flow
CCI	Controlled Cortical Impact
CD34	Hematopoietic Progenitor Cell Marker
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
CRASH	Corticosteroid Randomisation after Significant Head Injury
CSF	Cerebrospinal Fluid
CT	Computerized Tomography
CVR	Cerebrovascular Resistance
C5b9	Complement Membrane Attack Complex
DAMP	Damage-Associated Molecular Pattern
DWI	Diffusion-Weighted Image
ED1	Macrophage, Activated Microglia
ES cell	Embryonic Stem Cell
GCS	Glasgow Coma Scale
GFAP	Glial Fibrillary Acidic Protein
GLT-1	Glutamate Transporter-1
GOS(e)	Glasgow Outcome Scale (extended)
(h)iPS	(Human) Induced Pluripotent Stem Cell
ICP	Intracranial Pressure
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-	Interleukin-
IMPACT	International Mission for Prognosis and Analysis of Clinical Trials
MAP	Mean Arterial Blood Pressure
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
NSE	Neuron-Specific Enolase
OX42	Microglia Marker
PRx	Pressure-Reactivity Index
Q_A	Albumin Quotient
SMI-71	Blood-Brain Barrier Endothelial Marker
S_0	Non-Diffusion Weighted Image
TBI	Traumatic Brain Injury
TGF-β	Transforming Growth Factor β
TNF-α	Tumor Necrosis Factor α
VEGF-α	Vascular Endothelial Growth Factor α

1 INTRODUCTION

The first known attempts to treat injuries within the head were in pre-historic times, demonstrated by ancient skulls subjected to trepanation (1). Hippocrates (460-377 BC) compiled the first systematic assessment on head injuries - "On Wounds in the Head" (2). More than 2000 years later, traumatic brain injuries (TBI) and the injury processes involved still constitute an enigma for the medical profession. This thesis addresses and expands our current knowledge on TBI through clinical and experimental studies, specifically focusing on the blood-brain barrier (BBB) disruption and neuroinflammation that ensue a severe TBI.

1.1 CLINICAL ASPECTS OF TRAUMATIC BRAIN INJURY

TBI denotes a heterogeneous group of injuries. Globally, large discrepancies in societal resources impact how TBI occurs and how it is managed, both of which hold consequences for TBI prognosis. Unless otherwise stated, the assumed context below is severe TBI in high-income countries, where patients are treated in neurocritical care units for their injuries.

1.1.1 Definition and Classification

TBI is defined as "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (3). Thus, the head is struck or penetrated by an exogenous force, or else undergo a movement so that the brain itself is struck against the cranium. This is verifiable as temporary or enduring neurological symptoms. Alternatively, the altered brain function manifests through additional assessments, such as biofluid analyses of brain markers of injury or radiological examinations (3) detecting structural injuries.

The injury panorama following TBI is heterogeneous, thus warranting subgroup classifications. Clinically, the Glasgow Coma Scale (GCS) (4) joint with neuroradiological findings comprise the gold-standard approach (5). The GCS is a summarized score of the level of consciousness, combining neurological features of best eye (4 points), motor (6 points), and verbal (5 points) response, ranging from 3 (worst) to 15 (best). GCS $\leq$ 8, where the GCS can be attributed to the TBI, is classified as a severe TBI. Further classification commonly necessitates the use of neuroradiology, where computerized tomography (CT) is the gold-standard modality in the acute phase (6).

Aside from GCS and neuroradiology, additional injury severity scoring tools have gained popularity within the research field. The most commonly used tools are the Abbreviated Injury Scale (7) and its derivative scores, e.g. the Injury Severity Score (8). As patients with a TBI are commonly subject to multi-trauma, these scoring systems compile intra- and extracranial injuries. The Abbreviated Injury Scale provides organ-system specific scores, from which the three most severely injured body regions are used to derive the Injury

Severity Score. This possibly renders the Abbreviated Injury Scale more pragmatic to use in a TBI context since it allows for comparison between head-injury severity and other injuries' severity.

1.1.2 Epidemiology and Outcome

Epidemiological TBI data is hampered by substantial data quality issues (9,10), why caution is advised when interpreting it. Globally and independent on severity (9), TBI annually afflicts between 10 and 50 million people (9,11). Adjusting for age and by geographical region, Sweden and Western Europe demonstrate an incidence of 282 and 292 per 100,000 citizens, respectively (5). Of these, 10-15% are claimed to be more severe injuries (12). In Sweden, age-adjusted TBI mortality rate was 9.2 per 100,000 citizens in 2012 (13). Hence, even when restricting the discussion to severe TBI, it is globally amongst the most high-frequent etiologies of mortality and persistent disability (14).

Severe TBI holds a semi-desolate prognosis, with a mean mortality of ~35% (15). Across Europe, 37% of all injury-related deaths can be attributed to TBI (9). Recently, it was estimated that age-standardized TBI-related years of life lost in Europe was 259.1 per 100,000 citizens (16). From a historical perspective, current TBI outcomes are the result of a substantial improvement stretching up until the 1990s. Following this, prognosis improvements have largely stagnated (15). The underlying reason for this is unclear, but one hypothesis is the increasing number of older, frailer patients that sustain a TBI (10,15).

Among TBI survivors, residual neurologic function and the extent of neurologic disability vary, which can be systematized using outcome classification systems (17). Traditionally, functional outcome following TBI has been defined by Glasgow Outcome Scale (GOS) stretching from 1 (deceased) to 5 (full recovery) (18), or GOS extended (GOSe) stretching from 1 (deceased) to 8 (upper good recovery) (19). Both GOS and GOSe constitute the recommended outcome metrics as deemed by various international bodies (20). Both being ordinal scale variables, they are commonly dichotomized into "favorable" (GOS 4-5 (21), GOSe 5-8 (22)) and "unfavorable" outcome. Global data collection initiatives such as the International Mission for Prognosis and Analysis of Clinical Trials in TBI (IMPACT) (23) has developed a prognostic model for 6-month outcome following TBI using GOS and GOSe. The model comprises clinical, radiological, and laboratory variables (24). Cumulatively, these variables merely explain ~35% of outcome variance following TBI (25), highlighting that other yet unknown variables contribute importantly to patient outcome. For researchers attempting to discern new potential outcome predictors accounting for the residual 65% in explained variance, the new variable should be tested together with current prognostic variables included in e.g. the IMPACT. If the new variables confer additional, independent outcome information, they may be of interest to study further. GOS and GOSe have thus proven useful for multiple reasons. Yet, they have also been critiqued for not fully

capturing the complexity of sequelae seen after TBI (17), why more granular outcome metrics might be expected to gain popularity in the future although there is still some debate surrounding this (26).

1.1.3 Structural Injury Panorama

Upon arrival, a TBI patient is clinically assessed with regard to GCS, pupillary features (reactivity and diameter) (12), and neurological symptoms (27) followed by CT examination. The latter distinguishes between focal and diffuse injuries (28). Between 25 and 60 % of severe TBI patients exhibit an intracranial hematoma (29,30), defined anatomically as either epidural, subdural, intraparenchymal (traumatic intracerebral hemorrhage) (30), contusion hemorrhage (31), or traumatic subarachnoid hemorrhage (32) **(Figure 1)**. The most common focal intracranial pathology is traumatic intracerebral hemorrhage, seen in 13-35% of severe TBI patients (33), followed by acute subdural hematoma with an incidence of 12-29% (34), and epidural hematoma that accounts for 2.7-4% of all severe TBIs (35). Diffuse injuries comprise diffuse/traumatic axonal injuries (36), but also edema (31,32). In severe TBI, as many as 90% of patients exhibit diffuse axonal injury (37), of which lesions in the ventral brainstem have been associated with particularly poor prognosis (38,39). Many severe TBI patients present with multiple different lesions (29). For example, concurrent lesions of diffuse axonal injury type and other lesions have been observed in 67% of severe TBI cases (37).

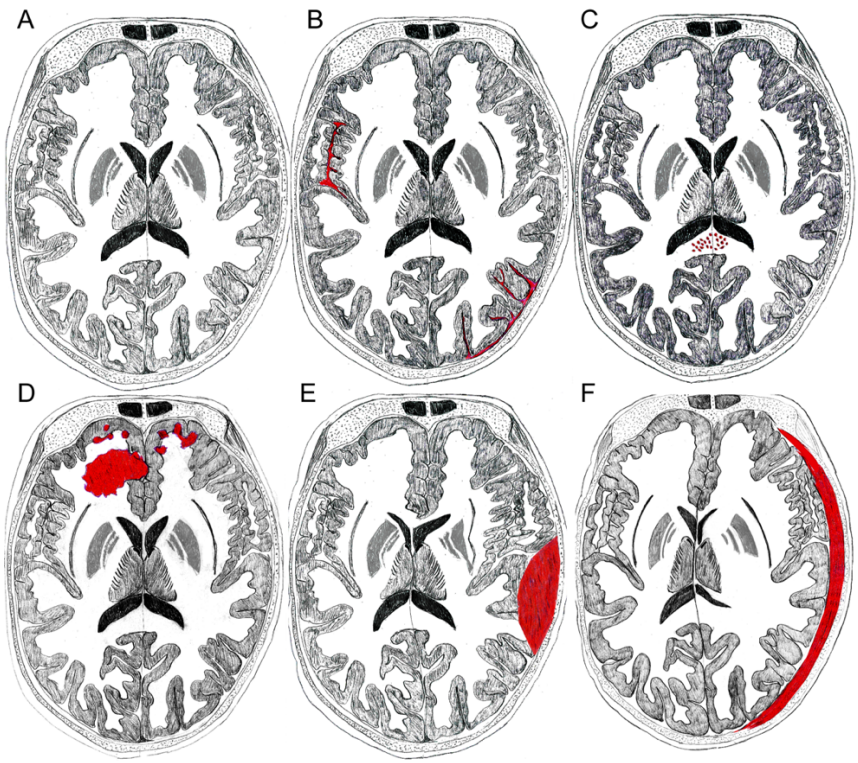

Figure 1: Hemorrhagic Lesions following a Severe TBI. A normal brain is shown in **(A)**. The following lesions are depicted: traumatic subarachnoid hemorrhage **(B)**, diffuse axonal injury **(C)**, intracerebral contusions and an intracerebral hemorrhage **(D)**, epidural hematoma **(E)**, and (acute) subdural hematoma **(F)**.

Different CT classification models have been developed in severe TBI (40). The superior imaging modality for diffuse axonal injury is magnetic resonance imaging (41), which is a limitation to all CT scores. The internationally most recognized CT scores are the Marshall CT classification (40), and Rotterdam CT scores (6), of which the Rotterdam CT score confers superior mortality prediction compared with the Marshall CT classification (6) (which was actually not originally developed as a prognostic tool (42)). For discrimination between favourable and unfavourable outcome, the more recently developed Stockholm CT score confers the highest accuracy (43). Other CT models comprise the Radboud CT model (44) and the Helsinki CT score (42). The Stockholm, Helsinki, Rotterdam scores, and Marshall CT classification were recently externally validated and compared (45). Both the Stockholm and Helsinki CT scores were superior to the Rotterdam CT score and Marshall CT classification, but the Stockholm CT score seemed to be the overall stronger radiologic tool for outcome prognostication (45). Of note, all of these are developed on blunt head traumas, why external validation of these CT scores in two penetrating TBI cohorts was recently undertaken (46), showing that both exhibit prognostic utility in this cohort as well.

1.1.4 Clinical Management

Clinical TBI management evolves around the primary injury (inflicted by the trauma) and secondary insults, that ensue swiftly or slowly (47). In-hospital management of TBI patients strive to hinder and treat secondary insults (12,47), in order to improve clinical outcome (48).

1.1.4.1 Clinical Dilemmas

Secondary insults can be either systemic or intracranial (49), and entail among else hypoxemia, hypo-/hypercarbia, hypotension, metabolic disturbances, hyponatremia, seizures, vasospasm, and increments in intracranial pressure (ICP) (48–50). If left untreated, secondary insults may result in a secondary injury of predominantly ischemic nature (49). The reason for this can be derived from the Monro-Kellie doctrine, stating that within an enclosed cranium the sum of the intracranial constituents is constant (48,51). Thus, the intracranial volumes (cerebrospinal fluid [CSF], blood, brain parenchyma, and pathological constituents) are confined to a restricted space. Upon intracranial volume alterations that yield ICP increases, CSF, venous, and arterial blood are displaced from the brain. If arterial blood flow is diminished, inadequate cerebral perfusion results in ischemia and subsequent infarctions (50). The arterial blood flow needed to meet the metabolic demands of the brain, the cerebral blood flow (CBF) (47), can be defined mathematically (Eq 1, Eq2).

$$CBF = \frac{(MAP - ICP)}{CVR} \quad \text{(Eq 1)}$$

$$CPP = MAP - ICP \quad \text{(Eq 2)}$$

In short, CBF relies on cerebral perfusion pressure (CPP) divided by cerebrovascular resistance (CVR) (Eq 1, 2) (47,52). CPP itself is the difference between mean arterial (blood) pressure (MAP) and ICP (Eq 2) (47,50). The CVR is dependent on among else the partial pressure of carbon dioxide and CPP, and is (under homeostasis) strictly maintained through cerebral autoregulation (50). This results in stable CBF across a broad range of CPP (and MAP) alterations (52–54). In the setting of a severe TBI, efforts are taken to maintain cerebral homeostasis as failure to detect physiological deterioration could impact outcome negatively (48). This usually encompasses multi-disciplinary decision making, involving both surgical, medical, and neurocritical care management (9,12).

1.1.4.2 Surgical Management

Surgical approaches following TBI primarily strives to remove mass-lesions, decrease ICP, or insert neuromonitoring equipment (9), the latter to detect derangement of intracranial homeostasis. Both epidural hematoma (55) and acute subdural hematoma (56) seem to benefit from surgical removal if causing a clinically relevant mass effect, even though neither has been evaluated in any randomized controlled trial (34,35). In contrast, traumatic intracerebral hemorrhage has not shown similar benefits of surgery as the STITCH trial (30) could not find any difference in 6-month outcome (primary study endpoint). However, secondary endpoint analysis revealed that patients with GCS 9-12 benefited from early surgery, whereas for patients presenting with GCS 13-15, watchful observation seemed sufficient (30). For diffuse TBI patients with therapy-refractory ICP, decompressive craniectomy is a tentative surgical strategy (9,12). One randomized study showed worse long-term outcome following early surgery (57), whereas another demonstrated decreased mortality following “last-resort” surgery (58). However, in the latter study (58), this was at the expense of increased extent of vegetative state patients, posing a grand ethical dilemma.

1.1.4.3 Neuromonitoring and Medical Management

In adjunct to surgical treatment a plethora of medical treatment options aim to counteract secondary insults (12). In order to tailor these treatment strategies, TBI patients obtain (neuro)monitoring equipment (9,12), of which ICP and CPP monitoring (59) are key. However, there are other neuromonitoring modalities not yet within international guidelines. Some noteworthy examples are: brain tissue oxygenation (60), cerebral microdialysis (61,62), electroencephalography, intraparenchymal temperature monitoring, transcranial doppler, near-infrared spectroscopy (12), and serial sampling of brain-enriched protein biomarkers of tissue fate (63). A multi-modal approach is considered superior, as monitoring data will comprise of several proxy metrics of intracranial conditions (9).

In spite of rigorous monitoring and versatile treatment options, 50% of TBI-related mortality is believed to be inferred by post-traumatic brain-swelling leading to increased ICP (64). A pivotal part of neuro-critical care monitoring is thus continuous ICP measurement (48,65), where values $\geq$ 22 mmHg should prompt treatment according to the most recent Brain

Trauma Foundation Guidelines (59). One multicentre randomized study (65) failed to show an effect of ICP monitoring on long-term outcome, but study design issues complicates interpretation (66). As such, ICP measurement is still recommended by the Brain Trauma Foundation Guidelines (59). Moreover, CPP can be calculated from concurrent ICP and MAP measurements (48), and normally acts as a surrogate for CBF. The Brain Trauma Foundation recommends CPP thresholds of 60-70 mmHg, depending on autoregulatory state (59), as ~50% of TBI patients suffer from a perturbed autoregulation (67). Autoregulation is difficult to assess (68), why proxy metrics such as the pressure-reactivity index (PRx) (69), have gained popularity (48). This has enabled optimization of individual patient autoregulation (12), by targeting CPP to minimum PRx (68). Recent results from a multicenter randomized controlled trial has demonstrated the safety and relative feasibility of such treatment (70). ICP versus CPP guided treatment have also been studied (71). An ICP-based treatment was superior when autoregulation was perturbed, and CPP-based treatment was superior when autoregulation was intact (71).

Aside from conventional monitoring, fluid ”biomarkers” (brain-enriched proteins of tissue fate), have gained interest (72), and are regionally implemented clinically (73,74). Biomarkers of current interest comprise S100B, neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), ubiquitin carboxy-terminal hydrolase-L1, tau, and neurofilament-light (75). S100B (76), NSE (77), GFAP, and neurofilament-light (75) have emerged as independent severe TBI outcome predictors. An extension of biomarker monitoring is neuroinflammatory marker measurement (62,78). Neuroinflammation can be assessed through chemical assays in brain tissue, CSF, brain extracellular fluid, or blood, but also through imaging modalities (62). Using these, cytokine/chemokine profiles following trauma have been shown to organize temporally (79), but also into patient clusters, predictive of outcome (80). Modulation of cytokines responses shift cytokine/chemokine profiles (81), in a complex fashion that warrants further research before conclusions concerning clinical efficacy can be drawn.

1.2 TBI PATHOPHYSIOLOGY

The primary injury and the secondary insults which may culminate in secondary injuries depend intracranially on multiple parallel, intertwined, and complex cellular injury processes (32,82,83). These are believed to be amenable to treatment (83,84), why understanding of them at the molecular level might prove pivotal in improving outcome for TBI patients.

A plethora of intracellular injury processes exist, and entail e.g. vascular injuries, hypoxia/ischemia, metabolic derangements, mitochondrial dysfunction, ion homeostasis perturbation, excitotoxicity, BBB injury, edema, neuroinflammation, and free

radicals/reactive oxygen species (28,82,83,85,86). It would be unfeasible to discuss all these jointly. However, two secondary injuries – BBB disruption and neuroinflammation – are inter-dependent, operate at the same anatomical unit, and are possible therapeutic targets to diminish ICP. They also relate to edema development following TBI. These will therefore be discussed below.

1.2.1 Blood-Brain Barrier Disruption following TBI

The adult central nervous system (CNS) comprise three barriers, namely the blood-brain barrier (BBB), the blood-CSF barrier, and the arachnoid barrier (87,88). Of note, a potentially fourth type of barrier denoted *the glymphatic system* was recently described *in vivo* (89), and emerging clinical data (90,91) support the existence of a human glymphatic system. For simplicity, this section primarily discusses the BBB. Other barriers will be mentioned where applicable.

The BBB between the CNS and the periphery was discovered in the Ehrlich and Goldmann experiments where different dyes were found to stain/not stain the CNS based on the site of dye injection (87). Anatomically, the BBB constitutes multiple layers of cells and extracellular material so that it forms a unit of tissue organized (orderly from the capillary blood vessel lumen into the parenchyma): endothelial cells, endothelial basement membrane with pericytes, meningeal epithelium and basement membrane, and astroglial basement membrane together with astrocyte end-feet (92). The endothelial cells lack fenestrations and form a sealed border through tight junction proteins (93,94). These unique anatomical features of cerebral endothelial cells at the BBB (or equivalently choroid plexus epithelial cells at the blood-CSF barrier interface (92)) restrict passive diffusion from the periphery into the CNS (93,95,96). The scope of the BBB has recently broadened into "the neurovascular (gliovascular) unit" (93,94,97), which refers to the intrinsic BBB function to relay stimuli from one compartment (peripheral or CNS) and yield a response in the opposite one (93), pivotal for CNS function (97).

The BBB is injured following TBI (84,94,97). In animals, the temporal trajectory and duration of BBB disruption, differs across injury models (98,99). Clinically, BBB disruption is commonly assessed through the CSF:serum albumin quota (Q_A), the current gold standard technique (100–102). In patients, BBB disruption has been reported to cease within hours following TBI (103,104), whereas other reports claim that BBB integrity can be compromised for days to weeks (105,106), and possibly even years (97,107). One tentative explanation for these data discrepancies are that BBB disruption can be caused by both the initial trauma and through downstream secondary insults (97), one of which is post-traumatic edema.

1.2.2 Post-Traumatic Cerebral Edema following TBI

TBI-induced cerebral edema is defined as an increased brain water content (108), and sub-categorized into vasogenic, ionic, and cytotoxic components. Historically, the vasogenic counterpart was believed to be of primary importance following TBI (64). Later work instead suggested post-traumatic brain edema to be primarily cytotoxic i.e. due to intracellular water accumulation (108), and even independent of BBB integrity (98). This was claimed to hold true both in the experimental (64,98) and clinical setting (109). Yet, it would be impossible to infer brain edema exclusively to cellular swelling, as a net-increase in brain water content could not be caused by a mere water redistribution within the CNS (110,111). In accordance, current data support the existence of both vasogenic and cytotoxic edema in the context of TBI (94,99), possibly at different time points following injury (94,112).

