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# **ABNORMAL COAGULATION AND PLATELET FUNCTION IN SEVERE TRAUMATIC BRAIN INJURY**

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# Abnormal coagulation and platelet function in severe traumatic brain injury

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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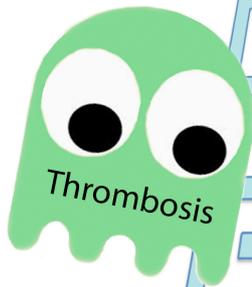
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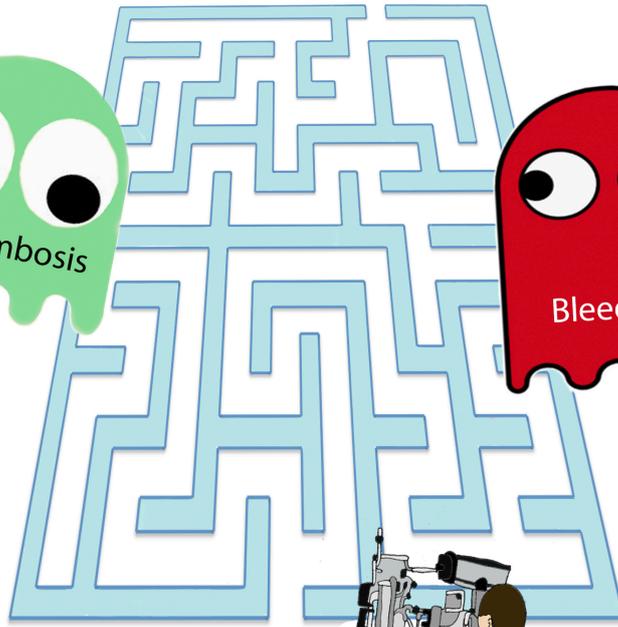
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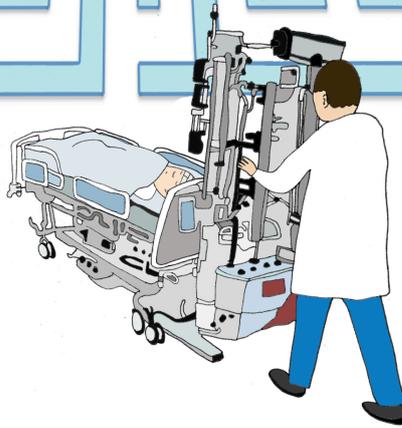




Thrombosis



Bleeding



## **ABSTRACT**

Traumatic brain injury (TBI) is associated with a high mortality and severe long-term morbidity in survivors. TBI often affects previously healthy young persons, and represents one of the most common causes of death among younger patients. The outcome in general trauma patients has improved over the recent decades. Unfortunately, lesser improvements have been achieved in the treatment of TBI. The pathophysiology of TBI is complicated, and changes in the haemostatic system are important parts of the complex response that occurs following TBI. Development of so-called secondary brain injuries with bleeding complications follow the trauma and contributes to the adverse outcome.

In the present thesis patients with severe isolated TBI were studied, with a focus on abnormalities in coagulation and platelet function. Cerebrovenous blood samples were collected repeatedly and compared to samples from the arterial circulation, in order to investigate the pathophysiological processes within the damaged brain. In agreement with previous studies, we observed that changes in the haemostatic system developed in hours to days following TBI. Intracerebral inflammation was also present in the TBI patients, which may modify coagulation responses to injury. Signs of “platelet dysfunction”, with a decreased platelet response to arachidonic acid, was observed in the patients, and over time a bleeding tendency developed. This “platelet dysfunction” was associated with bleeding complications. We also investigated circulating microparticles (MPs) released from platelets, endothelial cells and leukocytes using flow-cytometry. We found that activation of platelets took place when blood passed the injured brain, as there was a transcranial gradient in platelet MPs exposing the platelet activation marker P-selectin. We also found that endothelial derived MPs exposing tissue factor were generated in the injured brain and released into the circulation, whereas leukocyte derived MPs exposing tissue factor seemed to accumulate in the brain. In order to identify new brain specific markers of injury we assessed circulating MPs exposing antigens from brain tissue (from astrocytes and neurons) using flow cytometry. These MPs were higher in plasma from TBI patients compared to healthy controls, but there was a considerable variability between individuals, and also with-in the patients over time. More research is needed before MPs derived from brain tissue can be used as biomarkers in TBI. Monitoring of coagulation and platelet function in TBI may provide information regarding which patients that will develop bleeding complications and need hemostatic (procoagulant) treatment. Solid evidence that this improves patient outcome is, however, lacking at present.

