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A clinical study

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ISBN 978-951-29-8315-5 (PRINT)
ISBN 978-951-29-8316-2 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Punamusta Oy, Vantaa, Finland 2020

Dedicated to
my beloved parents
and
late Emeritus Professor Rashiduddin Ahmad

UNIVERSITY OF TURKU
Faculty of Medicine
Neurology, Department of Clinical Neurosciences, Division of Neurosurgery
IFTAKHER HOSSAIN: Acute biochemical diagnostics of mild traumatic brain injury
Doctoral Dissertation, 125 pp.
Doctoral Programme in Clinical Research
December 2020

ABSTRACT

Traumatic brain injury (TBI) is a global health burden. Most cases diagnosed with TBI are mild traumatic brain injury (mTBI), however, no unanimous definition of mTBI exists. Although most of the patients with mTBI recover well, a group of patients develop persistent post-injury symptoms. Blood biomarkers could be used as the surrogate markers of injury and could assist in assessing the true severity and prognosis of the eventual brain damage.

Three studies were conducted for this project. Firstly, blood levels of glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) were analysed in patients with orthopedic trauma without any central nervous system (CNS) insults compared to patients with computed tomography (CT)-negative mTBI at multiple time points after admission and during follow-up visits. The second study correlated the admission levels (≤24 hours) of GFAP and neurofilament light (NF-L) with outcome in patients with mTBI to explore the prediction abilities of these blood biomarkers. In the last study, the prognostic value of the neurodegenerative biomarkers, total tau (T-tau) and β-amyloid isoforms 1–40 (Aβ40), and 1–42 (Aβ42) were investigated using admission samples. Combinations of biomarkers panels were formed to study the sensitivity and specificity of these biomarkers for outcome prediction (studies II and III). A multiparameter panel including the clinical parameters and blood biomarkers was devised to study the best prediction model (study III).

This project, focusing on the acute biochemical diagnostics of mTBI, reported that GFAP and UCH-L1 are not specific biomarkers for CT-negative mTBI. However, we found that the early levels of GFAP and NF-L are significantly correlated with the outcome in patients with mTBI. The admission level of NF-L has a significant predictive value for mTBI, also in a multi-variate model. Finally, the admission levels of T-tau were significantly correlated with the outcome in patients with mTBI.

Keywords: traumatic brain injury, orthopedic injury, glial fibrillary acidic protein, ubiquitin carboxy-terminal hydrolase L1, neurofilament light, total tau, β-amyloid 1–40, β-amyloid 1–42, outcome

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Neurologia, Kliiniset neurotieteet, neurokirurgia
IFTAKHER HOSSAIN: Lievän tapaturmaisen aivovamman biokemiallinen diagnostiikka
Väitöskirja, 125 s.
Turun kliininen tohtoriohjelma
joulukuu 2020

TIIVISTELMÄ

Tapaturmaiset aivovammat ovat maailmanlaajuinen terveysongelma. Suurin osa aivovammoista on lieviä. Suurin osa lievän aivovamman saaneista potilaista toipuu hyvin, mutta osalle jää vammasta kuitenkin pitkäaikaisia jälkioireita. Verestä mitattavia merkkiaineita, biomarkkereita, voidaan käyttää mahdollisesti apuna aivovamman vakavuuden, aivovaurion luonteen ja potilaan toipumisennusteen arvioinnissa.

Tähän väitöskirjahankkeeseen kuuluu kolme kliinistä tutkimusta. Ensimmäisessä analysoitiin gliaalisen fibrillaarisen happaman proteiinin (GFAP) ja ubikitiini-karboksiterminaalisen hydrolaasi L1:n (UCH-L1) veripitoisuudet ortopedisen vamman saaneilla potilailta, joilla ei ollut taustallaan aivovammaa tai muuta aivotapahtumaa, ja vertasimme niitä lievän aivovamman saaneiden potilaiden vastaaviin veripitoisuuksiin. Näillä aivovammapotilailla ei ollut poikkeavia löydöksiä pään tietokonetomografiassa (TT) eli he olivat TT-negatiivisia. Toisessa tutkimuksessa analysoitiin lievän aivovamman saaneilta potilailta 24 tunnin kuluessa sairaalaan saapumisesta veren GFAP:n ja kevyen neurofilamentti -proteiinin (NF-L) pitoisuudet, ja tutkittiin niiden korrelaatiota potilaiden myöhempään toipumisen tasoon. Kolmannessa tutkimuksessa analysoitiin kokonais-taun, beeta-amyloidi 1–40:n (Aβ40) ja beeta-amyloidi 1–42:n (Aβ42) veripitoisuudet lievän aivovamman saaneilta potilailta 24 tunnin kuluessa sairaalaan saapumisesta. Jälleen tutkittiin merkkiaineiden ennustearvoa myöhemmän toipumisen tason suhteen.

Tämän lievien tapaturmaisten aivovammojen biokemiallista diagnostiikkaa tutkivan väitöskirjahankkeen tärkeimmät löydökset ovat: i) verestä mitattujen GFAP:n ja UCH-L1:n pitoisuudet eivät ole spesifejä lievälle TT-negatiiviselle aivovammalle, ii) sairaalaan saapumisvaiheessa mitatut GFAP:n ja NF-L:n veripitoisuudet korreloivat merkittävästi lievän aivovamman toipumisennusteen kanssa, ja iii) sairaalaan saapumisvaiheen kokonais-taun, Aβ40:n ja Aβ42:n veripitoisuuksilla ei kyetä ennustamaan lievän aivovamman saaneen potilaan toipumista.

Avainsanat: tapaturmainen aivovamma, ortopedinen vamma, gliaalinen fibrillaarinen hapan proteiini, ubikitiini-karboksiterminaalinen hydrolaasi L1, kevyt neurofilamentti, kokonais-tau, beeta-amyloidi 1–40, beeta-amyloidi 1–42, toipumisennuste

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Abbreviations

AD	Alzheimer's disease
AUC	area under receiver operating characteristics curve
Aβ40	β-amyloid isoform 1–40
Aβ42	β-amyloid isoform 1–42
BBB	blood-brain barrier
CBF	cerebral blood flow
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computed tomography
CTE	chronic traumatic encephalopathy
DAI	diffuse axonal injury
DTI	diffusion tensor imaging
DWI	diffusion-weighted imaging
ED	emergency department
EDH	epidural hematoma
ELISA	enzyme-linked immunosorbent assay method
FLAIR	fluid-attenuated inversion recovery
GCS	Glasgow coma scale
GFAP	glial fibrillary acidic protein
GOS	Glasgow outcome scale
GOSE	Glasgow outcome scale extended
HI	head injury
ICBT	iterative combination of biomarkers and thresholds
ICH	intracerebral hemorrhage
ICU	intensive care unit
IQR	interquartile range
ISS	injury severity score
IVH	intraventricular hemorrhage
LLoD	lower limit of detection
LLoQ	lower limit of quantification
LOC	loss of consciousness

moTBI	moderate traumatic brain injury
MRI	magnetic resonance imaging
mTBI	mild traumatic brain injury
N4PA	Neurology 4-Plex A assay
NF-L	neurofilament light
NPV	negative predictive value
OR	odds ratio
pAUC	partial area under receiver operating characteristics curve
PCS	post-concussion syndrome
PTA	post-traumatic amnesia
P-tau	hyperphosphorylated tau protein
ROC	receiver operating characteristics curve
RPCSQ	Rivermead Post-Concussion Symptoms Questionnaire
S100B	S100 calcium-binding protein B
SD	standard deviation
SDH	subdural hematoma
Simoa	ultrasensitive single molecule array
sTBI	severe traumatic brain injury
SWI	susceptibility weighted imaging
TBI	traumatic brain injury
tSAH	traumatic subarachnoid hemorrhage
T-tau	total tau protein
UCH-L1	ubiquitin carboxy-terminal hydrolase L1
USA	The United States of America

List of original publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals I – III:

I **Posti, J.P., Hossain, I.**, Takala, R.S.K., Liedes, H., Newcombe, V., Outtrim, J., Katila, A.J., Frantzén, J., Ala-Seppälä, H., Coles, J.P., Kyllönen, A., Maanpää, H.R., Tallus, J., Hutchinson, P.J., Van Gils, M., Menon, D.K., Tenovuo, O., and TBIcare Investigators. Glial Fibrillary Acidic Protein and Ubiquitin C-Terminal Hydrolase-L1 Are Not Specific Biomarkers for Mild CT-Negative Traumatic Brain Injury. Journal of Neurotrauma, 2017; 34, 1427 – 1438. https://doi: 10.1089 / neu.2016.4442.

II **Hossain, I.**, Mohammadian, M., Takala, R.S.K., Tenovuo, O., Lagerstedt, L., Ala-Seppälä, H., Frantzén, J., van Gils, M., Hutchinson, P., Katila, A.J., Maanpää, H.-R., Menon, D.K., Newcombe, V.F., Tallus, J., Hrusovsky, K., Wilson, D.H., Blennow, K., Sanchez, J.-C., Zetterberg, H., and Posti, J.P. Early Levels of Glial Fibrillary Acidic Protein and Neurofilament Light Protein in Predicting the Outcome of Mild Traumatic Brain Injury. Journal of Neurotrauma, 2019; 36, 1551 – 1560. https://doi: 10.1089 / neu.2018.5952.

III **Hossain, I.**, Mohammadian, M., Takala, R.S.K., Tenovuo, O., Azurmendi, Gill, J., Frantzén, J., van Gils, M., Hutchinson, P.J., Katila, A.J., Maanpää, H.R., Menon, D.K., Newcombe, V.F., Tallus, J., Hrusovsky, K., Wilson, D.H., Gill, J., Blennow, K., Sanchez, J.C., Zetterberg, H., Posti, J.P. Admission levels of total tau and β-amyloid isoforms 1-40 and 1-42 in predicting the outcome of mild traumatic brain injury. Frontiers in Neurology, 2020; 11:325 https://doi: 10.3389 / fneur.2020.00325.

1 Introduction

Traumatic brain injury (TBI) is among key reasons behind mortality, morbidity and disability in the United States of America (USA) and European populations (Maas et al., 2017b). TBIs are mostly caused by traffic accidents, falls, violence, sports, and war (Asemota et al., 2013; Khan et al., 2015). With the medical cost of TBI, in the tens of billions for both Europe and USA, TBI is expected to become, globally, the third most important health burden by 2020, with increasing socioeconomic consequences (Mathers & Loncar, 2006). TBIs can be classified as mild, moderate, and severe (Maas et al., 2017b; Menon & Maas, 2015), but the diagnostic tools to predict the true severity and the outcome still remain quite old (Lingsma et al., 2015). Methods such as level of consciousness, conventional computed tomography (CT), magnetic resonance (MR) imaging, or newer MR methods, e.g. diffusion tensor imaging (DTI), functional MRI (fMRI), and susceptibility weighted imaging (SWI), can be helpful in evaluating the severity of the injury (Jagoda et al., 2008a; Shenton et al., 2012), but there is no single specific neuroimaging or any other test for accurate diagnosis and evaluation of TBI (Maas et al., 2017a; Wang et al., 2018b; Zetterberg & Blennow, 2016). In addition, sophisticated imaging tests are costly, not readily available and have an uncertain diagnostic value.

In other fields of medicine, investigations of blood biomarkers are used in conjunction with other investigations for precise diagnosis and an effective treatment plan. Brain derived enzymes, proteins and protein degradation products have been tested in numerous studies (Diaz-Arrastia et al., 2014; Thelin et al., 2017; Yue et al., 2019; Zetterberg et al., 2013b). So far, S100 calcium-binding protein B (S100B) in severe TBI (sTBI), and GFAP in mild TBI (mTBI), have consistently demonstrated the ability to predict injury and outcome in adults (Neselius et al., 2013; Ramos-Cejudo et al., 2018a; Thelin et al., 2017), though they are not entirely brain specific (Zetterberg et al., 2013a; Zetterberg & Blennow, 2016), and the studies using well characterized cohorts and highly sensitive immunoassays for the blood biomarker analysis are scarce (Menon & Maas, 2015).

Glial fibrillary acidic protein (GFAP) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) have shown great promise as blood biomarkers for TBI (Luoto et al., 2017). Serum levels of GFAP have been found to significantly predict CT-positive

brain injury, with satisfactory sensitivity and specificity for acute TBI (Dadas et al., 2018; Papa et al., 2012; Wang et al., 2018b). A recent multicenter observational trial reported negative predictive value (NPV) and high sensitivity of GFAP and UCH-L1 for the prediction of acute TBI on head CT, therefore, could significantly reduce the use of head CT (Bazarian et al., 2018). Also, according to multiple studies, the levels of GFAP and UCH-L1 could be promising markers of the existence and severity of TBI, particularly for complicated mTBI, moderate TBI (moTBI), and sTBI (Metting et al., 2012; Papa et al., 2012; Posti et al., 2019). However, currently the specificity of GFAP and UCH-L1 for the diagnosis of mTBI is uncertain (Kiviniemi et al., 2015; Meyer-Schwesinger et al., 2009; Papa et al., 2016b; Viale et al., 1988). There are only few studies comparing the levels of GFAP and UCH-L1 between patients with CT-negative mTBI and orthopedic trauma as controls.

The standard tool to assess acute TBI, head CT, is not sensitive enough to detect microbleeds and diffuse axonal injury (DAI) (Topal et al., 2008). Even though most of the patients with mTBI have good recovery, unfortunately, there are patients who do not fully recover, and suffer from disabling symptoms, cumulatively known as post-concussion syndrome (PCS) (Maas et al., 2017b; Shahim et al., 2016). Regrettably, clinically validated models are still unavailable for predicting the outcome of mTBI (Lingsma et al., 2015; Ponsford et al., 2008). There are few studies that explored the prognostic abilities of the blood biomarkers, using the admission samples. If the admission samples could be used in the acute setting to predict the outcome of the patients with mTBI, it would greatly assist the clinicians to stratify the group of patients who might need observation, further treatment, follow-up and rehabilitation. A recent study showed that the levels of neurofilament light (NF-L) 7–10 days after a bout in amateur boxers correlated with the number of head impacts during the match (Neselius et al., 2013). Moreover, there are promising studies, using cerebrospinal fluid (CSF) samples, reporting the utility of NF-L protein for the evaluation of concussion (Shahim et al., 2017; Shahim et al., 2016). From a clinical point of view, CSF sampling is invasive and, therefore, unrealistic for the assessment of patients with mTBI in the emergency department (ED). The efficacy of the admission samples of NF-L in blood, for the outcome prediction of mTBI, has not been studied earlier.

Recently, the neurodegenerative axon terminal biomarkers, tau and β-amyloid isoforms 1–40 (Aβ40), and 1–42 (Aβ42), have been investigated to establish whether a correlation exists between neuronal damage and PCS, following repeated mTBI (Shahim et al., 2016b). Although CSF levels of these biomarkers have shown promising results, there are no studies reporting a correlation between the axon terminal biomarkers' plasma levels at admission and outcome of mTBI.

For all the reasons mentioned above, we carried out a prospective, observational clinical research project, including three studies. Firstly, we investigated the

diagnostic ability of GFAP and UCH-L1 levels in blood, to differentiate patients with orthopedic injury from CT-negative mTBI. We also explored the extracranial sources of these blood biomarkers to study their specificity for TBI. Secondly, we studied the early blood levels of GFAP and NF-L and whether they could predict the outcome of mTBI. Thirdly, we investigated if the levels of total tau (T-tau) and Aβ40 and Aβ42, during the first 24 hours after admission, could correlate with outcome in patients with mTBI. Importantly, for the last two studies, ultrasensitive single molecule array (Simoa) technology was used to analyse the blood biomarker levels.

2 Review of the literature

2.1 Traumatic brain injury

2.1.1 Definition

TBI could be defined as an alteration in brain function, or other evidence of brain pathology, caused by an external force (Menon et al., 2010). This external force could be a traumatic, direct or indirect, biomechanical force to the head. TBI is a heterogeneous disease (Lingsma et al., 2010). Although the term "head injury" (HI) has been synonymously used for TBI in literature, HI might be only confined to injury of the skull without causing any pathological abnormalities in the brain.

Four clinical signs, listed below, are principally considered for the diagnosis of TBI (Giza et al., 2013; Menon et al., 2010; Signoretti et al., 2011):

- loss of consciousness (LOC)
- loss of memory i.e. post-traumatic amnesia (PTA)
- alteration in mental status
- and / or focal neurological deficits

2.1.2 Epidemiology

TBI, the silent epidemic, is one of the greatest public health problems worldwide. It has been reported that approximately 60 million new TBI cases occur annually around the world (Dewan et al., 2019). In the European Union (EU), a minimum of 2.5 million new cases of TBI are reported each year. For the USA, it has been estimated that approximately 3.5 million new cases of TBI occurs yearly (Coronado et al., 2012). A study using standardised Eurostat data found that 1.5 million patients were discharged from the hospital and 57 000 patients died in 2012 due to TBI in the EU (Majdan et al., 2016). The US Centers for Disease Control and Prevention (CDC) reported that over 2 million patients with TBI are treated and discharged from an ED, anually, and almost 56 000 deaths as a result of TBI (Taylor et al., 2017). In Finland, the incidence of hospitalized TBI and the mortality rate, is approximately

100 / 100 000 and 18 / 100 000, respectively (Koskinen & Alaranta, 2008). By comparison, a systematic review of the epidemiology of TBI showed an overall incidence of 790 / 100 000 in New Zealand, 344 / 100 000 in Asia, 235 / 100 000 in Europe, 226 / 100 000 in Australia, 160 / 100 000 in India, and 103 / 100 000 in USA (Feigin et al., 2013; Tagliaferri et al., 2006). However, these contrasting figures possibly reveal national variations in healthcare and registration systems, rather than actual differences in incidence.

TBI causes significant healthcare and societal costs, but an estimation of an accurate global cost of TBI is not available (Maas et al., 2017b). Studies from the Brain Injury Outcomes New Zealand In the Community (BIONIC), using estimation by extrapolation of new cases worldwide of mTBI (52–56 million) and moTBI – sTBI (2.2–3.6 million) per year, suggest that the global economic burden of TBI could range between US$362 billion–US$445 billion in 2017 (Ao et al., 2014). Unfortunately, low-income and middle-income countries (LMICs) are facing a greater burden of TBI than high-income countries. Due to lack of funded studies and lack of appropriate multicenter research efforts, evidence-based management of TBI is still not well established in LMICs (Kolias et al., 2019).

Over 80%–90% of patients who sustain TBI are classified in the mild end of the spectrum, including those injuries labelled as concussions (Levin & Diaz-Arrastia, 2015). A systematic review conducted by the World Health Organization (WHO) Collaborating Centre Task Force on mTBI reported that the annual incidence of mTBI was in the range of 100–600 / 100 000 (Donovan et al., 2014). The incidence is likely underestimated, since a large percentage of asymptomatic patients with mTBI are not presented to the ED. While most patients with a single mTBI fully recover, many do not, leading to prolonged suffering, impaired quality of life, and increased risk of post-traumatic sequelae (Carroll et al., 2014). Despite the fact that data from prospective hospital-based mTBI studies are limited, it is estimated that reduced and lost productivity after mTBI accounts for the largest component of the economic costs of brain trauma each year (Maas et al., 2017b). The most frequent causes of mTBI are falls, motor-vehicle accidents and sports-related concussions (Coronado et al., 2012). It is important to note that almost half of the patients with TBI are under the influence of alcohol at the time of the injury (Posti et al., 2019; Salim et al., 2009, Parry-Jones et al., 2006). Alcohol intoxication increases the probability of premature death. It has been reported that head injury, regardless of TBI, under the influence of alcohol decreases life expectancy by approximately 9 years, and the risk of alcohol related death later in life is 14.7%, in case of moTBI and sTBI (Puljula et al., 2016). A significant amount of people affected by mTBI are teenagers, young adults, and the working class. However, due to the aging population of the developed countries and owing to the vulnerability to fall, the incidence of

mTBI is also high in elderly patients, which reflects a demographic shift of TBI (Roozenbeek et al., 2013).

2.1.3 Pathophysiology

Brain damage, due to trauma, could be divided into two main categories: focal injury and diffuse injury. Focal injury includes cortical or subcortical contusions and lacerations, in addition to intracranial hemorrhage. Focal injuries are generally found in severe cases of TBI due to a serious direct impact to the brain. On the other hand, stretching and tearing of the brain tissue cause diffuse injury, which is commonly seen in case of milder spectrum of TBI. Thus, it does not need any direct impact or crush injury to the surface of the brain to cause diffuse injury. DAI is the main form of diffuse injury (Johnson et al., 2013a; Vieira et al., 2016), which occurs due to acceleration / deceleration forces that lead to shearing of axons.

TBI with acceleration or deceleration forces to the brain causes a neurometabolic cascade that affects the brain function (Blennow et al., 2012; Giza & Hovda, 2014). The initiating event of this cascade is stretching and disruption of neuronal and axonal cell membranes (Geddes et al., 2003). Such membrane defects trigger a deregulated flux of ions, including an influx of calcium and efflux of potassium (Prins et al., 2013). The enhanced release of excitatory neurotransmitters, specifically glutamate, is accelerated by the aforementioned events. Glutamate binds to N-methyl-D-aspartate (NMDA) receptors and this creates advancing depolarization, which eventually causes an influx of calcium ions (Giza & Hovda, 2014). An imbalance in cellular ions distorts the normal glucose metabolism. This trauma-induced hypermetabolism reflects the effort of cells to restore normal ionic balance, which is disrupted by pathological ionic flows through ion channels. Neuronal glucose consumption increases, which in turn diminishes energy stores, and causes calcium influx into mitochondria. Impaired oxidative metabolism, anaerobic glycolysis, lactate production, and reactive oxygen species cause acidosis and edema (Blennow et al., 2012; Giza & Hovda, 2014). This all causes neuronal dysfunction that is thought to reflect to the acute symptoms of TBI. The disrupted state can last for days and the consequences of the neurometabolic cascades (Figure 1) of TBI have been reported as an evolving phenomena (Ng & Lee, 2019).

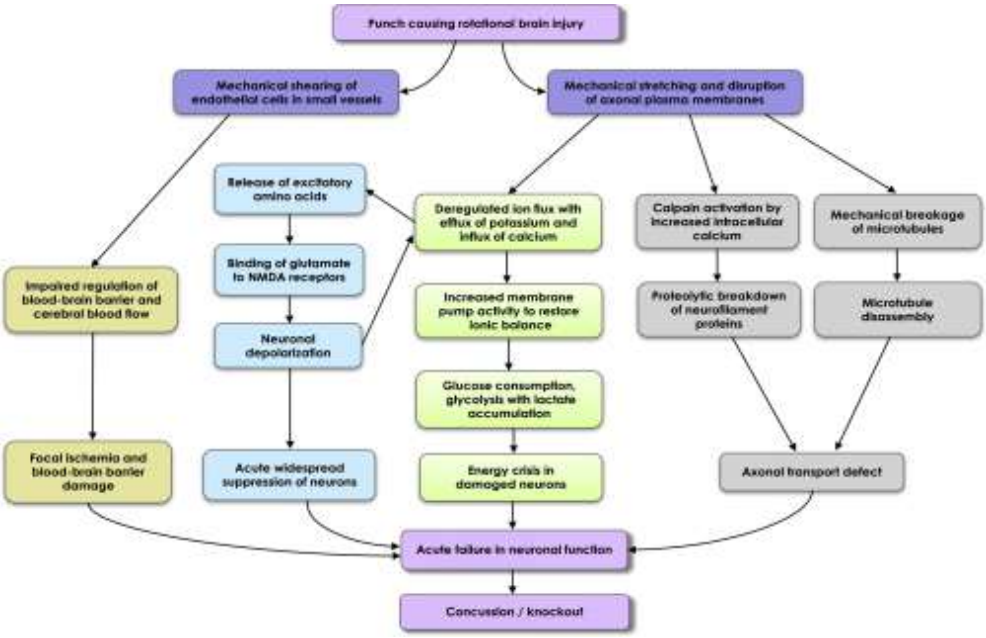


Figure 1. Molecular Pathophysiology of mild traumatic brain injury. (NMDA = N-methyl-D-aspartate) (Blennow et al., 2012). Reprinted with permission from Neuron.

2.1.4 Classifications

Different systems could be used for the classification of TBI. Most often TBI has been classified by one of four main systems (Gravesteijn et al., 2020; Maas et al., 2017a):

- clinical indices of severity
- pathoanatomic type
- physical mechanism
- classification by pathophysiology

Besides, classification by prognostic modelling has been also mentioned in the current literature (Saatman et al., 2008).

2.1.4.1 Classification by injury severity

There are several classical parameters, which are used to classify TBI severity in the acute phase based on its clinical presentation, i.e. GCS, PTA, and duration of LOC. Historically, TBI has been classified as mild, moderate or severe by using the GCS (Narayan et al., 2002), a system used to assess coma and impaired consciousness

(Teasdale & Jennett, 1974). TBI severity could be classified according to the GCS score as follows: 13–15, mild; 9–12, moderate; 3–8, severe (Teasdale & Jennett, 1974). Another useful index of the severity of TBI is PTA, which is the interval between the initial injury and the complete orientation of the patient, when the patients can form new memories, and later recall these memories. PTA classification of TBI is mild (0–1 day), moderate (>1 to <7 days) and severe (>7 days) (Forslund et al., 2019a; Walker et al., 2018a).