Vasogenic edema formation initiates upon BBB disruption, when blood leak from vessels and accumulate in the brain parenchyma (97,113) **(Figure 2)**. In experimental studies, BBB disruption peaks at 1-3 hours after injury (113), whereafter BBB closes promptly (64,98). In accordance, the contribution of vasogenic edema to overall edema diminishes. Edema progression, however, ensues as cells in the vicinity of the injury region die, whereupon they leak intracellular content e.g. neurotransmitters, and ions. As local osmolarity thus increases, additional water movement from the vessels to the brain parenchyma yields an ionic edema (112) **(Figure 2)**. Importantly, both these processes increase total brain water content. Following this early phase of vasogenic and ionic edema, cytotoxic edema ensues and remains dominant throughout the first week following the TBI (64).

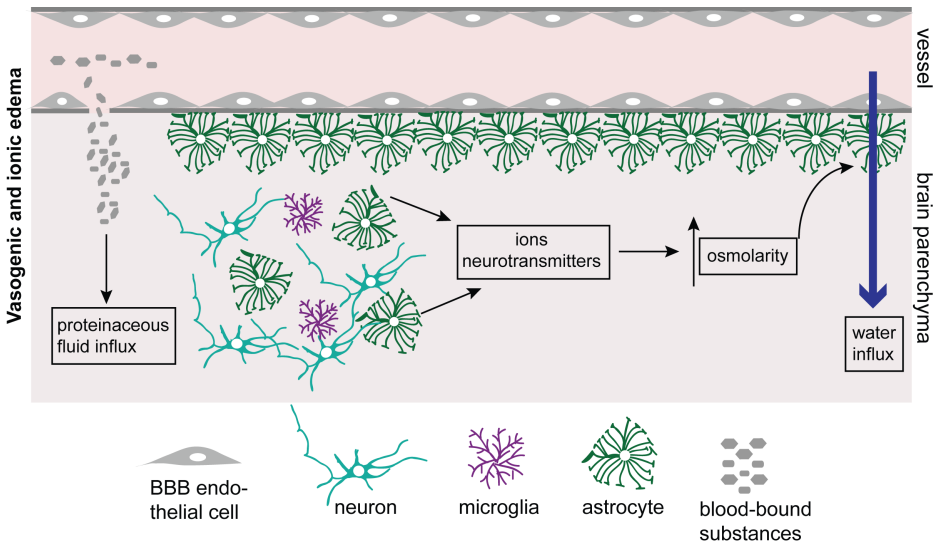

Figure 2: Vasogenic and Ionic Edema following Severe TBI. Following trauma, BBB integrity is compromised (vasogenic edema, left in panel), whereupon proteinaceous fluid rich in otherwise blood-bound molecules leak into the brain parenchyma. In parallel, CNS cells die upon which intracellular substances leak to the CNS extracellular milieu, increasing local osmolarity and thus triggering additional water movement from CNS vessels into the brain parenchyma (ionic edema, right in panel). Both processes increase net brain water content. **Abbreviations:** BBB, blood-brain barrier, CNS, central nervous system; TBI, traumatic brain injury.

In contrast to vasogenic edema, cytotoxic edema **(Figure 3)** is caused by restricted blood flow into the lesion core. This yields a depletion of nutrients and notably adenosine triphosphate locally. When adenosine triphosphate-dependent cellular ion pumps fail, ions accumulate intracellularly, with consequent intracellular water inlet (113). The cell type predominantly afflicted by these processes is the astrocyte (113,114), although both neurons and BBB endothelial cells are also subjected to cytotoxic swelling (112). The swelling of these cells can lead to oncotic cell death (113), thus potentially creating a vicious cycle whereby additional BBB disintegration ensues causing a delayed vasogenic edema with exacerbated cytotoxic edema (112) due to an increasing cell death. The delayed vasogenic edema has been suggested to occur after 3-7 days following injury (112,113,115). The total duration of edema following TBI is not fully elucidated. One experimental study reported persistent edematous changes using magnetic resonance imaging even at 30 days following injury (116).

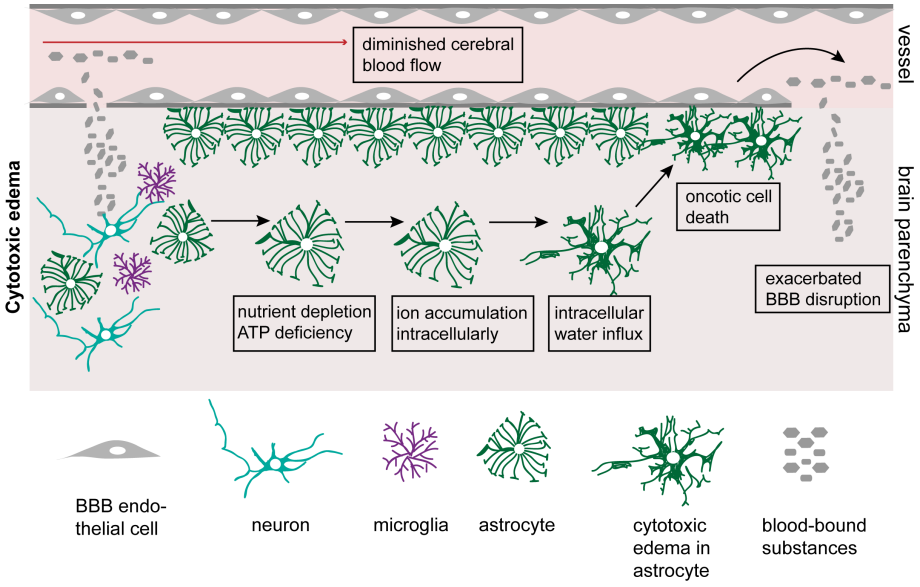

Figure 3: Cytotoxic Edema following Severe TBI. Cytotoxic edema onset occurs upon diminished cerebral blood flow into the lesion core. This generates nutrient depletion, and importantly ATP deficiency locally. As cellular ATP-driven pumps fail, intracellular ion accumulation ensues which eventually increases intracellular water content. Of note, this puts cells at risk for oncotic cell death, which can exacerbate BBB disruption (right in panel). **Abbreviations:** ATP, adenosine triphosphate; BBB, blood-brain barrier.

Importantly, post-traumatic cerebral edema has also been linked to neuroinflammation (111,117). Interestingly, neuroinflammatory signaling have been implied in brain edema and inhibition of it in edema mitigation (118–120).

1.2.3 Neuroinflammation following TBI

Acute neuroinflammation affects, aside from edema, also BBB disruption (97), and might even by triggered by it (85). Neuroinflammation has emerged as a key cellular injury process subsequent to TBI (83,84,121), following decades of conviction that the CNS was immune privileged (122). The concept of “neuroinflammation” denotes CNS specific inflammatory events (123), and is today believed to be an intricate sequence of processes, with some being

deleterious and some beneficial for the injured CNS (124). As there is an elaborate interdependency between BBB disruption and the neuroinflammatory response, I discuss these in parallel below chronologically. I omit chronic neuroinflammation from the discussion. Although this is an emerging field of important implications for neurodegenerative diseases (125) it is currently predominantly explored in mild TBI.

1.2.3.1 Early Innate Reaction

Acute neuroinflammation ensues the trauma immediately **(Figure 4)**, due to tissue injury (124) and CNS barrier breakage (83,85). BBB disruption allows inlet of otherwise blood-borne substances, such as complement (84,126,127), albumin, thrombin, and fibrinogen (94), which triggers the innate immune system (125). Further, substances released from damaged CNS tissue and/or the disrupted BBB, so called damage-associated molecular patterns (DAMPs) (85,124,126) trigger the immune response. The subgroup of DAMPs that are endogenously derived are referred to as alarmins, and comprise among else adenosine triphosphate, high-mobility group protein B1, and various interleukins (IL-) such as e.g. IL-33 (124). The DAMPs cause a cascade of early innate immune system effects. Within the CNS, the DAMPs activate glial cells (microglia and astrocytes) momentarily (124).

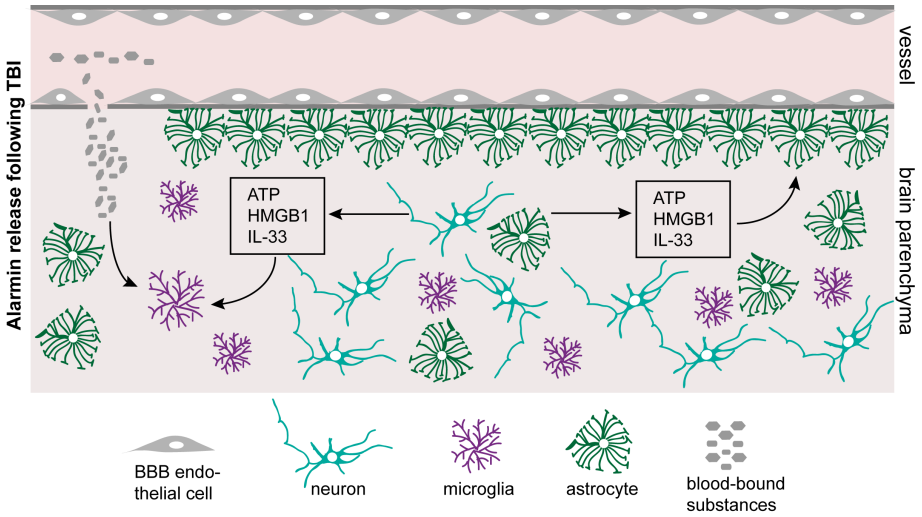

Figure 4: Alarmin Release and Early Initiation of the Innate Neuroinflammatory Response. BBB disruption and cell death within the CNS leads to the inlet and leakage of DAMPs and alarmins, which trigger the neuroinflammatory cascade. Importantly, microglia and astrocytes are immediately evoked in response to DAMPs. **Abbreviations:** ATP, adenosine triphosphate; BBB, blood-brain barrier; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group protein B1; IL-, interleukin.

Microglia, a latent immune surveillance cell of the CNS (123), are chemotactically attracted to sites of alarmin release (124) and respond by rapid morphological alteration (85). This enables microglia to shield the injury site from the healthy CNS **(Figure 5)** (83), but also to initiate phagocytosis (123,128) thus enabling debris clearance. Importantly, microglia activation also induces cytokine production (83,123), comprising e.g. IL-1β, IL-6, tumor necrosis factor α (TNF- α), and reactive oxygen species (85). This leads to increasing BBB permeability (129), and down-stream consequences.

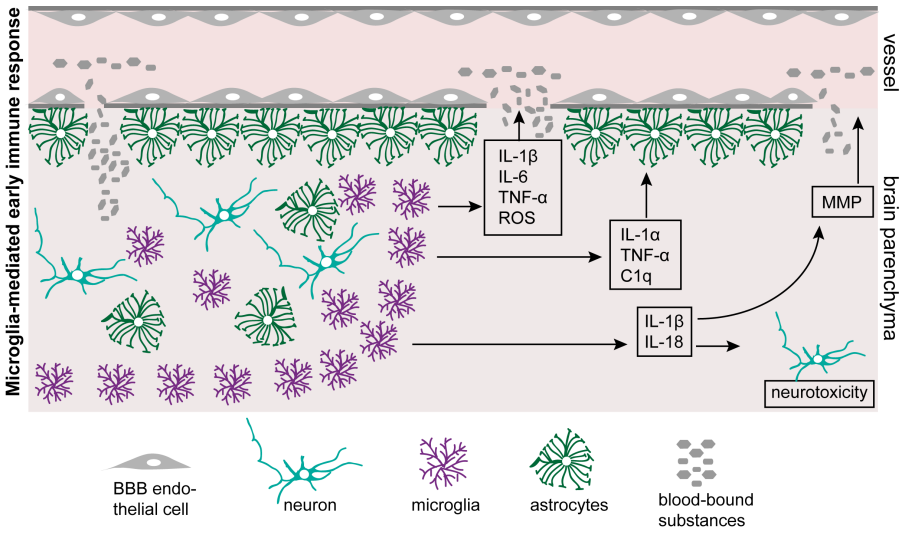

Figure 5: Microglia-Mediated Early Neuroinflammatory Response. Microglia are chemotactically attracted to the lesion site in response to DAMPs, where they shield the injury region. They also initiate pro-inflammatory cytokine signaling, that can cause e.g. exacerbated BBB disruption, and neurotoxicity. In addition, recent data suggests that microglia activates astrocytes. **Abbreviations:** BBB, blood-brain barrier; CNS, central nervous system; DAMP, damage-associated molecular pattern; IL-, interleukin, MMP, matrix metalloproteinase; ROS, reactive oxygen species; TNF-, tumor necrosis factor.

Alarmins, DAMPS, and mechanical triggers also activate astrocytes **(Figure 6)** (130), possibly through the Signal Transducer and Activator of Transcription 3-signaling pathway (131). This leads to feedback loops promoting incremental alarmin release, and astrocyte-mediated production of chemoattractant signals that recruit peripheral immune cells (124). Recent evidence suggests that microglia may also trigger astrocytes through microglial-mediated cytokine secretion of IL-1α, TNF-α and complement component (C) 1q (132). Further, astrocytes secrete IL-6 and matrix metalloproteinase (MMP-) 9, which promotes BBB disruption (88,133) and has been implicated in vasogenic edema formation (85).

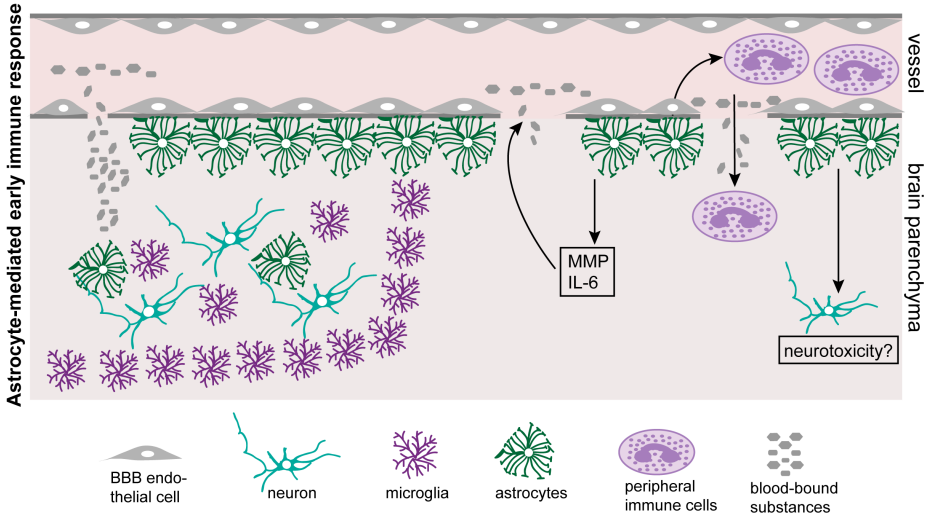

Figure 6: Astrocyte-Mediated Early Neuroinflammatory Response. In response to DAMPs, astrocytes release chemoattractant signals which promote leukocyte recruitment. Astrocytes also secrete pro-inflammatory substances e.g. IL-6, and MMP-9, possibly contributing to exacerbated BBB disruption. **Abbreviations:** BBB, blood-brain barrier; DAMP, damage-associated molecular pattern; IL, interleukin; MMP, matrix metalloproteinase.

The immediately triggered neuroinflammatory response following TBI is ensued by further inflammatory responses that span the first minutes to hours following TBI (124). Microglia-derived cytokines and cytokines produced as a consequence of alarmin release trigger the activation of the inflammasome (124). The inflammasome is a cytoplasmic oligomerized protein complex localized to microglia (134,135), and possibly other CNS cells (124) as well as peripheral immune cells migrating to the CNS (134,136) although this is somewhat debated (135). Inflammasome activation leads production of IL-1β and IL-18 in a caspase-1 mediated process (124,134). It is still not clear which CNS cells that confers the vast majority of IL-1β and IL-18 production following TBI (126). Nonetheless, both these cytokines are considered potently pro-inflammatory (123,124) and detrimental in the context of TBI (85). The effects of IL-18, although not conclusive elucidated, comprise microglial-mediated MMP production, stimulation of further cytokine production, direct/indirect neurotoxic effects, either through recruitment of peripheral immune cells (neutrophils, monocyte-derived macrophages) or directly by stimulating neuronal apoptosis (137). Similarly to IL-18, IL-1β exerts neurotoxic effects (83), secrete neutrophil chemoattractants (85), and induce MMPs (94,138,139). The latter causes increased BBB permeability and peripheral immune cell recruitment (85,94,133). In addition, IL-1β also stimulates reactive oxygen species (122), thus exacerbating the inflammatory cascade.

1.2.3.2 Late Innate Reaction

Also initiated as early as a few hours (140), but peaking within 24-48 hours (126) following TBI is peripheral immune cell infiltration into the CNS. The first cell to appear is the neutrophil (125), promoted through upstream secretion of chemoattractant molecules and e.g. reactive oxygen species (94), that upregulates adhesion molecules (85) facilitating migration. Interestingly, the expression of the brain endothelial intercellular adhesion molecule 1 correlates with the extent of BBB disruption (97), why BBB disruption can be assumed to be integral to this process. The role of neutrophils highlights the duality of neuroinflammation following TBI – on the one hand they exert neurotoxic effects through degranulation (137) and aggravated BBB disruption (128) through e.g. MMPs (94,133), but on the other hand they have also been shown to exert neuroprotective actions (124).

The involvement of the innate immune system continues throughout the first days following TBI, when monocytes from the periphery are recruited to the CNS (124). Locally, they become macrophages (121). Monocyte-derived macrophage recruitment is partly mediated through the chemokine (C-C motif) ligand 2 (85,128), acutely secreted by astrocytes in response to the alarmin IL-33 (124). Similarly to neutrophils, macrophages exert both detrimental and protective effects following TBI (124). Among the beneficial effects exerted is the production of neuroprotective cytokines such as IL-10 (124). Historically, this duality was referred to as polarization states M1 (deleterious) and M2 (adaptive/beneficial), but this is today considered an oversimplification *in vivo* (85).

1.2.3.3 Adaptive Immune Responses

In the subacute phase lingering into the chronic phase (days to weeks) following TBI, there is an onset of an adaptive immune response following TBI (124,125). During this period, among else, T cells from the periphery enter the CNS (126,128) and although it confers some (neuro)protective effects such as IL-4 production (126), there are also other processes such as auto-immune T cells (125) and autoantibodies (141) for which data is beginning to emerge.

In summary, TBI encompasses a vast immune response, and many possible therapeutic avenues lie in the acute phase. Within the neurovascular unit, multiple processes are intimately intertwined in a complex meshwork. One cell type with key relevance for both BBB function, edema, and neuroinflammation is the astrocyte, why we further will indulge in this previously largely overlooked CNS cell.

1.2.4 Astrocytes in the Healthy and Injured CNS

1.2.4.1 Astrocytes in Health

The astrocyte – originally (and erroneously) described as a supportive cell (142–144) – is the most abundant cell within the CNS (145,146) comprising roughly half all human CNS cells (147,148). Astrocytes in the healthy CNS encompass immense heterogeneity (149), and in order to appropriately define them, one must consider morphological, antigenic, functional and locational characteristics. Common to most subgroups, astrocytes are stellate cells with multiple processes emanating from the cell soma (148), originally classified into either protoplasmic or fibrous (147). Today, more than 10 different subtypes have been described (88) in the non-injured CNS. Notably, astrocytes do not unite in a common molecular signature (142). The intermediate filament protein GFAP was previously believed to be an astrocyte-specific marker (150), but it is now well-established that there are GFAP negative astrocytes (142,151) and that some non-astrocytic cells express GFAP (152). Among other markers investigated, the one most likely to be "pan-astrocytic" is aldehyde dehydrogenase 1 family member L1 (147,151). Other astrocytic markers, showing large promise is glutamate transporter-1 (GLT-1) (153), aquaporin-4 (AQP4) (154), and SOX9 (151). For SOX9, however, an important draw-back is that it is also expressed by neural progenitor cells (151).

Astrocytes also exhibit heterogeneity by harnessing the capability to exert a diverse set of functions within the CNS. These comprise, but are not limited to: neurotrophic support (148,155), synapse development and functional maintenance (147,156), and ion as well as neurotransmitter homeostasis (130,156). Of particular interest here, is the capability of astrocytes to establish, maintain, function with/repair the BBB (143,148), and interact in the regulation thereof as part of the neuro-/gliovascular unit (88). This is partly due to the water channel AQP4, that while operating in concert with the inward rectifier potassium channel Kir4.1, regulates water inlet into the CNS at the astrocytic end-feet (88) lining the BBB.

AQP4-expressing astrocytes at the BBB interface gained even more attention recently, following the discovery of the glymphatic system (89). In a seminal paper, Iliff and colleagues demonstrated how CSF is transported and cleared para-vascularly, while by-passing the CNS interstitial fluid in a transport mechanism that is AQP4-dependent (89). Recent data supports the existence of a glymphatic system in humans (90,91), although further studies are warranted.

1.2.4.2 Reactive Astrocytes and TBI

Following CNS injury, astrocytes undergo alterations morphologically, transcriptionally, and functionally (130,143,157). These are commonly referred to as "reactive astrocytes", or interchangeably "reactive astrogliosis" (156). Collectively, reactive astrogliosis encompasses four key elements: situation-specific astrocyte alterations (i) developing following all CNS insults (ii) with varying features depending on insult-severity (iii), that can comprise functional alterations of deleterious or protective nature (iv) (157). Given this definition, it is not surprising that reactive astrocytes similarly to healthy astrocytes exhibit vast heterogeneity (144,158,159).