## LIST OF SCIENTIFIC PAPERS

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# CONTENTS

1.	Introduction	
	1.1. Pathophysiology of TBI	1
	1.2. Cerebral oedema	2
	1.3. Neuroinflammation	2
	1.4. Complement activation	4
	1.5. Apoptosis	4
2.	Coagulation under normal conditions and in TBI	7
	2.1 The coagulation cascade	9
	2.2 The coagulation system in TBI	12
	2.3 Tissue factor in TBI	16
	2.4 Fibrinogen in TBI and general trauma	17
	2.5 Microthrombosis <i>vs.</i> bleeding in TBI	18
3	Platelets	21
	3.1 Platelet activation	21
	3.2 Platelet receptors and platelet inhibitors	22
	3.3 Measurement of platelet function	25
	3.4 Platelets in general trauma and TBI	26
4	Viscoelastic methods for the monitoring of coagulation	29
5	Microparticles	31
6	Potential treatment strategies in TBI-induced coagulation	32
7	Aims	37
8	Material and methods	37
9	Results and conclusions	41
10	Future perspectives	59
11	Summary in Swedish	62
12	Acknowledgements	63
13	References	67

## LIST OF ABBREVIATIONS

AA	Arachidonic Acid	LMPs	Leukocyte-derived Microparticles
ADP	Adenosine DiPhosphate	MAC	Membrane Attack Complex
AIS	Abbreviated Injury Score	MEA	Multiple electrode aggregometry
APTT	Activated Partial Thromboplastin Time	MPs	Microparticles (microvesicles)
AQP4	Aquaporin-4	NETs	Neutrophil Extracellular Traps
ASA	Acetylsalicylic Acid	NICU	Neuro-Intensive Care Unit
AT	Antithrombin	NSE	Neuron-Specific Enolase
ATP	Adenosine Triphosphate	PAR	Protease-activated Receptor
BBB	Blood Brain Barrier	PHI	Progressive Haemorrhagic Injury
C5b-9	Complement Membrane Attacking Complex	PMPs	Platelet-derived Microparticles
CNS	Central Nervous System	PT	Prothrombin Time
COX	Cyclooxygenase	ROTEM	Rotational Thromboelastometry
CSF	Cerebrospinal Fluid	SIRS	Systemic Inflammatory Response Syndrome
DAMP	Danger-Associated Molecular Pattern	TAT	Thrombin-Antithrombin complex
DIC	Disseminated Intravascular Coagulation	TBI	Traumatic Brain Injury
DVT	Deep Venous Thrombosis	TEG	Thromboelastography
EMPs	Endothelial-derived Microparticles	TF	Tissue Factor
F1+2	Prothrombin Fragment 1+2	TFPI	Tissue Factor Pathway Inhibitor
FDP	Fibrin Degradation Products	TM	Thrombomodulin
GCS	Glasgow Coma Score	TXA	Tranexamic acid
GFAP	Glial Fibrillary Acidic Protein	VWF	von Willebrand factor
GOS	Glasgow Outcome Score		
IL-6	Interleukin-6		
INR	International Normalized Ratio		

# 1 INTRODUCTION

Traumatic brain injury (TBI) is the most dangerous kind of trauma, associated with high mortality and severe long-term morbidity in survivors. In a recent review, the incidence rate of 235 cases per 100,000 people yearly was reported in Europe. The Brain Trauma Foundation database gives a mortality rate of 23% in severe TBI cases ( $GCS \leq 8$ ) [1-3]. The death rate in Nordic countries, except in Finland, is about 10 per 100,000 inhabitants yearly, constituting 1/3 of all trauma deaths [4]. It often affects previously healthy young persons, and represents one of the most common causes of death among younger patients [5-8]. The outcome in cases of TBI has improved in recent decades, but to a lesser extent compared with improvements in other kinds of trauma. According to a review of TBI care performed by Stein et al., spanning over a 150-year time frame, no improvements in patient outcome were achieved between 1990 and 2009 [9]. This illustrates the magnitude of the problem, which remains challenging despite many scientific and organizational efforts.

## 1.1 Pathophysiology of TBI

Despite the relatively small volume of damaged tissue, head trauma may alter the functions of vital centres of the brain directly on impact or cause fatal complications later. The initial damage to the brain occurs instantaneously on the site of incident, and is denoted *primary brain injury*. This damage is irreversible and cannot be treated by therapeutic interventions. Additional damage may occur later, due to poor perfusion and reduced oxygen delivery, with ensuing pathological processes such as activation of inflammation and coagulation with the development of subsequent microthrombosis, apoptosis and brain oedema [10]. These processes lead to *secondary brain damage*, which is a condition potentially open to therapeutic intervention. The goal of modern intensive care in TBI is to optimize the environment of the damaged brain tissue. Through improved macro- and microcirculation with increased oxygenation, optimal metabolic substrate delivery and minimized oedema formation, the survival of brain tissue in the border zone (even denoted as the penumbra area) may be improved [11-14].