While the use of the GCS score has entered routine clinical practice, PTA is still rarely prospectively assessed in clinical settings. Perhaps unsurprisingly, there is often poor concordance between the GCS and PTA. Many patients with TBI who might, by the GCS criteria alone, be considered "mild", have prolonged PTA durations indicating a more severe injury (Dikmen et al., 2001; Hart et al., 2016). These two tools provide complementary information about brain function and often lead to quite different estimates of clinical severity. Failure to assess either of these accurately and in a standardized fashion may be a major contributor to disparate and often inaccurate severity classification and prognosis after a TBI. Notably, both the features and duration of the GCS and PTA have been shown to be poorly aligned with pathophysiological substrates of TBI (King et al., 1997a; Zuercher et al., 2009). The assessment of TBI severity in the acute care setting is also often hindered by several confounders (Zuercher et al., 2009). Regrettably, the current concept of severity, especially for mTBI, is poorly defined and may be used in variable contexts. This issue will be elaboratively discussed in the further chapters of this book, since it focuses on the acute diagnostics of mTBI.

2.1.4.2 Classification by pathoanatomic type

As mentioned in the pathophysiology, TBI could be classified as focal and diffuse brain injury. Many TBI cases might have both aspects. Cerebral contusion, cerebral laceration, epidural hematoma (EDH), subdural hematoma (SDH), intracerebral hemorrhage (ICH) and intraventricular hemorrhage (IVH) are included in focal injuries. Diffuse injuries cover DAI, diffuse ischemic and vascular injury, and cerebral edema.

2.1.4.3 Classification by physical mechanism

TBI can be classified according to contact or "impact" loading and / or noncontact or "inertial" loading. The direction and degree of each type or combination of loading forces might foresee severity and type of injury (Cloots et al., 2008; Demann & Leisman, 1990).

2.1.4.4 Classification by pathophysiology

According to the pathological mechanism, TBI is conventionally divided into two phases: primary and secondary brain injury. The primary injury refers to the mechanical damage to the brain parenchyma that occurs at the time of injury. The primary injury advances over time, reaching its ictus in the following hours, which induce pathophysiological changes in the brain, causing the secondary injury. The secondary brain injury, with early phases overlapping the primary injury, takes place in the subsequent hours and days. Secondary brain injury processes include – hypoxic-ischemic injury, cerebral edema, metabolic dysfunction, alterations in vascular permeability, diminished blood flow, DAI, vasospasm, hydrocephalus, and the consequences of intracranial hypertension (Haddad & Arabi, 2012; Rosenfeld et al., 2012). Further exacerbation of secondary injuries by systemic insults, such as: coagulopathy, hypertension, hypotension, hypoxemia, hyperthermia, hyperglycemia, hypoglycemia, hypercapnia, hypocapnia, anemia, hypernatremia, hyponatremia, and acid-base disorders (Chesnut et al., 1993; Unterberg et al., 2004). Therefore, the emphasis of TBI treatment is on preventing the consequences of primary brain injury and preventing or even reversing secondary brain injury. It is possible to treat the secondary injury, but the primary injury is only preventable (Maas et al., 2017b; Murray et al., 1999; Stallones et al., 2008).

2.1.5 Outcome

Variability in outcomes is partly caused by distinct mechanisms of injury, which are not captured by current global injury severity classification schemes. For instance, EDHs are life threatening as an acute presentation of sTBI, but rapid treatment often provides improved outcomes. In contrast, DAI might be underestimated in the acute setting, giving an impression of a mild injury, but is often associated with long-term disability. Although local visible traumatic lesions may produce recognizable symptoms, the overall outcome is mostly dependent on the extent and severity of the diffuse damage in the brain networks (van Eijck et al., 2018), which is largely invisible for routine clinical imaging (Amyot et al., 2015; Brandstack et al., 2013). Ideally, the uncertainty in predicting long-term outcomes that results from pathophysiological variability, should be reflected in the injury classification, so that inappropriate and potentially inaccurate or over optimistic clinical decision-making could be avoided.

Although many patients have good recovery within weeks or months following mTBI, 5%–30% patients with mTBI suffer from neurologic, cognitive, and / or neuropsychiatric symptoms for one year post-injury or longer (Borg et al., 2004; Lingsma et al., 2010; Saatman et al., 2008; Sharp & Jenkins, 2015). All of these persisting symptoms or findings are known as PCS, which could be defined as a

collection of post-traumatic symptoms, and could be allocated into the three domains: cognitive (poor concentration, forgetfulness, or slowed processing speed), somatic (headaches, dizziness, blurred or double vision, nausea, photophobia or phonophobia, disrupted sleep habits, or fatigue), or emotional (depression, restlessness, irritability, or frustration) (Ponsford et al., 2008; Ponsford et al., 2014). Be noted that PCS symptoms could be widely present among normal population (Polinder et al., 2018).

The International Classification of Diseases, Tenth Revision (ICD-10) recommends that a diagnosis of PCS must include an HI, severe enough to result in LOC, and also three subjective symptoms present for at least four weeks. Significant clinical impairment needs to be caused by these symptoms. Since the current classification of TBI, based on the GCS, does not measure the degree of severity precisely, it is complex to identify the group of patients with mTBI who are prone to develop PCS (Sharp & Jenkins, 2015).

The factors predicting PCS have been divided into three categories by the WHO, Collaborating Centre Task Force on Mild Traumatic Brain Injury (Caplain et al., 2017; Carroll et al., 2004).

- The individual: gender (female), marital status, educational level, age >40 years, pre-existing disabilities, previous neurological disease, prior HI, psychiatric illness, and significant life stressors
- Injury: road traffic accident
- Consequences: GCS <15, LOC, PTA >20 min, presence of nausea or memory problems following the injury, polytraumas

Recently, it has been studied that more acute symptoms, premorbid psychiatric problems, such as anxiety or poorer premorbid physical health, are associated with poor recovery following mTBI (Mooney et al., 2005; Rabinowitz et al., 2015). Additionally, seeking compensation has been identified as an important factor in patients with persisting symptoms, which is related to the medicolegal issues of mTBI (Ponsford et al., 2014; Willemse-van Son et al., 2007). Patients with mTBI having post-injury symptoms, causing poor quality of life, have a significantly higher risk of a negative vocational outcome than those with no complaints or better recovery (Bullinger et al., 2002; Colantonio et al., 2016; Walker et al., 2006). Unfortunately, in the setting of acute trauma care, it is not always feasible to gather all the risk factors for the early identification of patients who are likely to develop worse outcome.

Since the assessment of TBI outcome is complex and multifactorial, therefore, the predictions could only be made by combining the above-mentioned variables in a multivariate model. This has led to the development of prognostic models and CT-

based scoring systems. These concepts will be discussed in the following sections of this book.

2.1.6 Diagnostic tools for traumatic brain injury

According to the one of the studies by the CENTER-TBI group, TBI has been most of the times diagnosed by the ED physicians and neurosurgeons (Cnossen et al., 2017). A careful as well as focused history taking and performing an accurate physical examination are the first and foremost steps for the assessment of patients with TBI. Several pre- and post-injury factors need to be considered during the initial evaluation of the patients with mTBI, considering the issues that the acute diagnostic features could be minimised or exaggerated by the other confounders, such as alcohol intoxication.

2.1.6.1 Assessment of consciousness

2.1.6.1.1 Glasgow Coma Scale

The GCS was originally developed as a tool to assess the level of consciousness (Teasdale & Jennett, 1974). The three components of the GCS are eye opening, verbal response and motor response (Table 1). Each of these three parts are individually scored according to the best response and the resulting points give a patient score between 3 (indicating deep unconsciousness) and 15 (normal consciousness). Although the total score is generally presented, however, clinically it is important to provide the individual scores (particularly the motor score, since it is strongly associated with TBI outcome) (Teasdale et al., 2014). A GCS score of 13 to 15 points after 30 minutes from the injury is considered as mTBI (Borg et al., 2004; Sharp & Jenkins, 2015). The GCS is a robust tool, which gives an approximation of the initial severity of TBI, and even in mTBI, the probability of a more severe injury increases as the GCS score decreases. In various studies the incidences of traumatic intracranial abnormalities stratified by the GCS score are as follows: 13 points: 28%–51%, 14 points: 12%–52%, 15 points: 6%–34% (Saboori et al., 2007; Thiruppathy & Muthukumar, 2004).

The traditional approach to define mTBI by the GCS is not beyond questions and recent multicenter, large data-based studies have reported that the GCS is not in any way an absolute measure of TBI severity, and it has been also studied that a GCS of 13 is in many ways closer to moTBI than mTBI (Majdan et al., 2015; Mena et al., 2011; Perrin et al., 2015). Currently, a survey of 71 neurotrauma centers participating in the CENTER-TBI study analysed that 40 centers (59%) defined mTBI as a GCS score between 13 and 15 and 26 (38%) defined it as a GCS score between 14 and 15

(Cnossen et al., 2017). The GCS also correlates poorly with clinical outcome of TBI for various reasons. A GCS score of 5 some minutes after the injury has a different significance than one recorded hours or days after the injury. The GCS may be assessed at various standpoints after the injury, with variable delays from the incident, and in variable circumstances. Although the GCS has proven to be useful in the acute phase of sTBI, it performs sub optimally while the observation of the patient lasts over the most acute phase (e.g. in the intensive care unit) (Wijdicks et al., 2005). Additionally, the routine use of early intubation and sedation during transport further complicates interpretation of the GCS (Barker et al., 2014).

Table 1. Glasgow Coma Scale (Teasdale & Jennett, 1974, 1976)

Behaviour	Response	Score
Best eye-opening response (E)	Spontaneous	4
	To verbal comment	3
	To painful stimuli	2
	None	1
Best verbal response (V)	Oriented	5
	Confused	4
	Inappropriate words	3
	Incomprehensible sounds	2
	None	1
Best motor response (M)	Obey commands	6
	Localizes painful stimuli	5
	Flexion withdrawal from pain	4
	Abnormal flexion	3
	Abnormal extension	2
	None	1

Total GCS score, E + V + M = 3 – 15, where 15 = fully concious and 3 = deeply unconcious

2.1.6.1.2 Loss of consciousness

The duration of unresponsive state due to TBI is defined as LOC (Blyth & Bazarian, 2010). The period of LOC must be 30 minutes or less in case of mTBI (Carroll et al., 2004). Notably, a GCS score of under nine is universally regarded as unconsciousness (Teasdale et al., 2014; Teasdale & Jennett, 1974). The mechanism of LOC caused by TBI is not completely known. It has been reported that temporal impairment in one or more parts of the ascending reticular activating system, which is located in the central pons, midbrain, hypothalamus, and thalamus is associated with the causal of LOC (Blyth & Bazarian, 2010; Olson & Graffagnino, 2005). It

has been also studied that LOC in patients with mTBI is associated with injury to white matter tracts (Levin et al., 2008).

In the clinical practice, the retrospective assessment of LOC is challenging. Objective assessment is limited because in most cases LOC has resolved by the time the patient reaches the hospital, meaning direct assessment is not possible. In more severe cases, patients have often been sedated and intubated in the field, again making assessment of duration impossible. Retrospective assessment of LOC by patient report is also unreliable, as patients often report that they have lost consciousness, when in fact they have amnesia for the post-traumatic period (Sherer et al., 2015). Hence, there is often a lack of accuracy in the assessment of whether LOC occurred and how long it lasted.

2.1.6.1.3 Post-traumatic amnesia

There is no uniform definition for PTA in the literature. It is a transient state of disorientation, confusion and memory impairment caused by an HI (Friedland & Swash, 2016; Menon et al., 2010). The duration of PTA is assessed using tools such as the Galveston Orientation and Amnesia Test (GOAT) and Westmead PTA scale (Levin et al., 1979; Meares et al., 2011). The acute cognitive effects of TBI can be estimated by the presence and duration of PTA. Numerous studies have shown that PTA is the best clinical predictor of long-term cognitive outcome after TBI (Hart et al., 2016; Königs et al., 2012; Ponsford et al., 2016; Walker et al., 2018b). PTA is characterized by variable impairments of cognition, including memory and attention, confusion, excessive sleepiness, restlessness and agitation (Marshman et al., 2013). Its measurement can provide valuable additional prognostic information and its duration is more closely related to long-term outcome than the GCS (Dahdah et al., 2016; Forslund et al., 2017; Perrin et al., 2015).

The pathophysiological basis for PTA is surprisingly poorly understood, although recent work provides evidence that PTA is caused by a transient disconnection between parts of the limbic system involved in memory encoding, in particular a disruption in the functional connectivity between the medial temporal lobe and other parts of the default mode network that resolves with the emergence from PTA (De Simoni et al., 2016). Hence, PTA may be produced by impairments to the hippocampus and parahippocampus' roles in supporting memory encoding and consolidation through a transient functional disconnection to other brain regions involved in memory process. The severity and location of DAI may be important in producing this disconnection, as white matter damage within the cingulum connections of the parahippocampal gyrus are associated with prolonged PTA duration (De Simoni et al., 2016). Dysfunction within the frontal lobes is also likely

to contribute to PTA, possibly reflecting a transient global disruption of functional connectivity (Metting et al., 2010).

There are problems with the clinical assessment and interpretation of PTA (Dahdah et al., 2016; Sherer et al., 2015). The presence and duration of PTA are infrequently assessed properly in EDs, inpatient wards, or intensive care units (Cota et al., 2019). Various tools have been developed for the assessment of PTA, but none has been universally accepted for clinical use. Very few studies have compared the reliability and reproducibility of different tools to measure PTA. Clinical estimates of PTA are often done retrospectively, but these may be inaccurate due to recall bias (Friedland & Swash, 2016). These retrospective assessments may give both longer and shorter estimates for PTA than prospective evaluations (Roberts et al., 2016). In addition, there are different severity classifications also based on the length of PTA (Greenwood, 2002; Russell & Smith, 1961).

Confounders for the use of GCS and PTA to assess injury severity

The assessment of TBI severity in the acute care setting is often confounded by difficulties, for instance, collecting or interpreting the GCS or PTA. These include language issues, inexperienced evaluators, intoxication, drug effects on the level of consciousness, and retrospective bias among other things (Ala-Seppälä et al., 2016; Dikmen et al., 2001). These confounds often lead to skewed estimates of the TBI severity in either direction. For example, drowsiness, confusion, and amnesia might be attributed to intoxication leading to an underestimation of TBI severity, or vice versa. The potential importance of these confounders for long-term outcome may only become apparent after the acute period. However, how the confounders have possibly influenced the acute assessment is usually impossible to determine reliably afterwards.

A patient who arrives unconscious and is diagnosed with sTBI may regain consciousness rapidly and recover quickly, especially in the setting of clinical confounders such as alcohol intoxication. A person who fell on the ground, hitting the head and convulsing immediately, may be deeply unconscious for a while but recover rapidly, if the lowered consciousness was actually post-ictal and not caused by the head trauma. Recognition of such confounders is not straightforward, and seldom are the initial severity assessments corrected to account for erroneous classification after the fact (Cloots et al., 2008; Sharp & Jenkins, 2015).

2.1.6.2 Medical history

Given that the abovementioned assessment tools are always not sensitive enough to diagnose mTBI, a proper medical history plays a vital role in the diagnosis. In

addition, the conventional neuroimaging methods could be negative for the assessment of the milder spectrum of TBI. The evaluation of these patients should include history of pre-existing diseases, e.g. psychiatric and neurological conditions, medications, previous TBI, any neurosurgical operation, and socioeconomic history, including alcohol abuse (Ponsford et al., 2008; Silverberg et al., 2020).

Medication (e.g., antithrombotic agents), certain pre-existing medical conditions (e.g., coagulopathies) and previous neurosurgery (e.g., cerebral shunt) increase the risk of having an intracranial hemorrhage even after mTBI (Menon et al., 2010; Undén et al., 2015). Alcohol abuse has a strong correlation with the development of intracranial haemorrhage (Haydel et al., 2000). Acute alcohol intoxication is also correlated with lower GCS score and could mimic a presentation of TBI (Scheenen et al., 2016). The initial assessment of mTBI could be affected by various types of medication, such as sedatives (e.g., benzodiazepines), analgesics (e.g., opioids), and antiemetics (e.g., dopamine antagonists). In case of polytrauma patients it is well known that even without TBI such patients could suffer from the same types of symptoms (McDonald et al., 2016; Stulemeijer et al., 2006). Considering this, it is important to evaluate the extracranial injuries, otherwise, mTBI could be misdiagnosed.

The acute symptoms of mTBI could be mimicked by different co-existing neurodegenerative diseases, e.g. Alzheimer's disease (AD), and underlying mental health problems (Iverson, 2005; Ramos-Cejudo et al., 2018b). For these reasons, it is crucial to differentiate the acute post-mTBI symptoms from the aforesaid, which could have the similar presentations.

2.1.6.3 Neurological and physical examination

A thorough neurological examination needs to be done for the evaluation of the patients with TBI. This includes evaluation of the level of consciousness and mental status, assessing the motor and sensory functions, including the cranial nerve examination, and the evaluation of balance and coordination. Besides, focal neurological signs should be checked as these signs are correlated with increased risk of intracranial lesions in TBI (Hyam et al., 2009; Vos et al., 2012). Signs of skull base fractures include hemotympanum, periorbital ecchymosis ("raccoon eyes" or "panda eyes"), mastoid ecchymosis (Battle's sign), CSF rhinorrhea and otorrhea should be checked (Haydel et al., 2000). Suspicion of skull fractures indicates the need of emergency head CT (Menon & Maas, 2015; Undén et al., 2015; Vos et al., 2012). It is important to note that a normal neurological examination does not totally exclude the possibility of a TBI (Vilke et al., 2000). The indications for the neuroimaging for the evaluation of patients with mTBI will be discussed in the further sections of this book.

In addition to the neurological examination, an effective physical examination should be done, as patients with mTBI might have different extracranial injuries (Lingsma et al., 2015; Stulemeijer et al., 2006). Concomitant cervical spine injuries are not uncommon in TBI (Morin et al., 2016) and clinically relevant cervical spinal injuries can be excluded with a proper physical examination done according to the international guidelines (Stiell et al., 2001).

2.1.6.4 Neuroimaging

Head CT became the key component in the diagnosis and acute assessment of TBI shortly after the introduction of CT in 1974 (Haydel et al., 2000). MRI is also used, generally in the subacute phase. Some more sophisticated and advanced neuroimaging techniques, such as DTI and fMRI are increasingly promising techniques, which are currently primarily used for research purposes for mTBI (Jagoda et al., 2008b; Sugiyama et al., 2009).

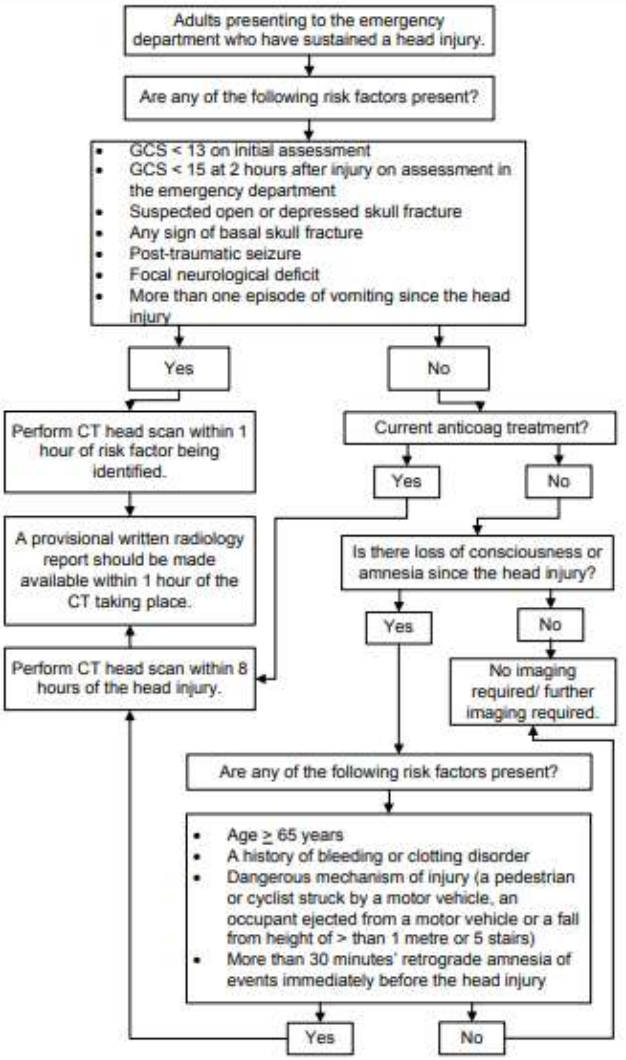
2.1.6.4.1 Traumatic intracranial lesions

Lesions such as contusion, DAI, EDH, SDH, traumatic subarachnoid hemorrhage (tSAH), ICH, IVH, and secondarily brain ischemia and edema are all macroscopic changes in the brain that can be a result of TBI. However, microscopic changes, including microbleeds, are not uncommon. For mTBI patients, the majority and most important traumatic lesions include SDH, tSAH, and focal cerebral contusions (Haydel et al., 2000). The injury severity has a great incidence and clinical impact on the above-mentioned lesions (Demann & Leisman, 1990).

2.1.6.4.2 Computed tomography

Since cranial CT scan can promptly identify a small subset of patients that require immediate neurosurgery, this is the modality of choice in the ED for the assessment of TBI. According to the studies of mTBI, for acute CT-positive intracranial lesions, the incidence rate ranges from 4.7%–38.9% (Saboori et al., 2007; Silverberg et al., 2020; Stiell et al., 2005; Thiruppathy & Muthukumar, 2004). To guide CT imaging decision making, and to predict the need for neurosurgical procedures, multiple guidelines have been validated and published, since 2000 (Ananthaharan et al., 2018; Haydel et al., 2000; Jagoda et al., 2008a; Vos et al., 2012). Guidelines improve the cost-effectiveness and optimization of hospital resources usage, by reducing the number of unnecessary head CT scans (Jagoda et al., 2008a; Morton & Korley, 2012). For adult patients with TBI, the Scandinavian guidelines are the newest guidelines for the initial management of moderate, mild, and minimal head injuries

(Undén et al., 2013). If there are no signs of skull fracture or altered mental status, 6–8 hours of hospital observation will be a sufficient, alternative to a CT scan (Norlund et al., 2006). The decision between observation and a head CT are influenced by worsening symptoms and risk factors. For patients with normal



neurological examination, mental status, and an available companion, home observation is recommended (National Institute for Health and Clinical Excellence, 2007)

Figure 2. **S**election criteria for adults for CT head scan according to National Institute for Health and Clinical Excellence (NICE): Guidance. Head Injury: Triage, Assessment, Investigation and Early Management of Head Injury in Children, Young People and Adults. Reprinted from National Institute for Health and Care Excellence (UK), 2014.

2.1.6.4.3 Delayed intracranial hemorrhage

After CT-negative finding, the most unpredictable complication is a delayed intracranial hemorrhage. This complication of mTBI is almost non-existent (Isokuortti et al., 2014), but require neurosurgical care in some cases (Feigin et al., 2013). It has been reported that patients with normal initial neurological examination and a GCS <15 are not free from risk of developing delayed intracranial injury (Nishijima et al., 2012, 2013).

Firstly, to determine which patients are suffering from these complications, and secondly, the possibility of identifying them before hospital discharge, are the two most important issues. For the reduction of unnecessary hospital observation, timely identification could conserve ED resources and decrease cost of treatment. Unfortunately, there are no clinically validated objective tools currently available for such purposes. (Zetterberg & Blennow, 2016) Because of amnesia or a history of losing consciousness, a large percentage of mTBI patients were hospitalized in the past, discharged within a few days with a brain concussion diagnosis. Due to increased use of CT scan, this policy has been questioned within the last decades (Geijerstam et al., 2004).

Delayed bleeding has multiple causes (Hamilton et al., 2010; Heino et al., 2019). The capacity of the brain to regulate cerebral blood flow (CBF) optimally, can be reduced through disturbed cerebral autoregulation. Bleeding may persist in minor contusions, due to disturbed autoregulation of CBF. Medication affecting the coagulation or blood coagulation disorders are also potential causes of delayed intracranial hemorrhage. Venous injuries are assumed to delay the signs of increased intracranial pressure (ICP) compared to arterial bleeding, since venous bleeding is naturally slower than arterial bleeding. It is still unclear if the use of anticoagulant medication after an initial normal CT scan in mTBI patients is a risk factor for delayed bleeding (Engelen et al., 2009). Although repeat imaging after an observation period is still part of the European guidelines (Vos et al., 2012), heterogeneous protocols for managing TBI patients have been developed globally in trauma centers (Maas et al., 2017b; Menon & Maas, 2015).