The main morphological alterations *in vivo* associated with reactive astrogliosis are cell soma/process hypertrophy (131,144,156), upregulation of GFAP (144,156) and other intermediary filament proteins (131). These morphological alterations do not represent a homogenous reactive astrocyte phenotype (156). In fact, different types of CNS insults inflict discrepant transcriptional alterations in astrocytes (160). Conversely, when examining astrocytic heterogeneity across brain regions following the same inductive stimuli, an even broader heterogeneity has been demonstrated (159) at single-cell resolution. These results were elaborated on in a pivotal article from the Ben Barres group, where one reactive astrocyte phenotype was shown to be neurotoxic following cytokine-mediated microglia activation (132). This phenotype was denoted "A1" and its corresponding, primarily (neuro)protective, counterpart "A2" (132). These findings have recently been corroborated for human cells (161). Albeit still not conclusively elucidated, the underlying mechanism is suggested to be a deleterious astrocytic gain-of-function, possibly mediated through saturated lipids (162). This illustrates the spectrum of reactive astrocyte heterogeneity but also the duality of reactive astrogliosis – it is not exclusively deleterious or beneficial – but rather serves different roles during different circumstances (131,144,157).

The duality of reactive astrogliosis is illustrated through the longitudinal sequence of events that ensues a TBI. Depending on the severity of trauma, early features of reactive astrogliosis include hypertrophy, cellular swelling, and proliferation (163). Data is somewhat conflicting regarding astrocyte migration, with data supporting astrocytic migration (164), while more recent data indicates the opposite (143). These astrocyte alterations occur during the first weeks following a TBI and are paralleled by BBB disruption and other inflammatory events

(163), as described above. Next, depending on lesion severity (157), reactive astrocytes form the astroglial scar (163). This scar locates at the border-zone between healthy and injured tissue (156). Over the weeks ensuing the trauma, it becomes compacted and permanent (163). The scar encapsulates the injury region at the expense of suppressing axon regeneration (143,144), possibly partly due to astrocyte-mediated production of keratan sulfate and chondroitin proteoglycans (131). Theoretically, astrocyte ablation would thus reduce the scar and improve axonal regrowth. However, astrocyte ablation has been shown to be detrimental for neuronal survival, while increasing infiltration of peripheral immune cell infiltration, and impairing BBB repair. In fact, reactive astrocytes lining the BBB have been described to be able to both increase and repair BBB disruption (130). The role of AQP4 in this process is emerging and described below.

1.2.4.3 The Role of AQP4-expressing Reactive Astrocytes Lining the BBB following TBI

As previously stated, astrocytes in health are intimately related with the BBB through its AQP4 expressing end-feet and through its important function within the glymphatic system. Following injury, AQP4 expression is globally increased (164–166), but the AQP4 polarization towards the end-feet is lost (167,168), so that AQP4 instead localizes around the cell soma (166). Concomitantly, the glymphatic system is suppressed with consequent intracerebral accumulation of neurodegenerative compounds (169). Moreover, it was recently suggested that the glymphatic system was imminent for brain protein biomarker clearance (170), and that not only TBI, but also routine clinical management such as CSF drainage, and altered sleep cycle patterns, could affect biomarker clearance to blood (170). This could potentially affect the clinical utility of brain enriched protein biomarkers in serum.

AQP4 expression alterations could also be of importance for post-traumatic brain edema. AQP4 was first implicated in brain edema through experiments where AQP4 was impaired using either transgenic knock-out animals in stroke models (171) or pharmacological inhibition in TBI animals (64), with consequent brain water content reduction. Edema reduction has also been discerned using AQP4 deficient mice following TBI (172). This strongly implies AQP4 inhibition for mitigation of cytotoxic edema. This would suggest therapies directed at AQP4 inhibition in order to halt post-traumatic edema, which has been attempted but proven difficult (173). In addition, this is not necessarily beneficial following TBI, which is also accompanied by vasogenic edema. In fact, AQP4 is instrumental for vasogenic edema clearance (174,175), and thus potentially deleterious to inhibit as it would hinder water removal. Importantly, Kitchen and colleagues recently demonstrated that this deleterious effect might be circumvented by targeting the calmodulin-dependent subcellular localization of AQP4 using an already Food and Drug Administration approved substance (trifluoperazine) with consequently improved outcome following a spinal cord injury model of edema (176), but additional studies are warranted.

Another avenue by which edema might be mitigated following severe TBI is through neuroinflammatory modulation. In fact, neuroinflammation might influence both AQP4 and edema development. One tentative pathway is through High-Mobility Group Protein B1, which through microglia activates IL-1β, which activates Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells, that in its turn increases AQP4 expression (165). In accordance, edema is reduced when IL-1β is inhibited, either through the usage of IL-1β deficient mice (177), or by administration of anti-IL-1β (118).

1.3 SUMMARY, KNOWLEDGE-GAP, AND OVERARCHING HYPOTHESES

Above, I summarize severe TBI from a translational viewpoint, highlighting the fact that TBI still today holds a semi-desolate prognosis, and that a large extent of current TBI-mortality can be inferred to deteriorated ICP. This is possibly mediated at least in part through interplay between BBB disruption and neuroinflammation, linked to one another through the involvement of astrocytes. It therefore seems possible that a more elaborate understanding of these cellular injury mechanisms both could refine severe TBI pathophysiology models and outcome prognostication, while offering potential new treatment avenues.

2 AIMS

The overall aim of this thesis was to characterize pathophysiological processes that ensue a severe TBI, and that are currently not commonly targeted for clinical intervention. We chose to focus on BBB disruption and neuroinflammation as these astrocyte-dependent processes might interplay in the aftermath following severe TBI and therefore could constitute eligible future treatment targets.

Specifically, we aimed to:

Paper I Determine the longitudinal development of BBB disruption following severe TBI in humans and whether clearance from brain to blood of CNS-enriched proteins is affected by BBB disruption.

Paper II Delineate if the CSF and blood proteome, focusing on structural and inflammatory proteins, in humans is associated with BBB disruption, and if this is of importance for long-term functional outcome.

Paper III Describe the relation between neuroinflammation, post-traumatic brain edema, and AQP4 retraction from the BBB endothelium by developing a multi-modal imaging system in an experimental rodent model of severe TBI.

Paper IV Define how neuroinflammatory astrocytes, from a CNS niche in which injury is associated with particularly poor prognosis, affect motor neurons *in vitro*.

3 MATERIALS AND METHODS

Table 1 contains an overview of the methods utilized across this thesis. Rather than reiterating procedural details that can be found in **papers I-IV**, I provide a theoretical rational of selected methods below, including a discussion of their strengths and limitations where applicable.

Table 1: Overview of Methods Employed in the Thesis

Method Type	Method specification	Study Applicability
Clinical Data Resources		
	Karolinska TBI Database	I, II
Surgical techniques		
	Controlled-cortical impact	III
	Animal handling	III
***In vitro* techniques**		
	Embryonic stem cell techniques	IV
	Motor neuron differentiation	IV
	Astrocyte differentiation	IV
	Neuroinflammatory modulation *in vitro*	IV
	Subventricular zone stem cell culture	IV
	Subventricular zone stem cell differentiation into astrocytes	IV
	Induction of neuroinflammation using soluble proteins	IV
Analytical Techniques		
	Immunohisto-/-cytochemistry	III, IV
	Confocal microscopy	IV
	Flow-cytometry	IV
	Fluorescence-activated cell sorting	IV
	Magnetic resonance imaging	III
	Multiplex suspension bead antibody array	II
	Other protein quantification techniques	I, II
	Library preparation for RNA sequencing	IV
Selected Statistical and Bioinformatic Techniques		
	Cross-correlations	I

Table 1: Overview of Methods Employed in the Thesis		
Method Type	**Method specification**	**Study Applicability**
Selected Statistical and Bioinformatic Techniques (continued)		
	Longitudinal regression modelling	I
	Dimensionality-reduction techniques	II
	Clustering-techniques	II
	Pathway analysis	II
	Proportional odds regression modelling	II
Softwares		
	MetaMorph	IV
	FIJI	III
	Python	III
	CRAN/R	I-IV

3.1 SELECTED METHODS AND METHODOLOGICAL CONSIDERATIONS

P**aper I** and **II** were to a large extent dependent on statistical analyses. **Paper I** comprised longitudinal data on severe TBI patients, whereas **paper II** comprised cross-sectional data on numerous proteins sampled from severe TBI patients, warranting different statistical considerations. In the experimental **paper III**, we utilized the controlled cortical impact model on rodents. The inferred TBI was then used to develop a multi-modal imaging system. In **paper IV**, we differentiated stem cells into astrocytes and motor neurons *in vitro*.

3.1.1 Paper I: Longitudinal Statistical Techniques

In **paper I**, n = 17 patients with a severe TBI were recruited. From all patients, we collected CSF and blood samples in addition to standard clinical data and patient demographics. Of these, one patient was excluded as no CSF-albumin samples had been obtained. The remaining n = 16 patients had an external ventricular drain, from which CSF (S100B and NSE) was analyzed at 6–12-hour intervals. Concomitantly, arterial blood was analyzed. All laboratory assays were undertaken at the Karolinska University Laboratory. S100B and NSE (in CSF) and NSE (in blood) were analyzed on a Liaison XL system (Diasorin, Saluggia, Italy) through an immunoluminometric assay. S100B (in blood) was analyzed through an electrochemiluminescence immunoassay (Elecsys, Roche Diagnostics, Basel, Switzerland). The different platforms utilized for S100B measurements occurred as a consequence of local procurements and technical aspects, precluding analysis of CSF samples on the Elecsys platform. The different platforms were likely of minor importance in the current study, where relative relationship was the focus of study and as the assays have shown robust association as well as run similarity (178). In addition, albumin from plasma and CSF was analyzed

repeatedly. The latter was used to derive the albumin quotient (Q_A), i.e. the quotient between CSF-albumin and plasma-albumin, which constitutes the literature gold standard for assessing the extent of BBB disruption (100,101,179). This is motivated in theory, as albumin has a 200 times lower concentration in CSF compared with blood, owing to its lack of intracranial synthesis and catabolism (100,180). Yet, Q_A and CSF-albumin hold important limitations, of which age-dependency (100), intraventricular hemorrhage (105), and administration of intravenous fluids can affect albumin values. Despite these limitations, Q_A is robustly characterized and utilized, leading us to also employ it throughout **paper I** and **paper II**.

The data described above constituted a uniquely high sampling time-resolution. However, this also means that measurement values for each patient were consecutively dependent. This needed to be considered in regression models. For all assessments, we used R (181), through the interface RStudio®. We constructed a within-group correlation structure for all patients, thus accounting for the assumption that a measured variable at timepoint *t* in one patient depends on the value observed for the variable at timepoint *t-1*. We constructed this correlation structure as a time series model, for which we assessed stationarity, autocorrelation, and partial autocorrelation. We found the data to be stationary using the Kwiatkowski-Phillips-Schmidt-Shin test in the tseries package (182) in R. We could thus model the time series as an autoregressive moving average process (ARMA) (183). An ARMA process is dependent of *p*, the autoregressive component, and *q*, the moving average component. These can be estimated using partial autocorrelation function (for *p*) and autocorrelation function (for *q*) (183). We estimated the underlying ARMA structure by testing n = 25 different ARMA combinations. We chose the optimal structure using the Akaike Information Criterion. For both S100B and NSE we decided on an ARMA(1,1) process, i.e. a first order autoregressive model with a moving average of 1. Model fit was evaluated graphically using the forecast package in R (184). The ARMA(1,1) process was included as a within-patient correlation structure in longitudinal regression models, where $S100B_{blood}$ and NSE_{blood} were modelled to be dependent on time from trauma, BBB disruption, and the $biomarker_{CSF}$ value. For all analyses, we used the nlme package in R (185). We used a marginal model for S100B as the variance-covariance matrix generated when using a linear mixed model was not positive-definite (186). We also excluded the first 12 hours from analysis, as this time-interval has been associated with an extracranial S100B peak (76,77). For NSE, we used a linear mixed model. Here, patient #17 constituted a suspicious outlier. We thus constructed NSE models both including and excluding patient #17, with overall similar results. Hence, patient #17 did not infer any major alterations regarding overall conclusions. Model assumptions were examined graphically and included variance homogeneity and normality, correlation between fitted/observed values, as well as individual assessments of residual autocorrelation and partial autocorrelation.

3.1.2 Paper II: Proteomic Data Analysis and Cross-Sectional Statistical Techniques

In **paper II**, we recruited in total n = 190 TBI patients, from which we collected CSF and blood samples in addition to clinical data. Of these, n = 4 patients were excluded *a priori* due to non-reliable sample data. Of the remaining, n = 90 patients had matched samples from CSF and serum and were used as the main study cohort. The remaining n = 96 patients had exclusively serum samples and were used as a validation cohort, thus enabling independent validation of key findings from the main cohort. In addition, we recruited n = 15 healthy control subjects. We assessed proteins in CSF and serum for all patients, including the healthy control subjects. CSF acquisition was enabled through external ventricular drains for TBI patients, and through lumbar puncture for control subjects. As CSF protein content varies along the rostro-caudal axis (187,188), some caution in interpretation is warranted. In addition, one might critique CSF to be but a proxy metric for intracranial injury processes, as CSF might fail to depict intracellular events (189). Yet, CSF provides global CNS data, whereas e.g. tissue biopsies or microdialysis merely convey information pertaining to a limited region within the brain (12,189).

3.1.2.1 Reflections on Proteomic Measurement Techniques in TBI Studies

We depicted 177 proteins by utilizing a suspension bead antibody array on 220 antibodies (190,191). Antibodies were selected using the Human Protein Atlas (www.proteinatlas.org) (192). Here, sample proteins are bound to antibodies attached to color-coded beads, detected using a FlexMap3D instrument (Luminex Corporation), for which the read-out is (median) fluorescent intensity. Protein selection was made using available data from previous proteomic TBI studies, protein data on CNS enrichment, or neuroinflammation studies (78,193–200). This method allows for multiplexed protein screens in large number of patients (201) and has previously shown applicability for both serum (191,201), and CSF (190). Utilizing this technique, we could include a larger patient cohort than previous TBI proteomic efforts (189,194–196,202–208). This comes at the expense of the protein selection procedure, yielding an inherent bias in the data. Even though non-biased proteomic approaches such as mass-spectrometry could theoretically assess more proteins than we did, this method often fails to detect low-abundance proteins (209).

3.1.2.2 Deriving Clinically Relevant Information from Proteomic Data

Statistical approached were different in **paper I** compared with **paper II**. The study design in **paper II** was cross-sectional, and included more patients than **paper I**, thus allowing for multivariable analyses including outcome prediction. In addition, the many proteins included in **paper II** warranted quality control considerations, protein analysis, and multiple-testing correction strategies in order to not infer a type I error in the data. For all assessments, we used R (181), through the interface RStudio®.

Quality control procedures entailed assessing bead count, and intensity across both samples and proteins. Blank and pooled samples served as sample negative and positive controls respectively. For analytes, an empty sample was used as negative control, and hIgG or rIgG as positive controls. This yielded exclusion of n = 4 patients, but no analytes (**Figure 7**).

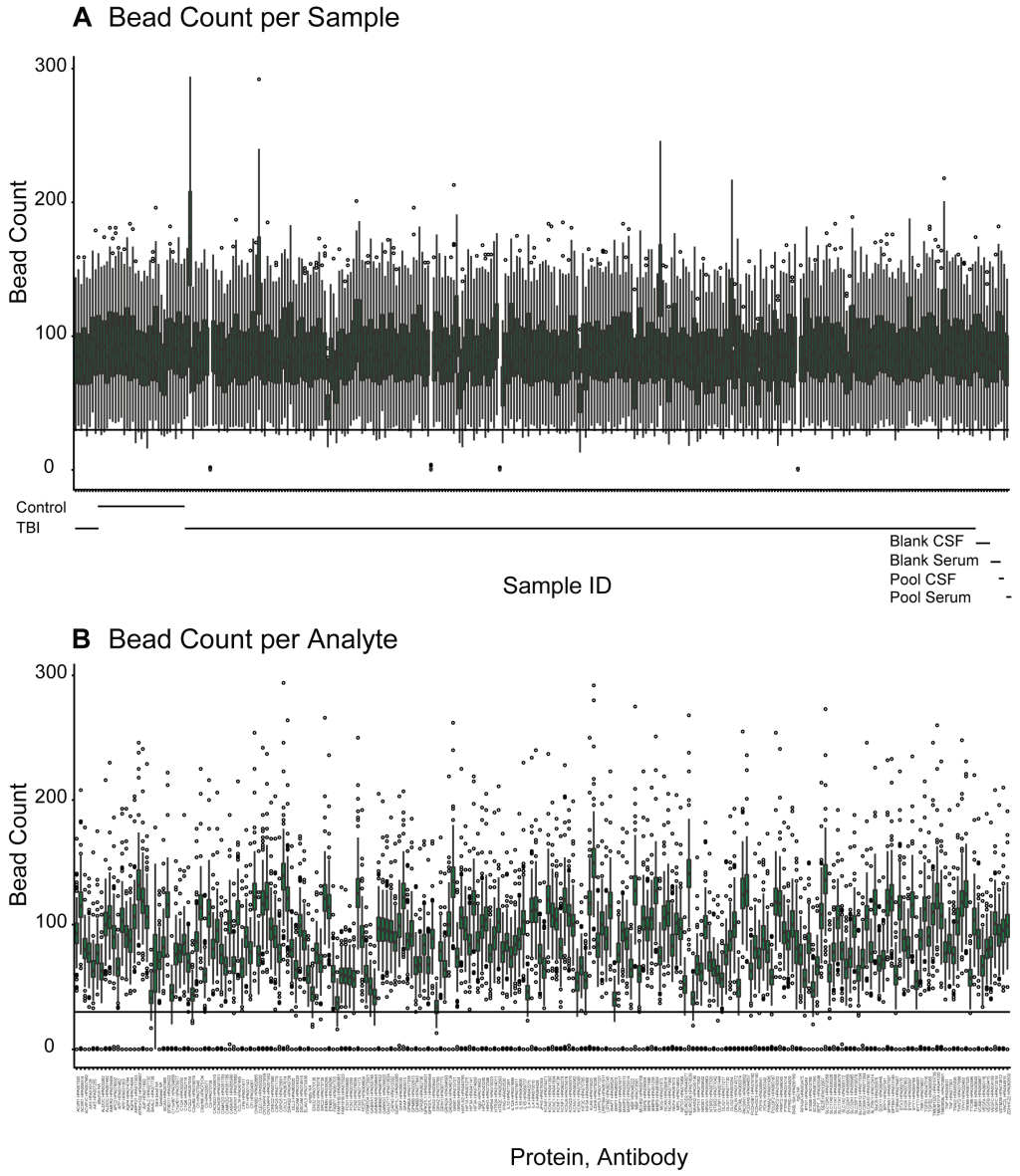

Figure 7: Bead Quality Control Yielded Exclusion of Four Patients but No Analytes. We assessed bead count across all patient samples **(A)** and analytes **(B)**. Four TBI samples had bead count < 30, why these four patients (n = 8 samples) were excluded from analyses. All analytes exhibited median bead count ≥ 30. **Abbreviations:** CSF, cerebrospinal fluid; TBI, traumatic brain injury.

Intensity measurements across samples demonstrated that CSF samples in general had slightly higher intensity compared to serum (**Figure 8A-B**), thus warranting background subtraction to normalize all samples. When comparing intensities across all analytes, one protein was excluded due to borderline non-detectable fluorescence (**Figure 8C**).

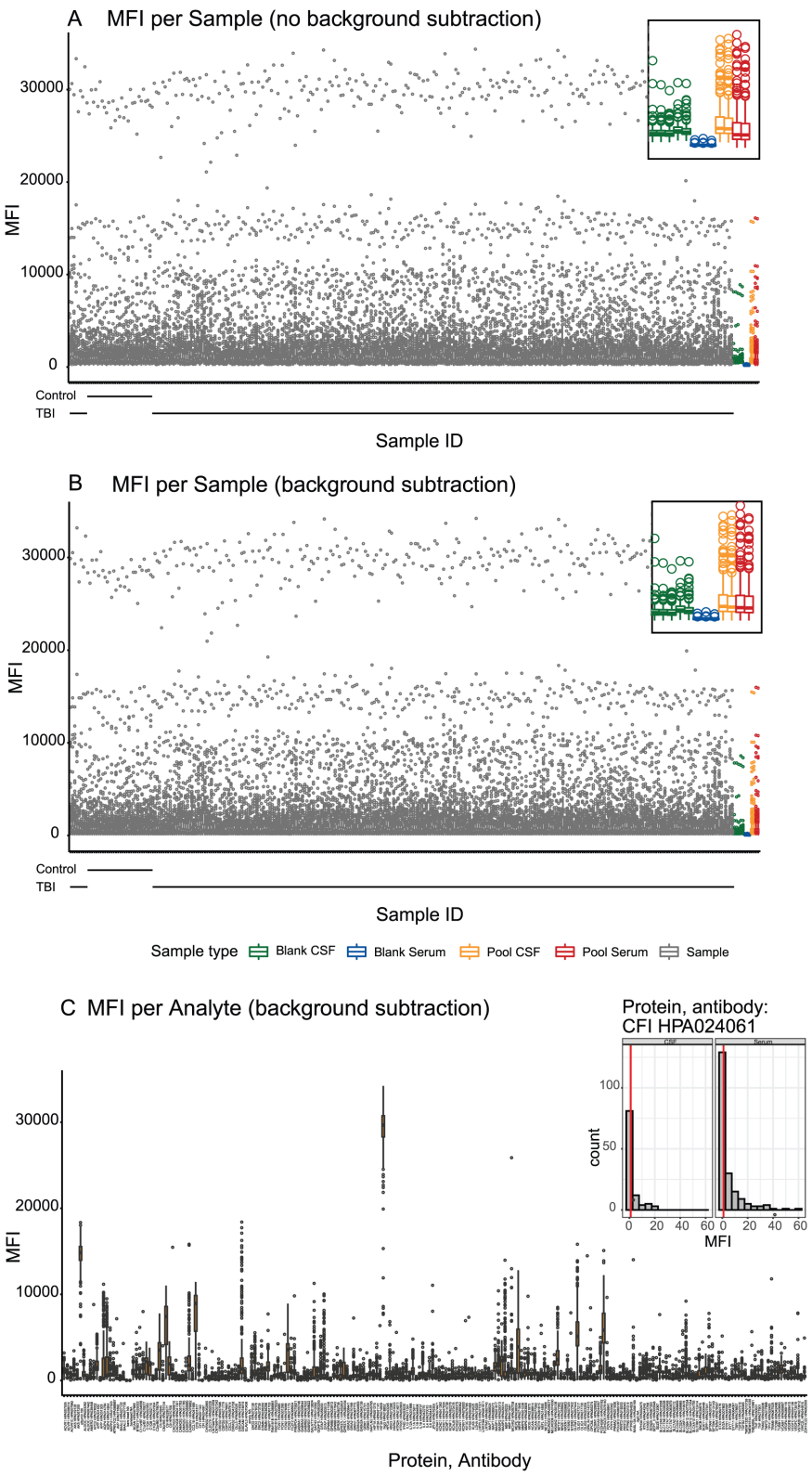

Figure 8: Intensity Assessments across Samples and Analytes. Median fluorescent intensity (MFI) was assessed per sample **(A, B)** and analyte **(C)**. CSF samples **(inset A)** had higher MFI than serum samples. To overcome this, background subtraction was executed with improved results **(inset B)**. Following background subtraction, MFI pe analyte was examined. Analytes that exhibited MFI < MFI for the positive control rIgG were examined in detail, whereupon CFI HPA024061 was excluded **(C, inset)**. **Abbreviations:** C, CSF; S, serum; CSF, cerebrospinal fluid; MFI, median fluorescent intensity.