## **1.2 Cerebral oedema**

Cerebral oedema is a common phenomenon in TBI and an important mechanism contributing to secondary brain damage. It accounts for up to half of the mortality among all TBI victims [15]. Cerebral oedema may be fatal as a result of an uncontrolled rise in intracranial pressure (ICP), causing general or local hypoperfusion of the brain. Five types of oedema have been described: vasogenic, cytotoxic, hydrostatic, osmotic and interstitial oedema, where vasogenic and cytotoxic mechanisms are the most important in TBI. Vasogenic oedema is localized in areas where the blood-brain barrier (BBB) is damaged and protein-rich fluid accumulates in the extravascular space, e.g. in areas of cerebral contusions. Cytotoxic oedema is a condition dependent on intracellular swelling, which in turn is caused by disturbances in ion-pumping mechanisms, mainly due to cerebral hypoxia and adenosine triphosphate (ATP) depletion. Various molecular mechanisms are involved in the pathophysiology of cytotoxic and vasogenic oedema. For example, water-channel proteins, aquaporins (AQPs) play a key role in this process, especially AQP4, which is predominantly expressed on astrocytes in proximity to cerebral microvessels [16]. AQPs constitute a potential therapeutic target in the control of cerebral oedema. Interestingly, AQP4 activators may have the potential to attenuate vasogenic oedema, while AQP4 inhibitors may be protective against cytotoxic oedema [16]. Hydrostatic oedema arises as a result of an increase in vascular transmural pressure when cerebral perfusion pressure is high. Osmotic oedema may be caused by iatrogenic hemodilution resulting in hyponatremia. Lastly, interstitial oedema may be caused by obstruction in the drainage of cerebrospinal fluid (CSF), e.g. as a result of hydrocephalus, although this is uncommon in the acute phase following TBI.

## **1.3 Neuroinflammation**

Inflammatory processes can be considered to be normal responses to trauma, and inflammation of the CNS following TBI is no exception. Neuroinflammation may, however, play a dual role. On the one hand it may lead to repair and regeneration of damaged brain tissue, but on the other it may cause generation and release of neurotoxic substances which may promote additional injury to the brain tissue. This dual nature of neuroinflammation is a phenomenon dependent on interplay between the innumerable cell types and molecular mediators. The inflammatory processes start acutely, i.e. within minutes after the traumatic impact, and may last for a very long time, up to several months or even years after the incident [10, 17]. The triggering mechanisms include release of so-called damage-associated

molecular pattern molecules (DAMPs), also called danger signals. Endogenous DAMPs released by dying cells or by the immune system are sometimes called alarmins [10]. Among the alarmins are high-mobility group box-1 protein (HMGB1), ATP, S100 proteins, interleukin-1 $\alpha$  and uric acid [18].

Several cell types are involved in the neuroinflammatory response to brain injury. *Microglial cells* are responsible for maintaining CNS homeostasis, neuronal plasticity and learning. Following trauma, microglial cells have been shown to clean up the debris and tighten the gaps between damaged astrocytes [19].

*Monocytes* are multipotent cells of the innate immune system and may differentiate into *macrophages* after invading damaged tissue. Macrophages participate in phagocytic processes; they can act as antigen-presenting cells and they can also release various cytokines. After TBI, the concentration of macrophages in the damaged brain reaches its maximum 24–48 hours later [20]. Experimental data derived from work on knockout mice suggest a pathogenic role of macrophages in the chronic phase after TBI, contributing to the development of cognitive sequelae [21]. Cognitive dysfunction, in turn, is one of the leading causes of disability in TBI patients [22]. *Neutrophils* are the most abundant cell type among the leukocytes and like monocytes are involved in phagocytosis. They may release various biologically active molecules like metalloproteinases and growth factors, and apart from being defenders against invasive microbes they are also important in wound healing. Neutrophils are recruited into the injured area within minutes, and can exert protective effects, which may lead to reduction of meningeal and parenchymal cell death [23]. However, harmful effects may also come about through release of proteases, cytokines (e.g. tumour necrosis factor) and reactive oxygen species (ROS), which may lead to neuronal cell death [24].

The characteristics and course of the inflammatory process are influenced by multiple factors, of which the time point of surgical treatment is one important factor. For example, in patients who underwent evacuation of haemorrhagic contusions less than 24 hours from trauma, the inflammation was mainly intravascular and the cellular response consisted largely of neutrophil activation (polymorphonuclear cells). In patients subjected to surgery 3–5 days after the trauma, the inflammation was parenchymal and the cellular response was dominated by macrophages, reactive microglia and T-lymphocytes [25]. This illustrates the complexity of the neuroinflammatory response, but also the theoretical possibility to influence the reactive process and lead it into the desirable and most favourable path. Furthermore, neuroinflammatory mechanisms tightly interact with the coagulation system in both

directions (inflammation influences coagulation and *vice versa*). Brain tissue is very rich in tissue factor (TF), and injury leads to formation of TF-FVIIa complexes and subsequently elicits a proinflammatory response. It has been shown that TF-FVIIa can induce synthesis of fibrinogen and increase its plasma levels, increase IL-6 levels and activate the complement system [26]. This interplay is described in more detail later in this thesis (Chapter 2).