2.1.6.4.4 Magnetic resonance imaging

CT scan of head is the golden standard of the evaluation of acute TBI, but it has been reported that conventional MRI has superior sensitivity in the identification of certain acute lesions, including microhemorrage, DAI and small contusions, compared to CT. According to the current research, the ability of the conventional MRI in detecting a wide range of acute lesions are 0%–43% (Eierud et al., 2014; Hughes et al., 2004; Uchino et al., 2001; van der Horn et al., 2018; Yuh et al., 2013). Nevertheless, the use of conventional MRI has been limited, since positive MRI

findings did not always show sensible predictive value for long term outcome (Amyot et al., 2015; Z. Morris et al., 2009). In acute mTBI, routine brain MRI is uncommon, due to limited availability and high cost, despite the high sensitivity. In current clinical practice, MRI is mainly used at the subacute stage for the evaluation of patients with mTBI, who have persistent symptoms. MRI sequences in the TBI Common Data Elements (CDE) include: (i) 3D T1-weighted, (ii) 3D T2-weighted, (iii) T2-weighted fast spin echo, (iv) T2-weighted fluid-attenuated inversion-recovery (FLAIR), (v) diffusion weighted echo planar imaging, (vi) 3D susceptibility weighted imaging (SWI), and (vii) 2D gradient-echo (Haacke et al., 2010). The SWI sequence has been reported as the most sensitive tool for the detection of hemorrhagic lesions (Liu et al., 2014; Tao et al., 2015). Following the limitations of the current imaging methods, more advanced and promising neuroimaging techniques have been developed for the better assessment of mTBI (Jagoda et al., 2008b). DTI is sensitive to the direction and magnitude of non-random water diffusion in the brain and offers a non-invasive and quantitative measurement of brain white matter microstructural properties and connectivity (Alexander et al., 2007). Unlike CT or conventional MRI, DTI is sensitive to microstructural axonal injury (Niogi & Mukherjee, 2010), the neuropathology that is thought to be most responsible for persistent cognitive and behavioral impairments that often occur after mTBI (Dadas et al., 2018; Shahim et al., 2016b; Zetterberg & Blennow, 2016). DTI produces two summary metrics, fractional anisotropy (FA) and mean diffusivity (MD), and two orthogonal metrics, axial diffusivity (AD) and radial diffusivity (RD) (Alexander et al., 2007; Sugiyama et al., 2009). FA and MD have been the main focus in the studies of DTI for mTBI and have shown promising results to evaluate DAI in patients with mTBI at sub-acute and chronic phases (Yin et al., 2019). However, DTI is still recognized as a research tool and not yet applicable in the clinical use. To conclude, there is a need for well characterized larger cohort mTBI studies for translating these advanced imaging methodologies into routine clinical practice.

2.1.7 Prognostic tools for traumatic brain injury

2.1.7.1 Outcome measures

The current functional outcome measures, namely, the Glasgow outcome scale (GOS) (Zuercher et al., 2009) and the Glasgow outcome scale extended (GOSE) (Dams-O'Connor et al., 2015) do not include cognitive, psychosocial, health-related quality-of-life, and other patient-reported outcomes. However, since there is no clinically validated prognostic models for the outcome prediction of mTBI, these outcome measures are still mostly used in the outcome evaluation.

2.1.7.1.1 Glasgow Outcome Scale

The GOS is the most common tool for the assessment of functional outcome following TBI, developed by Bryan Jennett and Michael Bond in 1975 (McMillan et al., 2016). This grading consists of five levels where GOS 1 = death and GOS 5 = good recovery (Table 2). The appeal of using the GOS is related to its simplicity, reliability, validity, stability, flexibility of administration (face-to-face, over the telephone and by post), short administration time, cost-free availability, and ease of access (Corral et al., 2007; Dams-O'Connor et al., 2015). Although the GOS is generally assessed at 6 months, further functional outcome trajectories have been reported beyond 12 months (Dams-O'Connor et al., 2015; Forslund et al., 2019b; Puffer et al., 2019).

2.1.7.1.2 Glasgow Outcome Scale Extended

GOS has been criticized for its wide-ranging use of "dependent" state (GOS = 3) and to improve this, the extended GOS (GOSE) was introduced in 1981 (Jennett et al., 1981). It is an 8-levels ordinal scale that is divided into upper and lower levels of good recovery, moderate disability, severe disability, vegetative state, and death. The advantage of the GOSE is to better describe disabilities following TBI, compared to the GOS.

Table 2. Glasgow Outcome Scale (GOS) (Jennett & Bond, 1975)

Score	Description
5	Good recovery, normal life resumed, even with persistent disabilities
4	Independent, disabilities cause to work in a sheltered environment, able to use public transport indepently and manage own personal hygiene
3	Dependent, physical and / or mental disabilities demand daily support
2	Vegetative state, unaware of self and surroundings
1	Death

Table 3: Glasgow Outcome Score Extended (GOSE) (Jennett et al., 1981)

Score	Description	
8	Upper good recovery	Minor psychological or neurological deficits allowed, but normal life resumes with the capasity to work. Level 8: deficits are not disabling; Level 7: minor disabling deficits.
7	Lower good recovery	

6	Upper moderate disability	Some personality or memory deficits and / or disabilities, independent at home, dependent outside. Level 6: able to return to work, possible special arrangement; Level 5: not able to return to work.
5	Lower moderate disability	
4	Upper severe disability	Patient is dependent and demand daily support. Level 4: Patient can be left alone >8 hours; Level 3: Patient needs assistence in an 8 hour timespan.
3	Lower severe disability	
2	Vegetative state	Unaware of self and surroudings, reflex responses only with spontaneous eye-opening periods
1	Death	

2.1.7.1.3 Other tools

RPCSQ/RPQ

Rivermead Post-concussion Symptoms Questionnaire (RPCSQ) consists of 16 items, which denote the most commonly reported symptoms after mTBI. The cognitive (RPCSQ cognitive), emotional (RPCSQ emotional), and physical (RPCSQ somatic) domains are covered by this instrument and has been reported to be effective for diagnosing post-TBI symptoms (King et al., 1995). To calculate the RPCSQ score, the patients are requested to rate the degree to which each item has become more of a problem during the former 24 hours compared to before the TBI. The responses are then rated on a 5-point Likert scale as follows: 0 = not experienced at all; 1 = no more of a problem; 2 = a mild problem; 3 = a moderate problem; and 4 = a severe problem. The RPQ items are then summed to a total score, without ratings of 1.

The Disability Rating Scale (DRS) (Rappaport et al., 1982), the Functional Independence Measure (FIM), supplement Functional Assessment Measure (FAM) (Corrigan et al., 1997) and Patient Health Questionnaire-9 (Fann et al., 2005) are other functional scales for the assessment of functional outcome after brain injury. These outcome scales consist of detail interviews and questions related to the patients' quality of life after TBI.

2.1.7.2 CT scoring systems

To date, there are several different CT scoring systems, which could be used for the evaluation, as well as for the outcome assessment of patients with TBI.

2.1.7.2.1 Marshall CT score

The leading scoring system used is the Marshall scoring system (Marshall et al., 1992). It is generally divided into three basic levels, which include no visible injury, diffuse (3 steps, based on severity) and focal injury. This division is mainly linked to mortality and derived from patients from the Extensive Traumatic Coma Data Bank (TCDB) study (Thelin et al., 2017). Another parameter illustrated in the scale is "Evacuated mass lesion". The authors perceived a rise in unfavorable outcome as diffuse swelling exacerbated, implying it as an indirect sign of increased ICP (Marshall et al., 1992). Later studies have shown a limited capacity of outcome of Marshall CT Score compared to Rotterdam, Stockholm, and Helsinki CT scoring systems (Nelson et al., 2010; Thelin et al., 2017; Yao et al., 2017).

Table 4: Marshall CT classification (Marshall et al., 1992)

Grade	Description
Diffuse injury I	CT scan detects no visible intracranial pathology
Diffuse injury II	Cisterns with midline shift of 0–5 mm and / or lesion densities present; no mixed or high-density lesion >25 cm^3 may include foreign bodies of bone fragments
Diffuse injury III	Cisterns with midline shift of 0–5 mm absent or compressed; no mixed or high-density lesion >25 cm^3
Diffuse injury IV	Midline shift >5 mm; no mixed or high-density lesion >25 cm^3
Evacuated mass lesion (V)	Any lesion surgically evacuated
Non-evacuated mass lesion (VI)	Mixed or high-density lesion >25 cm^3; not surgically evacuated

2.1.7.2.2 Rotterdam CT score

Maas et al. introduced the Rotterdam CT Score by applying the variables of Marshall CT Score (Maas et al., 2005). This system re-evaluates the parameters and ranks them in a scale from 1 (best) to 6 (worst). In this system, tSAH, basal cistern compression and midline shift (>5 mm) are isolated as unfavorable parameters, while the presence of EDH is a more favorable parameter regarding correlation with outcome. EDH has displayed to be a more positive predictor of outcome in greater numbers of patients (Maas et al., 2007). This is due to the conjecture that, if treatment is provided rapidly in these patients, the brain parenchyma will remain comparatively unaffected.

2.1.7.2.3 Stockholm CT score

The Stockholm CT-score was published in 2010 and, in contrast to the other systems, applies a continuous scale to grade severity (Nelson et al., 2010). This scoring system utilizes the magnitude of midline shift as the total of all focal and diffuse lesions. The presence of EDH is also a positive factor. The system has its own categorization of tSAH. In addition to that, it is the only CT scoring system that takes the noticeable DAI on CT Scan into account, as a forecast of poor outcome (Nelson et al., 2010).

2.1.7.2.4 Helsinki CT score

Raj et al. proposed the Helsinki CT score in 2014 to predict the long-term outcome, including unfavorable functional outcome and mortality (Raj et al., 2014). The authors categorized the types of mass lesions (SDH, ICH, and EDH), emphasized the predictive value of IVH, and introduced the suprasellar cisterns (SSCs) status (divided into normal, compressed, or obliterated), into a CT scoring system for the first time (Raj et al., 2014; Thelin et al., 2017). It has been reported that the Helsinki CT score performed better than both the Marshall and Rotterdam CT scoring systems for predicting outcome (Yao et al., 2017; Thelin et al., 2017).

The above-mentioned head CT scoring systems include features of radiologic CDE originally introduced in 2010 (Haacke et al., 2010), which includes controlled terms and standardized definitions to characterize the different types of pathoanatomic lesions encountered on imaging of patients with TBI.

2.1.7.3 Prognostic models

TBI is not a single event, but rather an evolving process and it affects multiple outcome domains (Lingsma et al., 2011, 2015; Maas et al., 2017b; Ponsford et al., 2008; Shahim et al., 2016b; Zetterberg & Blennow, 2016). The outcome of TBI does not only necessarily depend on the quality of acute care, but also on patients and injury characteristics, for example, mechanism of injury, injury severity, presence and severity of extracranial injuries, patient's age, underlying diseases, and socioeconomic condition (Willemse-van Son et al., 2007). Different prognostic models have been developed combining patient and injury characteristics at presentation for the long-term outcome prediction. Most of the prognostic models have been developed for moTBI and sTBI (Maas et al., 2017b). Unfortunately, current approaches to predict long-term outcome after TBI are also limited in their accuracy. There are a range of tools for predicting outcomes after TBI which use a multivariate approach to combine many factors that potentially influence outcomes, e.g. Corticosteroid Randomisation After Significant Head injury (CRASH) model and International Mission for Prognosis and Analysis of Clinical Trials in TBI

(IMPACT) calculator (Perel et al., 2008; Steyerberg et al., 2008). Variables assessed include age, pupillary reactivity, presence of secondary injuries, comorbidities, and brain imaging findings. By combining different predictors, emerging assessment tools have achieved superior predictive value in patients with moTBI or sTBI (Roozenbeek et al., 2012). The complexity of assessing TBI is highlighted by the fact that, even by using combination models, we are able to explain only 35% of the variance outcome after sTBI (Maas et al., 2015). Multivariate models derived from large study populations performed fairly well (80%–90 % accuracy) in predicting mortality or poor outcome (Roozenbeek et al., 2012), but outcomes are much more complex than being dead or severely disabled (Maas et al., 2017b; Menon & Maas, 2015). Notably, death, complete, or incomplete recovery are inappropriate endpoints for the prognostic analysis in patients with mTBI. In the prognostic modelling of patients with mTBI, the utility of the GOS is not certain due to the fact that a considerable number of patients diagnosed with mTBI could have outcome score in the upper segment of the GOS categories, however, might live with disabilities and poor quality of life (Maas et al., 2017b). New clinical tools are needed for evaluation of injuries at both ends of the TBI spectrum. In case of mTBI (as well as TBI in general), factors such as pre-trauma cognitive achievement, personality traits, coping ability, resilience and availability of financial and social support systems have significant predictive value for outcome and quality of life (Lingsma et al., 2015; Ponsford et al., 2008; Ponsford et al., 2014). Research has shown that the current prediction algorithms for mTBI perform poorly in explaining the outcome, also compared to prediction models for more severe cases (Cnossen et al., 2018). A recent large prospective study, analysing the prognostic factors of mTBI, reported that psychological factors in combination with pre-injury mental health problems were the most significant predictors for recovery at 6 months following mTBI (van der Naalt et al., 2017). Another recent pilot study of the TRACK-TBI investigator group developed a prediction model, where the demographic and clinical variables at baseline could predict post-concussion symptoms six months following mTBI (Cnossen et al., 2017). Development and validation of such prognostic models in larger cohorts for mTBI, using sensitive endpoints and the variables involved in TBI pathophysiology, have been strongly recommended (Maas et al., 2017b).

2.2 Blood biomarkers in the assessment of TBI

2.2.1 Definition

A biomarker is defined as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson et al., 2001).

Biomarkers could be proteins, metabolites or other substances such genetic markers, which could act as surrogate markers of injury, and assist in diagnosis, prognosis and quantifying the risk of developing a disease. In other fields of medicine, biomarkers are already playing an important role in the managements of diseases. For example, the protein Troponin-T is used to diagnose myocardial infarction (Hamm et al., 1997).

In an ideal scenario, the following criteria should be met by a biomarker of brain injury (Diaz-Arrastia et al., 2014; Saatman et al., 2008; Shahim et al., 2016a; Wang et al., 2018a; Zetterberg et al., 2013a; Zetterberg & Blennow, 2016):

- Have a high sensitivity and specificity for brain injury
- Help to stratify patients by severity of injury
- Have a fast appearance in obtainable body fluids
- Could deliver valuable insights about complex injury mechanisms
- A passive release from the central nervous system (CNS) without any stimulated active release
- An infinite passage via the blood-brain barrier (BBB)
- Have properly characterized bio-kinetic properties
- Could assist in evaluating the progress of disease as well as in assessing the response to treatment
- Might aid in the prediction of functional outcome

To date, these aforementioned criteria have not been fulfilled by any TBI biomarkers, however, with the development of proteomics, several exist with a great promise to aid the clinicians in the assessment of TBI.

2.2.2 Clinical needs

TBI is a heterogeneous and complex disease. Although, the severity of TBI has been traditionally classified by the GCS, the milder end of "the most complex disease in the most complex organ" reflects a gradually evolving process that arises a diagnostic dilemma (Maas et al., 2017b; Zetterberg et al., 2013a). According to the current literature, a patient diagnosed with mTBI is not free from the risks of developing further intracranial lesions and a subgroup of these patients are prone to develop chronic symptoms (Chenoweth et al., 2018; Cnossen et al., 2018; Maas et al., 2005; McMahon et al., 2014). Inside the busy environment of an ED, the working physician usually discharge a patient with mTBI, who has a negative CT and no significant neurological symptoms (Menon & Maas, 2015; Saboori et al., 2007). Unfortunately, there is no concrete evidence that such a patient will not present with

disabling symptoms in the future but will have complete recovery. In an ideal setting, the patients with mTBI presenting with neurological symptoms require careful observation, imaging, and possibly further follow-ups. However, not all the centers treating a vast burden of TBI have enough resources, as well as manpower, to follow-up the patients for an extended period of time (Kolias et al., 2019; Tropeano et al., 2019). Besides the shortcomings of the current neuroimaging methods in clinical practice for the diagnosis of acute mTBI, disappointingly, there is no unanimous definition of mTBI and the performance of the tested model for the outcome prediction of mTBI is fairly poor (Lingsma et al., 2015; van der Naalt et al., 2017). Considering these issues, it is difficult to diagnose and to stratify the patients with milder range of TBI demanding proper rehabilitation. Concussion, often described synonymously as mTBI, (Sharp & Jenkins, 2015) is common in contact sports (Shahim et al., 2016a). Athletes participating in different contact sports are the vulnerable group to suffer from repeated TBI and there is no robust objective evidence ensuring a safe duration of time for return to play (Shahim et al., 2014). Not only for the acute diagnostics of mTBI, but also for the assessment of moderate to severe TBI, there is no clinically validated objective test that could mirror the multidimensional pathophysiology of TBI. Such a test, assessing TBI, might guide the treating physician to monitor the treatment efficacy and to perform further intervention as early as possible to prevent a permanent damage. Since the collection of CSF samples is invasive and not completely realistic in the case of mTBI, blood biomarkers are preferred. Application of a body fluid biomarker, with high sensitivity, adequate specificity and well-defined bio-kinetic properties would be able to aid the management of TBI in the following ways:

- To better stratify the patients with TBI, which might lead to a precise classification
- To develop an automated point of care device for the cost-effective assessment of TBI
- To avoid unnecessary CT-imaging
- To predict any intracranial lesions as a surrogate marker of imaging
- To be used as a reliable discriminant of CT-positive and CT-negative brain injury in clinical practice
- To identify patients with TBI in case of polytraumas
- To decide the group of patients who might need advanced imaging e.g. MRI
- To identify the patients with DAI in the acute setting – this group consists of the grey zone of TBI, given the fact that there are no exact criteria on

how to radiologically diagnose traumatic axonal injury, and neuropathological examination is the only exact method for diagnosing DAI at this moment

- To construct a proper rehabilitation plan to prevent PCS and to ensure better quality of life
- To explore the return to play duration for the contact sports and, thus, to hinder the process of neurodegenerative disease
- To use as an advanced neuromonitoring tool to evaluate the treatment effects
- To be included in a multifactorial prediction model to provide realistic prognosis information to the patients and their families
- Serves as a cost-effective tool

2.2.3 Current state – biomarkers assessing TBI and their association with neuroimaging

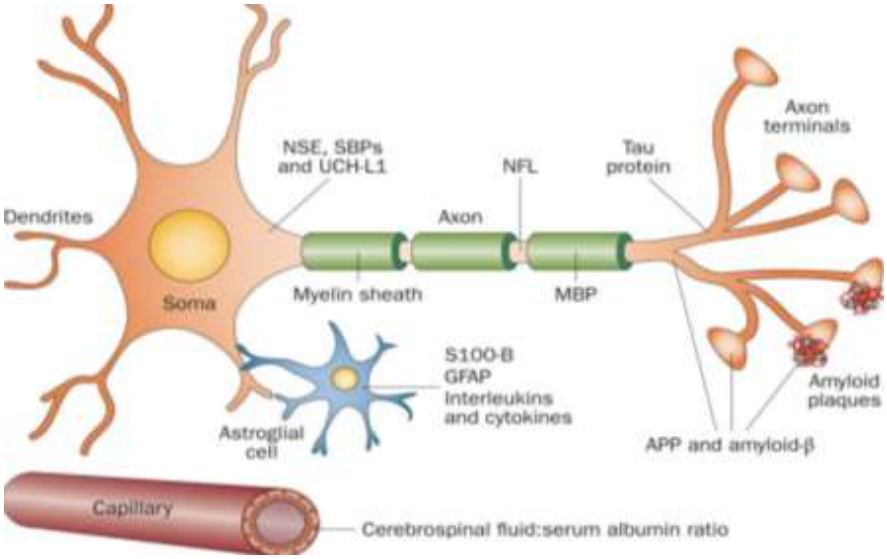


Figure 3. Possible blood biomarkers of traumatic brain injury. (Zetterberg et al., 2013). Reprinted with permission from Nature Reviews Neurology.

2.2.3.1 S100B

S100B is a small protein that belongs to a family of intracellular, calcium-binding proteins (Donato, 2001), that predominantly presents in astrocytes in the CNS (Thelin et al., 2017). Historically, S100B is the most studied biomarker for the assessment of TBI. The latest published Scandinavian guidelines uses S100B for the screening of mTBI patients to select those who need cranial CT (Bogoslovsky et al., 2016; Undén et al., 2013). It was recommended that adult patients after mTBI, with a GCS of 14 and no risk factors (anticoagulant therapy or coagulation disorders, post-traumatic seizures, clinical signs of depressed or basal skull fracture, focal neurological deficits) or a GCS of 15 with loss of consciousness or repeated (≥2 times) vomiting and no other risk factors, will be sampled for analysis of S100B if less than 6 hours have elapsed following trauma. The recommendations suggest that if S100B is less than 0.10 μg/L, the patient might be discharged without a CT (Ananthaharan et al., 2018). Despite having excellent NPV for CT, the clinical utility of S100B in TBI is limited due to less brain specificity (Dadas et al., 2018; Thelin et al., 2017). Interestingly, S100B released from extracerebral origin appears to have a faster clearance than S100B released from the CNS (Thelin et al., 2017).

2.2.3.2 GFAP

GFAP, a cytoskeletal monomeric filament protein (Eng et al., 1971), present in astrocytes located both in white and gray brain matter (Yoon et al., 2017), has performed as a more reliable biomarker for a focal than for a diffuse injury (Mondello et al., 2011; Papa et al., 2012). According to the previous studies, the admission blood levels of GFAP were correlated with both the initial GCS scores and brain imaging findings (Luoto et al., 2017). It has been also reported that serum levels of GFAP were increased in those patients with clinically diagnosed mTBI with abnormal CT compared with those patients with mTBI with normal CT (Diaz-Arrastia et al., 2014; Wang et al., 2018a). Additionally, it has been found that the patients with axonal injury, identified by MRI, and the patients who required neurosurgical intervention followed by TBI, had significantly higher levels of GFAP (Diaz-Arrastia et al., 2014; Papa et al., 2012). Although GFAP is not entirely brain specific (Hainfellner et al., 2001; Jessen et al., 1984; Middeldorp & Hol, 2011), the levels of GFAP could discriminate both patients with mTBI and moTBI from healthy controls and from patients with orthopedic injury without TBI (Papa et al., 2012). It is shown that a slight increase in GFAP levels have better ability to diagnose TBI accurately compared to the levels of S100B, especially if patients have orthopedic injuries, since chondrocytes release S100B (Zetterberg & Blennow, 2016). Notably, GFAP has been reported as a significant predictor of CT-positive brain damage having a good sensitivity and specificity in acute TBI (Luoto et al., 2017). Current

ongoing large-scale initiatives, CENTER-TBI, and TRACK-TBI, are important in verifying the potential of GFAP as a marker in acute TBI triage. Recently, the TRACK-TBI investigators reported that the blood levels of GFAP within 24 hours of injury has the significant discriminative ability to identify MRI abnormalities in patients with normal CT findings (Yue et al., 2019). From a practical point of view, a rapid capillary blood-based GFAP screening test would be beneficial for patient management in a pre-hospital environment.

2.2.3.3 UCH-L1

UCH-L1 is involved in either adding or removing ubiquitin from proteins targeted for metabolism, abnormal proteins, and proteins damaged by oxidation (Liu et al., 2002). Given that UCH-L1 is produced by a neighbouring cell type of astrocytes, namely neurons, it is considered a suitable counterpart for GFAP in TBI diagnostics (Diaz-Arrastia et al., 2014; Papa et al., 2016b). UCH-L1 is found to be more abundant after diffuse than after focal injury (Papa et al., 2012). The superior sensitivity and specificity for diagnosing TBI was obtained when GFAP was combined with UCH-L1, thus supporting the idea that a combination of biomarkers may be superior compared to using each alone for the diagnosis and prognosis of TBI (Bogoslovsky et al., 2016). Patients with mTBI had higher levels of serum UCH-L1 compared to non-brain-injured patients with orthopedic traumas and to healthy controls. It is important to note that UCH-L1 was able to discriminate between CT-positive and CT-negative mTBI and between healthy controls and patients with full spectrum of TBI (Dadas et al., 2018; Diaz-Arrastia et al., 2014; Papa et al., 2016b; Papa et al., 2012). However, several groups have presented contradictory results in which UCH-L1 levels were unable to distinguish healthy controls from patients with mTBI when different immunoassays were used (Dadas et al., 2018; Wang et al., 2018a). Another contributing factor for the variations of such results could be that there is no standard-general definition of mTBI, which most possibly create the methodological dissimilarities among the studies.