We also assessed protein correlations, both between compartments and within samples, between compartments between samples (within analytes), and within compartments for individual analytes portrayed through two antibodies (**Figure 9**). For proteins represented by one antibody, low correlation between serum and CSF is difficult to evaluate. For proteins represented by different antibodies (i.e. *sibling antibodies* (210)), low between-compartment

correlations for one antibody but not the other led to exclusion of the low-correlating antibody. In total, we excluded n = 9 of in total n = 42 sibling antibodies using this strategy.

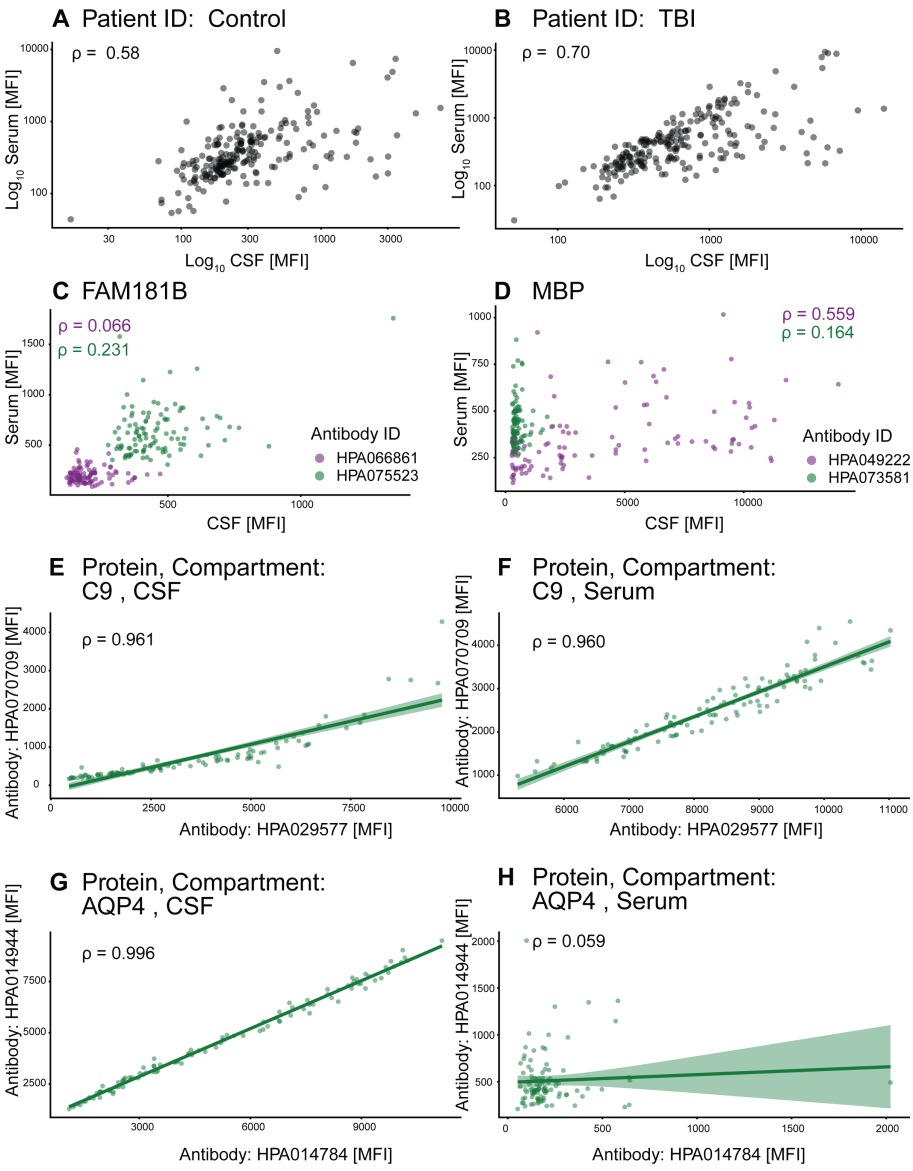

Figure 9: Patient Samples Correlated between CSF and Serum, whereas Individual Analytes Had a variable CSF/Serum Correlation. Individual samples showed good protein correlations between serum and CSF **(A, B)**. In contrast, analytes **(C,D)** had variable CSF-serum correlations. For proteins depicted through multiple antibodies, this was examined in greater detail **(E-H)**. Here antibodies showed either acceptable within-compartment correlations **(E,F)**, or good correlations within one compartment **(G)**, while not in the other **(H)**, leading to additional analyte exclusion. **Abbreviations:** CSF, cerebrospinal fluid; MFI, median fluorescent intensity; TBI, traumatic brain injury.

Following data quality control, we integrated protein data with clinical data analysis techniques. Protein characterization was enabled through the Human Protein Atlas data using both protein/RNA, and Brain Atlas data (192,211,212). We conducted dimensionality reduction analyses using t-distributed stochastic neighbor embedding (213,214) to identify protein patterns. We used hierarchical clustering techniques to discern the presumptuous importance of BBB disruption for protein levels, and pathway analysis (215) to understand

how the deduced proteins related to one another. Throughout these analyses, multiple testing correction was conducted using the Bonferroni method, Holm method (216), or the false-discovery rate (217).

Uniquely for **paper II**, we conducted outcome analysis. We applied a hypothesis-driven approach and defined proteins of interest for outcome analysis to be: i) proteins altered upon hierarchical clustering analysis in CSF and that were enriched in TBI patients in CSF, or ii) serum, and iii) proteins that were elevated/decreased in CSF/serum in TBI patients following BBB disruption. Aligning with the TBI field, we used GOS as dependent variable and constructed a proportional odds regression analysis through the rms package in R (218). Univariable analysis was conducted with the protein level of the individual protein as independent variable. If significant in univariable analysis, we proceeded with multivariable analysis, in which we included the previously implicated IMPACT variables (24), comprising age, GCS, pupillary reactions, pre-hospital hypoxia/hypotension, and the Stockholm CT score. When applicable, step-down modelling was performed to see how proteins performed jointly in the regression models. Of note, results are presented as both p-value and the relative variable's contribution to overall model fit (Δpseudo-R^2), as defined by Nagelkerke (219). This stems from the notion that currently available prognostic TBI variables explain ~35% (25) of TBI outcome. A new prognostic TBI marker should therefore provide independent information (i.e. be significant), while also increasing the absolute amount of explained variance to the model in order to be of potential interest.

3.1.3 Paper III: The Controlled Cortical Impact Model and Development of a Multi-Modal Imaging System for Protein Assessment within Edema Subtypes

3.1.3.1 The Controlled Cortical Impact Model

In **paper III**, we used the controlled cortical impact (CCI) technique to mimic a focal severe TBI in rats. This method was originally described by Lighthall (220), and translated to the rodent by Dixon and colleagues (221). Both the animals operated in **paper III** (199,222) and the operating procedure (223) have been described previously by us. In brief, the CCI is inferred by craniotomizing an anesthetized rodent, discarding the bone-flap. A pneumatic piston (Precision Systems and Instrumentation) infers the contusion towards the dural surface following an apparatus calibration ("tare"). Intra- and postoperatively, animals are administered systemic and local analgesia. It is also beneficial to monitor vital parameters intra-operatively to minimize the risk for uncontrolled secondary insult development.

The injury panorama seen after CCI mimics clinical aspects of human severe TBI (224). In **paper III**, we assessed edema and its relationship with (neuro)inflammation. Both of these

cellular injury mechanisms ensue a severe TBI inferred by CCI (224,225). In addition, the CCI model allow strict control of injury parameters e.g. piston velocity, contusion depth and dwell time (224,226), thus reducing experimental heterogeneity. This comes at the expense of inflicting a craniotomy without re-attachment of the extirpated bone-flap. Although recommended by Dixon and colleagues (227), this strategy is unphysiological compared with closed skull injuries, as it ameliorates ICP development and secondary injuries (228,229).

3.1.3.2 A Multi-Modal Imaging System for Parallel Assessment of Edema Subtypes and Protein Expression

Animals exposed to either a sham or TBI surgery (n = 10) in **paper III** underwent magnetic resonance imaging (MRI) followed by immunohistochemistry (IHC) assessments *ex vivo*. Notably, each experimental animal underwent both assessments, which allowed us to match each MRI section with the anatomically equivalent IHC section, which we did for n = 15 injury regions. Through computational methods, we aligned these images with one another, thus allowing for assessment of protein expression within different edema subtypes. Softwares used throughout these procedures were FIJI (230), specifically the plugins TrakEM2 (231), and Image Calculator (232). In addition, we used Python™ (233), through the interface PyCharm community (version 2020.1.2) (234).

The three most critical aspects for parallel assessment of protein expression within edema subtypes were i) image registration, ii) edema delineation, and iii) calculation of AQP4 retraction from BBB endothelium. Image registration is a method consisting of aligning images obtained under different conditions (235). Image registration comprises detection of common image elements between a reference image and a second image, followed by procedures that align these and evaluate registration quality (235). For this, we used TrakEM2 (231), considered to be a state-of-the-art technique for image registration, although other registration tools also exist, some of which have been claimed to outperform TrakEM2 registration accuracy (236). TrakEM2 was developed for registration of large volumes of consecutive electron microscopy 2D-tiles (231), but we instead registered fewer images from two different imaging modalities. We used a landmark-based approach (237), i.e. we chose intrinsic study subject features and used these for alignment **(Figure 10)**.

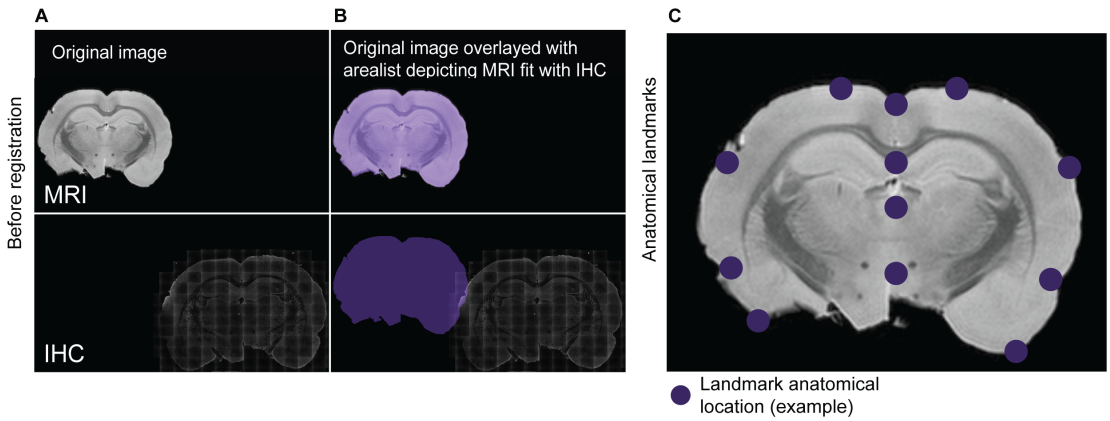

Figure 10: Registration of Immunohistochemical Images to Magnetic Resonance Images. Raw-data IHC and MRI images differed morphologically **(A)**. The MRI served as anatomical ground-truth depiction. When the MRI section surface area was overlayed upon the IHC image, discrepancies were even more clear **(B)**. Using anatomical landmarks **(C)**, IHC images were registered to the MRI image. **Abbreviations:** IHC, immunohistochemistry; MRI, magnetic resonance imaging.

Following landmark annotation **(Figure 10C)**, we tested four different global mapping models (235,238), that entailed the methods *translation, rigid, similarity,* and *affine*. A translation transformation allows image displacements in two dimensions, a rigid transformation in addition allows for image rotation, while a similarity transformation also enables isotropic scaling, while an affine transformation allows for all of the aforementioned techniques but also for shear transformation (238). We used the transformation that yielded the visually best image alignment (235) **(Figure 11)**.

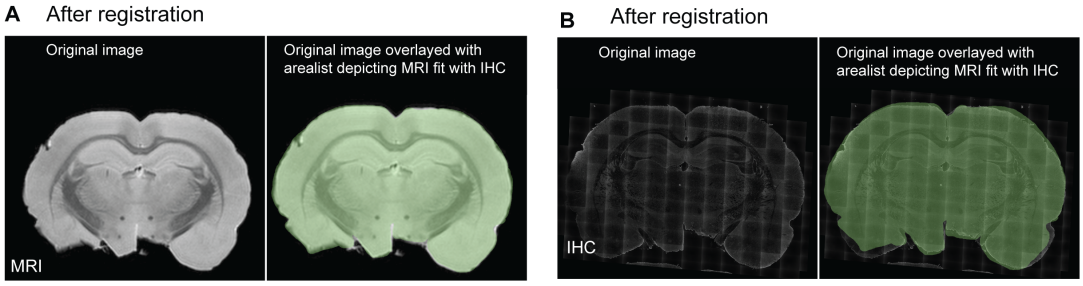

Figure 11: Image Registration Quality Control. MRI images served as ground-truth images **(A)**. Following image registration **(B)**, IHC images were harmonized to the MRI images with regard to size, scale, rotation and shear effects. The robustness of the procedure is highlighted by overlaying the modified IHC image with the MRI image **(B, right panel)**. **Abbreviations:** IHC, immunohistochemistry; MRI, magnetic resonance imaging.

Edema was defined sequentially **(Figure 12)**, using diffusion and non-diffusion weighted MRI images comprising the non-diffusion weighted sequence (S_0), the apparent diffusion coefficient (ADC), and the diffusion weighted imaging (DWI) sequences. Utilizing the S_0 image, we distinguished between "contusion" i.e. necrotic tissue and all types of edema ("total edema"). A contusion was defined as a hypo-intensity, and conversely edema as a hyper-intensity, in-line with current literature (116,239,240). A cytotoxic edema within the total edema area was defined as an ADC hypo-intensity, with a concurrent DWI hyper-

intensity. The opposite criteria were used to define a vasogenic edema. Remaining, non-categorized pixels within the total edema area following this was deemed to entail a "mixed edema" (111). To examine protein expression within these regions, the exact same region was cropped from the immunohistochemical image using Python.

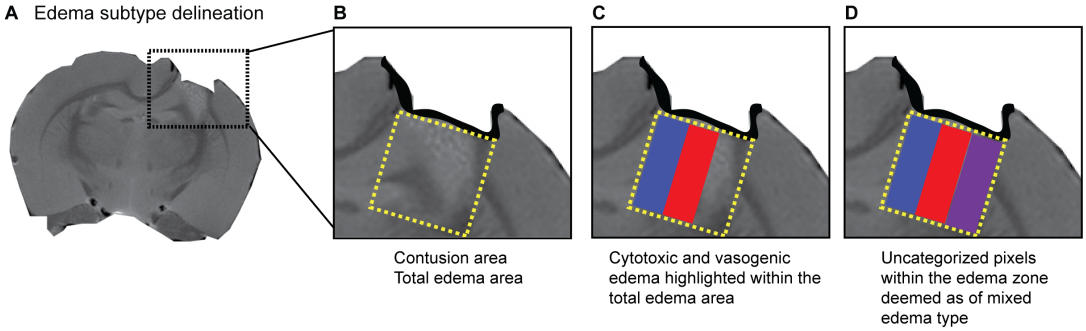

Figure 12: Edema Subtypes Were Defined Sequentially, Using a Standardized Approach. The total edema area (yellow dotted) and the contusion (necrotic, black-shaded) area were defined using non-diffusion weighted images of the injury zone **(A,B)**. The total edema area could be defined into a cytotoxic or vasogenic component depending on ADC and DWI sequences **(C)**. Remaining, non-categorized pixels within the total edema region were considered to comprise a mixed type edema **(C,D)**. Note that this image was simplified for schematic and examplifying purposes. **Abbreviations:** ADC, apparent diffusion coefficient; DWI, diffusion-weighted imaging.

In order to assess AQP4 retraction from astrocytic end-feet lining the BBB, we calculated AQP4 loss from the BBB endothelium using the FIJI plugin Image Calculator. Under homeostasis, the BBB is delineated by BBB-endothelial staining (SMI-71+) (241), and AQP4+ astrocytic end-feet (114) **(Figure 13A)**. Following TBI, AQP4 retracts towards perivascular astrocytic soma (166), so that only the endothelial staining remains **(Figure 13B)**. The ratio between SMI-71 and AQP4 thereby alters so that AQP4 withdrawal increases the SMI-71/AQP4-ratio towards infinity, which on the image-level yields an increased fluorescent signal **(Figure 13C)**, that can be quantified for regions of interest using Python.

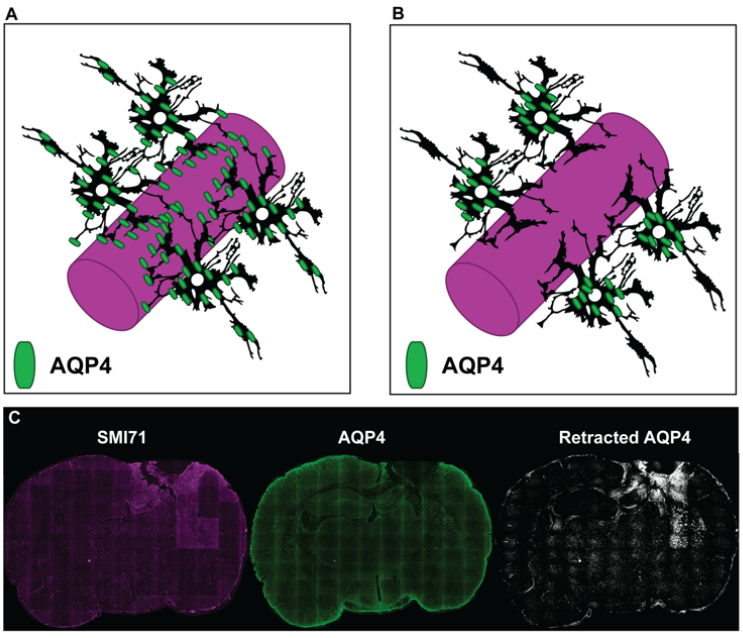

Figure 13: Theoretical Rationale and Assessment of AQP4 Retraction from the Injury Zone. Under homeostasis **(A)**, AQP4 (green) covers the BBB endothelium (magenta). Following injury **(B)**, AQP4 is believed to retract towards the astrocytic soma **(B)**. We quantified this by dividing the BBB endothelial marker SMI-71 with AQP4, yielding increased intensities **(C)** in regions with AQP4 retraction. **Abbreviations:** AQP4, aquaporin-4; BBB, blood-brain barrier.

3.1.4 Paper IV: Stem Cell-Based Disease-Modelling

We used a stem-cell based model system for **paper IV** in order to examine neuroinflammatory astrocytes. The advent of stem-cell techniques, first through embryonic stem cells (ES cells) (242,243), later followed by induced pluripotent stem cells (iPS cells) (244) and human iPS (hiPS) cells (245,246), have created a novel platform for disease-modelling, drug screening, and cell therapy (247). Human ES cell studies have largely been replaced with the hiPS equivalent, as hiPS cells overcome two of the most pressing issues with ES cells, namely ethical aspects and graft-host rejection (247). We used mouse ES cells, as **paper IV** was not aimed at cell replacement but disease-modelling. For such studies, mouse ES cells provide swifter experimental turn-over while allowing translation into either the *in vivo* or hiPS cell *in vitro* setting.

We derived ES-astrocytes and ES-motor neurons by mimicking neuronal differentiation *in vivo*. In the developing CNS, both temporal and spatial cues determine cell fate (248). The process is initiated by neurogenesis (249). We differentiated ES-motor neurons and ES-astrocytes that *in vivo* are situated in the ventral brainstem, where ES-motor neuron genesis depends on Sonic hedgehog and retinoic acid (250). We used the widely recognized Wichterle protocol (250) to generate ES-motor neurons **(Figure 14)**.

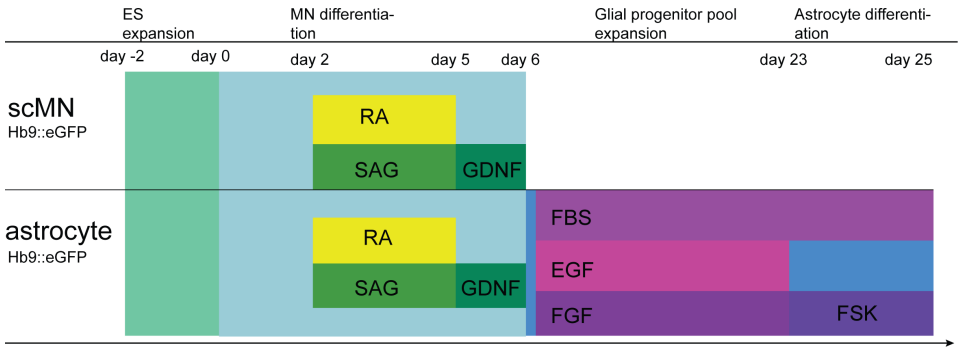

Figure 14: Directed Differentiation of Embryonic Stem Cells into Motor Neurons and Astrocytes with Ventral Brainstem Identity. Neurogenesis *in vivo* was mimicked by using the Wichterle protocol (250). The gliogenic switch seen *in vivo* was then induced by exchanging medium for FBS and culturing glial progenitors in the presence of first proliferative growth factors and then forskolin to prompt astrocytic differentiation. **Abbreviations:** EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; FGF, fibroblast growth factor; FSK, forskolin; GDNF, glial cell-line derived neurotrophic factor; MN, motor neuron; RA, retinoic acid; SAG, smoothened agonist; sc, spinal cord.

Having employed this protocol, resulting cells comprised interneurons, post-mitotic motor neurons, and glial progenitors **(Figure 15)**.

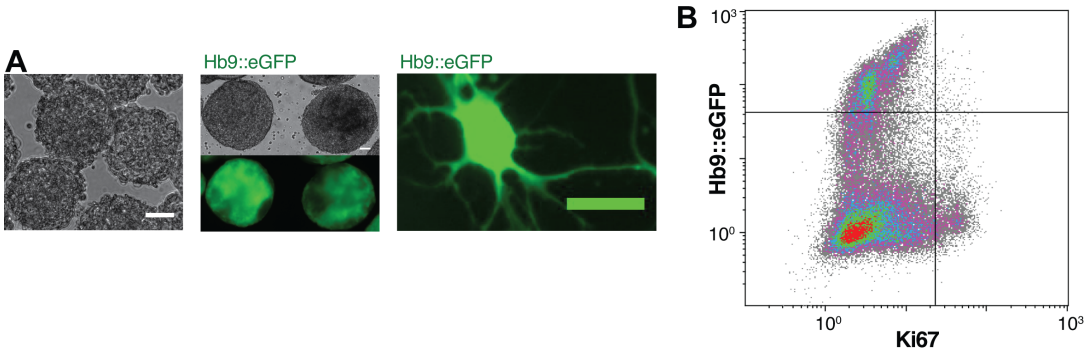

Figure 15: Directed Differentiation of Glial Progenitors following Motor Neuron Differentiation. Following the first eight days in culture, embryoid bodies **(A, left and middle panel)** were dissociated into single cells of post-mitotic motor neurons as deemed by Hb9::eGFP positivity **(A, right-sided panel)**. Within these cultures, glial progenitors were found **(B, lower right quadrant)**, that could be further differentiated into astrocytes. **Scale bars left to right**: 50 µm, 50 µm, 25 µm. **Abbreviations:** eGFP, enhanced green fluorescent protein.

This is well in-line with the *in vivo* setting, where astrocyte and oligodendrocyte development ensue neurogenesis through a "gliogenic switch" (249). We therefore expanded the glial progenitor pool **(Figure 14)**. We applied previously described protocols for directed astrocyte differentiation (251,252), that we adapted. Notably, we prompted astrocytic differentiation through addition of a panel of differentiation factors previously implicated in astrocytic differentiation (251–256). Following screening, we used the cyclic adenosine monophosphate activator forskolin in the established differentiation protocol (176).

The rationale for differentiating ES cells into mature cell types is that ES-derived cells, e.g. ES-astrocytes, are sufficiently similar to *bona fide* astrocytes. In addition, in the CNS, cell access is difficult, which makes stem cells translationally more relevant than primary cultures. Previous work has shown that astrocytes differentiated from hiPS cells are immature (257), and that longer culture times are required for maturation (258). To overcome this, over-expression of astrocytic transcription factors (259), or sorting methods (161), have been suggested to improve the yield of homogenous, mature astrocytes. We did not attempt any of these strategies, which entails an important limitation in **paper IV**. Cultures undertaken are likely not entirely homogenous and some cells are expected to retain immature features. Astrocytic sorting techniques would probably have improved this. Yet, we validated the ES-astrocytes robustly using versatile techniques, encompassing gene validation by RNA-sequencing the cells and validating them against previously published data (260). Here we used a protocol originally adapted for single-cell RNA sequencing (261,262), but that operates equally well for bulk-RNA sequencing (263) which we undertook. We further used immunocytochemistry to validate protein expression of astrocytic markers using among else the canonical astrocytic markers GFAP (150), AQP4 (264), and GLT-1 (153). We also assessed the ES-astrocytes functionally with regard to hallmark functional features, such as inflammatory responses (132,265). Finally, we undertook primary culture validation of our protocol, using subventricular zone stem cells. Taken together, stem cell-based systems have

already shown to be valuable due to their translational versatility. Using this approach we could differentiate astrocyte-like cells that carried multiple resemblances with *bona fide* astrocytes.

3.2 ETHICAL CONSIDERATIONS

All human data studies (**paper I, II**) within this thesis were conducted in accordance with Swedish Law and the Declaration of Helsinki. Ethical approvals were granted through the regional branch of the Swedish Ethical Review Authority. All experimental studies (**paper III, IV**) were conducted in accordance with Swedish legislation, and as stipulated in the Code of Regulations of the Swedish Board of Agriculture (266). Ethical approval was granted by the Swedish Board of Agriculture's regional Stockholm County branch ethics committee. **Table 2** entails an overview of applicable ethical permits for this thesis.

Table 2: Ethical Approvals Paper I-IV

Ethical approval number	Paper relevance	Permit applicability	Additional information
2009/1112-31/3	I	TBI patients	NA
2005/1526-31/2	II	TBI patients	NA
2014/1201-31/1	II	Control patients	NA
N369/12	III	Rodent experiments	Amendment/renewal under #N126/13
N275/15	IV	Cell harvesting	Appeal: N38/16. Amendment/renewal under #9182-2018.
N104/14	IV	Embryonic stem cells.	NA

3.2.1 Ethical Reflections on Clinical Studies

Preclinical research within the TBI field has generated promising therapeutic targets and tentative pharmacological compounds that have failed upon translational attempts (128,267). This highlights the importance for studies in humans, which were undertaken in **paper I** and **paper II**.

The most pressing ethical issue in **paper I** and **paper II** is patient inclusion procedures. In both studies, study inclusion was granted by consent from a next-of-kin. This is common practice in clinical severe TBI studies, where the patient is unconscious and therefore incapacitated to provide informed consent themselves. This strategy enables the clinical field to obtain information regarding the acute injury processes that ensue a severe TBI. The Swedish law 2003:460 (268) also supports this line of reasoning. Specifically, the 20 § accompanied by §§ 21 and 22, regulates how research *without* informed consent should be effectuated. The next-of-kin system, although imperfect, is therefore what enables these types of studies.

Patients included in both **paper I** and **paper II** were subjected to standard treatment. Neither study conferred any particular benefit to study participants. In **paper I**, patients were monitored somewhat more intensely than what is conventional. Here, all patients had external ventricular drains, from which samples were taken at a high temporal resolution. This could theoretically have inferred an augmented risk for meningitis/ventriculitis (269). This was overcome through a four-way stopcock and a LiquoGuard® CSF-pump (Möller Medical GmbH), which allowed CSF collection in an enclosed system, thus minimizing the risk for iatrogenic contamination and subsequent infection. In **paper II**, samples were taken with a lower temporal resolution, thus not necessitating the CSF-pump system.

3.2.2 Ethical Reflections on Experimental Studies

For experimental studies, animals (rats) were used for experimental surgical procedures (**paper III**) and mice for stem-cell acquisition (**paper IV**). All experimental animals were housed at the Karolinska Institutet animal facility on a 12-hour dark/light cycle, with *ad libitum* access to water and food. Ethical principles guiding animal experiments were derived from the Code of Regulations of the Swedish Board of Agriculture (266), within which the "3R Principle" is described. In short, the 3Rs promote researchers to Replace (animals in research with other models when possible), Refine (experiments to improve animal well-fare), and Reduce (the numbers of animals used). Below these are contextualized within the surgical TBI model (**paper III**) and to a minor extent the cell culture procedures (**paper IV**).

Animals cannot be fully replaced in TBI research since patient and injury heterogeneity often precludes deduction of pathophysiological relationships. For this, reproducible injury models are needed. Further, *in vitro* models lack the complexity of an *in vivo* system, thus necessitating animal models. We used rodents, a traditional research animal in TBI studies. A moral-philosophical aspect of animal research is that animals with lower cognitive capacity should be used (270). Rodents fulfil these criteria but still retain acceptable CNS similarity with humans. Since TBI research involves a physical trauma, minimization of animal suffering and distress is imperative. All animals included (rats) were anesthetized and administered both systemic and local analgesia intraoperatively. Post-operatively, they were monitored closely for signs of pain, and provided analgesia if needed. Naturally, a pre-set

threshold for sacrifice was set and in case of post-operative complications, animals were sacrificed prematurely in order to avoid suffering for the individual animal. We used *ex vivo* data derived from previously published animal experiments (199,222) thus significantly limiting the number of animals used. In addition, we applied multiple *ex vivo* methods to the same animals. This was critical for the research question and had the positive benefit of reducing the number of animals needed.

Lastly, we have endeavoured in the use of *in vitro*-models (**paper IV**). Even though these models cannot encapsulate the complexity of an organism, they can serve as screening tools and mechanistic deduction so that hypothesis-generation is optimized before animal-experiments are attempted. In line with this, we used stem cell-derived astrocytes as they share many similarities with astrocytes differentiated from primary culture cells (in our hands assessed using subventricular zone stem cells). We pursued the majority of our experiments using embryonic stem-cell derived cells, thus significantly limiting the number of animals used compared with primary culture experiments.

4 RESULTS

In order to detect a biological effect ("signal") in human severe TBI data, its magnitude must be stronger than the "noise" inferred by patient and injury heterogeneity. In contrast, experimental models are at risk of over-simplification (271), due to artificial model homogeneity and failure to account for translational complexity (271,272). Experimental models thus risk to over-interpret small biological effects, that will not have meaningful impact when translated into the clinical setting. Accordingly, the majority of translational attempts within the TBI field have failed (272). With this in mind, I sought to first delineate biological phenomena of prognostic importance in human observational data, and thereafter characterize these using experimental models. This thesis is therefore structured from a "bed to bench" perspective.

Utilizing this approach, I have found that a severe TBI is accompanied by BBB disruption (**paper I**), that comprises a novel predictor of long-term functional outcome following TBI (**paper II**), thus stressing its clinical relevance. The underlying mechanism of this is not fully portrayed here, but I show a strong relationship between BBB disruption and neuroinflammation, using human subject data (**paper II**). I further analyze neuroinflammatory protein expression *in situ* in an experimental rodent model (**paper III**), where I find that cytotoxic edema regions with presumably more intact BBB are associated with an innate neuroinflammatory response. In these regions, astrocytes retract AQP4 from the BBB (**paper III**). Astrocytes also acquire other attributes upon neuroinflammation, which I demonstrate in **paper IV**, where astrocytes stimulated with clinically relevant neuroinflammatory stimuli adopt a neurotoxic phenotype *in vitro* (**paper IV**).

4.1 SEVERE TRAUMATIC BRAIN INJURY IS ACCOMPANIED BY BLOOD-BRAIN BARRIER DISRUPTION, WHICH CONSTITUTES A NOVEL PROGNOSTIC MARKER FOR LONG-TERM OUTCOME

Across **paper I-III**, we discerned BBB disruption following severe TBI. We assessed BBB integrity using the current clinical gold-standard metric (101,179) – the albumin quotient Q_A (100) in **paper I-II**. In contrast, for the experimental **paper III**, we measured BBB integrity indirectly using both MRI sequences and *in situ* protein expression. In the cross-sectional **paper II**, n = 23 TBI patients (32%) suffered a BBB disruption as per current clinical definitions (**Figure 16**).

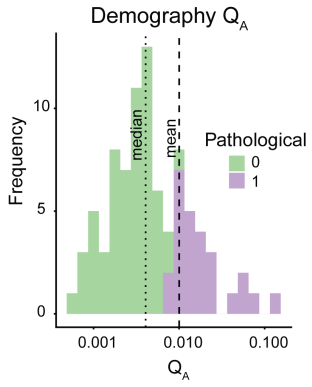

Figure 16: A Severe TBI Is Accompanied by BBB Disruption. In the cross-sectional **paper II**, we found that a subset of patients suffered a BBB breakage, quantified using Q_A with current clinical reference intervals. Of note, CSF samples were in median obtained two days following trauma, possibly causing the number of patients with BBB injury to be falsely too low. **Abbreviations:** BBB, blood-brain barrier; Q_A, albumin quotient.

In **paper I**, the BBB disruption could be seen to extend longitudinally for at least the first week following the TBI (**Figure 17**). Of note, not all patients suffered a BBB disruption as defined per Q_A reference intervals. This highlights that a severe TBI for at least a subset of patients is accompanied by a BBB breakage, that is maintained following the trauma.

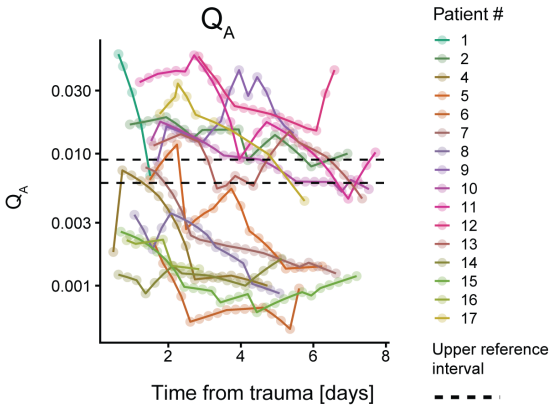

Figure 17: A TBI-Induced BBB Disruption Persists Longitudinally. In the longitudinal **paper I**, we examined Q_A for around a week following the TBI. Although Q_A could be seen to decay somewhat throughout the study period, BBB-injury persisted for around seven days for several patients. **Abbreviations:** BBB, blood-brain barrier. Q_A, albumin quotient; TBI, traumatic brain injury.

Whereas the data from **paper I** indicates that the BBB injury inflicted at the time of trauma is the main contributor to this BBB disruption, the experimental **paper III** brings further complexity. Here, we found that TBI-inflicted edema was maintained for at least 28 days following trauma, and moreover, that the contribution of vasogenic edema increased longitudinally (**Figure 18**). As a delayed vasogenic edema has been linked to exacerbated BBB disruption through post-traumatic cellular injury processes (113), this indicates that BBB disruption following TBI is a dynamic process caused concomitantly by the inducing trauma and other injury processes. Taken together, BBB disruption accompanies a severe traumatic brain injury. BBB disruption can be either maintained, or in the case of ongoing cellular injury-processes, exacerbated, longitudinally.

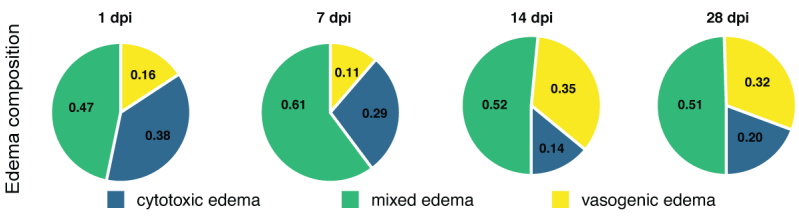

Figure 18: Delayed Vasogenic Edema in Experimental TBI. A delayed vasogenic edema suggests that secondary injuries contribute to post-traumatically maintained edema. **Abbreviations:** dpi, days post injury.

Importantly, we show that BBB disruption is of clinical importance, which is novel. In **paper I**, we show that two brain-enriched proteins of tissue fate were cleared differently from CSF to blood, at least in part dependent on the BBB. We assessed the astrocytic and neuronal proteins S100B and NSE. Overall, we found stronger correlations between blood, CSF, and Q_A for S100B than for NSE. As one might expect a time-shift/lagged clearance from brain to blood, we also assessed cross correlations. Here, we could see a discretely delayed clearance of S100B from CSF to blood, which coincided with an identically lagged correlation between Q_A and $S100B_{blood}$, speaking in favor of a BBB-induced delayed clearance from CSF to blood (**Figure 19A-C**). We could not observe the same phenomenon for NSE (**Figure 19D-F**).

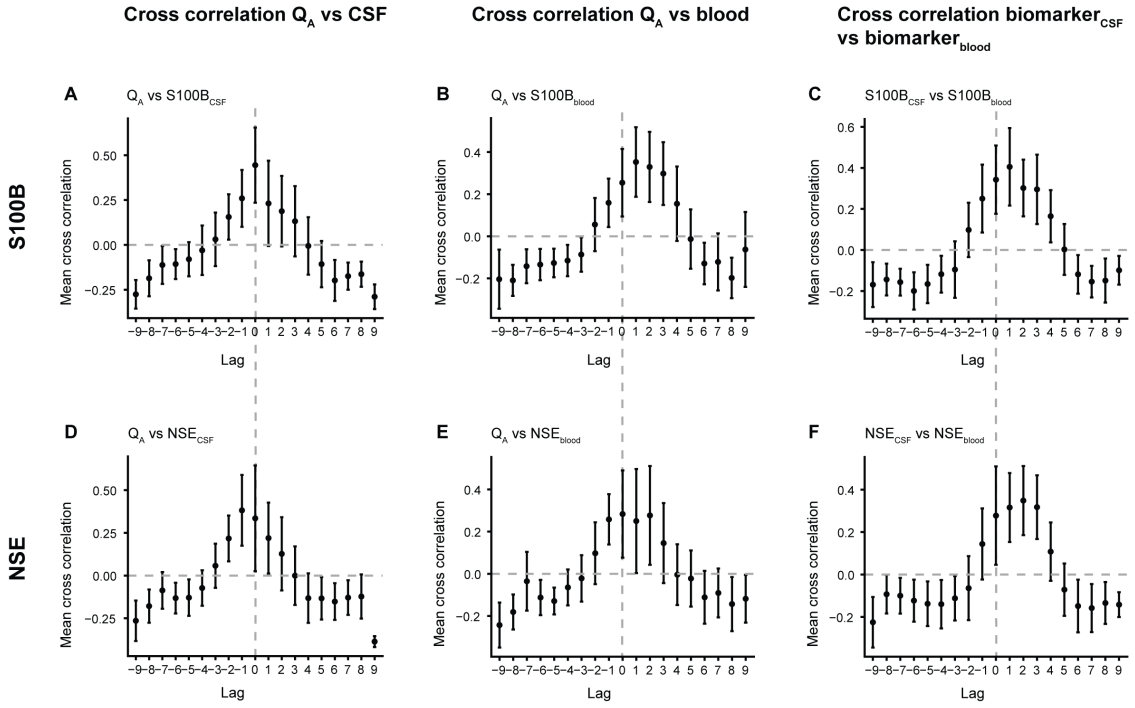

Figure 19: S100B and NSE Exhibit Discrepant Cross-Correlation Patterns. S100B **(A-C)** is detected in blood ~12h later than in CSF, attributed to delayed BBB clearance **(B)**. NSE **(D-F)** is also cleared with a delay to blood, but this could not be related to intracranial NSE release **(D)** or BBB clearance **(E)**. **Abbreviations:** BBB, blood-brain barrier; CSF, cerebrospinal fluid; NSE, neuron-specific enolase.

The discrepancy in cross correlation patterns provide an indication that S100B and NSE are cleared through different routes from the injured CNS. In order to demonstrate this more robustly, we conducted longitudinal regression analyses. Here we found that S100B blood levels exhibited a curvilinear longitudinal decrease, that was also dependent on $S100B_{CSF}$ and Q_A **(Figure 20A)**. In contrast, for NSE, only time from trauma predicted NSE concentrations **(Figure 20B)**. Taken together, our findings indicate that S100B and NSE are cleared differently from the injured CNS, and importantly, that S100B clearance is related to BBB disruption as measured through Q_A.