2.2.3.4 Tau

Tau, a microtubule-associated protein that is located in the axons of CNS neurons, serves as a structural element in the axonal cytoskeleton (Olivera et al., 2015; Rubenstein et al., 2015). Though tau could be mostly found in the brain, extracranial sources exist, for example, liver, kidney and testis (Morris et al., 2011). It is identified as a neurodegenerative biomarker, (Jack et al., 2019; Kim et al., 2018) and has been widely studied for the development of axonal pathology following TBI (Neselius et al., 2013). Phosphorylation of tau is a normal event in healthy neurons,

but hyperphosphorylation and aggregation into neurofibrillary tangles is a characteristic of AD and chronic traumatic encephalopathy (CTE) (Zetterberg & Blennow, 2016). However, it has been studied that many people with the neuropathology of CTE do not appear to have a progressive tauopathy (Iverson et al., 2018). In addition, it has been recently demonstrated that CTE pathology could be present in people who did not experience sub-concussive blows to the head, or multiple concussions (Iverson et al., 2019). It has been widely studied that the elevated levels of plasma T-tau are correlated with the outcome of repeated mTBI or concussion (Neselius et al., 2013; Shahim et al., 2014). In case of sTBI, serum tau and admission CSF tau levels were reported as significant outcome predictors (Liliang et al., 2010). Previous studies, using the traditional enzyme-linked immunosorbent assay (ELISA) method, reported that T-tau was unable to differentiate CT-positive and CT-negative mTBI groups (Zetterberg et al., 2013a). These findings are reasonable, since traditional immunoassay methods are not sensitive enough to analyse especially the low levels of tau in blood (Zetterberg & Blennow, 2016). Lately, using the ultrasensitive Simoa platform, (Kuhle et al., 2016) it has been studied that acute plasma hyperphosphorylated tau protein (P-tau) is a more sensitive biomarker compared to T-tau for the outcome prediction of TBI (Rubenstein et al., 2017). Furthermore, using this new assay method, it has been examined that the acute levels of plasma tau could differentiate patients with complicated mTBI from controls (Zetterberg & Blennow, 2016). Significantly elevated levels of plasma tau were also reported in case of ice hockey players compared to their pre-season levels (Shahim et al., 2014). However, to date, no recent large studies could find that admission levels of plasma T-tau were able to differentiate incomplete and complete recovery in case of single and uncomplicated mTBI.

2.2.3.5 NF-L

NF-L protein is a relatively new and less studied blood biomarker for traumatic axonal injury (Kuhle et al., 2016; Wilson et al., 2016). NF-L is mainly expressed in the long myelinated white matter axons (Shahim et al., 2014; Shahim et al., 2016; Zetterberg et al., 2013b) and a significant relationship between DTI of DAI following sTBI and the levels of NF-L in the CSF has been reported, indicating that the levels of NF-L could predict the degree of axonal injury as well as the outcome following TBI (Shahim et al., 2016). The elevated levels of plasma NF-L in case of mTBI have been reported for contact sports athletes, although such studies did not report the correlation between the levels of NF-L and white matter integrity due to the unavailability of DTI data (Shahim et al., 2017; Shahim et al., 2016a). Significant elevation in the serum levels of NF-L have been reported after TBI, where the levels

steadily increased up to 10–12 post-injury days. In addition, the admission levels as well as the levels of several time-points were correlated with the outcome of TBI (Skillbäck et al., 2014). It has been shown that the levels of NF-L were significantly elevated for contact sports athletes, for example, professional hockey players suffering from symptoms following repetitive mTBI (Shahim et al., 2017). Two groups of contact sports players could be differentiated using the levels of CSF NF-L, where one group had rapidly resolving symptoms and the other group continued to have prolonged concussion symptoms (Shahim et al., 2016a).

2.2.3.6 Aβ40 and Aβ42

Aβ40 (Tsitsopoulos & Marklund, 2013a) and Aβ42 (Johnson et al., 2010; Tsitsopoulos & Marklund, 2013b) have been studied as potential biomarkers of axonal damage in TBI (Johnson et al., 2013b; Marklund et al., 2014), since they reflect amyloidogenic amyloid precursor protein (APP) metabolism. A histologic hallmark of AD is Aβ pathology, primarily consisting of aggregated Aβ42 peptides, and TBI has been suggested to be one of the risk factors for AD (Ramos-Cejudo et al., 2018a). Studies have reported that Aβ pathology (amyloid plaques) was found in boxers having dementia pugilistica (Roberts et al., 1990) and in a proportion of other contact sport athletes having CTE (Blennow & Nellgård, 2004). Although ventricular CSF levels of Aβ40 and Aβ42 were elevated during the first week after sTBI (Olsson et al., 2004), no changes in Aβ40 or Aβ42 were reported in mTBI, where CSF samples were collected by lumbar puncture (Neselius et al., 2013). However, for repetitive mTBI, post-injury subjective symptoms were associated with the reduction of CSF levels of Aβ40 and Aβ42 (Olsson et al., 2004; Tsitsopoulos & Marklund, 2013b).

2.2.3.7 Other biomarkers

Besides the abovementioned body fluid biomarkers, neuron-specific enolase (NSE), interleukin 10 (IL-10), heart-fatty acid binding protein (H-FABP) etc. have been also studied recently for the different severities of TBI and provided promising results (Dadas et al., 2018; Mondello et al., 2018; Wang et al., 2018a). However, most of those pilot study findings using small sample sizes need to be replicated in larger cohorts.

2.2.4 Kinetics

Contradictory results have been reported on the kinetics of the blood biomarkers. The following table summarizes the half-life of the mostly studied biomarkers for mTBI.

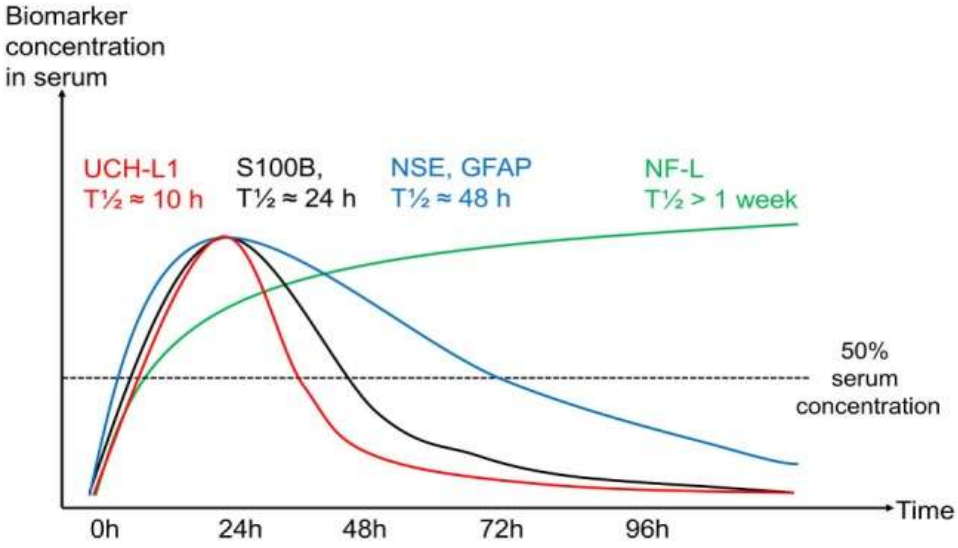


Figure 4. Kinetics of the blood biomarkers for traumatic brain injury (Thelin et al., 2017). Reprinted with permission from Frontiers in Neurology.

Table 5. Kinetics of blood biomarkers (Thelin et al., 2017).

Biomarker	Suggested serum half-life (depending on TBI severity)
S100B	2–24 hours (2–6 hours in case of mTBI)
GFAP	24–48 hours
UCH-L1	10 hours
TAU	10 hours, second peak after 36 hours
NF-L	>7 days
AB40 AND AB42	>24 hours

2.3 Blood-brain barrier and the glymphatic system

The mechanism of the passage of the blood biomarkers from the brain to blood is still not completely understood. The BBB disruption and the currently discovered glymphatic system are the mostly studied routes.

BBB

BBB, abutting the vessels of the brain, composed of tightly connected endothelial cells and astrocytes, connected by tight junctions, becomes disintegrated in TBI (Dadas & Janigro, 2018). The BBB manages to create a tightly regulated environment in the CNS by controlling ingress of immune cells and blood-borne metabolites. On top of that it controls the cerebral environment by necessary influx of vital substrates and efflux of waste materials. In BBB transportation, the astrocytic podocytes, along with microglial cells and basal cell membrane of the endothelium, are indispensable as it acts as a bridge between the brain parenchyma and micro vessels. Breakdown of functional integrity of the BBB due to injury leads to functional changes in the pericontusional area and raised permeability to high molecular weight protein such as albumin (Thelin et al., 2017). This is primarily due to functional changes. Rat models of TBI have shown an increased permeability 4–6 hours after injury along with a secondary peak after 3 days. However, in humans, elevated albumin quota, the ratio CSF:serum, is observed up to a week following TBI. The disruption of BBB also results in edema development (Dadas & Janigro, 2018).

The glymphatic system

The glymphatic system is a recently discovered route that connects the interstitial fluid of the brain, CSF, and venous outflow. It is so-named due to its link between the glial cells and aquaporin-4 dependant perivascular pathways. Thus, it is believed to act as a lymphatic drainage from the brain (Sullan et al., 2018). It has been proposed that this para-arterial influx of CSF through brain extracellular fluid to a paravenous outflow, is the principal path of efflux of cerebral protein debris, driven by arterial pulsations. TBI demonstrates a loss in perivascular polarization of aquaporin-4 up to a period of 28 days. This results in a reduced outflow of tau proteins in TBI (Plog et al., 2015). A recent study reveals the fact that the glymphatic system acts unaided from the BBB integrity following brain injury. It further shows that proteins of cerebral origin mainly drain through the glymphatic system from the injured brain (Piantino et al., 2019; Sullan et al., 2018).

3 Aims of the study

The specific aims of this study were:

- To investigate the levels of GFAP and UCH-L1 in patients with acute orthopedic injuries without CNS involvement, and to relate them to the type of extracranial injury, head MRI findings, and the levels of GFAP and UCH-L1 in patients with CT-negative mTBI. Following this, our aim was to explore the performance of GFAP and UCH-L1 in discriminating patients with orthopedic trauma and CT-negative mTBI.
- To correlate the levels of GFAP and NF-L during the first 24 hours after admission with outcome in patients with mTBI to find out their potential for clinical use in assessing mTBI.
- To investigate if the admission levels of T-tau and Aβ40 and Aβ42 correlate with outcome in patients with mTBI.

4 Materials and methods

The studies described below were part of the EU-funded TBIcare project (Evidence-based Diagnostic and Treatment Planning Solution for Traumatic Brain Injuries).

4.1 Study population

Study I

This prospective two-center study was conducted at the Turku University Hospital (Finland) and the Addenbrooke's Hospital Cambridge (United Kingdom). Patients with TBI of all severities as well as a control group of patients with acute orthopedic injuries were recruited in the project.

In this study, all 73 patients with acute orthopedic trauma and those 93 patients with mTBI who had head CT with no pathological brain parenchymal findings, were analysed. The mTBI group included three patients with skull base fracture, but CT-negative concomitant parenchymal findings. The orthopedic group's inclusion criteria comprised of age ≥16 years, acute orthopedic nontrivial injury, or injuries with the absence of acute CNS involvement. Any suspected acute TBI (head injury signs, any suspected TBI signs at the time of injury, possible TBI symptoms), prior brain disease or TBI, polytrauma requiring intensive care, or trivial injuries not requiring emergency measures or follow-up, were excluded. In the case of sustained brain injury that fulfilled the American Congress of Rehabilitation Medicine criteria for TBI (Sharp & Jenkins, 2015) and that patient's lowest recorded GCS was ≥13, mTBI was diagnosed. However, excluded from the mTBI group were patients with GCS of 13 and concomitant multi-trauma requiring intensive care or deteriorating patients with GCS of 13. Also, the patient was excluded if suspected that the signs of TBI could be caused by confounders (inebriation, medications). The patients did not receive anaesthetics before the initial sample was obtained, except for one patient who was sedated with propofol before the blood samples were obtained, because of an orthopedic operation following orthopedic injury. No patients with elevated GFAP and / or UCH-L1 levels went under general anaesthesia.

Head MR imaging for the control group

As part of the TBIcare study protocol, within the first 4 weeks after the injury, majority of the patients with orthopedic trauma (n = 52 / 73) underwent head MRI, and again at the follow-up visit 3–10 months after the injury. If there were any doubt of clinical stability, safety, and contraindications (e.g. because of internal fixation), then a treating clinician outside the study personnel made the decision whether MRI could be performed or not. FLAIR, diffusion-weighted imaging (DWI), T2, T13D, DTI, and SWI were included in the 3T MRI sequences.

Studies II and III

For these studies, 107 patients with mTBI (GCS ≥13) with available blood samples within 24 hours upon arrival at the ED of Turku University Hospital, Finland, were recruited.

Patients included in these studies fulfilled the following criteria: GCS ≥13, age ≥18 years, clinically diagnosed TBI and acute head CT indications according to NICE criteria (National Institute for Health and Clinical Excellence, 2007) . Patients with age <18 years, chronic subdural hematoma, penetrating or blast-induced injury, suspected TBI or TBI not requiring head CT, pre-existing brain disease causing the inability to live independently, >2 weeks from injury, reasons preventing follow-up visits (not living in the district), inability to speak native language, or no received consent were excluded from the studies.

Notably, patients with mTBI with available GFAP and NF-L were used for study II and the patients with mTBI with available T-tau, Aβ40, and Aβ42 were used for study III, all obtained within 24 hours after arrival to the ED.

4.2 Analysis of the blood biomarkers

4.2.1 Analysis of GFAP and UCH-L1

Study I

The first blood samples for GFAP and UCH-L1 were obtained after arrival to the ED. The following blood samples were collected on days 1, 2, 3, and when available on day 7 after being admitted. Similar samples were also collected 3–10 months after the injury on the follow-up visit. First samples were marked as day 1 samples if the patient was recruited more than 24 hours after admission, since patients were not recruited during the night. Admission due to prolonged symptoms in numerous patients with mTBI made it possible to obtain longitudinal samples from these

patients. After being centrifuged for 10 minutes at 10 000 rpm at 4°C, the samples were frozen immediately for further analysis at –70°C.

Randox Laboratories Ltd (Crumlin, County Antrim, United Kingdom) conducted proteomic analysis with Randox Biochip technology. In this technology, an array of discrete test regions of immobilized antibodies specific to different cerebral immunoassays is contained in a solid-state device. The samples were individually prepared. A rise in the emitted chemiluminescent signal due to increasing binding of antibody labelled with horseradish peroxidase, because of higher levels in a specimen. Digital imaging technology was used to detect the light signal generated on a biochip from all the test regions which was compared to a stored calibration curve. The calibration curve was used to calculate the presence of concentration of analyst in the sample. Simultaneous quantitative testing for UCH-L1 and GFAP was conducted through the Evidence Investigator Cerebral Custom Array IV (Randox Laboratories Ltd).

The lower limit of quantification (LLoQ) for GFAP was 0.16 ng/mL and for UCH-L1 was 0.3 ng/mL. The upper limits were 100 ng/mL and 50 ng/mL for GFAP and UCH-L1, respectively. The coefficient of variation was 3%–4% for the GFAP assay and 6%–7% for the UCH-L1 assay. A value of zero were assigned to samples where biomarker levels were not detectable.

4.2.2 Analysis of GFAP and NF-L

Study II

An HD-1 Simoa instrument with Human Neurology 4-Plex A assay (N4PA) were used to measure plasma GFAP and NF-L levels, according to manufacturer instructions (Quanterix, Lexington, MA). Board-certified laboratory technicians, blinded to clinical data, performed one round of experiments by means of one reagents batch. The coefficients of variations for GFAP were 3.1% at 113 pg/mL and 3.8% at 86 pg/mL, and for NF-L were 4.4% at 13.9 pg/mL and 6.1% at 7.1 pg/mL, as a result of quality control (QC) sample analyses in each run.

4.2.3 Analysis of T-tau, Aβ40, and Aβ42

Study III

Like the above-mentioned analysis, this analysis also used the Human N4PA on a Simoa instrument to analyse the plasma T-tau, again following manufacturer's instructions (Quanterix, Lexington, MA). The analysis for T-tau showed that the

calibration range was 0.136 pg/mL to 112 pg/mL, the lower limit of detection (LLoD) was 0.024 pg/mL, whereas the LLoQ was 0.053 pg/mL. A duplex Simoa immunoassay (Quanterix, Lexington, MA, USA) was used to measure the concentrations of plasma Aβ40 and Aβ42. The analysis for Aβ40 has shown a calibration range between 0 pg/mL to 90.0 pg/mL, with LLoD of 0.045 pg/mL and LLoQ of 0.142 pg/mL. For Aβ42, a calibration range between 0 pg/mL to 11.0 pg/mL, with LLoD of 0.142 pg/mL and LLoQ of 0.69 pg/ml, was shown. Blinded to clinical data, the board-certified laboratory technicians performed the measurements. Importantly, no samples were below the LLoDs and LLoQs.

Time elapse (Studies II and III)

The interval between the time of the injury and the time when the first sample was taken was defined as time elapse. Be noted that the samples were not always drawn within 24 hours from the injury, even though they were obtained within 24 hours of admission. In the multiparameter prognostic panel analysis, time elapse was used as a dichotomous variable, exceeding 24 hours and less than 24 hours.

4.3 TBI severity and outcome grading

Studies II and III

The emergency physician assessed the patients' lowest GCS scores in the ED, if not already assessed by paramedics during transport or at the accident scene. The lowest GCS score of each patient was used to grade the severity of TBI (Takala et al., 2016). It is important to note that the severity grading in this thesis is not based on the Finnish National Current Care Guidelines (Käypä hoito) or WHO guidelines – it is not a clinical diagnosis. This is due to the fact that in the biomarker literature, most of the studies have used only admission GCS for severity grading. The Injury Severity Score (ISS) (Baker et al., 1974) was used to assess the overall injury severity of the patients. The Rivermead method (King et al., 1997b) was used at the outcome visit to assess the duration of PTA. Marshall et al. proposed a descriptive system (Marshall et al., 1992), which was used for the analysis of CT scans, where normal CT corresponds to class I, diffuse injuries to classes II–IV, and mass lesions to classes V and VI. Patients were divided into CT-positive and CT-negative groups according to this classification.

4.4 Outcome

Study II

The GOSE was used to assess the outcome 6–12 months after the injury (Wilson et al., 1998). Outcomes were categorised into four categories: unfavorable outcome (GOSE 1–4), favorable outcome (GOSE 5–8), incomplete recovery (GOSE <8), and complete recovery (GOSE 8). The severity and presence of mTBI-related symptoms were assessed by using the RPCSQ (King et al., 1995).

Study III

Similar to study II, the GOSE was used to assess the outcome 6–12 months after the injury, but in this study only two outcome categories were specified: incomplete recovery (GOSE <8) and complete recovery (GOSE 8). The RPCSQ was also used to assess the severity of present mTBI symptoms.

One specific experienced neurologist at the Turku Brain Injury Centre evaluated every patient.

4.5 Ethics declarations

Ethics approval and consent to participate

For study I, the ethical review board of Cambridgeshire 2 Research Ethics, the Norfolk Research Ethics Committee, and Hospital District of South-West Finland approved the study protocol.

For studies II and III, the study protocol was approved by the Hospital District of South-West Finland's Ethical review board.

For all the above-mentioned studies, verbal and written information were given to patients or their next of kin and written informed consent was obtained.

4.6 Statistical analyses

Study I

Through visual inspection of histograms and by using the Kolmogorov-Smirnov test, the normality of GFAP, UCH-L1, age and injury severity score were assessed. Because these variables were not normally distributed, further analyses were

performed by using nonparametric methods. The χ^2 test for gender and the Mann-Whitney U test for age and injury severity score were used to study background variables differences between patients with mTBI and orthopedic injury patients. The correlation between GFAP and UCH-L1 on different days in orthopedic injury patients, was assessed by Spearman correlation coefficient. Mann-Whitney U test for gender and Spearman correlation coefficients for age and injury severity score, were used to assess background variables and biomarkers' association on arrival.

Diagnostic thresholds were defined based on values obtained in extracranial orthopedic injury patients, rather than values from healthy subjects, to distinguish CT-negative mTBI from extracranial injury, given that the ability to distinguish of these protein biomarkers was explored. To provide a basis in the orthopedic injury population, undertaking more detailed exploratory analysis in subjects with high levels of GFAP and UCH-L1, an individual biomarker's cut-off value was set at the 95th percentile, which is theoretically determined based on a previous publication (Biberthaler et al., 2006).

In the orthopedic injury population, MRI findings were also analysed. MRI findings were classified into three categories: normal MRI findings, abnormal MRI findings, and MRI not done. The Kruskal-Wallis test were used to study the differences in biomarkers among above-mentioned categories. The differences between the levels of GFAP and UCH-L1 in mTBI patients and othopedic injury patients were studied using the Mann-Whitney U test. The receiver operating characteristics curve (ROC) and the area under the ROC curve (AUC) was used to evaluate the differentiation ability of the biomarkers for these two patient groups. The Wilcoxon signed rank test was used to compare GFAP and UCH-L1 levels on day 1 and at the follow-up. The Kruskal-Wallis test was used to study the differences in biomarker levels among patients with CT-negative mTBI and concomitant orthopedic injuries, isolated CT-negative mTBI, and orthopedic injuries.

Matlab R2012b (MathWorks, Natick, MA) and IBM SPSS Statistics 22 (IBM Corp, New York) were used for data analysis.

Study II

Subjects' demographics are presented as mean ± standard deviation (SD). Visual inspection of data histograms and the Kolmogorov-Smirnov test were used to assess the normality of distribution of biomarkers levels. In the statistical analyses nonparametric tests were used, since the GFAP and NF-L levels were not normally distributed. Data are presented as interquartile range (IQR) and medians. The Spearman rank correlation coefficient was used to analyse correlations between outcomes and biomarkers levels. The correlation between biomarker levels with age and gender was evaluated by means of the Pearson's correlation coefficient. The

comparison of biomarker levels between outcome groups was conducted by the Mann-Whitney U test. The prognostic ability of the biomarkers was studied through multi-variate logistic regression analysis, for dichotomized outcomes prediction.

The following variables were included in the regression analysis: levels of GFAP and NF-L, lowest GCS score, pupillary reactivity, time elapse, age, PTA, ISS, and Marshall score. Categorical variables were pupil reactivity and Marshall score. In multi-variate logistic regression, the reference categories were reactive pupils and Marshall class I denoting CT-negative finding. In the multi-variate logistic regression models, GFAP and NF-L were used together in the same models and independently with other variables. The prognostic ability of the biomarkers was evaluated using AUC. AUC of 0.5–0.7 was considered poor, AUC of 0.7–0.8 was considered adequate, and AUC of 0.8–1.0 was considered very good (Zetterberg et al., 2013b). A p value <0.05 was considered statistically significant. For the prediction of dichotomized outcomes, by using the ROC curve at the sensitivity >90%, cut-off values were defined. In the whole study population, the correlation of GFAP and NF-L levels was assessed by Pearson's correlation coefficient.

Furthermore, the correlation of GFAP and NF-L in the four different outcome groups were measured. These groups, as previously defined, are unfavourable, favourable, incomplete, and complete outcome. MATLAB R2015b (Math Works, Natick, MA) and IBM SPSS Statistics 22 (IBM Corp, Armonk, NY) were used for data analysis. In addition, PanelomiX software (Robin et al., 2013) was used to generate panels of biomarkers, based on their best cut-off values. These panels of biomarkers were used to differentiate unfavourable and favourable outcome, as well as incomplete and complete recovery, through assessing the performance of combining the GFAP and NF-L biomarkers. To achieve a sensitivity of >90%, cut-off values were selected.

Study III

Demographics data are presented as mean ± SD or percentages. The distribution normality was assessed through visual inspection of data histograms and by using the Kolmogorov-Smirnov test. Data are presented as medians and IQR. In the statistical analyses nonparametric tests were used, since the T-tau, Aβ40 and Aβ42 levels were not normally distributed. The correlations between the outcomes and the levels of biomarkers were assessed by the Spearman rank correlation coefficient. Pearson's and Spearman rank correlation were used for analysis of the correlations of age and gender with biomarker levels, respectively. The correlation between the amyloids and the levels of T-tau was also assessed by using the Spearman correlation coefficient, in the incomplete and complete recovery groups, as well as in the whole cohort. The biomarker levels between the different outcome groups were compared

using the Mann-Whitney U test. The investigation of a biomarker's independent predictive power for outcome beyond the clinical predictors, either alone or in combination with other biomarkers, was performed using a multivariate logistic regression analysis. To examine whether a biomarker had better predictive ability in combination with other biomarkers, compared to any biomarker alone, a biomarker panel analysis was used. The following variables were included in the regression analysis: levels of T-tau, Aβ40 and Aβ42, time elapse, worse recorded GCS score, PTA duration, age, sex, educational level, ISS, and Marshall CT classification. Division of educational level was higher level professional and academic, lower level professional, and basic school education. The following variables were considered as categorical variables: time elapse, sex, educational level, and Marshall CT classification. In multivariate logistic regression, the reference categories were: time elapse >24 hours, female sex, basic school education, and Marshall class I (denoting CT-negative finding). In the analysis, all other variables were numerical variables. In the multivariate logistic regression models, T-tau, Aβ40, and Aβ42 were used independently and together with other variables in the same models. AUC was also used to study the biomarkers' prognostic ability. AUC of 0.5–0.7 was considered poor, AUC of 0.7–0.8 was considered adequate, and AUC of 0.8–1.0 was considered very good (Zetterberg et al., 2013b), and a p value of <0.05 was statistically significant. The ROC curve at clinically compatible sensitivity >90% was used to define cut-off values for dichotomized outcomes prediction. MATLAB R2016b (Math Works, Natick, Massachusetts) and IBM SPSS Statistics 22 (IBM Corp, Armonk, New York) were used for data analysis.