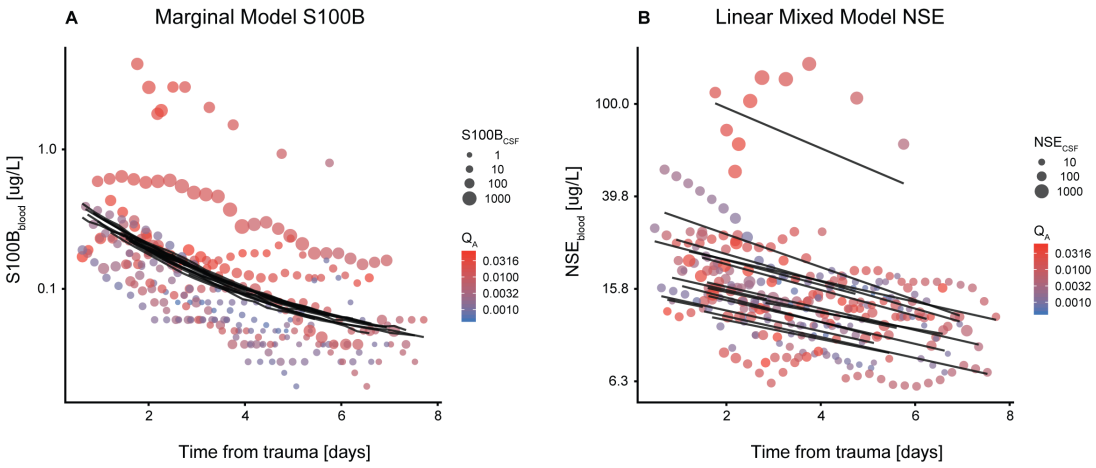

Figure 20: Longitudinal Regression Modelling of Biomarker Clearance for S100B and NSE. For S100B **(A)**, time from trauma, Q_A, and $S100B_{CSF}$ predicted blood levels of S100B. In contrast, NSE in blood **(B)** was only predicted by time from trauma. **Abbreviations:** NSE, neuron-specific enolase; Q_A, albumin quotient.

The sample size included in **paper I** was not powered for outcome analysis. In contrast, we had a uniquely large cohort and a control cohort in **paper II**. We assessed the CSF and serum proteome following a severe TBI and compared protein levels with healthy subjects' values. We found clear discrepancies between the blood and CSF proteome following trauma (**Figure 21A**). Strikingly, the CSF proteome was related to BBB disruption, indicating that BBB disruption plays a role in pathological processes ensuing the trauma (**Figure 21B**).

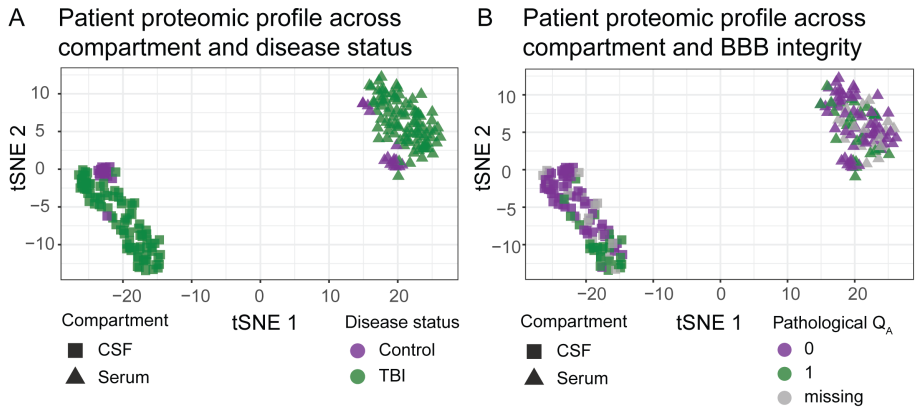

Figure 21: The CSF Proteome Is Affected by both TBI and BBB disruption. As expected, a severe TBI elicited proteomic differences compared with healthy control patients **(A)**, that was discernible in both CSF and serum. Patient-specific attributes e.g. BBB disruption following TBI, also yielded proteomic differences, particularly in the CSF compartment **(B)**. **Abbreviations:** BBB, blood-brain barrier; CSF, cerebrospinal fluid; TBI, traumatic brain injury; tsne, t-distributed stochastic neighbor embedding.

We demonstrated that Q_A constituted an independent predictor of GOS ($p = 0.044$, ΔNagelkerke's pseudo-$R^2 = 8.89\%$). This is novel, and important. We thus demonstrated the occurrence and dynamics of BBB injury following TBI, and related BBB disruption to long-term prognosis. We next sought to characterize the relationship between acute neuroinflammatory responses in the injured brain and whether they were of associative importance for BBB breakdown.

4.2 INNATE NEUROINFLAMMATORY RESPONSES DEMONSTRATE AN ASSOCIATION WITH BLOOD-BRAIN BARRIER DISRUPTION CLINICALLY AND EXPERIMENTALLY

We examined the relationship between BBB disruption and neuroinflammation in **paper II** and **paper III**. In **paper II**, we used fluid proteomic profiling of CSF and serum following severe TBI which we compared with a healthy control cohort. In **paper III**, we conducted *in situ* analysis of protein expression within edema subtypes determined through MRI following an experimental CCI model in rats. In **paper II**, n = 114 of the examined n = 177 proteins correlated with Q_A through the CSF/serum ratio. Correlated proteins exhibited in median a correlation coefficient τ = 0.33 (0.29-0.40), of which nine of the ten proteins with highest correlation coefficient were complement proteins. The other proteins were, as assessed through the Human Protein Atlas (212), predominantly CNS enriched or inflammatory in nature (**Figure 22A**). When we assessed these proteins in higher detail, we found protein levels in CSF (but not serum) to be associated with Q_A (**Figure 22B**). Interestingly, protein levels clustered both depending on Q_A and dichotomized GOS, but not patient genetic attributes in the form of Apolipoprotein E ϵ4-allele carriership. Upon pathway analysis of these proteins, we found predominantly enrichment of inflammatory pathways (**Figure 22C**).

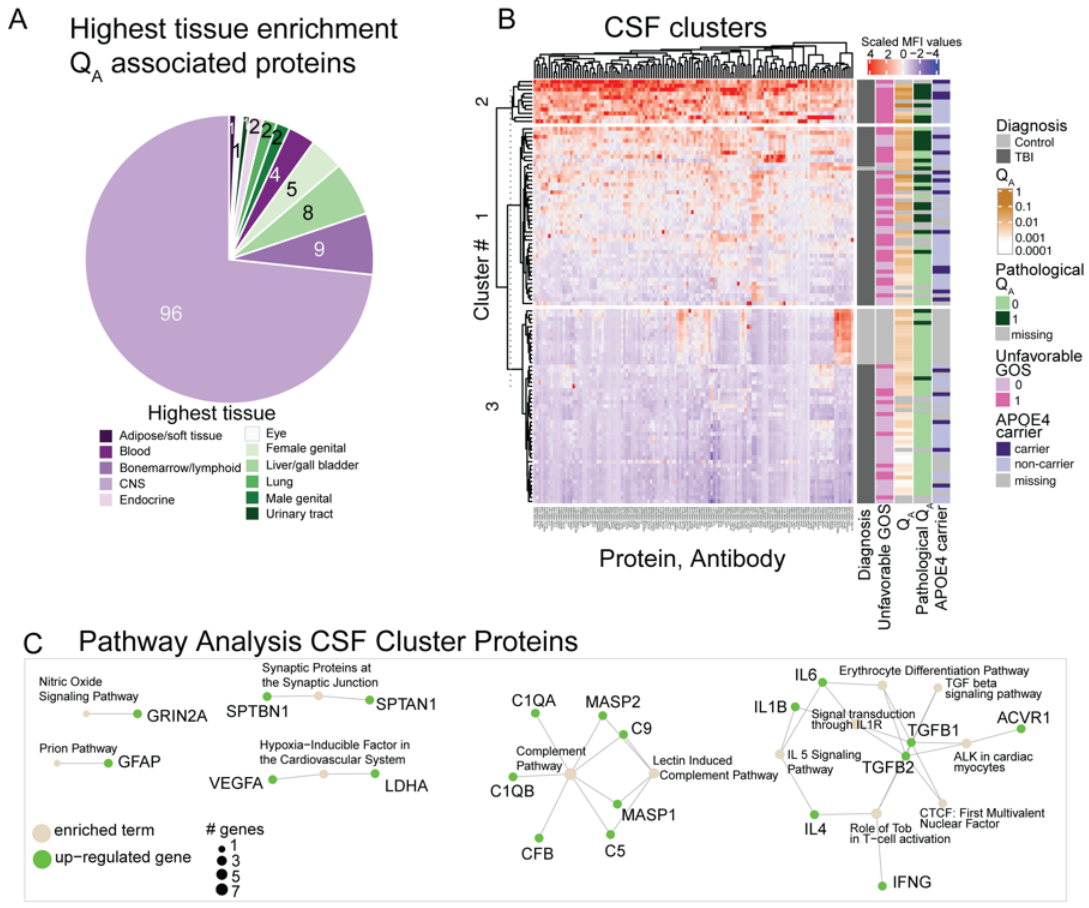

Figure 22: BBB Disruption Is Related to Innate Immune Pathways, Notably the Complement Cascade. Among the n = 114 proteins that were correlated with Q_A, many were CNS- or immune system-enriched **(A)**. Interestingly, protein levels clustered depending on Q_A **(B)**, and seemed related to outcome. Pathway analysis between cluster-specific proteins implicated structural but importantly also inflammatory pathways **(C)**. **Abbreviations:** APOE4, apolipoprotein E ϵ4-allele; GOS, Glasgow Outcome Score; Q_A, albumin quotient.

Notably, only n = 5 proteins exhibited significantly altered levels against dichotomized Q_A. In CSF, these entailed the inflammatory proteins complement factor B, C9, Ficolin 1, and IL-6. We conducted outcome analysis for numerous combinations of hypothesis-guided proteins. This included among else proportional odds regression analysis of proteins that were altered depending on if the patient had an intact or disrupted BBB. Upon multivariable modelling, merely complement factor B in serum ($p = 0.003$, $\Delta R^2 = 9.2\%$) and C9 in CSF ($p = 0.014$, $\Delta R^2 = 7.4\%$) were retained following step-down procedures. Taken together, this implies an intimate relationship between BBB disruption and neuroinflammation, where complement cascade proteins seem to be of particular interest.

In contrast to the global neuroinflammatory assessments undertaken in **paper II**, we examined local lesion zone expression of neuroinflammatory proteins in **paper III**. We delineated edema subtypes using MRI. The entire edema area was defined as a hyper-intensity on a non-diffusion weighted MRI. We next defined a vasogenic edema as a hyper-intensity on an ADC image, together with a concurrent hypo-intensity on the DWI. The opposite held true for cytotoxic edema. Following image alignment between MRI and IHC images using image registration, we assessed local protein expression within each defined edema subtype. Using this unique methodological approach, we could corroborate previous findings on both cytotoxic and vasogenic edema. We found a moderate correlation ($\rho = -0.29$) between the BBB endothelial marker SMI-71 and vasogenic edema **(Figure 23A)**, indicating that vasogenic edema is associated with a disrupted BBB. As this would lead to the inflow of proteinaceous fluid into the brain parenchyma (112), we also assessed local amount of immunoglobulin G (IgG), for which we found a weak correlation with vasogenic edema (**Figure 23B**, $\rho = 0.11$). Both these findings are well in-line with current literature, stipulating that vasogenic edema is related to BBB disruption (113).

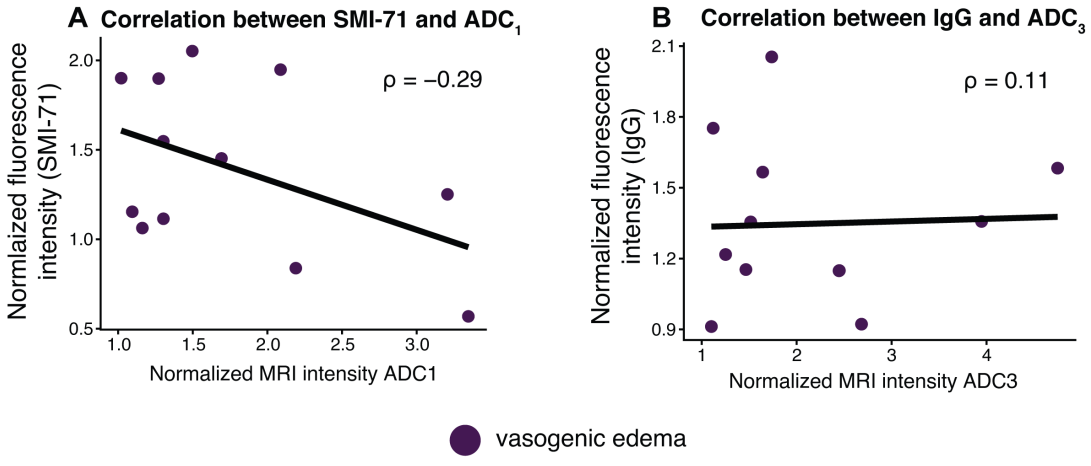

Figure 23: BBB Breakdown Correlates with Vasogenic Edema. The BBB-endothelial marker SMI-71 was negatively correlated with ADC, indicating that vasogenic edema co-occur with BBB breakdown (A). Accordingly, extravasation of otherwise vessel-restricted IgG into the brain parenchyma was (weakly) correlated with ADC (B). **Abbreviations:** ADC, apparent diffusion coefficient; IgG; immunoglobulin G; MRI, magnetic resonance imaging.

In contrast, cytotoxic edema has been more sparsely studied. As cytotoxic edema itself could lead to (or indicate) oncotic cell death, thereby causing exacerbated BBB disruption, and a delayed vasogenic edema (112,113), we characterized this further. We found cytotoxic edema to be correlated with innate immune markers such as macrophages (ED1+, also a marker for activated phagocytic microglia), hematopoietic progenitor cells (CD34+), as well as a microglia-specific marker (OX42) (**Figure 24A-C**). Microglia is well-known to act in concert with complement (107), and in accordance, we found a strong correlation between the complement membrane attack complex (C5b9) and the cytotoxic edema region (**Figure 24D**, $\rho = 0.75$). One interpretation for this, albeit speculative, is that the cytotoxic edema that ensues the TBI is accompanied by a potent (innate) inflammatory reaction, that leads to a delayed BBB breakdown, thus explaining the correlation between e.g. complement and Q_A (**paper II**) as well as the correlation between complement expression and cytotoxic edema (**paper III**).

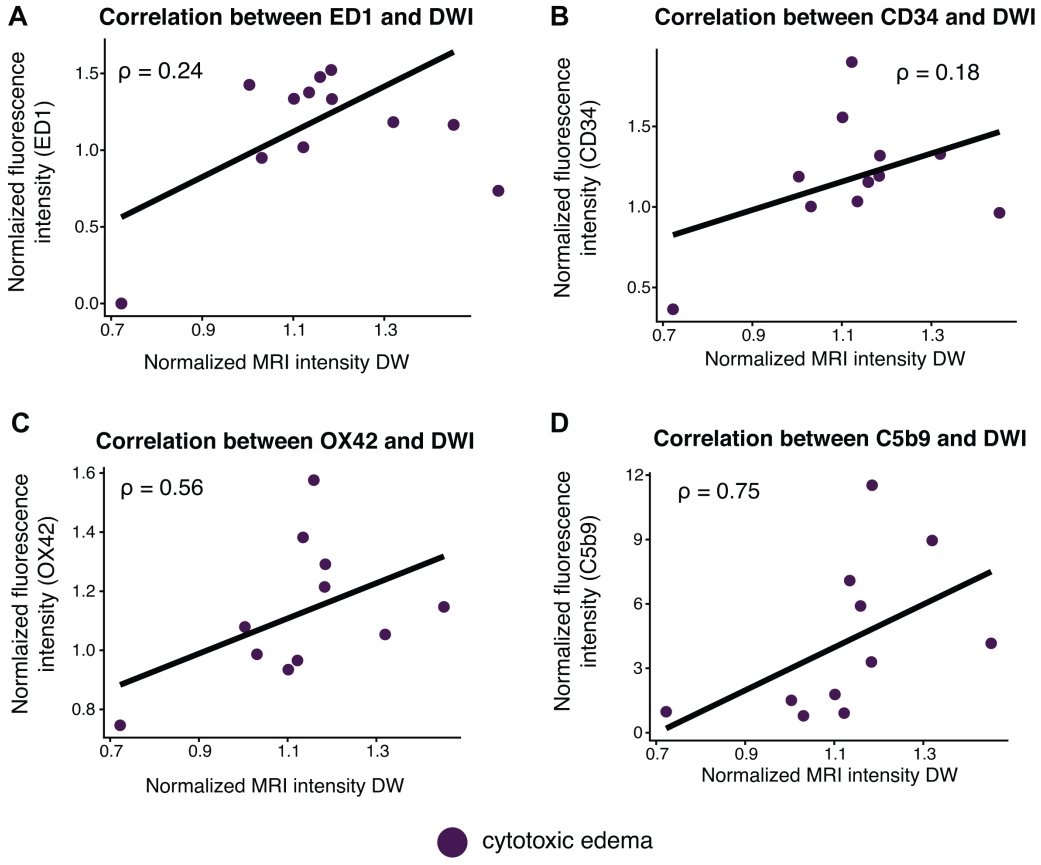

Figure 24: Innate Neuroinflammatory Proteins Are Correlated with Cytotoxic Edema. A positive relationship was seen between markers for macrophages and hematopoietic progenitor cells and cytotoxic edema **(A, B)**, indicating that cytotoxic edema co-occurs with peripheral immune cell recruitment. Moreover, both the microglia-marker OX42 and the complement membrane attack complex C5b9 correlated positively with cytotoxic edema **(C, D)**, highlighting that cytotoxic edema progression is also related to *in situ* innate immune reactions. **Abbreviations**: DWI, diffusion-weighted imaging.

As mentioned, one of the key cells in the BBB and the gliovascular unit is the astrocyte, specifically highlighted in both **paper III** and **paper IV**. Astrocytes are positioned at the abluminal BBB surface where they elongate their foot-processes (end-feet), which are AQP4-

covered towards the outer BBB surface (273). Interestingly, following TBI *in vivo*, AQP4 has been implied to be retracted from the astrocytic end-feet to the cell soma (166,167). In **paper III**, we assessed AQP4 retraction, and found a wide-spread retraction of AQP4 across all edema subtypes, that however was dominant in the cytotoxic edema area (**Figure 25**), which also emanated as a predictor for AQP4 retraction upon regression modelling ($\beta = 0.14$, $p = 0.003$, $R^2 = 0.39$).

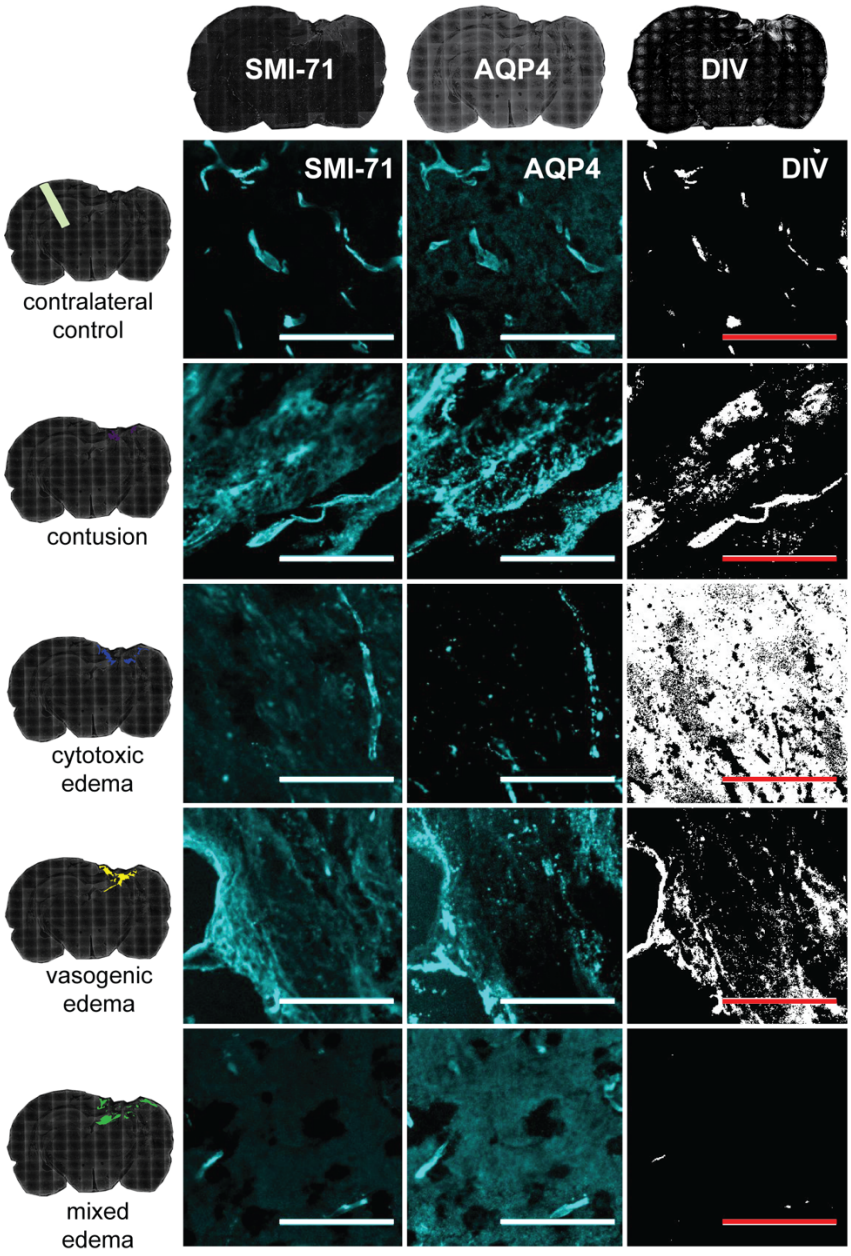

Figure 25: Cytotoxic Edema Is Associated with AQP4-Retraction from Astrocytic End-Feet Lining the BBB. Widespread AQP4 retraction from BBB vessels was seen following TBI, most clearly in the cytotoxic edema area (middle row). **Abbreviations:** AQP4, aquaporin-4, BBB, blood-brain barrier; DIV, the ratio between SMI-71 and AQP4. **Scale bar:** 100 µm.

Interestingly, this relationship was time-dependent, and the extent of AQP4 retraction decreased longitudinally together with cytotoxic edema ($p = 0.024$, $p = 0.001$, $p =$ NS for 7-, 14-, and 28-days post injury as interaction effect with cytotoxic edema). We associated AQP4

retraction with co-occurring protein expression and found C5b9, IgG, OX42, transforming growth factor β (TGF-β), and vascular endothelial growth factor α (VEGF-α) to be moderately correlated with the extent of AQP4 retraction. Upon regression modelling, C5b9 and IgG constituted independent, albeit interacting predictors of AQP4 retraction ($\beta = 0.015$, $p = 0.022$ for the interaction) when modelled together with SMI-71 and AQP4. When constructing a regression model independent of AQP4 and SMI-71, IgG remained significant ($p = 0.005$), while VEGF-α ($p = 0.009$), TFG-β ($p = 0.049$), and OX42 ($p = 0.001$) were also significant predictors of AQP4 retraction. Taken together, we found that the neuroinflammatory response is potent in regions with cytotoxic edema, and that this was associated with AQP4 retraction. This strongly implicates the astrocyte as an important cell linking BBB disruption, edema, and neuroinflammation.

4.3 ASTROCYTES ADOPT A NEUROTOXIC PHENOTYPE UPON NEUROINFLAMMATORY STIMULATION, OF IMPORTANCE FOR CELLULAR INTERPLAY WITHIN DEFINED CNS NICHES

Astrocytes hold versatile functions, among else the astrogliotic response to neuroinflammatory stimulus, that at the BBB interface seemed to be strongly related to AQP4 retraction in **paper III**. In **paper IV**, we extended our studies of neuroinflammation with a specific focus on astrocytes, and how reactive (i.e. astrogliotic) astrocytes affect neurons following clinically relevant pro-inflammatory stimulation. We examined astrocytes and motor neurons *in vitro*, using cells that were derived from ES-cells using directed differentiation. We chose to differentiate cells with a positional identity in the ventral brainstem, as this region is associated with especially poor prognosis following diffuse/traumatic axonal injury (36,38). Further, even though astrocytes have been implied in mediation of neurotoxic effects (132,161), no studies have assessed astrocytes and neurons residing in the same brainstem niche.

We verified the positional identity of the ES-derived astrocytes and ES-motor neurons using RNA-sequencing. Along the rostro-caudal axis, we found all brainstem/hindbrain Hox 4 paralogs (*Hoxa4-Hoxd4*) (257,274) to be non-significant between both cell types, indicating a common origin. Further, ventral identity was assessed using the dorsoventral genes *Nkx6.1*, *Pax6*, *Slit1*, *Reln* (275). We could also discriminate between ES-motor neurons and ES-astrocytes by assessing upregulation of canonical motor neurons genes, such as *Mnx1* for motor neurons ($\log_2$ fold change 7, $p_{adjusted} < 0.001$), and *Slc1a3, Fabp7, Fgfr3, Slc1a2*, and *CD44* for astrocytes as implied in a landmark study by Cahoy and colleagues (260). As we to some extent developed a new protocol for astrocyte-differentiation, we also verified the robustness of the protocol by applying it to subventricular zone-stem cells, that we successfully differentiated into subventricular zone-astrocytes (**Figure 26**).

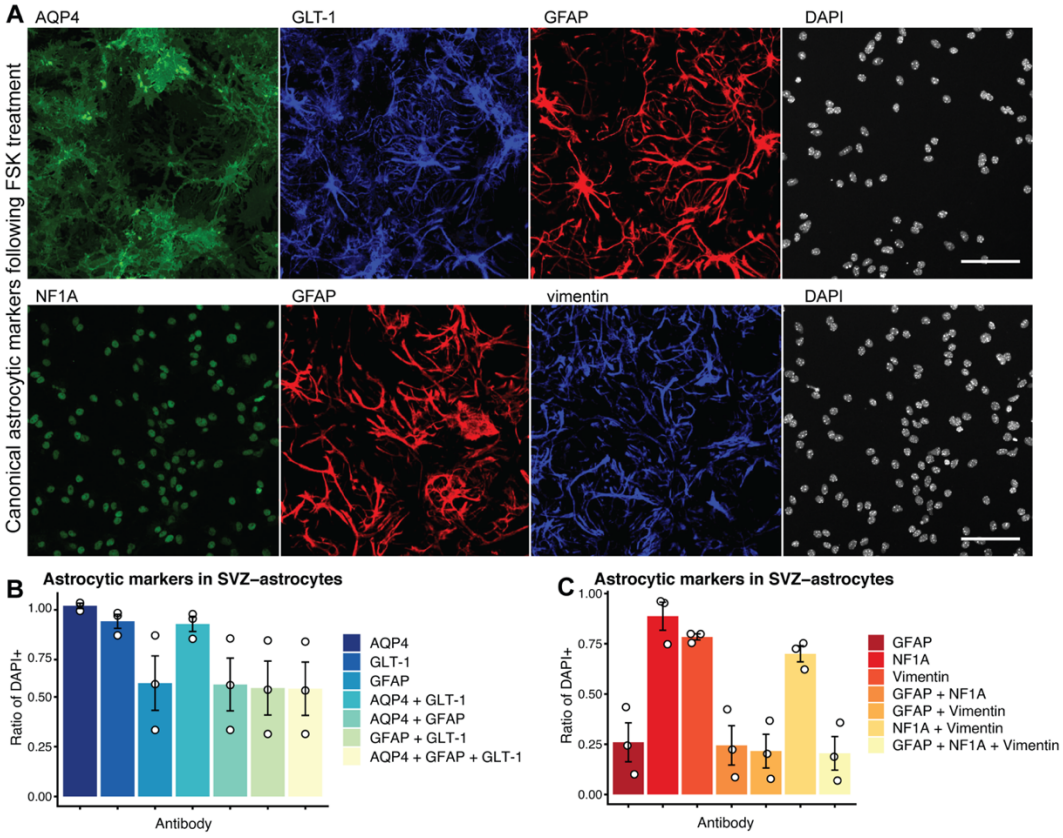

Figure 26: Validation of the Astrocyte Differentiation Protocol Using Subventricular Zone Stem Cells. SVZ-derived astrocytes exposed to the same culture protocol as ES-astrocytes expressed numerous canonical astrocytic markers at the protein level **(A)**. With the notable exception of GFAP, the majority of proteins were expressed in >75% of cells with consequent high extent of co-localization **(B, C)**. **Abbreviations:** ES-astrocytes; embryonic stem cell-derived astrocytes; FSK, forskolin; NF1A, nuclear factor 1A; SVZ, subventricular zone. For other protein abbreviations, please see list of abbreviations. **Scale bar:** 100 µm.

We used the previously implicated cytokines IL-1α and TNF-α for activation of the ES-astrocytes, which we evaluated through the increased phosphorylation of c-Jun, a down-stream product in the c-Jun N-terminal kinase pathway (**Figure 27A**) at 2 and 24 hours (**Figure 27B-I**).

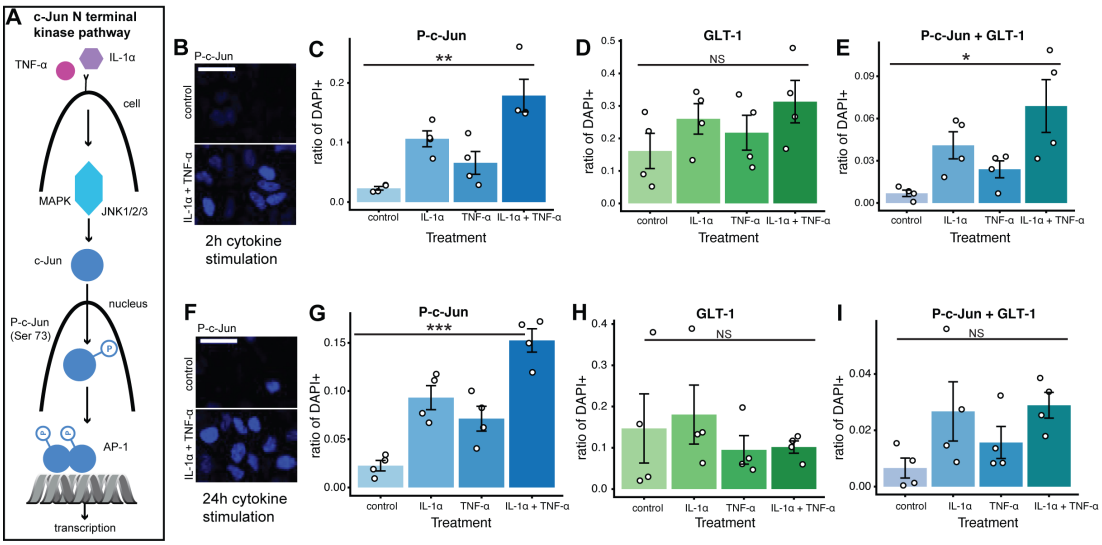

Figure 27 (from previous page): ES-astrocytes Increase the Expression of Phosphorylated c-Jun following an Inflammatory Stimulus with IL-1α and TNF-α. We verified that cells responded to inflammatory stimulus by evaluating the c-Jun N terminal kinase pathway downstream product c-Jun phosphorylation (P-c-Jun) **(A)**. Following both 2h **(B-E)** and 24h **(F-I)** of cytokine-stimulation, P-c-Jun was increased in ES-astrocytes. Notably, this did not affect expression of canonical astrocytic markers such as GLT-1 **(D, H)**. The inflammatory effect was not restricted to glial progenitors, as also mature ES-astrocytes were seen to be affected **(E, I)**. **Abbreviations:** GLT-1, glutamate transporter 1; IL-1α, interleukin 1α; MAPK, mitogen activated protein kinase; P-c-Jun, phosphorylated c-Jun; TNF-α, tumor necrosis factor α. **Scale bar:** 25 µm.

Interestingly, following co-culture between neuroinflammatory ES-astrocytes and FACS-sorted ES-motor neurons, fewer ES-motor neurons survived (**Figure 28**).

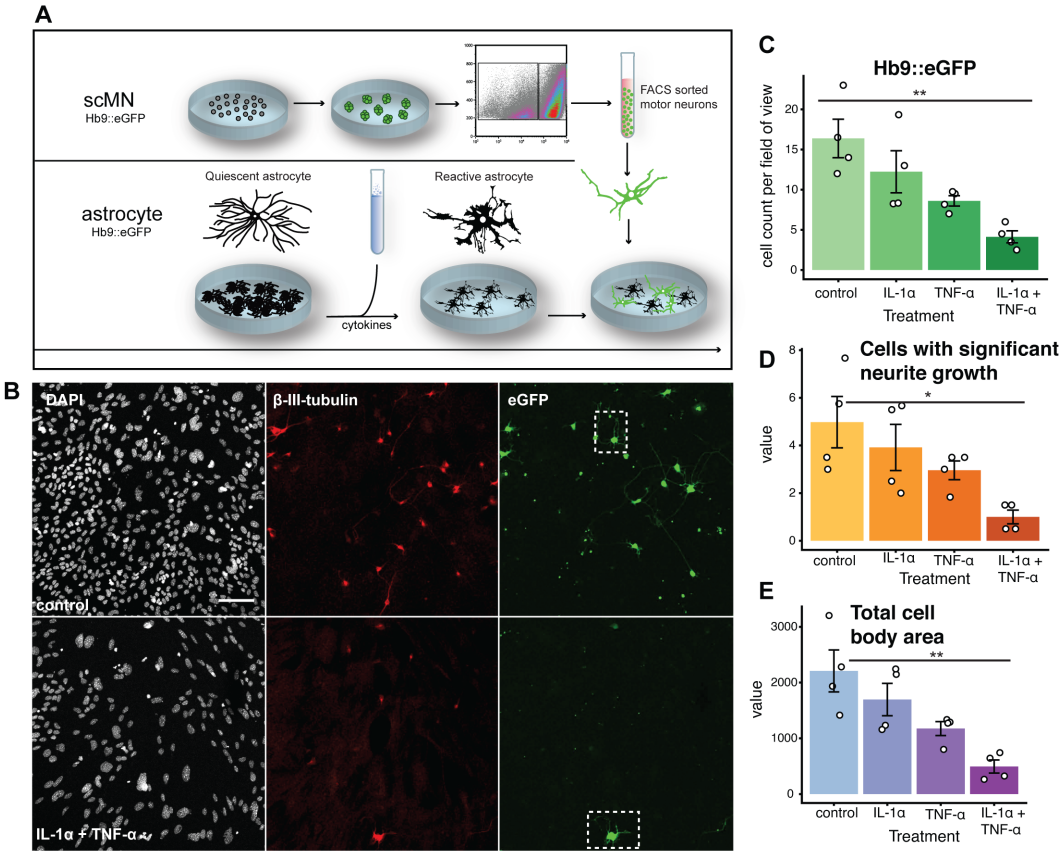

Figure 28: ES-Astrocytes Adopt a Neurotoxic Phenotype following Induction with IL-1α and TNF-α. We cultured ventral brainstem ES-astrocytes and ES-motor neurons in parallel **(A)**. ES-motor neurons were FACS sorted and plated on top of ES-astrocytes stimulated with inflammatory substances. Following co-culture, ES-motor neuron survival decreased when cultured together with IL-1α and TNF-α-treated ES-astrocytes **(B)**. The effect was ES-motor neuron specific **(C)**, and affected both absolute cell count as well as morphological metrics **(C-E)**. **Abbreviations:** FACS, fluorescence-activated cell sorting; IL-1α, interleukin 1α; MN, motor neuron; sc, brainstem/spinal cord, TNF-α, tumor necrosis factor α. **Scale bar:** 100 µm.

We elaborated on these findings by exposing the ES-astrocytes to neuroinflammatory factors derived as important markers from **paper II**, namely complement component C1Q, C5, IL-1β, IL-6, and TGF-β2. Interestingly, and novel, IL-1β and IL-6-stimulated ES-astrocytes adopted a neurotoxic fate in a contact-dependent co-culture system with ES-motor neurons (**Figure 29**). In a cell-autonomous system, toxic effects seemed to be at play, but were less clear.

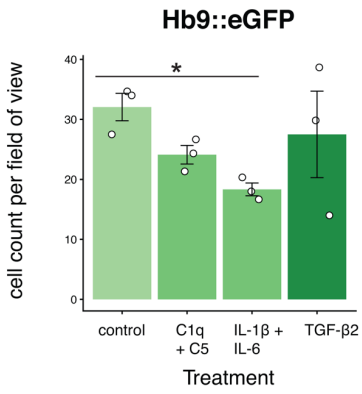

Figure 29: Clinically Relevant Neuroinflammatory Substances Cause ES-Astrocytes to Adopt a Neurotoxic Fate. We stimulated ES-astrocytes with substances derived from **paper II** and subsequently co-cultured them with ES-motor neurons. Following IL-1β and IL-6 stimulation, ES-astrocytes again adopted a neurotoxic phenotype. **Abbreviations:** C1q, complement component 1q; C5, complement component 5; IL-, interleukin; TGF, transforming growth factor.

We assessed the "neurotoxic astrocytes" using RNA-sequencing. Upon gene set enrichment analysis, we found pathways implicated in MYC, endoplasmic reticulum stress and cell cycle mechanisms to be of particular interest (**Figure 30**).

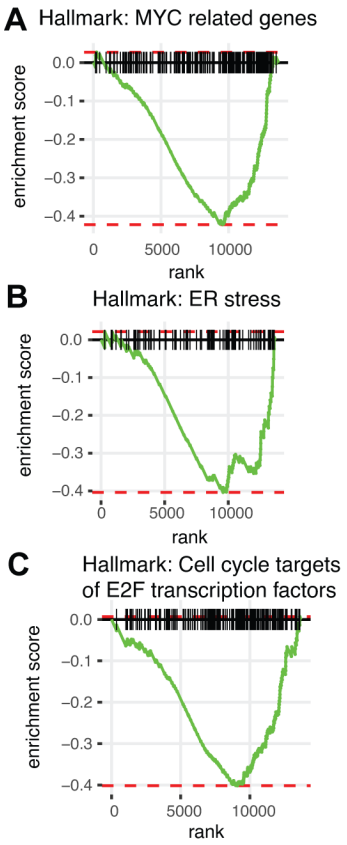

Figure 30: Neurotoxic ES-Astrocytes Alter Gene Regulation of MYC, Endoplasmic Reticulum Stress, and Cell Cycle Pathways. We compared the transcriptome of neurotoxic and control ES-astrocytes using bulk polyA+ RNA sequencing. Here, pathways **(A-C)** implicated in MYC, endoplasmic reticulum stress, and cell cycle targets were differentially regulated. This provides a hypothesis-generating framework for why ES-astrocytes adopt a neurotoxic phenotype. Within these gene sets we found numerous genes implicated in reactive astrocytes as well as TBI. **Abbreviations:** ER, endoplasmic reticulum.

Within the leading edges of these gene sets, we found numerous genes previously implicated in reactive astrocytes, TBI (also in **paper II**), and traumatic axonal injury (159,189,276), e.g. *Ccl2*, *Cdkn1a, Gins1, Hspa5, LDHA, Mthfd2, Prdx3, Prdx4, Ppia, Psma1, Tubb2a, Vegfa,* and *Ywhaq*.

5 DISCUSSION

This thesis characterizes pathophysiological injury processes that ensue a severe TBI through clinical and experimental studies. I focus particularly on BBB disruption and neuroinflammation, that share the involvement of astrocyte-mediated processes. I have found BBB disruption to occur following a severe TBI and to extend throughout the first week after injury, possibly because of the eliciting trauma but also through additional secondary insults. This is of clinical relevance, both for biomarker clearance from the injured CNS and for long-term prognosis. I further establish an important association between BBB disruption and neuroinflammation, which seems to be strongly related to complement-system mediated mechanisms, in themselves also of importance for long-term prognosis. I expand on the relationship between BBB disruption and neuroinflammation by studying the lesion vicinity following an experimental TBI. Here, I show that retraction of the astrocytic AQP4 from the BBB interface is associated with innate immune mechanisms in cytotoxic edema regions, emphasizing astrocytes as a key cell type for both BBB disruption and neuroinflammation. Lastly, I examine astrocytes in isolation through an *in vitro* system, where I demonstrate that astrocytes following clinically relevant neuroinflammatory stimuli adopt a neurotoxic phenotype. These findings implicate neuroinflammation as a treatment target in the aftermath following severe TBI, expanded upon below.

5.1 OUTCOME PREDICTION FOLLOWING SEVERE TBI IMPROVES UPON INCLUSION OF CELLULAR INJURY MECHANISMS

We characterized BBB disruption and neuroinflammation following severe TBI in humans **(paper I and II)**. We found BBB disruption measured through Q_A to occur in at least a subset of patients, extend throughout the first week following a severe TBI, and comprise a novel predictor of long-term outcome. BBB disruption affected clearance from brain to blood of S100B. This is important as blood levels of S100B in many centers directs clinical management (72). Presumptuous sources of erroneously high or low S100B concentrations, e.g. BBB disruption must therefore be considered. In contrast, NSE clearance from brain to blood did not seem reliant on BBB disruption. Although this might be a mere reflection of extracranial sources of NSE, limiting its clinical utility as a biomarker (77), it might also indicate that brain-derived proteins are cleared through different mechanisms from brain to blood. As S100B and NSE exhibit different properties, stretching from size (277–279), half-life (63), and cellular sources (77,280–282), we can at this point primarily speculate that they are cleared differently. Future studies must explore this further, particularly focusing on the glymphatic system, which has previously been implicated in biomarker clearance (170).