As used in Study II, PanelomiX toolbox (Robin et al., 2013) was also used to form a multiparameter prognostic panel using clinical information and the levels of T-tau, Aβ40, and Aβ42 for the best prediction of incomplete recovery. The clinical information included GCS, GOSE, time elapse, age, sex, educational levels, duration of PTA, ISS, and CT findings. To ensure a sensitivity of >90%, cut-off values were selected. Focusing only on a portion of the ROC curve, the partial AUC (pAUC) was used for the prognostic panels as a local comparative approach (Turck et al., 2010).

4.6.1 Panels of biomarkers analyses

PanelomiX (Studies II and III)

As mentioned in the statistical analysis sections of studies II and III, medical biomarkers can be combined into panels to increase their predictive power. However, the increased application of panels and their implementation into clinical practice are hampered, because of the lack of implementing rigorous validation standards and interpretable results generated by ready-to-use tools.

PanelomiX, the computational toolbox, uses a method called iterative combination of biomarkers and thresholds. Thresholds that provide optimal classification performance are selected to combine clinical scores and biomarkers in this method (Robin et al., 2013). PanelomiX uses the random forest method to select a subset of parameters and thresholds to accelerate the calculation for a big quantity of biomarkers. ROC analysis and cross-validation are used to analyse the performance and robustness of the panels (Turck et al., 2010).

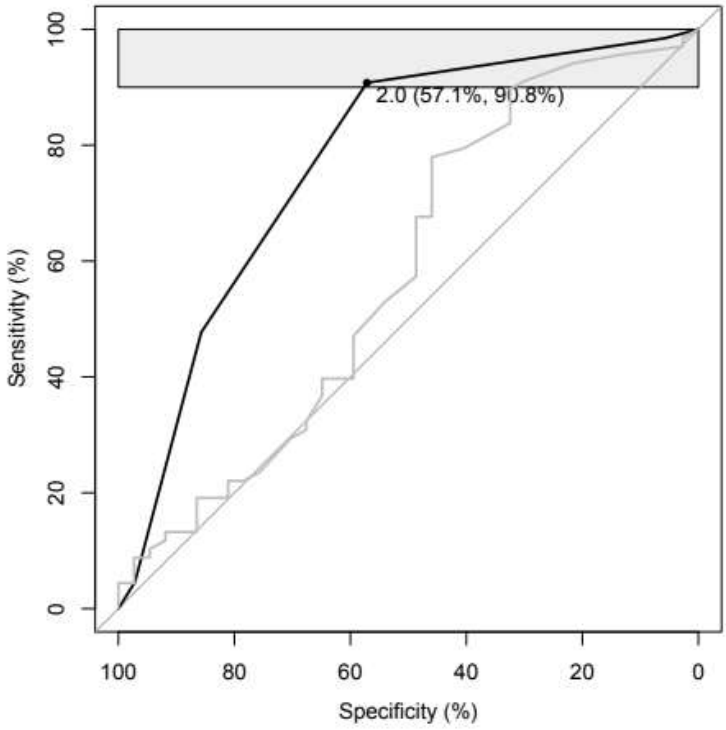


Figure 5. Combination of biomarkers using PanelomiX toolbox.

To conclude, the PanelomiX software integrate biomarkers and examines the performance of panels to improve patient stratification, compared to single markers or additional classifiers.

5 Results

5.1 GFAP and UCH-L1 in orthopedic injury

5.1.1 Study subjects

Table 6 illustrates the injury types, mechanisms of injuries, ISS, as well as the demographic features of the two study groups. In the orthopedic injury cohort (n = 73), the average patient age was 46.7 ± 18.3 years (mean ± standard deviation) and the majority of the patients were female (55%). In this cohort, the most frequent injuries were ankle fractures, lower or upper extremity soft tissue contusions and bruises, and wrist fractures (29%, 14%, and 10%, respectively). In the CT-negative mTBI subgroup, the mean age of the patients was 42.1 ± 18.6 years, and the majority of the patients were male (63%). A total of 43 patients (47%) in the mTBI subgroup had concomitant orthopedic injury.

Table 6. Demographics of the subjects and extracranial injuries (From the original publication I)

	Orthopedic trauma	**mTBI**	**p value**
N	73	93	
Age	46.7 ± 18.3	42.1 ± 18.6	0.084
Gender			0.019
Male	33 (45.2%)	59 (63.4%)	
Female	40 (54.8%)	34 (36.6%)	
Mechanism of injury			
Ground level fall	47 (64.4%)	34 (36.6%)	
Head against object	0 (0.0%)	39 (41.9%)	
Acceleration / deceleration	5 (6.8%)	21 (22.6%)	
Fall from height	3 (4.1%)	20 (21.5%)	
Direct impact blow to head	0 (0.0%)	10 (10.8%)	
Unknown/other	7 (9.6%)	2 (2.2%)	
Missing	6 (8.2%)	1 (1.1%)	
Crush	5 (6.8%)	0 (0.0%)	

Violence	0 (0.0%)	5 (5.4%)	
Injury severity score	3.9 ± 3.4	7.1 ± 6.6	0.002
Extracerebral injury			
No extracerebral trauma	0 (0.0%)	49 (52.7%)	
Other superficial injuries of upper or lower extremity	4 (5.5%)	8 (8.6%)	
Ankle fracture (complex, bi- or trimalleoli)	11 (15.1%)	0 (0.0%)	
Ankle fracture (simple)	10 (13.7%)	1 (1.1%)	
Upper or lower extremity contusion	10 (13.7%)	0 (0.0%)	
Superficial injury of head (scalp, ears)	0 (0.0%)	10 (10.8%)	
Spinal fracture (without spinal cord injury)	0 (0.0%)	9 (9.7%)	
Forearm fracture (elbow included)	5 (6.8%)	4 (4.3%)	
Wrist fracture (radial or ulnar bone)	7 (9.6%)	1 (1.1%)	
Humerus fracture	6 (8.2%)	0 (0.0%)	
Maxillary or orbital fracture	0 (0.0%)	6 (6.5%)	
Hand fracture (fingers included)	3 (4.1%)	2 (2.2%)	
Superficial injuries involving multiple body regions	5 (6.8%)	0 (0.0%)	
Rib fracture (one, multiple and flail chest)	1 (1.4%)	4 (4.3%)	
Open wound of head (scalp, ears)	0 (0.0%)	4 (4.3%)	
Clavicle fracture (all types)	1 (1.4%)	3 (3.2%)	
Injury of muscle and / or tendon of upper or lower extremity	3 (4.1%)	1 (1.1%)	
Skull base or calvarial fracture	0 (0.0%)	3 (3.2%)	
Hip fracture	3 (4.1%)	0 (0.0%)	
Pelvic fracture (simple, one location)	1 (1.4%)	2 (2.2%)	
Shoulder or elbow luxation	2 (2.7%)	1 (1.1%)	
Knee fracture (femur, tibia, and fibula included)	2 (2.7%)	0 (0.0%)	
Crushing injury of wrist and hand	2 (2.7%)	0 (0.0%)	
Dislocation, sprain and strain of joints and ligaments at neck level (excl: rupture or displacement (nontraumatic) of cervical intervertebral disc)	0 (0.0%)	2 (2.2%)	
Renal contusion or laceration	0 (0.0%)	2 (2.2%)	
Pelvic fracture (complex)	1 (1.4%)	1 (1.1%)	
Foot or toe fracture	1 (1.4%)	1 (1.1%)	
Pelvic, abdominal, or dorsal muscle injury	1 (1.4%)	0 (0.0%)	
Thoracic contusion	0 (0.0%)	1 (1.1%)	
Dislocation, sprain and strain of joints and ligaments of head	0 (0.0%)	1 (1.1%)	
Fracture of shaft of femur	0 (0.0%)	1 (1.1%)	

Values are expressed as mean ± standard deviation or number of subjects (percentage of subjects); N: number of subjects; Percentages do not sum up to 100% because some subjects had several mechanisms or several extracerebral injuries. Orthopedic, patients with orthopedic injury; mTBI, patients with mild traumatic brain injury

5.1.2 Levels of GFAP and UCH-L1

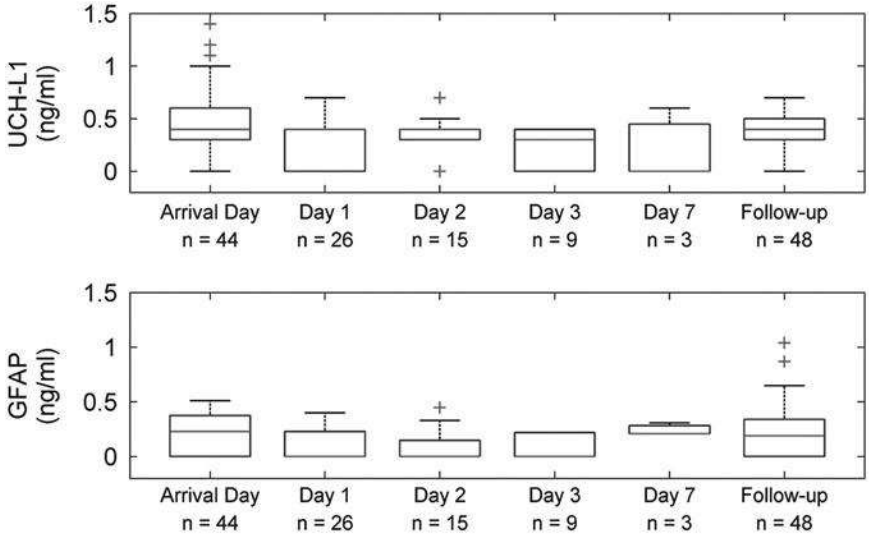


Figure 6. Levels of ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) on different days in patients with orthopedic trauma (y-axis zoomed). Box plots represent medians in nanograms per milliliter and interquartile ranges. (From the original publication I)

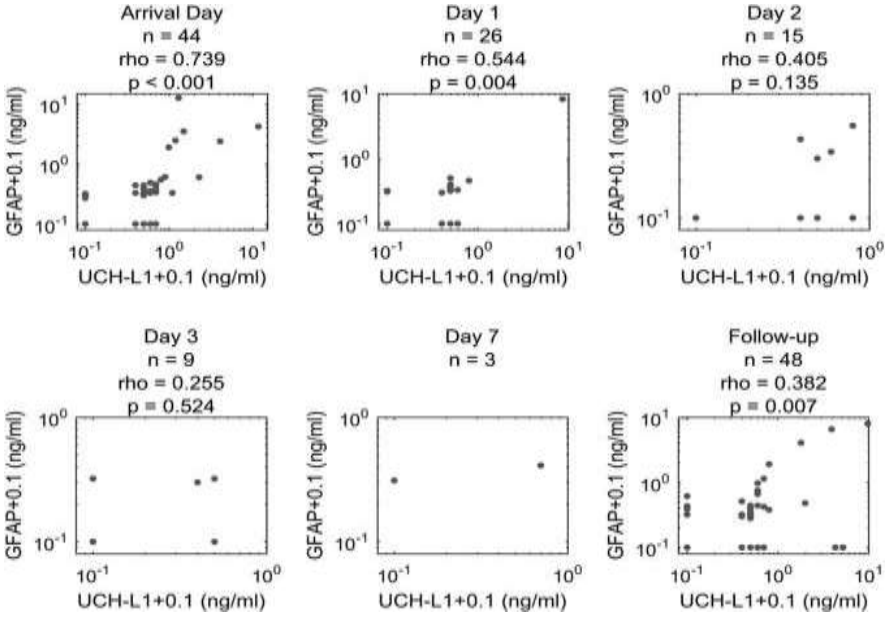


Figure 7. Spearman correlations coefficients (q) between ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) levels in patients with orthopedic trauma on different days. p, p value of rho; n, number of subjects. (From the original publication I)

The GFAP and UCH-L1 levels were available for most of the orthopedic injury patients on arrival, the following day as well as at the follow-up visit (Figure 5).

Spearman correlation coefficients between GFAP and UCH-L1 and scatter plots of these biomarkers in the same subjects are shown in Figure 6. In this figure, the number of plotted dots seems to be less than the number of subjects in the panels, since the levels of GFAP and UCH-L1 were identical in multiple subjects. On arrival (Spearman $\rho = 0.739$, $p < 0.001$), day 1 (Spearman $\rho = 0.544$, $p = 0.004$), and at the follow-up visit (Spearman $\rho = 0.382$, $p = 0.007$), significant correlations were found between GFAP and UCH-L1 levels. Regarding orthopedic injuries, female patients had significantly higher levels of UCH-L1 than male patients ($p = 0.036$). This was the only association between biomarker values and demographic features.

Orthopedic injury patients who presented with GFAP or UCH-L1 levels in the 95th percentile (n = 6, 8%, GFAP >3.61 ng/mL or UCH-L1 >2.74 ng/mL) are shown in Table 7, along with their demographic features, previous and current injuries, comorbidities, and MRI findings. All patients with raised GFAP or UCH-L1 levels in the 95th percentile were female, of which four out of six patients (67%) showed raised levels for both these biomarkers, and their injuries were either in the upper or lower extremities. A total of six patients were in the 95th percentile, of which five patients (83%) had distal part fractures and one had superficial injuries of the extremities. Five (83%) out of the six patients, after showing raised levels of either one or both biomarkers during the first week post injury, again showed a high level at the follow-up visit (Table 7). Only one patient in the CT-negative group showed biomarker levels, defined in the orthopedic controls, that were in the 95th percentile (GFAP >3.61 ng/mL or UCH-L1 >2.74 ng/mL). This 21-year-old male had no extracranial injuries and was previously healthy. On arrival day, his GFAP level was 0 ng/mL and his UCH-L1 level was 3.50 ng/mL. On the follow-up visit, his GFAP level was 1.31 ng/mL and his UCH-L1 level was 0.80 ng/mL. There was no difference in biomarker levels between the three patients with skull base fracture with no intracranial abnormalities and other mTBI patients.

5.1.3 MRI findings

A total of 52 (71%) orthopedic trauma patients underwent head MRI, of which 30 patients (58%) had normal MRI findings. Only one old contusion was suspected in one case, but either nonspecific ischemic-degenerative changes, or other insignificant abnormalities were shown in the rest of the patients. No imaging changes comparable with acute TBI were found. Normal findings were observed in four of the six patients (67%), who underwent head MRI, having levels of GFAP and UCH-L1 in the 95th percentile. Only one mTBI patient, whose arrival day sample showed UCH-L1 level in the 95th percentile, had normal head MRI findings.

Table 7. Patients with extracranial injuries with levels of GFAP and UCH-L1 in the 95th percentile (≥3.61 ng/ml and ≥2.74 ng/ml for GFAP and UCH-L1, respectively) and demographic details, comorbidities, current and previous injuries, and MRI findings. T13D, T2, FLAIR, SWI, DWI and DTI sequences were utilized and used to confirm the absence of brain pathology. Patients with biomarkers with high levels on the follow-up visit and during the hospital stay have shaded background. (From the original publication I)

ID	Age	Gender	Comorb-idities	High level	Arrival day	Day 1	Day 2	Day 3	Day 7	Follow-up	Current injuries	Earlier injuries	MRI finding
Patient 1	30	Female	No	GFAP	12.57	N/A	N/A	N/A	N/A	N/A	Forearm fracture, upper or lower extremity contusion	Thoracic vertebrae fracture	Normal
				UCH-L1	1.2	N/A	N/A	N/A	N/A	N/A			
Patient 2	52	Female	No	GFAP	2.3	N/A	N/A	N/A	N/A	0	Humerus fracture	Concussion	N/A
				UCH-L1	4.0	N/A	N/A	N/A	N/A	5.1	-		
Patient 3	26	Female	No	GFAP	2.37	N/A	N/A	N/A	N/A	6.48	Wrist fracture	No	Normal
				UCH-L1	1.1	N/A	N/A	N/A	N/A	3.8	-		
Patient 4	39	Female	No	GFAP	4.1	N/A	N/A	N/A	N/A	3.95	Hand fracture	No	Normal
				UCH-L1	11.6	N/A	N/A	N/A	N/A	1.7	-		
Patient 5	38	Female	No	GFAP	N/A	8.13	N/A	N/A	N/A	7.96	Fibula ankle fracture	No	Normal
				UCH-L1	N/A	8.5	N/A	N/A	N/A	9.7	-		
Patient 6	24	Female	No	UCH-L1	N/A	N/A	N/A	N/A	N/A	4.2	Superficial injuries of upper and lower extremity	No	N/A

Values are presented as nanograms per milliliter. T13D, T2, fluid-attenuated inversion recovery (FLAIR), susceptibility-weighted imaging (SWI), diffusion-weighted imaging (DWI), and diffusion tensor imaging (DTI) sequences were utilized and used to confirm the absence of brain pathology. Patients with particular biomarkers with high levels at the follow-up visit and during the hospital stay have shaded background. GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1.

Table 8. Head MRI findings in the patients with orthopedic injury. T13D, T2, FLAIR, SWI, DWI and DTI sequences were utilized and used to confirm the absence of brain pathology. (From the original publication I)

MRI finding	N	%
Not done	21	28.8
Normal	30	41.1
Slight ischemic-degenerative lesions	7	9.6
Ischemic-degenerative lesions and atrophy	1	1.4
Old infarct, ischemic-degenerative lesions and atrophy	3	4.1
Ischemic-degenerative lesions and signs of old trauma	1	1.4
Venous angioma or cavernotic angioma	3	4.1
Calcification	2	2.7
Unspecified white matter lesion	5	6.8

T13D, T2, fluid-attenuated inversion recovery (FLAIR), susceptibility-weighted imaging (SWI), diffusion-weighted imaging (DWI), and diffusion tensor imaging (DTI) sequences were utilized and used to confirm the absence of brain pathology.

MRI findings in orthopedic injury patients are shown in Table 8. MRI findings were categorized into three classes: abnormal MRI findings, normal MRI findings, and MRI not done. For the orthopedic patients, the levels of GFAP and UCH-L1 within these three classes are shown in Table 9. No significant difference in levels of GFAP and UCH-L1 were observed among these classes.

Table 9. Levels of UCH-L1 and GFAP in the different magnetic resonance imaging classes in the population of patients with orthopedic injury (From the original publication I)

	MRI not done	N	Normal MRI	N	Abnormal MRI	N	p value
UCH-L1							
Arrival Day	0.60 (0.30; 0.78; 3.85)	11	0.40 (0.40; 1.00; 7.84)	18	0.40 (0.30; 0.50; 1.05)	15	0.425
Day 1	0.00 (0.00; 0.40; 0.50)	6	0.35 (0.00; 0.40; 0.70)	10	0.40 (0.30; 0.40; 8.50)	10	0.319
Day 2	0.40 (0.30; 0.40; 0.40)	5	0.30 (0.23; 0.33; 0.40)	5	0.50 (0.23; 0.70; 0.70)	5	0.299
Follow-up	0.60 (0.38; 4.43; 5.10)	5	0.40 (0.30; 0.50; 2.09)	28	0.40 (0.30; 0.48; 7.43)	15	0.215
GFAP							
Arrival Day	0.27 (0.19; 0.50; 2.28)	11	0.23 (0.20; 0.36; 3.82)	18	0.21 (0.00; 0.34; 9.53)	15	0.350
Day 1	0.11 (0.00; 0.23; 0.24)	6	0.10 (0.00; 0.22; 0.36)	10	0.00 (0.00; 0.30; 8.13)	10	0.992
Day 2	0.00 (0.00; 0.05; 0.20)	5	0.00 (0.00; 0.08; 0.33)	5	0.00 (0.00; 0.29; 0.45)	5	0.631
Follow-up	0.00 (0.00; 0.09; 0.34)	5	0.20 (0.00; 0.34; 4.20)	28	0.24 (0.00; 0.49; 6.19)	15	0.381

Values are presented as median in nanograms per milliliter (25th percentile; 75th percentile; 95th percentile); p value from Kruskal-Wallis test. Days 3 and 7 are not presented due to the low number of samples.

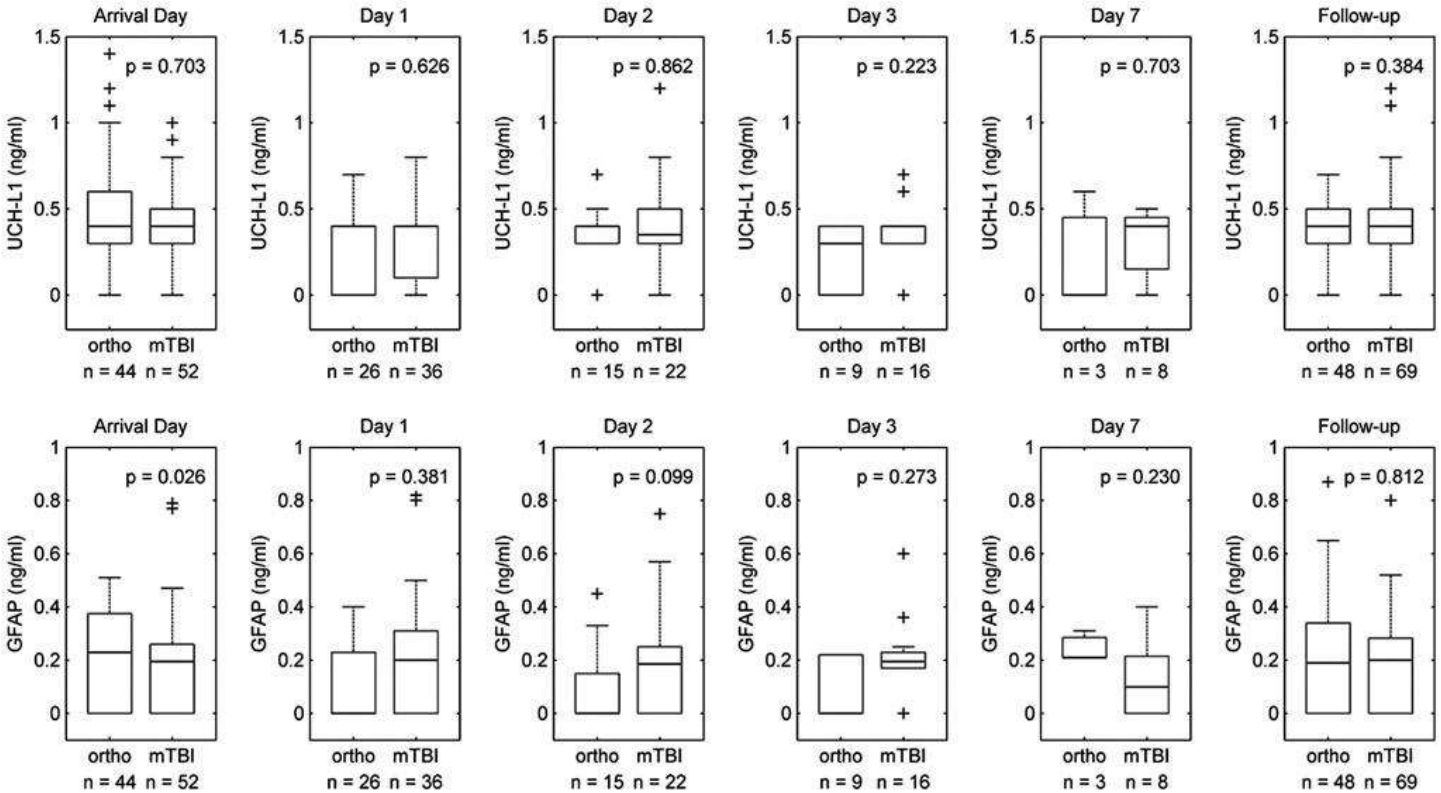


Figure 8. Spearman correlations coefficients (ϱ) between ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) levels in patients with orthopedic trauma on different days. p, p value of rho; n, number of subjects. (From the original publication I)

5.1.4 Comparison between orthopedic trauma and CT-negative mTBI groups

On arrival day, higher GFAP levels were observed in patients with orthopedic trauma compared to patients with CT-negative mTBI findings ($p = 0.026$); but in the following days no difference was observed (Fig. 7). Comparing patients with orthopedic injury and CT-negative mTBI findings, no significant difference was observed in UCH-L1 levels. Therefore, the GFAP levels in the arrival day sampling had the ability to discriminate the patient groups modestly in the ROC analysis (AUC = 0.629, 95% CI, 0.514–0.731; Fig. 8 and Table 10). Differences in GFAP and UCH-L1 levels over time are shown in Table 11. In patients with CT-negative mTBI, a significant decrease in UCH-L1 levels after arrival day were observed on day 1 and at the follow-up, while no significant differences in GFAP levels were observed. In orthopedic injury patients, no significant differences in GFAP and UCH-L1 levels were observed over time. Among patients with isolated CT-negative mTBI, orthopedic injury patients, and CT-negative mTBI patients with concomitant orthopedic injuries, no significant statistical differences in GFAP and UCH-L1 levels were found.

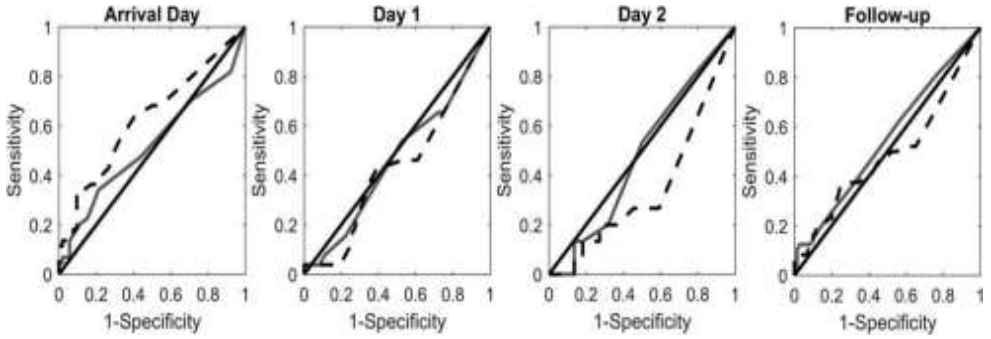


Figure 9. Receiver operating characteristic (ROC) curves for distinguishing orthopedic patients from patients with CT-negative mild traumatic brain injury Days 3 and 7 are not shown due to the sample size. GFAP, dashed line; UCH-L1, solid line. For numeral values see Table 6. (From the original publication I)

Table 10. Receiver operating characteristic curves for distinguishing orthopedic patients from patients with CT-negative mild traumatic brain injury. (From the original publication I)

	UCH-L1 (ng / mL)			**GFAP (ng / mL)**			**n (ortho)**	**n (mTBI)**
	AUC	95 % CI		AUC	95 % CI			
Arrival day	0.523	0.399	0.638	**0.629**	**0.514**	**0.731**	44	52
Day 1	0.464	0.310	0.604	0.437	0.300	0.576	26	36
Day 2	0.482	0.300	0.666	0.350	0.201	0.535	15	22
Follow-up	0.547	0.432	0.651	0.487	0.387	0.603	48	69

Days 3 and 7 are not shown because of the small sample size. Significant AUC in bold.
GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; AUC, area under the receiver operating characteristic curve; CI, confidence interval; n (ortho), number of orthopedic patients; n (mTBI), number of patients with CT-negative mild traumatic brain injury.

Table 11. Differences in UCH-L1 and GFAP Levels Over Time (From the original publication I)

	Day 1 - Arrival day	**p value**	**Follow-up - Arrival Day**	**p value**
Orthopedic injury				
n	11		27	
UCH-L1 (ng / ml)	-0.10 (-0.28; 0.00)	0.250	-0.10 (-0.28; 0.08)	0.336
GFAP (ng / ml)	-0.03 (-0.18; 0.00)	0.641	0.00 (-0.22; 0.07)	0.194
CT-negative mTBI				
n	14		37	
UCH-L1 (ng / ml)	-0.15 (-0.40; 0.00)	0.021*	-0.10 (-0.30; 0.00)	0.003*
GFAP (ng / ml)	0.00 (-0.01; 0.06)	0.922	0.00 (-0.16; 0.18)	0.658

The table shows changes in UCH-L1 and GFAP levels between arrival day and day 1 and arrival day and follow-up visit. n, number of subjects with proteomics levels at both time points; p values from Wilcoxon signed rank test. *p <0.05. Other values are expressed as median (25th; 75th percentile). GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; mTBI, mild traumatic brain injury.

5.2 Early levels of GFAP and NF-L in mTBI

5.2.1 Study subjects

In the mTBI cohort of 107 patients, with 73 male (68.2%) and 34 female (31.8%) patients, the mean patient age was 47.6 ± 20.2 years. The number of patients with CT-negative and CT-positive findings were 52 (48.6%) and 55 (51.4%), respectively. The GOSE scores were available for 105 patients (98.1%). Table 12 shows the patient characteristics. Regarding outcome, 15 patients (14%) had unfavorable outcome, 90 patients (84.1%) had favorable outcome, 68 patients (63.5%) had incomplete recovery, 37 patients (34.6%) had complete recovery, and 4 patients were dead (3.7%).

Table 12. Patient characteristics (From the original publication II)

Age (years)	47.64 ± 20.19
Sex	
Male	73 (68.2%)
Female	34 (31.8%)
Marshall Grade	
No visual pathology	52 (48.6%)
Diffuse injury	24 (22.4%)
Diffuse injury with swelling	1 (0.9%)
Diffuse injury with shift	1 (0.9%)
Mass lesions	29 (27.1%)
Pupil reactivity	
Unreactive	1 (0.9%)
Sluggish	2 (1.9%)
Reactive	99 (92.5%)
Missing data	5 (4.7%)
GOSE	
1	4 (3.7%)
2	0
3	6 (5.6%)
4	5 (4.7%)
5	7 (6.5%)
6	14 (13.1%)
7	32 (29.9%)
8	37 (34.6%)

Missing data	2 (1.9%)
Total	107 (100%)

Demographics are reported in mean ± standard deviation or percentages (%). GOSE = Glasgow Outcome Scale-Extended.

5.2.2 GFAP and outcome

Comparison of levels of GFAP between patients with unfavorable vs. favorable outcome, and incomplete vs. complete recovery are shown in figures 9A and 9B. There was not a significant difference between patients with incomplete recovery (median, 1467 pg/mL; IQR, 6453 pg/mL) and complete recovery (median, 612 pg/mL; IQR, 1996 pg/mL). The patients with unfavorable outcome (median, 4867 pg/mL; IQR, 24 667 pg/mL) had significantly higher levels of GFAP compared to the patients with favorable outcome (median, 875 pg/mL; IQR, 2280 pg/mL; p = 0.002).

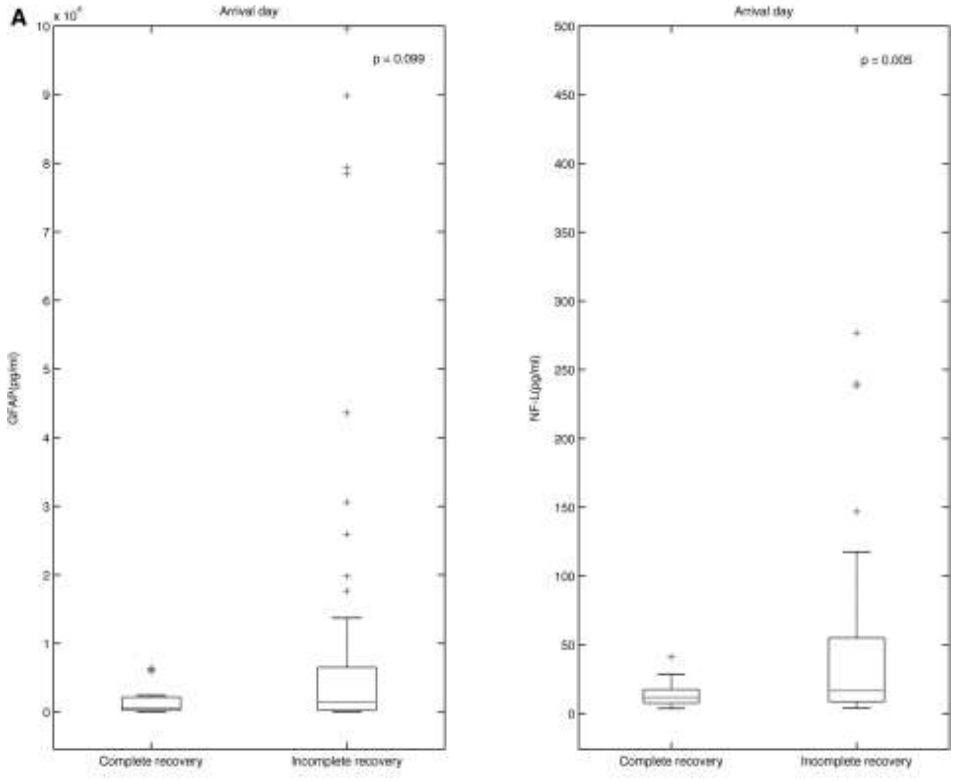


Figure 10A. Levels of glial fibrillary acidic protein (GFAP) and neurofilament light protein (NF-L) in patients with complete (Glasgow Outcome Scale-Extended [GOSE] 8) and incomplete (GOSE <8) recovery (y axis is zoomed). Box plots represent medians in picograms per milliliter and interquartile ranges. (From the original publication II)

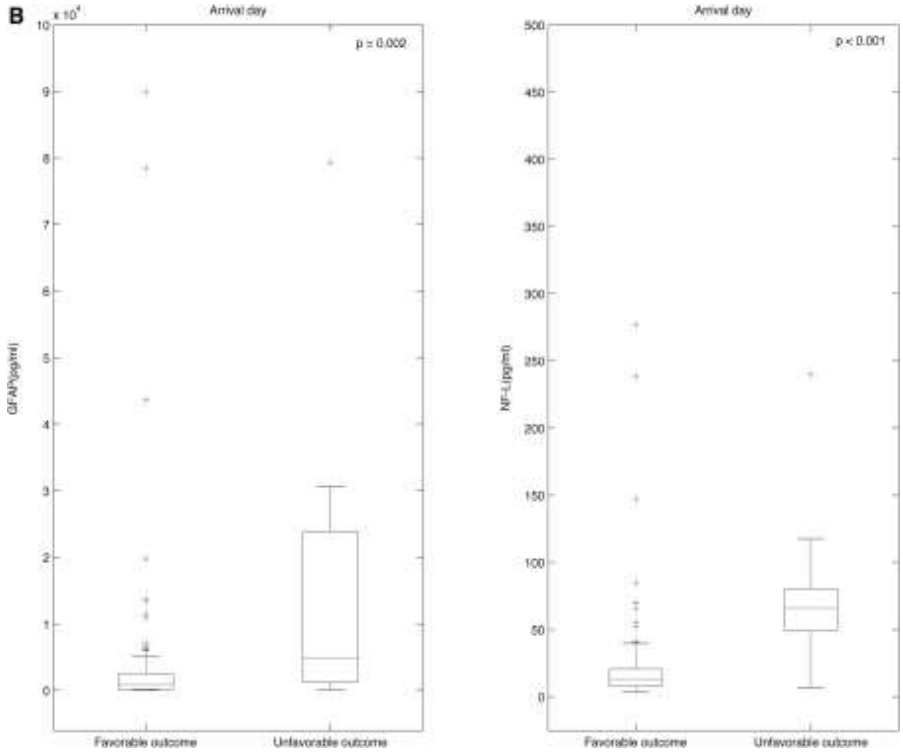


Figure 10B. Levels of glial fibrillary acidic protein (GFAP) and neurofilament light protein (NF-L) in patients with favorable (Glasgow Outcome Scale-Extended [GOSE] 5–8) and unfavorable (GOSE 1–4) outcome (y axis is zoomed). Box plots represent medians in picograms per milliliter and interquartile ranges. (From the original publication II)

A significant negative correlation existed between GFAP levels and GOSE score (Spearman ρ = –0.25; p = 0.01; Table 13). With an AUC of 0.755, GFAP could predict favorable outcome (95% CI, 0.628–0.882; p = 0.002), and with an AUC of 0.598, GFAP could predict complete recovery (95% CI, 0.489–0.706; p = 0.099; Fig. 10A, 10B).

Table 13. Correlation between biomarkers and GOSE and RPCSQ (From the original publication II)

Biomarkers	GOSE			RPCSQ (PRQ, total)		
	Spearman ρ	p-value	n	Pearson's r	p-value	n
GFAP	**-0.25**	**0.010**	105	0.030	0.769	96
NF-L	**-0.382**	**P <0.001**	105	-0.016	0.874	96

Statistically significant findings are in bold. GOSE: Glasgow Outcome Scale-Extended; RPCSQ: Rivermead Post Concussion Symptoms Questionnaire.

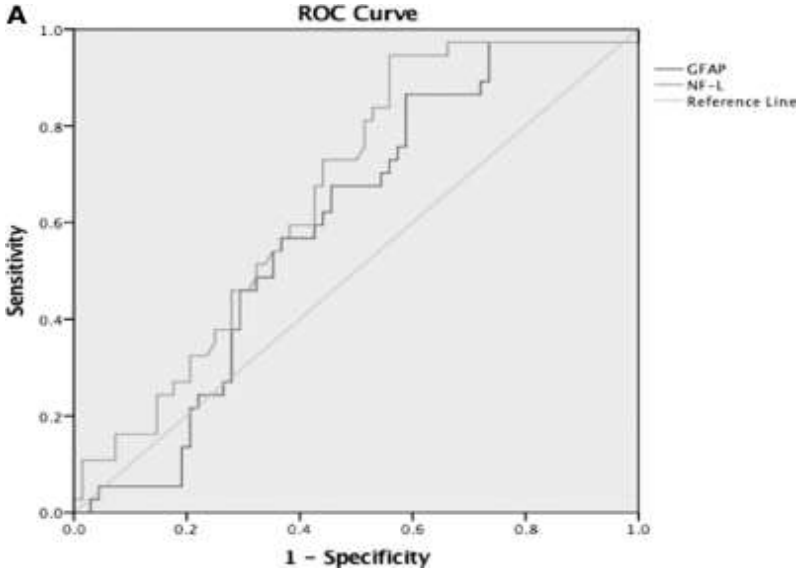


Figure 11A. Receiver operating characteristic (ROC) curves for predicting complete recovery (Glasgow Outcome Scale-Extended 8). Area under the ROC curve (AUC) for glial fibrillary acidic protein (GFAP), 0.598 (95% CI, 0.489–0.706, p = 0.099) and AUC for neurofilament light protein (NF-L), 0.665 (95% CI, 0.561–0.768, p = 0.005). (From the original publication II)

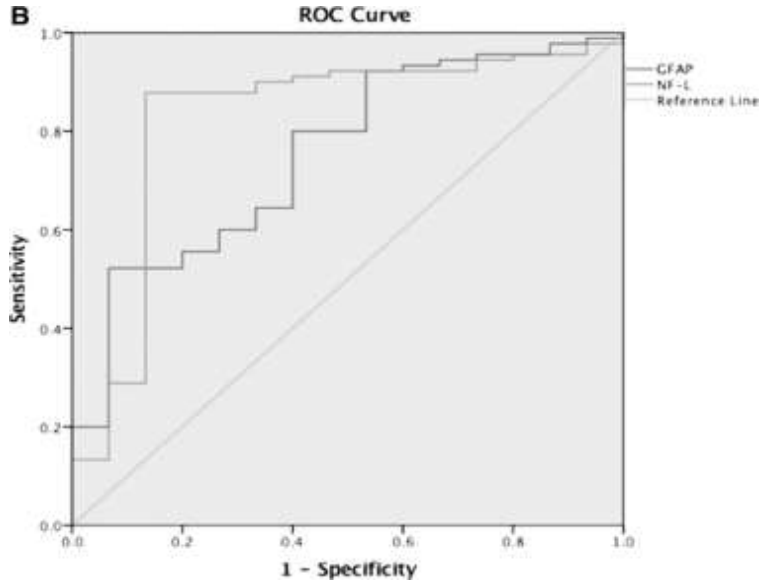


Figure 11B. Receiver operating characteristic (ROC) curves for predicting favorable outcome (Glasgow Outcome Scale-Extended [GOSE] 5–8). Area under the ROC curve (AUC) for glial fibrillary acidic protein (GFAP), 0.755 (95% CI, 0.628–0.882, p = 0.002) and AUC for neurofilament light protein (NF-L), 0.826 (95% CI, 0.694–0.958, p <0.001). (From the original publication II)

5.2.3 GFAP and outcome in CT-positive / negative mTBI

After dividing patients into CT-negative and CT-positive subgroups, there was no significant correlation of levels within these outcome subgroups, and there was no difference in GFAP levels between the outcome groups. GFAP was unable to predict outcome in multi-variate logistic regression model, neither independently, nor together with NF-L.

5.2.4 NF-L and outcome

Significantly higher levels of NF-L were observed in patients with incomplete recovery (median, 17 pg/mL; IQR, 47 pg/mL) compared to patients with complete recovery (median, 11 pg/mL; IQR, 10 pg/mL; $p = 0.005$). Patients with unfavorable outcome (median, 66 pg/mL; IQR, 35 pg/mL) also had significantly higher levels of NF-L than patients with favorable outcome (median, 13 pg/mL; IQR, 13 pg/mL; $p < 0.001$; Fig. 9A, 9B).

A significant negative correlation existed between the GOSE score and the NF-L levels (Spearman $\rho = -0.382$; $p < 0.001$; Table 12). With an AUC of 0.665 (95% CI, 0.561–0.768; $p = 0.005$) and an AUC of 0.826 (95% CI, 0.694–0.958; $p < 0.001$), NF-L could predict complete recovery and favorable outcome, respectively (Fig. 10A, 10B). Having GFAP in a multi-variate logistic regression model, the NF-L level was a complete recovery predictor (odds ratio [OR] = 1.008; 95% CI, 1.000–1.016; Table 14). Furthermore, the NF-L level was also a statistically significant complete recovery predictor in the model (OR = 1.006; 95% CI, 1.001–1.011).

Table 14. Logistic Regression Analysis of GFAP and NF-L to Distinguish Mild TBI Patients with Complete Recovery from Patients with Incomplete Recovery (From the original publication II)

Number of patients = 98	OR	95%CI	
Age	0.987	0.961	1.013
PTA	0.483	0.158	1.473
Time elapse	**4.037**	**1.264**	**12.897**
Worst GCS	0.672	0.285	1.581
ISS	0.960	0.902	1.021
Marshall II – V	0.417	0.121	1.442
Marshall V	0.232	0.052	1.037
Pupillary reactivity	**29.760**	**1.543**	**574.069**
GFAP	1.000	1.000	1.000
NF-L	**1.008**	**1.000**	**1.016**

Time elapse of more than 24 hours, Marshall I, and pupil reactive are used as reference category. Significant OR values in bold. GFAP, glial fibrillary acidic protein; NF-L, neurofilament light protein;

TBI, traumatic brain injury; OR: odds ratio; CI: confidence interval; PTA: post-traumatic amnesia; GCS, Glasgow Coma Scale; ISS, Injury Severity Score.

5.2.5 NF-L and outcome in CT-positive / negative mTBI

In the CT-negative mTBI subgroup, there was no difference in the levels of NF-L between the outcome groups, and there was no significant correlation between the levels and the outcome. However, the NF-L levels in patients with incomplete recovery (median, 52 pg/mL; IQR, 54 pg/mL) were significantly higher than patients with complete recovery (median, 15 pg/mL; IQR, 15 pg/mL; $p = 0.007$), within the CT-positive mTBI subgroup. Also, within this subgroup, significantly higher levels of NF-L were observed in patients with unfavorable outcome (median, 66 pg/mL; IQR, 35 pg/mL) compared to patients with favorable outcome (median, 20 pg/mL; IQR, 41 pg/mL; $p = 0.013$).

In the CT-positive mTBI subgroup, there was a significant negative correlation between GOSE score and the levels of NF-L (Spearman $\rho = –0.450$; $p = 0.001$). In this subgroup, with an AUC of 0.750 (95% CI, 0.593–0.908; $p = 0.007$) and an AUC of 0.720 (95% CI, 0.559–0.880; $p = 0.013$), NF-L could predict complete recovery and favorable outcome, respectively. Also, within this subgroup, in a multi-variate logistic regression model, the level of NF-L could significantly predict complete recovery (OR = 1.009; 95% CI, 1.001–1.016).

5.2.6 Cut-off values

By using the ROC curves of the full cohort, cut-off values for GFAP and NF-L were derived for predicting favorable outcome and complete recovery. The level of sensitivity was set to a minimum of 90%.

For predicting complete recovery, GFAP had a cut-off level of 6438.05 pg/mL, with a sensitivity of 97% (95% CI, 86–100) and a specificity of 26% (95% CI, 68–99). For the prediction of favorable outcome, GFAP had a cut-off level of 12189.85 pg/mL, with a sensitivity of 92% (95% CI, 85–99) and a specificity of 47% (95% CI, 16–68).

For predicting complete recovery, NF-L had a cut-off value of 28.15 pg/mL, with a sensitivity of 94% (95% CI, 82–99) and a specificity of 44% (95% CI, 32–57). For the prediction of favorable outcome, NF-L had a cut-off value of 53.6 pg/mL, with a sensitivity of 90% (95% CI, 82–95) and a specificity of 67% (95% CI, 38–88).

5.2.7 Combination of GFAP and NF-L and outcome

For complete recovery, with GFAP levels below 6438.05 pg/mL and NF-L levels below 28.15 pg/mL, the combination of the two biomarkers were used in a panel

with a sensitivity set to >90%. The optimal sensitivity was 94.6% (95% CI, 86.5–100.0) and specificity was 47.1% (95% CI, 35.3–58.8).

For favorable outcome, with GFAP levels below 980.75 pg/mL and NF-L levels below 41.85 pg/mL, the combination of the two biomarkers were used in a panel with a sensitivity set to >90%. The optimal sensitivity was 90% (95% CI, 83.3–95.6) and specificity was 86.7% (95% CI, 66.7–100.0).

5.2.8 Correlation between the levels of GFAP and NF-L

The levels of GFAP and NF-L were correlated significantly, except for unfavorable outcome. Pearson's r = 0.635 and p <0.0001 for the whole cohort. For incomplete recovery, Pearson's r = 0.496 and p <0.0001, for complete recovery, Pearson's r = 0.995, p <0.0001, and for favorable outcome, Pearson's r = 0.739 and p <0.0001.

5.3 Admission levels of T-tau and Aβ40 and Aβ42 in outcome of mTBI

5.3.1 Study subjects

In the mTBI cohort (n = 107), 72 male (68.6%) and 33 female (31.4%) patients were recruited, with the mean patient age of 47 ± 20 years (mean ± SD). The final study population was formed by the 105 patients with available GOSE score. Patients were divided into two subgroups, patients with CT-positive findings (n = 54, 51.4%) and patients with CT-negative findings (n = 51, 48.6%). Table 15 illustrates patients' characteristics.

Regarding outcome, 35% of patients had complete recovery (n = 37), 65% of patients had incomplete recovery (n = 68), and the mortality was 3.8% (n = 4). The time elapse was 28 ± 35 hours among the patients in whom the exact time of injury was available (n = 76). For the group of patients for whom time elapse was unknown, 11 patients were sampled <24 hours, and 18 patients were sampled >24 hours from the injury.

Table 15. Patient characteristics (From the original publication III)

Age (years)	47.46 ± 20.25
Sex	
Male	72 (68.6%)
Female	33 (31.4%)
Marshall Grade	
No visual pathology	51 (48.6%)
Diffuse injury	24 (22.9%)
Diffuse injury with swelling	1 (1%)
Diffuse injury with shift	1 (1%)
Mass lesions	28 (26.7%)
Pupil reactivity	
Unreactive	1 (1%)
Sluggish	2 (1.9%)
Reactive	98 (96.2%)
Missing data	4 (3.8%)
GOSE	
1	4 (3.8%)
2	0
3	6 (5.7%)
4	5 (4.8%)
5	7 (6.7%)
6	14 (13.3%)
7	32 (30.5%)
8	37 (35%)
Missing data	2 (1.9%)
Total	107 (100%)

Demographics are reported in mean ± standard deviation or percentages (%). GOSE = Glasgow Outcome Scale Extended.

5.3.2 The levels of T-tau and outcome

No significant differences were observed after comparing the levels of T-tau between patients with incomplete recovery (2.8 pg/mL, IQR 7.5 pg/mL) and patients with complete recovery (2.65 pg/mL, IQR 3.58 pg/mL; Figure 11). A significant negative correlation between GOSE score and the levels of T-tau were observed in all patients (Spearman $\rho = -0.231$, $p = 0.018$; Table 16A). The likelihood of complete recovery could not be predicted by the level of T-tau (AUC 0.56, 95% CI, 0.45–0.67; Figure 12A). It seemed that gender influenced T-tau (Table 16B). There was no significant correlation between the outcome and the levels of T-tau within the CT-negative

subgroup, and there was no difference in the levels of T-tau between the outcome groups. A significant negative correlation existed in the CT-positive subgroup between the ordinal GOSE score and the levels of T-tau (Spearman ρ = –0.288, p = 0.035). There was no correlation between the levels of T-tau and the RPCSQ scores (Table 16A).

Table 16A. Correlation between biomarkers and GOSE and RPCSQ (From the original publication III)

Biomarkers	GOSE			RPCSQ (PRQ, total)		
	Spearman ρ	p-value	n	Pearson's r	p-value	N
Amyloid β40	–0.082	0.410	104	–0.007	0.948	95
Amyloid β40	0.063	0.525	103	–0.015	0.889	94
Tau	**–0.231**	**0.018**	105	–0.013	0.900	96

Statistically significant findings are in bold. GOSE: Glasgow Outcome Scale Extended; RPCSQ: Rivermead Post Concussion Symptoms Questionnaire.

Table 16B. Correlation between biomarkers and Gender and Age (From the original publication III)

Biomarkers	Gender			Age		
	Spearman ρ	p-value	n	Pearson's r	p-value	n
Amyloid β40	0.034	0.731	104	0.180	0.068	104
Amyloid β40	–0.032	0.750	103	0.063	0.525	103
Tau	**0.252**	**0.010**	105	0.013	0.899	105

Statistically significant findings are in bold.