Further, BBB disruption was associated with a unique expression profile of neuroinflammatory proteins in CSF, some of which also constituted novel and independent outcome predictor proteins. This implicates BBB disruption and neuroinflammation as new

possible outcome predictors depicting disease progression in the acute/sub-acute phase following the trauma while the patient is in neurocritical care. Yet, such "pathophysiology-oriented" biomarkers rarely form part of current outcome prediction models. In fact, numerous outcome prediction models have been derived using TBI data, but many of these are hampered by inherent methodological issues, limiting their validity (283,284). In 2007, two larger-scale collaborations denominated "Corticosteriod Randomisation after Significant Head Injury" (CRASH) and IMPACT (23,285) developed outcome prediction tools that dominate the field today (286) and that are available as clinical scoring tools (285,287). Both CRASH and IMPACT exclusively use admission and demographic data (24,285) and they have been thoroughly validated externally upon creation (285,287), and recently (288). Yet, neither CRASH nor IMPACT has been widely implemented as clinical decision-making tools (284), or in guidelines, even though their predictive ability trumps prognostic scores implemented in other disciplines (286). One explanation for this might be that neither of these models account for the full complexity ensuing a severe TBI. We used the IMPACT models for benchmarking in **paper II**. The original IMPACT model had an estimated model fit described as Nagelkerke's pseudo-R^2 of ~35% (25), meaning that ~65 % of variance in outcome prediction is unaccounted for using currently included variables. We demonstrate how this predictive capacity could be improved, by incorporating variables depicting cellular injury mechanisms that underlie the pathophysiological course of TBI, such as BBB disruption, and neuroinflammation. Previously, disease course has been suggested as a tentative component in outcome prediction following TBI (25). Secondary insults upon admission (289) or throughout neurocritical care management (290) have been described as important outcome predictors amenable to clinical intervention with improved prognostic results (291). In this context, BBB disruption and neuroinflammation should be seen as cellular injury mechanisms that can lead to secondary insults such as ICP increments, but that also form part of the expected pathophysiological trajectory following a TBI. We here demonstrate their importance throughout severe TBI in humans, and how they influence outcome. This speaks in favor of cellular injury mechanisms as possibly underlying some of the outcome variance unaccounted for, and two of these being BBB disruption and neuroinflammation. Analogous to how prevention of secondary insult-directed treatment can improve outcome, modification of pathophysiological events such as BBB disruption and the neuroinflammatory response might also lead to prognostic improvement. In order to target future monitoring and in extension, treatment of these mechanisms specifically, an improved pathophysiological understanding is warranted.

5.2 BLOOD-BRAIN BARRIER DISRUPTION ENSUES TBI AND IS ASSOCIATED WITH NEUROINFLAMMATORY EVENTS

We delineated BBB disruption longitudinally following clinical TBI **(paper I)** and experimental TBI inferred using the CCI model **(paper III)**. In the clinical material, we found that BBB disruption developed already at the time of trauma and was maintained

throughout the first week. This indicates that BBB disruption occurs already during the primary injury inflicted by the mechanical trauma (97,113). Interestingly, not all patients seemed to be afflicted by BBB disruption, measured using Q_A, in either **paper I** or **paper II**. Even though this might indicate individual variations in cellular injury mechanisms, it might also be a source of error due to wash-out of Q_A, particularly in **paper II** where Q_A measurements was initiated sub-acutely. In spite of this, we demonstrate how BBB disruption was maintained for patients in **paper I** for at least the first week following trauma. Previously, BBB disruption measured through Q_A has been found to normalize rather steeply within the first week post-TBI (104,105), whereas others have found persistently deranged Q_A values (106), occasionally even extending into the long-term (97,107). This conflicting data suggests that also other mechanisms might be at play, exacerbating or alleviating BBB disruption throughout the course of disease. One of these might be events occurring at the neuro-/gliovascular unit, leading to exacerbated BBB disruption. We corroborated this hypothesis in **paper III**, where we modelled edema progression following experimental TBI. Here we found that although the total edema region diminished longitudinally, there was a shift towards a vasogenic edema at later time-points. This speaks in favor of the occurrence of a delayed vasogenic edema and hence a more prolonged disruption of the BBB, similarly to what has been previously described (112,116).

One mechanism potentially contributing to extended BBB disruption is neuroinflammation. We found that neuroinflammation was strongly associated with BBB disruption using fluid proteomics following human TBI **(paper II)** and with AQP4 retraction from the BBB *in situ* following experimental TBI **(paper III)**. In **paper II**, particularly complement proteins demonstrated a strong association with BBB disruption. In fact, among proteins that discriminated between patients with intact and disrupted BBB, the complement proteins complement factor B, C9, and Ficolin 1 stood out. Of these, both C9 and complement factor B were predictive of outcome. This implies the complement system strongly in post-traumatic BBB disruption, well in-line with the literature that has implied complement in both the brain parenchyma (195,197,292), and CSF (194,203,293,294) following TBI. The complement system proteins entail ~50 proteins (295), that circulate or else are membrane-bound (107). In the systemic circulation, complement proteins are derived from hepatic synthesis (107). Additionally, studies show that all complement system proteins can likely be locally produced in the brain (296,297), implying that complement activation following TBI can occur concomitantly through BBB leakage and local activation. Within the injured CNS, the complement system targets cells for destruction through opsonization, induce cell lysis through the membrane attack complex, and stimulates inflammatory mechanisms through anaphylatoxins (298). Activation occurs through three (classical, lectin, alternative) or possibly four different pathways (299), that independent of the inductive pathway culminates with cleavage of the protein C3 to C3a (anaphylatoxin) and C3b, where the latter forms a complex together with complement factor B thus enabling cleavage of C5 into C5a

(anaphylatoxin) and C5b (84). C5b can together with C6, C7, C8, and numerous C9 molecules form the membrane attack complex (C5b9) thus causing cell lysis (84).

Within the brain following TBI, the suggested mechanism for complement-mediated neuroinflammation is that C3b (and breakdown products) deposit on e.g. neuronal cell surfaces and thereby target them for subsequent phagocytosis and C5b9 formation (297,298,300). Meanwhile, the anaphylatoxins C3a and C5a activate infiltrating immune cells, microglia, and astrocytes (107,295,297), which migrate to the injury site. Both microglia and astrocytes have been implicated in the complement-mediated response (296,298,300,301). Whereas microglia has been implied to participate in phagocytosis (302,303), the role of astrocytes is less clear. Astrocytes have been shown to express complement proteins and anaphylatoxin receptors (107,295), synthesize complement system proteins (296), be involved in phagocytosis (298), be activated following complement activation (300), and reduce astrogliosis following complement inhibition (303,304). Moreover, astrocytes have been suggested to induce astrocytic scars following complement activation, and to be involved in exacerbation of BBB disruption through anaphylatoxin signaling (301). Anaphylatoxins may also promote vascular permeability, thus potentially exacerbating the BBB leakage, while promoting recruitment of phagocytic or lytic leukocytes (84,295,301). We assessed all complement pathways in **paper II**, and we implicated primarily complement factor B (the alternative pathway) and C9 (one of the proteins involved in C5b9 formation (301)) in relation to outcome. This is in-line with recent data, suggesting that C5b9 formation is critical in the acute phase following TBI, while inhibition of the alternative pathway has been shown to be critical for sustained complement inhibition following TBI (300). We thus associated BBB disruption and the neuroinflammatory response following TBI with a prognostically important altered complement regulation. We expanded on these findings in **paper III**, where we assessed the neuroinflammatory response *in situ* at the BBB interface and related this to retraction of AQP4 end-feet from the BBB. We found a strong association between both C5b9 and microglia with cytotoxic edema. This might indicate that cell death in this region, of presumptive importance for exacerbated BBB breakdown, is related to the neuroinflammatory response. It has been suggested that complement activation in this region is also associated with BBB disruption through shearing of the astrocytic end-feet in close proximity to the BBB (301). In fact, we found that C5b9 and microglia served as predictors in a model of AQP4 retraction from the BBB, strengthening the notion that BBB breakdown, and neuroinflammation are intertwined. In addition, we found the inflammatory mediators TGF-β and VEGF-α to also be predictive of AQP4 retraction from the BBB. Together with complement, both of these have been implicated in BBB breakdown (105,113,293,305). This makes the astrocyte a cell of particular interest within the neurovascular unit. In order to characterize this further, we assessed the effect of neuroinflammatory reactive astrocytes on neurons (**paper IV**).

5.3 NEUROINFLAMMATORY ASTROCYTES EXERT NEUROTOXIC FUNCTIONS FOLLOWING STIMULATION WITH CLINICALLY RELEVANT INFLAMMATORY MEDIATORS

In **paper IV**, we differentiated AQP4-expressing astrocytes with a ventral brainstem profile utilizing ES cells *in vitro*. We demonstrated that these cells respond similarly to primary culture astrocytes in response to neuroinflammatory triggers, and interestingly, that these ES-astrocytes adopted a neurotoxic phenotype when stimulated with pro-inflammatory factors discerned in **paper II**. Here, the ES-astrocytes exhibited neurotoxic attributes upon stimulation with IL-1β and IL-6, but not with complement components.

In neuroscience, there has been a growing interest in astrocytes, owing to their substantial involvement in many disease processes within the CNS (147), complicated by astrocyte heterogeneity in both health and disease (88,149,159,160). Astrocyte heterogeneity likely arises already during developmental processes (146), and hold functional implications (306,307). Therefore, we chose to differentiate astrocytes and neurons from one CNS niche. We chose the ventral brainstem niche, which in the setting of diffuse/traumatic axonal injuries, is particularly important (36,38,39). In this niche, we demonstrated that ES-astrocytes stimulated with IL-1α and TNF-α adopts a phenotype which is toxic to ES-motor neurons, in line with what has previously been demonstrated for forebrain astrocytes, and spinal astrocytes together with cortical neurons (132,161). We however show this for one anatomical CNS niche, which is novel and likely portrays the *in vivo* situation more stringently. By utilizing the neuroinflammatory factors delineated in **paper II**, we expanded the assessment of astrocyte-neuronal interactions by stimulating astrocytes with clinically relevant pro-inflammatory factors. Notably, complement component proteins did not elicit neurotoxic effects on motor neurons following astrocytic stimulation with C1q and C5. A multitude of causes might underlie this finding. As of now, we can say that astrocytic complement stimulation with a limited number of factors is non-sufficient to elicit astrocyte-mediated neurotoxicity. In contrast, we demonstrate how IL-1β and IL-6, of which IL-6 was significantly increased between patients with intact and disrupted BBB in **paper II**, inferred astrocyte-mediated neurotoxicity.

Numerous CNS cell types express the IL-1β receptor (78). Similarly, IL-6 is expressed by a multitude of cell types within the CNS (78). Interestingly, both these cytokines have been implied in BBB disruption in themselves and/or through astrocyte-mediated mechanisms. Injection of IL-1β *in vivo* has been shown to induce BBB permeability (308), highlighting that IL-1β might have a broad function in the CNS encompassing both inflammatory, BBB disruption, and edema mechanisms (129). When astrocytes specifically were stimulated with IL-1β, however, they produce MMPs indicating detrimental effects as MMPs degrade the BBB (133). Following human TBI, a strong correlation has been noted between IL-6 and MMP-9 (309), as well as between IL-6 and BBB disruption (310). This speaks in favor of

neuroinflammatory modulation in order to mitigate post-traumatic BBB disruption. The culture-system that we employed in **paper IV** did not mimic the full gliovascular unit, but rather astrocytic and neuronal interactions. In a seminal paper of Liddelow and colleagues (132), astrocytes were shown to exert a neurotoxic effect following stimulation with IL-1α, TNF-α, and C1q. This has been corroborated recently using astrocytes and neurons differentiated from human induced pluripotent stem cells (161), and by emerging work (311). We corroborated these findings, while expanding on them by also showing that human disease-relevant inflammatory mediators can exert similar effects. Taken together, we thus possibly uncover an additional local cellular injury process of importance following a severe TBI. Why astrocytes adopt a neurotoxic fate is not yet clear. Nearest at hand would be to assume that reactive astrocytes due to loss-of-function develop less neurotrophic attributes. We cannot rule this out in our data, as cultures with merely motor neurons overall exhibited a very low survival following fluorescence-activated cell sorting. Studies *in vivo*, deleting the "A1 genes", however, have found improved neuronal survival, arguing against the loss-of-function hypothesis (132). Other suggested mechanisms for astrocyte-mediated neurotoxicity involve gain-of-function hypotheses and are under investigation. Early work preceding the A1 delineation implied astrocyte-mediated neurotoxic effects through reactive nitrogen oxide-related pathways (312), but also through astrocyte-released MMP-1 (313). Recently, astrocytes were demonstrated to secrete lipids that confer the neurotoxic effect (162). Recent work which still has not undergone scientific peer-review interestingly implies IL-6 through autocrine-paracrine signaling mechanisms to be of particular importance for at least the astrocyte inflammatory state (311). More data concerning this is expected to emerge in the future, especially following the delineation of astrocyte heterogeneity in the reactive state (159). We present no definitive mechanistic answers, but we describe a genetic signature of the neurotoxic astrocytes with implications towards endoplasmic reticulum stress and altered MYC pathway regulation. Although this is but correlative in our data, we find interesting overlaps between genes in the leading edges of these gene sets and genes highlighted following TBI and traumatic axonal injury (189). Of note, even though these genes entail interesting translational avenues, the mouse and human astrocyte genome differ (314), why it will be beneficial to explore this further in hiPS cell systems. Yet, we show that the neuroinflammatory response that occurs in humans following TBI hold implications for how astrocytes fail to support neurons, possibly representing a novel cellular injury mechanism amenable to treatment.

6 CONCLUSIONS

Across this thesis I have characterized BBB disruption and neuroinflammation, two cellular injury mechanisms contributing to severe TBI pathophysiology. I link them together using both clinical and experimental data. In addition, I focus particularly on the astrocyte, a key cell type involved in both these processes.

Across the papers within this thesis, I have found that:

Paper I A severe TBI is ensued by a BBB injury that extends for at least a week among human subjects. BBB disruption is important as it influences how S100B, but not NSE, is cleared from the injured brain to blood.

Paper II BBB disruption following TBI is predictive of long-term prognosis in humans, possibly as BBB disruption is strongly associated with a neuroinflammatory response in the CSF, especially implicating complement proteins.

Paper III Neuroinflammation, including complement, is associated with cytotoxic edema, and post-traumatic AQP4-retraction from the BBB following a rodent model of severe TBI, which we delineated by developing a multi-modal imaging system.

Paper IV ES-astrocytes with a ventral brainstem identity may adopt a toxic phenotype for neurons when exposed to clinically relevant soluble inflammatory mediators.

Taken together, I demonstrate how BBB disruption is of clinical and prognostic importance and that BBB disruption is strongly associated with neuroinflammation. In the local lesion zone, astrocytes exhibit neuroinflammatory functions, of possible importance for both BBB disruption and local cell survival. My findings suggest that modulation of the neuroinflammatory response represents an important therapeutic avenue for future TBI studies, as it might concomitantly mitigate BBB disruption while also ameliorate otherwise deleterious neuroinflammatory processes in the local lesion vicinity.

7 FUTURE RESEARCH AVENUES

Much work remains before the findings presented above can be implemented into clinical practice. Below I highlight tentative approaches in order to steer treatment towards diminishing BBB disruption through neuroinflammatory modulation.

7.1 TBI MANAGEMENT WARRANTS TOOLS TO MONITOR BBB DISRUPTION AND NEUROINFLAMMATION

In order to treat BBB disruption and neuroinflammation, new monitoring tools are needed. We assessed BBB disruption through the albumin quotient Q_A across **paper I** and **paper II**, while we employed joint magnetic resonance imaging and protein expression in **paper III**. Shortcomings of Q_A has been highlighted above and within **paper I** and **II**. We therefore suggest that CSF-albumin alone could be used as a marker for BBB disruption (**paper I**). Importantly, CSF sampling is not readily available among patients without external ventricular drains, which could hamper its utilization. An alternative approach could be to assess BBB disruption radiologically, as has been suggested using dynamic enhanced contrast magnetic resonance imaging (315–318) and as we did in **paper III** experimentally. This also seems more reasonable than employing blood, as protein clearance from the CNS could be both dependent and independent of BBB disruption as we show in **paper I**. A key future priority should thus be to prospectively validate radiological protocols for BBB integrity. The optimal design for this experiment would be to recruit subjects with external ventricular drains, so that BBB damage could be assessed in relation to Q_A.

For neuroinflammation, it is ill-advised to argue against the use of CSF, since we in **paper II** demonstrated how neuroinflammatory proteins in CSF, but not blood, clustered to injury attributes. For neuroinflammatory assessments, access to CSF or other brain-derivative fluids therefore seems to be a priority. One approach to access brain extracellular fluid, without the need for an external ventricular drain, is to employ microdialysis. Microdialysis can be independently inserted, or as part of e.g. a triple lumen cranial access device, indicating that this might be a feasible avenue for a broader range of patients. Neuroinflammatory assessments through microdialysis was initiated ~20 years ago, first by measuring a handful of cytokines (319–321), and later expanded to broader cytokine screens (198,322). An important aspect of this is the microdialysis catheter probe size. Even though one study reports the usage of a 3000 kDa probe (319), the clinically most feasible is 100 kDa (198,322–324). Helmy and colleagues reported the cytokine recovery from the brain extracellular fluid to be inversely correlated with the molecular weight of the protein (322). In addition, the perfusion fluid used in the microdialysis catheter influences the cytokine recovery (322,325). Using microdialysis therefore seems a possible avenue for future neuroinflammatory assessments among TBI patients. Yet, as highlighted in **paper II**, the molecular size of e.g. many complement proteins exceed 100 kDa, therefore precluding

assessment of these in current set-ups. In addition, broader cytokine screens referenced above (198,322,324), were analyzed on a Luminex platform. In order to fully integrate these monitoring techniques into clinical routine, commercial assays need to be made more fully automated to better fit the hospital environment in terms of sampling volume and assay accuracy, or sampling on available platforms be done more readily. In order to undertake this work, neuroinflammatory mediators of interest need to be targeted. We provide strong associations between complement proteins in CSF and clinical outcome, that we also externally validate in **paper II**. We therefore suggest that nuanced neuroinflammatory monitoring should be a prioritized area within microdialysis research, in order to corroborate the findings done in CSF. Of particular interest is targeted complement monitoring, currently likely unfeasible using available microdialysis equipment.

7.2 PATHOPHYSIOLOGY-GUIDED, INDIVIDUALIZED TREATMENT

Across this thesis, I provide data that indirectly supports neuroinflammatory modulation among TBI patients. Among the putative inflammatory targets that I highlight, some are already eligible to pursue in clinical interventional trials, whereas others currently are premature to study outside the experimental setting. Independent of treatment target, substantial humility should accompany all studies of neuroinflammatory modulation following severe TBI, given its complexity. This is highlighted across **paper II-IV**. **Paper II** depicts global inflammatory aspects in CSF and implicates complement in BBB disruption and long-term outcome. In contrast, **paper III** and **paper IV** portrays the local inflammatory response at the BBB interface and in a vulnerable CNS niche. Here, both microglia- and astrocyte-mediated inflammatory aspects were of importance.

Previous neuroinflammatory modulation attempts have been undertaken following severe TBI. Notably, Helmy and colleagues completed a phase II prospective randomized controlled trial using the human recombinant IL-1-receptor antagonist (81,324). The first study (324) assessed safety and feasibility, while verifying that the study drug could reach the brain, as assessed per microdialysis measurements of the IL-1 receptor antagonist. In subsequent work, the authors found that the study intervention shifted the CNS cytokine profile towards a "M1 microglial phenotype" (81). Some important aspects of clinical trials in TBI research can be deduced from this. Firstly, a study drug needs to reach the CNS. Secondly, inflammatory reactions within the CNS are inter-related, thus making it difficult to foresee all possible treatment downstream effects. Thirdly, the prognostic consequence of a neuroinflammatory modulator is difficult to predict based on neuroinflammatory alterations in the CNS milieu, why studies need to be powered for clinical outcome such as magnitude of BBB disruption, or extent of secondary injury, before assessments can be made. In this thesis and through previous work, the complement system seems to be one of the best targets for neuroinflammatory modulation. In fact, an up-coming multi-center randomized controlled

safety-trial evaluating complement inhibition has recently been described (326). Here, TBI patients are planned to be treated with a C1-inhibitor, utilizing a drug already approved for treatment of hereditary angioedema. The study drug will be given intravenously, which raises some questions regarding BBB passage. Further, the planned study intervention targets all complement pathways and some steps within the coagulation cascade (326). Speculatively, this approach might decrease the risk for false negative results due to too narrow drug targets. In the planned study, merely a single dose of C1-inhibitor will be administered (326). Although reasonable for a safety trial, an important future study question is whether continuous complement inhibition is required as indicated by experimental data (303).

The neuroinflammatory responses assessed in the local lesion vicinity in **paper III** and **paper IV** are interesting but warrant further experimental characterization. In **paper III** the neuroinflammatory response seems to be related to retraction of AQP4 from the BBB interface, speculatively indicating that incremented BBB disruption is mediated through an inflammatory process. This could be assessed through both exacerbation/alleviation of inflammation using our multi-modal imaging technique. If our findings can be externally validated, the study should be pursued by mechanistically oriented studies. A similar line of reasoning can be applied to **paper IV**. The notion that astrocytes can acquire a neurotoxic phenotype is new (132), and currently incompletely characterized. Although emerging data (162) suggest underlying mechanisms, much work remains. Global astrocytic ablation *in vivo* has proved detrimental in previous work (327,328), suggesting that astrocytic modulation rather than ablation is an eligible strategy. Other important questions that need to be addressed are if all astrocytes or merely astrocytes in the lesion vicinity should be targeted, and – perhaps most importantly – if there is a beneficial evolutionary consequence of neurotoxic astrocytes. Improved neuronal survival might hold deleterious side effects that currently are unforeseeable, such as an increased risk for epileptic seizures.

Taken together and allowing for speculation, the long-term possibilities of pathophysiology-guided treatment following severe TBI is vast. A tantalizing future scenario would be to be able to steer global neuroinflammatory responses ensuing TBI into a favorable state, while concomitantly treat local, lesion-specific attributes. This would open an avenue for at this stage still far-fetched notions of cell replacement therapy using e.g. hiPS cells. The feasibility, efficacy, and safety of such ideas must be robustly validated through experimental, observational, and eventual clinical studies. Yet, when Hippocrates wrote the first essay on TBI, the state of the TBI field today likely would have seemed highly astonishing. Or, as the astrocyte-legend Ben Barres quoted Nobel Laureate Richard Axel: *Before you know, you must imagine* (147).

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