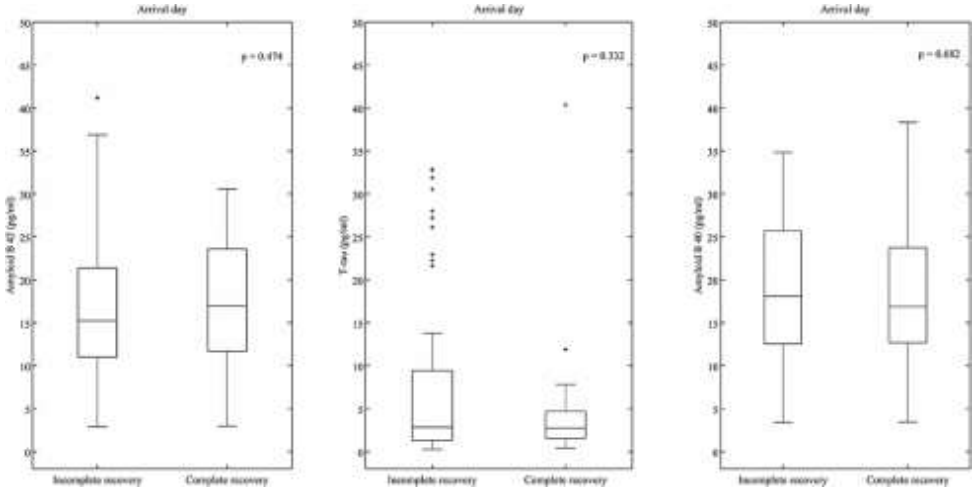


Figure 12. Levels of total tau (T-tau), β-amyloid isoform 1–40 (Aβ40), and β-amyloid isoform 1–42 (Aβ42) in patients with complete (GOS 8) and incomplete (GOS <8) recovery (y axis is zoomed). Box plots represent medians in picograms per milliliter and interquartile ranges. (From the original publication III)

5.3.3 The levels of Aβ40 and Aβ42 and outcome

There was no significant difference in the levels of Aβ40 between patients with incomplete (17.42 pg / mL, IQR 12.65pg / mL) and complete recovery (16.9 pg / mL, IQR 12.76 pg / mL). There was also no significant difference in the levels of Aβ42 between patients with incomplete (15.23 pg / mL, IQR 10.61 pg / mL) and complete recovery (16.94 pg / mL, IQR 12.36 pg / mL; Figure 11). The levels of Aβ40 and Aβ42 had no significant correlation with the GOSE score (Table 16A). Prediction of complete recovery were not possible by examining Aβ40 (AUC 0.52, 95% CI, 0.41 – 0.64) and Aβ42 (AUC 0.54, 95% CI, 0.43 – 0.63; Figure 12B, 12C). There was no difference in levels of Aβ40 and Aβ42 between the CT-positive and CT-negative outcome groups, and there was no significant correlation between the outcome and levels within these subgroups. There was no correlation between the levels of Aβ40 and Aβ42 and the RPCSQ scores (Table 16A).

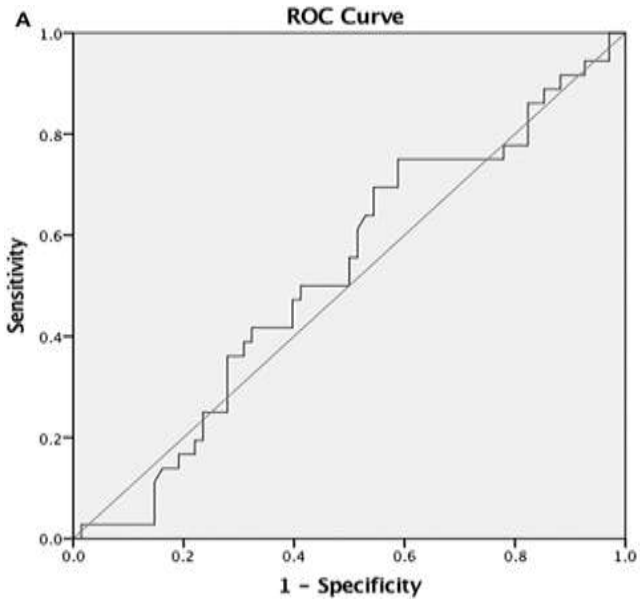


Figure 13A. Receiver operating characteristic (ROC) curves for predicting complete recovery (GOS 8). Area under the curve (AUC) for T-tau, 0.56 (95% CI 0.45–0.67). (From the original publication III)

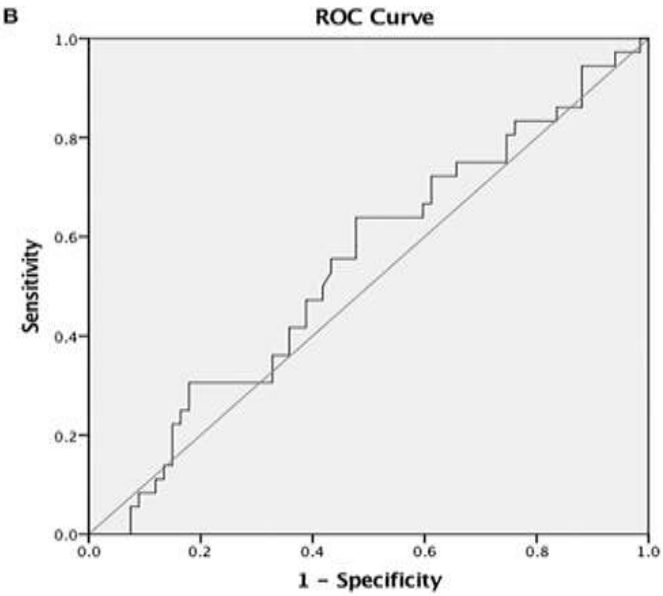


Figure 13B. ROC curves for predicting complete recovery (GOS 8). AUC for Aβ40, 0.52 (95% CI 0.41–0.64). (From the original publication III)

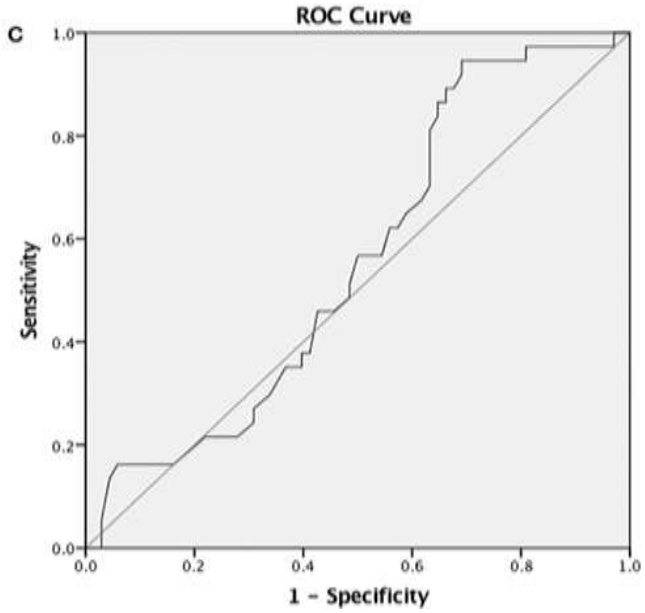


Figure 13C. ROC curves for predicting complete recovery (GOS 8). AUC for Aβ42, 0.54 (95% CI 0.43–0.63). (From the original publication III)

5.3.4 Combining T-tau, Aβ40 and Aβ42

T-tau were not able to predict outcome together with Aβ40 and Aβ42 or independently, or vice versa, using a conventional multivariate logistic regression model. For the evaluation of the ability of these three biomarkers to predict incomplete recovery, PanelomiX software was used.

The optimal sensitivity was 92.5% (95% CI, 85.1–98.5) and specificity was 27.8% (95% CI, 13.9–41.7), when the sensitivity was set to >90%, and levels of T-tau were above 0.55 pg/mL, Aβ40 above 20.26 pg/mL and Aβ42 above 23.9 pg/mL, for at least two of the three biomarkers.

5.3.5 Correlation among the levels of T-tau, Aβ40 and Aβ42

There was no significant correlation in levels of T-tau, Aβ40 and Aβ42 for incomplete and complete recovery subgroups, as well as in the whole population.

5.3.6 Best multiparameter panel for outcome prediction

Combining biomarker levels, clinical variables, and considering time elapse to predict the outcome, different panels were examined to find the best combination.

T-tau taken >24 hours from the injury, combined with ISS and age, was the best available panel. This panel had a sensitivity and specificity of 90.8% (95% CI, 83.1–96.9) and 57.1% (95% CI, 40–74.3), respectively, given that the variables were above their cut-off values (12.84 pg/ml for T-tau, 3.5 for ISS, and 22.5 years for age) for at least two of the three variables.

6 Discussion

6.1 Persistently elevated levels of GFAP and UCH-L1 in orthopedic injury, unrelated to TBI

In this observational, prospective, two-center study, the serum levels of GFAP and UCH-L1 were assessed in patients with CT-negative mTBI findings compared to patients with acute orthopedic injury. The main finding was that, in a clinically relevant sense, these biomarker levels were unable to discriminate these groups. Therefore, when determining whether patients with acute injuries have concomitant mTBI or not, these biomarkers do not provide diagnostic benefit. In addition, these levels of biomarkers were not able to distinguish patients with isolated CT-negative mTBI, patients with CT-negative mTBI with concomitant orthopedic injuries, and patients with orthopedic injuries. High levels of these biomarkers were prone to persist, comparing acute phase sampling to samples taken at follow-up visits several months later. This suggests that, irrespective of an injury, clearly higher levels can be found in some people. All the patients in the orthopedic injury subgroup with biomarker levels in the 95th percentile had injuries in the extremities and were female. Of the 52 patients in the othopedic injury subgroup who underwent head MRI, 30 patients had normal MRI findings, while the other 22 patients had nonspecific ischemic-degenerative changes indications or other insignificant abnormalities. None of these findings suggested acute TBI, which was carefully clinically excluded. In addition, only one patient had CT-negative mTBI findings together with high biomarker values but had normal MRI findings.

Many studies have explored the levels of UCH-L1 and GFAP in all TBI severity classes, though the mTBI validation is incomplete. The reason is that the controls are generally healthy volunteers, and poor characterization of patients and small numbers have been barriers when non-CNS trauma controls are used. Also, the diagnostic differentiation that is relevant for this study is for mTBI, but comparison in previous studies has mainly been with sTBI and moTBI. Promising results in diagnostical and outcome prediction potential of GFAP and UCH-L1 for TBI have been reported by several studies (Mondello et al., 2012; Diaz-Arrastia, et al., 2014; Nylén et al., 2006; Mondello et al., 2014; Okonkwo et al., 2013; Papa & Lewis, 2012). Some studies detected measurable but low levels of GFAP (Honda et al.,

2010; Papa et al., 2016a; Papa & Lewis, 2012; Welch et al., 2016) and UCH-L1(Mondello et al., 2012; Diaz-Arrastia et al., 2014; Papa et al., 2010, 2016b) in patients with orthopedic injury without any TBI. Two studies have shown that higher levels of GFAP (Papa & Lewis, 2012)and UCH-L1 (Papa et al., 2012) were observed in orthopedic trauma patients compared to the uninjured controls, and significant differences were found between all other groups (including orthopedic controls and different TBI severities) and uninjured controls (Papa et al., 2012; Papa & Lewis, 2012). Papa et al. found that in patients with CT-positive findings the breakdown product levels of GFAP were significantly higher compared to patients with CT-negative findings, irrespective of mTBI, moTBI or orthopedic injury. They also found that significantly higher breakdown product levels of GFAP were found in CT-negative mTBI and moTBI patients compared to patients with orthopedic injuries, although no median of p values were provided (Papa & Lewis, 2012). The performance of UCH-L1 was investigated by the same group, and they found that the levels of UCH-L1 were higher in patients with CT-negative TBI (mTBI or moTBI) compared to CT-negative trauma controls ($p = 0.057$) (Papa et al., 2012). The results of the current study are inconsistent with these findings. However, another study has also shown that patients with mTBI could not be discriminated from uninjured controls after analysing the levels of UCH-L1 in two different immunoassays (Puvenna et al., 2014). Papa et al. also found promising results for differentiation of patients with CT-positive and CT-negative mTBI and moTBI and patients with orthopedic injuries, regarding performance of UCH-L1 and GFAP levels (Papa et al., 2016b). They also detected high UCH-L1 levels (range 0.045–4.241 ng/mL) in some of their controls with orthopedic trauma, which agree with our findings, even though the TBI study populations has significant differences.

Cross-study comparisons are difficult, since different assay methods are used. However, key findings in our analysis relied on comparisons within the study by using a single analytic platform, so these should not be affected. We found significant correlations between the levels of UCH-L1 and GFAP in patients with orthopedic injuries, on arrival day, day 1 and at the follow-up visit. Biomarker levels over time showed one significant difference: the levels of UCH-L1 in patients with CT-negative mTBI were significantly lower on day 1 and follow-up compared to arrival day. In the orthopedic injury subgroup of our study there was no relationship found between the relatively high levels of UCH-L1 and GFAP in patients and their acute injury, since heterogeneous injuries were observed with elevated biomarker levels several months after the injury. The above-mentioned studies, excluding one study by Papa, et al., (Papa et al., 2016b) did not measure the levels and correlation of UCH-L1 and GFAP in patients with orthopedic injuries at different time points.

Interestingly, all the patients who had UCH-L1 and GFAP levels in the 95th percentile of the orthopedic injury subgroup were females, and at the follow-up visit,

majority of them had persistent high levels of these biomarkers. Throughout the orthopedic injury subgroup, female patients had significantly higher UCH-L1 levels than male patients. This observation is still unexplained. However, in a combined-gender analysis, higher levels of GFAP were observed in patients with orthopedic injury compared to patients with mTBI, while on arrival, the levels of UCH-L1 had no statistical difference.

Original presumptions were that both UCH-L1 and GFAP are CNS specific. Subsequently, UCH-L1 has been detected outside the CNS, in the cells of the kidney, ovaries, and testis (Kajimoto et al., 1992; Meyer-Schwesinger et al., 2009; Wilkinson et al., 1989), whereas the expression of GFAP was found in non-CNS and non-glial cells, such as liver stellate cells (Guido et al., 1997; Middeldorp & Hol, 2011), Schwann cells (Jessen et al., 1984), chondrocytes, (Hainfellner et al., 2001) lymphocytes (Middeldorp & Hol, 2011), fibroblasts (Hainfellner et al., 2001), and myoepithelial cells (Viale et al., 1988). Studies with knocked-out mice as subjects have reported that UCH-L1 plays an integral role in the function and structure of the neuromuscular junction (Chen et al., 2010). Regardless, UCH-L1 and GFAP have largely been considered TBI specific, regarding TBI diagnostics (Papa & Lewis, 2012; Papa et al., 2012). S100B is the most researched astroglial biomarker for TBI (Thelin et al., 2017; Undén et al., 2013), and is included in the Scandinavian guidelines for the initial management of minimal, mild and moderate traumatic head injuries (Undén et al., 2015). Since it has an effective NPV for pathological intracranial CT findings (Romner et al., 2000), it is possible to avoid unnecessary CT imaging, but its availability of extracranial sources, e.g. adipocytes and chondrocytes, has raised concerns regarding its utility (Olsson et al., 2011; Rothoerl et al., 1998; Savola et al., 2004; Thelin et al., 2017). Nevertheless, GFAP has been reported to be superior to S100B in the detection of intracranial injuries in multi-trauma patients with TBI (Luoto et al., 2017; Zetterberg & Blennow, 2016), and patients with mTBI (Papa et al., 2016a) and sTBI (Diaz-Arrastia et al., 2014) .

UCH-L1 and GFAP are not TBI specific biomarkers, even though they are mostly originated from the CNS. It has been reported that strokes and seizures resulted in elevated levels of UCH-L1 and GFAP (Gurnett et al., 2003; Ren et al., 2016). Studies on glioma (Jung et al., 2007) and ependymomas (Ilhan et al., 2011) have reported high plasma levels of GFAP, and a promising study investigated that there was a correlation between the levels of GFAP and the prognostic markers with high-grade gliomas. However, there was one subject included in the healthy control group of this study who had significantly higher levels of GFAP compared to the median of GFAP levels of the patients with high-grade glioma (Kiviniemi et al., 2015).

Previously, using the same assay method, our research group reported that the levels of UCH-L1 and GFAP could differentiate patients with unfavorable outcome

from favorable outcome. In that study, the reported cut-off value for unfavorable outcome of UCH-L1 and GFAP was 1.03 ng/mL and 1.26 ng/mL (Takala et al., 2016) respectively, while in another study a cut-off value for UCH-L1 of 1.89 ng/mL (Mondello et al., 2011) and for GFAP >1.5 ng/mL (Vos et al., 2004) to predict in-hospital mortality has been reported. Papa et al. reported the median levels of UCH-L1 and GFAP for the orthopedic injury patients, which are relatively comparable with our study findings. Our study, using The Evidence Investigator Cerebral Custom Array IV by Randox Biochip technology, found that patients with acute orthopedic trauma might often have overlapping levels of UCH-L1 and GFAP with the levels of patients with mTBI. Note that in this study we did not analyse the properties of these biomarkers and their ability to assess different subgroups of mTBI.

All patients with available MRI (67%) in the group of patients with GFAP and UCH-L1 levels in the 95th percentile, had normal MRI findings. These patients had neither intracranial tumors, nor any history of seizures or epilepsy related to their current injuries. According to these results, it is clear that an increase in the levels of these biomarkers, for the orthopedic patients, is not related to any acute brain disease or condition. Therefore, three possible interferences and explanations are raised.

First, previous studies reported that orthopedic injury patients could have higher levels of GFAP and UCH-L1 compared to the healthy control group. It supports the idea that these two biomarkers are not entirely brain specific. In other words, feasible extracerebral origins exists. AntiGFAP antibodies have been found in ligamentum and epiglottis flavum in vitro, by staining fibroblasts and chondrocytes (Hainfellner et al., 2001). Fellenberg et al. and Hsu et al. studied that, bone marrow-derived mesenchymal cells, and skin fibroblasts of patients with spinal muscular atrophy in vitro could be a rich source of UCH-L1, respectively (Fellenberg et al., 2010; Hsu et al., 2010). Thus, the expression of GFAP and UCH-L1 in fibroblasts and chondrocytes of extremity bone marrow and joint cartilage (which) leads to an increase of these biomarkers in peripheral blood following orthopedic fractures, which potentially explains our results. It has been also studied that both of these biomarkers could be expressed in neuromuscular junction and Schwann cells, which further leads to an explanation that our patients with orhopedic trauma developed peripheral traumatic neuropathy (Chen et al., 2010; Jessen et al., 1984). Unfortunately, this explanation remains theoretical, since clinical data describing the symptoms of neuropathy were unavailable and these patients did not undergo electrophysiological tests.

Secondly, although there is a minor possibility that according to the current definition of mTBI, which is not standardized, some orthopedic controls could have concomitant mTBI. However, it should be considered that the inclusion and exclusion criteria of orthopedic controls and patients with TBI were evaluated

meticulously during this prospective study. If any patient with orthopedic injuries had any suspicions or signs of head or neck trauma, indicating concussion or involved in high-energy trauma, the patient was excluded from this study. Following this, no patients, including those with elevated levels of GFAP and UCH-L1, had acute changes in their MRI.

Thirdly, it is well known that orthopedic injuries might develop CNS inflammation following the neurohumoral and cytokine storm due to the peripheral trauma. Therefore, there is a possibility that some of the orthopedic patients in this study had a non-traumatic CNS insult (Cape et al., 2014; Chuang et al., 2005).

According to current literature, the half-life of GFAP and UCH-L1 are approximately 10 hours and 24 hours, respectively (Thelin et al., 2017). Surprisingly, in this study, those patients with orthopedic injuries had elevated levels of these two blood biomarkers in acute setting, as well as several months later, during the follow-up visits. It conveys the information that many patients with orthopedic injuries had high levels of GFAP and UCH-L1, irrespective of TBI and acute injuries.

6.2 Significant correlation between early levels of GFAP and NF-L and outcome in mTBI

It was found that in this population, NF-L was useful in predicting complete recovery, and especially useful in predicting favorable outcome, using samples obtained within 24 hours after admission. GFAP also provided adequate favorable outcome prediction ability. The most important finding was that in a multivariate logistic regression model, with known level of GFAP and outcome predictors, statistically, NF-L was a significant complete recovery predictor for all patients with mTBI. Moreover, in patients with CT-positive mTBI, NF-L could predict complete recovery independently in a multi-variate logistic regression model using the same clinical variables. For the prediction of complete recovery, compared to the prediction ability of a single biomarker, a combination of these two biomarkers has shown increased sensitivity (94.6%) and specificity (47.1%). For predicting favorable outcome, the same combination also had higher sensitivity (90.0%) and specificity (86.7%), compared to a single biomarker. Significantly higher levels of NF-L were observed in patients with incomplete recovery, compared to the patients with complete recovery. Patients with unfavorable outcome had significantly higher levels of GFAP and NF-L, compared to patients with favorable outcome. The outcome assessed with GOSE had a strong negative correlation with the levels of GFAP and NF-L.

The correlation of the two biomarkers was weakest in patients with unfavorable outcome, since different structures and cell types express GFAP and NF-L, different responses arise from the exoskeleton of long axons and the cytoskeleton of astroglia

following the same physical forces. Therefore, such panels of biomarkers are needed to be developed for more reliable prediction of outcome following mTBI.

Higher levels of GFAP were observed in patients with incomplete recovery in one of our previous studies, assessing samples taken on Day 1 after the injury in a cohort with TBIs of all severities. Patients with unfavorable outcome had higher levels of GFAP on the arrival day, Day 1, and Day 2, and these levels had negative correlation with GOSE. Furthermore, the levels of GFAP upon arrival had the ability to distinguish between unfavorable and favorable outcome (Takala et al., 2016). A previous study showed that GFAP levels, within 12 hours from the injury, were higher in patients with unfavorable outcome than patients with favorable outcome (Zetterberg et al., 2013a). One study found that mortality in patients with TBI could be predicted by elevated levels of GFAP on Day 2 (Lumpkins et al., 2008), while another study reported GFAP levels were significant outcome predictors within 6 hours of the injury (Wiesmann et al., 2010). One study reported that GFAP breakdown products could not sufficiently predict complete recovery, but favorable outcome could be adequately predicted (McMahon et al., 2015). An interesting finding by Metting et al. (ref?) is that prediction was not possible from GFAP levels measured <3 hours after the injury, after comparing these results with the GOSE score obtained 6 months following mTBI (Luoto et al., 2017; Wang et al., 2018b). This result may relate to findings that have shown that the levels of GFAP only rise 16–24 hours after the injury (E. P. Thelin, Zeiler, et al., 2017). The Transforming Research and Clinical Knowledge in TBI (TRACK-TBI) study, where 83% of the study population were patients with mTBI, showed that patients with complete recovery could not be discriminated from patients with incomplete recovery, by observing the GFAP levels (Diaz-Arrastia et al., 2014). Including TBIs of all severities, two studies found that GFAP is able to discriminate patients with favorable and unfavorable outcome (Diaz-Arrastia et al., 2014; Takala et al., 2016). The TRACK-TBI investigators reported this aforementioned property of GFAP, with an AUC of 0.74 (95% CI, 0.61–0.87) (Diaz-Arrastia et al., 2014) and our prior study reported this property with an AUC of 0.723 (95% CI, 0.602–0.814) (Takala et al., 2016). Thus, the results of this study are consistent with the above-mentioned studies, with an AUC of 0.755 (95% CI, 0.628–0.882). Studies have shown that predictive ability may improve by combining multiple astroglia-derived biomarkers (Halford et al., 2017).

It has been reported that for sTBI, NF-L is a blood-based protein biomarker with high sensitivity (Shahim et al., 2016). The relationship between the NF-L levels in the CSF and DTI of DAI after sTBI has been investigated, and it is shown that the NF-L levels could predict the TBI outcome as well as the degree of the axonal injury (Skillbäck et al., 2014). A novel finding in this study is that, in the whole study population, as well as in the CT-positive mTBI subgroup, NF-L levels have a

predictive ability to discriminate patients with unfavorable outcome from favorable outcome and incomplete recovery from complete recovery. While other studies have shown that athletes with rapid recovery could be differentiated from athletes with prolonged symptoms by assessing the levels of NF-L (Shahim et al., 2017; Shahim et al., 2016a), in this study there was no correlation between the RPCSQ scores and the levels of either GFAP of NF-L. There was, however, a correlation between the GOSE scores and the NF-L levels. A combination of the levels of NF-L and GFAP obtained higher sensitivity and specificity than that of each individual biomarker.

Neither of the two biomarkers were able to predict recovery in the CT-negative subgroup. Potentially, inadequate statistical power could be the cause. Poor outcomes were less in the CT-negative subgroup, even though the CT-positive and CT-negative subgroups had similar population size. It is also a possibility that the factors that cause poor outcome can be different in the CT-positive and CT-negative subgroups. Rather than structural injury (detectable by structural neuroimaging or possibly by blood biomarkers), host factors, such as education, premorbid mental health, coping strategies and socioeconomic status (Lingsma et al., 2015; van der Naalt et al., 2017), could be causes of poor outcome in CT-negative patients.

In this mTBI study, available data showed that even though all patients had GCS ≥13, some injuries could be classified as more severe injuries, using other severity measures, such as PTA. For many patients it was not possible to assess the duration of PTA accurately. In addition, imaging findings in TBI have no standardized severity classification. Therefore, only GCS was used, since it is most often used, and it has least uncertainty.

6.3 Prediction ability of admission levels of T-tau for outcome in mTBI

This observational, prospective study investigated the circulating protein biomarkers T-tau, Aβ40, and Aβ42 and their performance for predicting outcome within the first 24 hours from admission, in a well characterized cohort including CT-positive and CT-negative subgroups, implementing highly sensitive modern immunoassays. A significant correlation between T-tau levels and outcome was found in the whole mTBI study population, including the CT-positive subgroup. However, comparing patients with complete and incomplete recovery, no significant difference was observed in the levels of T-tau, Aβ40, and Aβ42, and these levels could not usefully predict the likelihood of complete recovery. Furthermore, assessing the RPCSQ scores, there was no correlation between the biomarker levels and the severity of symptoms. Although, a multiparameter panel method suggested that T-tau levels sampled >24 hours from the injury may have predictive value (sensitivity of 90.8%

and specificity of 57.1%) for predicting incomplete recovery, when used in combination with the clinical parameters ISS and age.

This study's results agree with earlier studies that reported limited diagnostic value of serum tau for intracranial injury and outcome prediction of mTBI (Bulut et al., 2006; Kavalci et al., 2007). The TRACK-TBI investigators reported that acute P-tau levels, as well as the P-tau – T-tau ratio used in an assay platform with high sensitivity, outperformed individual T-tau levels in TBI outcome prediction (Rubenstein et al., 2017). The results of this study might have been different if P-tau levels were also measured, but only T-tau levels were available and analysed. The admission levels of plasma T-tau are not able to discriminate complete and incomplete recovery. A cause may be that in most cases of mTBI, mainly subcortical myelinated axons in the white matter are injured, but the unmyelinated cortical axons mainly express tau (Blennow et al., 2012; Shahim et al., 2016a). The samples were obtained within 24 hours after admission, therefore, it might be that the measurable levels of T-tau were not reflecting the cortical axonal injury yet, since this eventual injury is a slower process. Previous studies reported that there was no correlation between the levels of Aβ40 and Aβ42 and outcome, and these levels are not able to predict complete or incomplete recovery (Marklund et al., 2014; McKee et al., 2016; Olsson et al., 2004; Shahim et al., 2016a; Tsitsopoulos et al., 2017). This study results agree with these previous findings. No significant correlation was found between neurocognitive tests following mTBI and plasma levels of T-tau and Aβ42 in a recent study (Lippa et al., 2019), but since late levels of T-tau were used, the findings of study cannot be compared to the results of our study.

6.4 Strengths

Study I

Very few previous published studies included, mostly, a small number of orthopedic patients and healthy individuals as controls, to compare the levels of GFAP and UCH-L1 with CT-negative mTBI. This study explored the specificity of these blood biomarkers to discriminate the patients with orthopedic injury and CT-negative mTBI, using a well-characterized cohort. The samples were collected at multiple time points, including the follow-up visit. Additionally, their head MRI data were analysed at 4 weeks, as well as 3–10 months during the follow-up visit to study the reasons of persistently elevated levels of GFAP and UCH-L1 in the orthopedic trauma group. Following this, the study strengthens the idea that GFAP and UCH-L1 are not TBI specific biomarkers.

Study II

For the clinical translation of biomarkers, especially for the stratification of the patients with mTBI, the prognostic ability of the blood biomarkers needs to be evaluated by using the acute samples. The outcome of the patients with mTBI was predicted using their admission blood levels of GFAP and NF-L, which was a unique criterion of this study. A well characterized cohort with a considerable sample size and measuring the levels of these blood-based biomarkers by using Simoa technology, were the other strengths of this study. It has been reported that Simoa has higher sensitivity than electro-chemiluminescence–based assay or conventional enzyme-linked immunosorbent assay (ELISA) (Kuhle et al., 2016).

Study III

A strength of the study is that a multiparameter panel was developed which supported the idea that T-tau is a slow-raising biomarker. Additionally, the patient cohort was well characterized and collected prospectively. It is almost impossible to measure the blood levels of T-tau accurately in mTBI, using most of the immunoassays, since the concentrations are considerably low in peripheral blood (Zetterberg & Blennow, 2016), therefore, the Simoa technology was also used, as in Study II. Additionally, experimenting the prediction ability of the axon terminal biomarkers for mTBI, using the admission sample was a novel idea.

6.5 Limitations

Study I

Although, since this study reported for the first time that the levels of GFAP and UCH-L1 are persistently high in some patients, irrespective of their traumas, this study had some limitations. A major limitation of this study was the performance of Randox assay. The lower limit quantification ability of this assay method is inferior to the other current assays. A more sensitive assay could possibly be able to differentiate patients with orthopedic injury and CT-negative mTBI (Dadas et al., 2018; Papa et al., 2016b; Papa & Lewis, 2012). However, this limitation would not change the main finding of this study – some patients have elevated levels of GFAP and UCH-L1, irrespective of an injury. This limitation also emphasises the development of an ideal immunoassay for the clinical application of the blood biomarkers (Luoto et al., 2017; Wang et al., 2018b).

Another limitation is that we did not have the biomarker levels measured from healthy controls. This could have provided more insights on the biochemical

properties of these blood-borne biomarkers in injured and uninjured patients (Middeldorp & Hol, 2011).

Furthermore, patients were not recruited at night and their first samples were taken on day 1. Moreover, many patients with mTBI only came to the ED after several hours of initial injury when symptoms developed, which is a common phenomenon. Due to this, it might have had an effect on the levels of UCH-L1 and GFAP, considering their kinetics in blood (Halford et al., 2017; Mondello et al., 2018; Thelin et al., 2017).

Study II

The key limitation of this study is the lack of data for NF-L and GFAP levels >24 hours after admission. If later levels were present, the likelihood of recovery could be better predicted. There was variety in the time from injury to sampling, which may have influenced the biomarker levels, even though this was included in the analysis as a covariate. To assess the clinical significance of several measured levels, more concrete and detailed information of these biomarker kinetics after TBI is necessary.

Another limitation is the lack of information on the symptom's duration following mTBI in patients who recovered prior to outcome evaluation. Importantly, the mTBI cohort in this study is atypical to a general mTBI study population. The severity was only assessed by GCS, due to a large CT-positive subgroup, which is uncommon, but due to hospital admission, the patients were easily recruited.

Another recognized limitation is the variable of the GOSE assessment between 6–12 months, yet all patients was assessed by one experienced clinician. Studies have reported that patients with full recovery reach recovery shortly following mTBI, whereas most patients who have symptoms after 6 months will also still be symptomatic by 1 year (Van Der Naalt et al., 1999). Uncertainty arises in the outcome validation since it is not impossible to recover by 1 year after still being symptomatic at 6 months.

These biomarkers' prognostic ability has quite large confidence intervals, due to the considerably small sample size, especially considering large variability within mTBI, which should be noted when interpreting results. Larger sample sizes should confirm these results, even though unfavorable outcome percentage after mTBI (15/107; 14%) in this study is in correlation with the general concept of mTBI outcome.

Study III

The first limitation of this study is that only a single timepoint, within 24 hours after admission, data of T-tau, Aβ40 and Aβ42 levels were available. More information about the ability of the studied biomarkers to predict outcome could be revealed by a serial sampling kinetic study, which would estimate the timing of the peak values and the total efflux of a biomarker (Thelin et al., 2019). It has been reported that Tau is a long-term biomarker for mTBI, with first peak value <1 hour after the initial injury and second peak around 36 hours after the injury (Shahim et al., 2014). Within the initial 24 hours after the injury, Aβ42 becomes significantly elevated and remains fairly stable for several days (Mondello et al., 2014), however, contraindicatory studies have reported no significant increase of Aβ40 and Aβ42 following mTBI (Zetterberg et al., 2006). It was found that the levels of T-tau certainly performed best combined with clinical variables, and when taken >24 hours from the injury. Variability between patients of sample collection timing in relation to injury could have a negative influence on the ability of the studied circulating biomarkers to predict outcome. Although time elapse (the time from injury to sampling) was used as a covariate in the analysis, we might have missed the most accurate diagnostic time window for these biomarkers.

Secondly, a limitation to consider is the variability in assessing the GOSE 6 to 12 months after the injury. The limitation section of study II elaborated on this limitation.

Thirdly, the severity of injuries in our mTBI cohort is worse than the average mTBI population typically seen in the ED. This is due to the recruitment bias towards patients that were admitted in hospital. Therefore, several abnormal CT findings were found in the mTBI cohort. Additionally, some patients had PTA for >24 hours after the injury, which is an indication of more severe TBI according to many classifications, even though all the patients had GCS in the mTBI category. This issue is a reflection on the problems of classification of acute TBI by severity. The CENTER-TBI study (Maas et al., 2017b) has shown that approximately 33% of intensive care unit (ICU) treated cases were classified as mTBI according to GCS (Steyerberg et al., 2019). Hence, the nature of this study had to be considered when results were interpreted. In addition, the assessment of the duration of PTA was performed retrospectively at the outcome visit, which is less reliable compared to prospective evaluation.

When comparing earlier studies with this study results, it is important to mention that there was no collection of CSF samples and no patients in the cohort had injury mechanism caused by repetitive sports-related injury.

For the critical interpretation of the findings of studies II and III, it is obvious that the results were driven by patients with more severe injuries – especially those

who had mass lesions or multiple contusions. As discussed, to partially address this, CT-positive and CT-negative findings were analysed separately, and significant findings were found in the CT-positive subgroup. These findings should be evaluated in the light of the studies' injury classification.

6.6 Future directions

6.6.1 Can cut-off values of the biomarkers be defined by using a standard and sensitive immunoassay method?

Considering the future applications of the blood biomarkers, for the rapid assessment of mTBI, the following steps are recommended. Firstly, developing a standard assay with clearly defined cut-off values for abnormalities, is an emerging need. This assay should have high sensitivity for the detection of TBI, and adequate specificity for patients with orthopedic injuries and patients with a wide range of pre-existing neurological and medical problems. Secondly, to translate the research findings of the blood biomarkers in clinical practice, large multicenter studies with adequate control groups, including healthy subjects and patients with polytraumas, should be conducted. Hopefully, the current studies from CENTER-TBI (Maas et al., 2017b) and TRACK-TBI (Huie et al., 2020) would be able to provide more meaningful insights for the clinical applications of the biomarkers in TBI.

6.6.2 Could NF-L be useful to identify DAI?

This project has reported that, for mTBI, both NF-L and GFAP might be useful predictive biomarkers, but this important study findings need to be validated using several time-points of biomarkers sampling and in larger cohorts. Since this study reported that the early levels of NF-L could identify the patients with unfavorable outcome and incomplete recovery, in a well characterized cohort with mTBI, it might be that NF-L – mainly expressed in subcortical myelinated axons of white matter (Shahim et al., 2017; Zetterberg & Blennow, 2016), is able to provide more helpful information for the early diagnosis of DAI, the most common mechanism of mTBI. For this purpose, correlating the early levels of NF-L with sub-acute and late DTI metrics of the patients with mTBI is strongly recommended.

6.6.3 Can a prediction model be developed including biomarkers for mTBI?

This project focussed on biomarkers mainly expressed by axon terminals. However, a panel analysis was used to investigate their ability to predict outcome, since it is

apparent that T-tau, Aβ40, or Aβ42 indicate various types of axonal damage. It is an emerging need to develop a prediction model including circular biomarkers from different cellular origins, especially used in panels, since mTBI is a multifaceted cascade of neurometabolic changes. A recent study has shown that to detect the need for head CT scanning following TBI, single proteins' ability was outperformed by different cellular origin biomarkers used in panels (Posti et al., 2019). In a cohort where 70% of the patients had sTBI, it was found that a serum biomarker panel, consisting of different cellular originated proteins, improved the ability to predict outcome (Thelin et al., 2019).

T-tau has shown to have outcome prediction potential, but studies that can possibly include a P-tau – T-tau ratio and using considerably larger sample sizes are necessary. Future studies should also give emphasis on the serial sampling of the biomarkers, as their kinetics need to be known for clinical application.

7 Conclusions

7.1 GFAP and UCH-L1 – not CT-negative TBI specific

In this study, the most important finding was that some patients with orthopedic injury had higher levels of UCH-L1 and GFAP compared to patients with CT-negative mTBI. Persistent elevation of these biomarkers, up to several months after the initial injury, suggests that the origin of these biomarkers is not necessarily related to injury, and the source remains unknown. It was also found that these biomarkers are not specific for mTBI. The diagnostic utility of UCH-L1 and GFAP is seriously impaired in patients with orthopedic trauma with suspicions of TBI, due to their lack of specificity.

7.2 GFAP and NF-L – promising outcome predictors of mTBI

A significant correlation exists between the early levels of NF-L and GFAP and the outcome in patients with mTBI. The NF-L levels within 24 hours of admission has significant predictive value in mTBI, as well as in a multi-variate model.

7.3 T-tau – a possible predictor

The main finding of this study was the significant correlation between the admission levels of T-tau and the outcome in patients with mTBI. Neither T-tau, Aβ40, or Aβ42 used alone, nor used in different panels could provide complete recovery prediction in the mTBI cohort.

Acknowledgements

October 2020
Iftakher Hossain

Before thanking a group of excellent scientists as well as clinicians from Finland and abroad, it is important for me to tell about the background story of this MD, PhD thesis. Did I decide to do this thesis as a traditional one? Did I have the opportunities to collaborate with the great scientists from around the world in the field of traumatic brain injury (TBI) during my medical school? Did I have the facilities to become an active member of the Turku Brain Injury Centre smoothly? The answer is, no. I even did not have the desire to become a medical doctor back in 2004. Following an unfortunate spinal injury, my vision of life was changed. I was treated by one of the best neurosurgeons of Asia late Emeritus **Prof Rashiduddin Ahmad** FRCS, who for the first time inspired me to become a medical doctor as well as introduced me with a noble field of medical science – neurosurgery. After completing my medical school to pursue a proper and latest neurosurgical training, I moved to Finland. Completely different part of the world for a young guy coming from Dhaka, Bangladesh. If a single person would be thanked for my interest in neurosurgical research having special interest in TBI that is late Emeritus Professor Rashiduddin Ahmad. Thank you for saving my life, inspiring me to be my best version, to introduce me with neurosurgery and notably, to make a contact for me with another legend of neurosurgery, Emeritus **Professor Juha Hernesniemi**. Thanks, Juha for being kind to me when I arrived first time in "ice cold" Helsinki. Your simplicity shocked me in that evening of 2014. Gradually, I came to know that people of Finland are mostly not complex, and they usually keep their promises. These two qualities of Finnish people certainly helped me to go forward for my dream. Prof Hernesniemi should be thanked to recommend me to start a doctoral thesis since as an immigrant doctor in a new country neurosurgery is not an easy specialty to get into.

Professor Risto Roine, a charismatic person whom I met first time online. How? Via an email. Coming from a country where communicating via emails was not

common back in 2014, my skills of sketching a proper email was not good enough. However, Prof Roine showed me a green signal and he is the first person who opened the door of the Division of Clinical Neurosciences of Turku University Hospital, Turku, Finland for me. Risto, I do not have enough words to thank you. I never felt helpless in Turku, because I knew that you were always there to help me. Besides supporting me financially (for a clinical research assistant post) to stay in Turku when I was planning to apply for a doctoral candidate job in the University of Turku, Risto introduced me with two scholars, who played unapparelled roles in my doctoral thesis as well as to guide me to go for my dream career – neurosurgery.

Firstly, I came to know **Professor Olli Tenovuo**. From the beginning of my scientific career till today, I have the opportunity to meet very few clinician scientists who have such level of patience to guide the new generations. Thanks Olli to listen to me on the first day when we met, to introduce me with the complex science of TBI, for being available whenever I needed you, for evaluating my doctoral training A to Z by giving me your constructive feedbacks, and always being open minded to learn from each other. Given that I had to ensure my staying in Finland, Olli had to write the letters for the immigration office for me regularly. Being a kind and humble scientist, Olli introduced me with a field of great interest – TBI diagnostics. I started to read a lot of articles on body fluid biomarkers and neuroimaging. My passion for TBI science later ended up in a doctoral thesis protocol. What have I learned from Olli? To be a good researcher, you need to have the excellent networking skills to collaborate with the centre of excellence, having the most up-to-date evidence-based knowledge in your field, building a multidisciplinary research team and to do science as a team. Otherwise, you might publish a thesis book and could have a party, however, from the global aspect your work might not have any significant scientific impact. Collaborations, learning from each other and the hunger to learn new techniques turn us better human beings.

Secondly, **Adjunct Professor Jussi Posti**. How should I define you, Jussi? When I met Jussi for the first time in a national conference in Turku, I was taking a beginner level Finnish language course. Be noted that this is one of the most difficult languages and not widely spoken from the global perspective. Fortunately, I pronounced his name correctly and he warmly welcomed me. I was happy to meet my practical supervisor with whom I already exchanged several emails. At that moment, Jussi was going to complete his neurosurgical training and was an efficient post-doc. From this aspect, he was a perfect choice by Risto and Olli for supervising me.

Jussi, thank you for your patience to train me how to do science, how to be punctual, how to present myself in front of the bosses in our filed and notably, for being available. You have definitely established an example that a supervisor could

always provide a platform to bloom. I am especially grateful to you to correct me in a constructive way.

During my doctoral thesis, I have been fortunate to give eight international talks on the promising findings of my project and allied in the conferences organized by the European Association of Neurosurgical Societies (EANS) and the World Federation of Neurosurgical Societies (WFNS). As mentioned earlier, besides doing science it is vital for the PhD candidates to learn how to collaborate and to present their works as well. Jussi, thanks for teaching me how to give a talk just before the night of my first international oral presentation in Lund, Sweden in 2015. Following your guidance and my efforts, I was glad to give the talk in the plenary session of the EANS 2018 congress in Brussels, Belgium for receiving the prestigious Integra EANS Research Grant Award 2018. I could surely mention that as a practical supervisor, Jussi was proud of me that day. It is possible to be successful when we have a good team.

Besides conducting my doctoral studies, I was learning Finnish language, was affiliated with the Department of Neurosurgery of Turku University Hospital and Turku Brain Injury Centre and was trying my best to be integrated with the North European culture. From this point of view, I need to thank Jussi and his wife **Hanna**. Jussi has been an ideal example for me regarding work life balance. At the end of the day, it is important to be productive, however, we need to enjoy the life being open minded. You will be successful, but step by step – said by my dear supervisor Jussi Posti. Thanks for all the instructions for traveling, integrating with Finnish culture, not to give up if the project was not moving, for accepting my new ideas and for guiding me all the way to reach this point of my career. We will continue collaborations in academic and clinical neurosurgery.

Professor Jaakko Rinne, one of the charismatic vascular neurosurgeons and a great administrator that I have met in my life. Thank you, Jaakko, for being always so positive about me. Grateful to you to support my clinical career in neurosurgery and to inspire me to complete my doctoral thesis. I used to work late night in the lab as well as in the research room of the Turku Brain Injury Centre. To keep my clinical knowledge updated and to learn advanced scientific techniques of neuroimaging, I was attending the morning handover meeting of the Department of Neurosurgery. Jaakko's friendly smile and care helped me to go forward with my tasks. I would like to thank **Adjunct Professor Melissa Rahi** as well. Melissa, your warm greetings and teachings in the theatre inspired me to go forward with my goals.

My sincere gratitude to resident neurosurgeon **Mikko Visuri**. Thanks for your valuable guidance and help during the tough days of life when the project was moving very slowly, was dark and cold outside, feedbacks from the manuscript reviewers were harsh, I was feeling homesick and was looking for a good friend with whom I could discuss professional goals and values of life. As we agreed, this

Finnish brotherhood will continue. I should not forget to mention that you are the person who took me to the gym to achieve a healthier body and mind. Yes, during the PhD years we need to be fit to enjoy the work.

I would like to thank **Adjunct Professor Riikka Takala** for being an excellent tutor. Riikka's office door was always open to me. Whenever I sent an email or text message to Riikka, she was more than glad to help me. During the first phase of my doctoral studies, Riikka was helping me with the statistics and the critical review of the published articles. Riikka, I find you as an exceptional talent in your field. It is amazing to see that after doing such a stressful job in anaesthesiology and intensive care, you have the patience to teach new students like me. Your punctuality, kindness and perfectionist attitude altogether turned you a proper tutor and a great example for the upcoming generations.

Our research group co-ordinator, **Satu Honkala** deserves sincere thanks. Not only my PhD thesis, but also all other projects have been efficiently managed by her. I remember those days when Jussi and I had to work hard to form the TBIcare master file, we knew that Satu was always available to provide any unavailable information. Moreover, being a good friend Satu helped me to know about Finland. I am grateful to you for being a great colleague and warm friend.

Thanks to our research nurse, **Patricia Bertenyi** for her tough work to collect all the data. Nothing would be possible, if we did not have a well-managed data bank. Thanks, Patricia, for being a source of positive energy as well.

Ari Katila, a nice colleague and a fatherly figure for me. Ari, I am grateful to you for being nice to me. For being caring and to help me whenever I needed you. I remember the days of 2015 when I was sick for a few days. Thanks for guiding me to reorganise my lifestyle.

During my PhD years, I found a true friend for life. Our statistician and biomedical engineer, **Mehrbod Mohammadian**. I could proudly say that our multidisciplinary collaboration truly helped our research group to be productive. Mehrbod, thanks for helping us with the critical statistical analyses and to provide us your great feedbacks on neuroimaging analyses. A true friend is able to help you, but also has the ability to correct you when it is needed. You have shown this quality, my friend, Mehrbod. I am looking forward to working together in the near future again. Outside of our scientific works, mind stimulating conversations had a great impact on my life.

Since we have been able to collaborate with several centres of excellence in the field of TBI research, I had the opportunities to work with **Professor Peter Hutchinson, Professor David Menon, Professor Henrik Zetterberg, Professor Kaj Blennow, Professor Jean-Charles Sanchez** and **Associate Professor Virginia Newcombe**. It was a great pleasure to work with the most eminent scientists in this field. Thanks to all of you for your critical reviews on my publications. I would like

to thank Professor Hutchinson, known as Hutch, to allow me to work in Academic Division of Neurosurgery of the University of Cambridge for 6 months to complete my doctoral thesis following the **Integra EANS Research Grant Award 2018**. Following this, currently, I am fortunate to continue my academic and clinical training in Cambridge neurosurgery. I wish to continue to learn from all of you throughout my career.

Skhar Maruf, who would like to introduce himself as Shark needs to be thanked. Thank you, my friend, for all the life oriented thoughtful conversations. Hopefully, with time we will see a more multicultural openminded world where people will be evaluated equally. During my PhD years, our conversations helped me to think about the meanings of life.

Markus Matilainen, one of my best Finnish friends who helped me whenever I was in crisis. Markus, you are a true example of a friend in need is a friend indeed. Thank you to treat me as your own brother. Grateful to you for your expert opinions on statistics. I should thank you for being my first unofficial Finnish language teacher. In this regard, I also would like to thank my lovely friend, **Kata Husman**. Kata, thanks for your valuable time. You have been always there to talk. I am pretty sure that you miss our tennis matches after the long working days. Sitting in Cambridge, I miss you, friends. Thanks to brotherly figure, **Zia Akram** to share your expertise in academic and clinical neurosurgery besides being a good friend.

During 2014-2019, I have been nourished by all the great colleagues of the Division of Clinical Neurosciences of Turku University Hospital, Turku, Finland. My promising clinical project is the result of the hard work of a great team of the Turku Brain Injury Centre. I would like to thank each of you and grateful to you to accept me as a part of your great family. Besides all of the aforementioned people, I remember **Henna-Riikka Maanpää**, **Matti Sankinen, Anna Östberg, Ville Vuorinen, Anna Kotkansalo, Antti Puntala, Anneke van Vilet, Kaisa Pyhäranta,** and **Jaana Korpela** today. Thanks for your love and inspirations.

I would like to thank the **EANS, Finnish Medical Foundation, Turku University Foundation, Orion Pharmaceuticals, Marie Taponen Foundation,** and **the Hospital District of Southwest Finland** for funding these innovative studies.

The officials of the Doctoral Program in Clinical Research need to be thanked.
I would like to thank a few people who have been influencing my life a lot.
I would like to thank my great friend **Olya Filatyeva** for her great supports. Olya, you are a great human being. Without your friendship, I am not sure if I it would be possible for me to go forward in my life during the difficult days.

My sincere love and gratitude to my beautiful and kind girlfriend, **Inge**. To be successful in life, we need to be systematic and to be positive to overcome all the difficulties. I have found these qualities in you. Your love and kindness always help

me to go ahead in life. I admire your quality to explore the world being a cosmopolitan person.

Finally, the highest thanks of all goes to my mother, **Pervin**. I am here in my life for your efforts and sacrifices. I hope one day you will be proud of me. My father, **Karim**, has been a great example for me in life as well to remain calm in the difficult situations. Mom and dad, thank you for providing me a great upbringing, allowing me the opportunities to be an open-minded person and to support the idea – if you have to go far from your home for the goals, you can do it.

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UNIVERSITY OF TURKU

PunaMusta Oy, Vantaa, Finland 2020

ISBN 978-951-29-8315-5 (print)
ISBN 978-951-29-8316-2 (pdf)
ISSN 0355-9483 (print)
ISSN 2343-3213 (online)