#### **1.4 Complement activation**

The complement system is an important part of the innate immune system. It facilitates phagocytosis and has chemotactic effects on leukocytes. [18]. Activation of the complement cascade initiates cleavage of complement proteins 3, 4 and 5, leading to the formation of anaphylatoxins C3a, C4a and C5a with subsequent powerful chemotactic stimulation of leukocytes into the area of injury [27]. Furthermore, complement activation is directly involved in the cytolysis of alien cells or damaged host cells through formation of a complement-related membrane attack complex (MAC; C5b-9) [28]. The complement system is pathophysiologically interconnected with other inflammatory mechanisms and with the coagulation system. Low levels of activation (sublytic MAC levels) trigger microglia to release inflammatory cytokines such as IL-6, IL-8 and VEGF [29]. MAC has an activating effect on platelets, causing release of microparticles (MPs), and induces platelet membrane surface transformation, which results in increased platelet stickiness [30, 31]. After TBI, the complement cascade is activated and MAC accumulates in the border zones of cerebral contusions, causing neuronal death [32-35]. Interestingly, complement activation is not solely triggered by the primary trauma, but also through secondary effects of trauma involving hypoxia or circulatory instability [36].

#### **1.5 Apoptosis**

Apoptosis is the process of programmed cell death; it is an active, energy-demanding process where the cell participates in its own destruction. Apoptosis, in contrast to necrosis, results in minimal extracellular release of nuclear material and pro-inflammatory mediators [37]. The role of apoptosis in acute and subacute TBI has been increasingly recognized in the past 15 years. In acute ischemia, apoptosis is the predominant form of cell death, while necrosis accounts for the most of the cell death after TBI [38]. Intracellular calcium release might direct cellular responses towards either apoptosis or necrosis: “low intracellular calcium”

facilitates apoptosis, while “high intracellular calcium” induces necrosis [37]. Since apoptosis is an energy-demanding process, ATP depletion due to ischemia and/or mitochondrial dysfunction will direct the cellular response towards necrosis [39] [40]. The apoptotic process is regulated by a family of proteins called caspases, which can be activated via the TNF-induced pathway (through stimulation of surface receptors such as TNF-alpha receptor) or via a pathway triggered by mitochondrial activation [41]. Apoptosis constitutes a potential target for pharmacological interventions: inhibiting apoptosis in the area of mild-to-moderate injury may reduce loss of cellular function and thus limit the damage. However, in areas with severe injury it is desirable to redirect the process from necrosis to apoptosis in order to reduce responses that may cause long-term inflammation [41, 42].



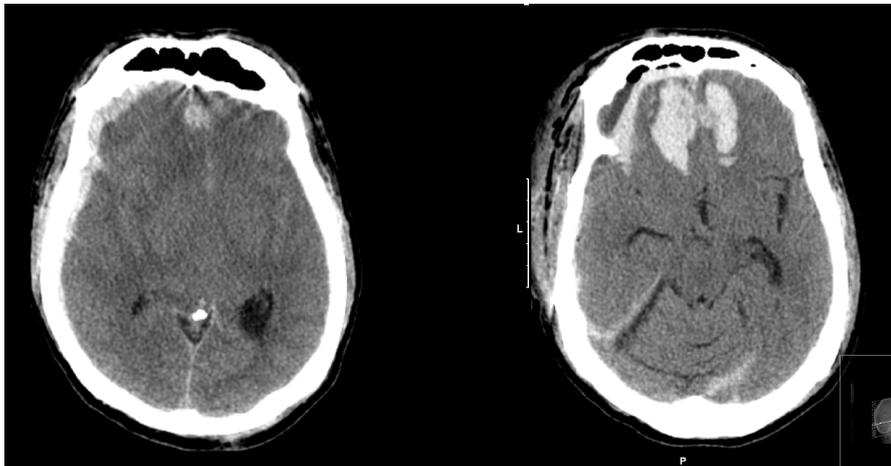
## 2 COAGULATION UNDER NORMAL CONDITIONS AND IN TBI

Coagulation disturbances in patients with TBI were recognized long ago but still pose many unsolved questions. The term “coagulopathy” is frequently used in the literature and represents a condition in which the blood’s ability to clot is impaired. The increasing number of publications in the area in recent years illustrates the awareness of coagulation-related problems within neurointensive care. Questions regarding incidence, significance and pathophysiological mechanisms of coagulopathy in TBI, perspectives of clinical coagulation monitoring, as well as possible treatment strategies, will be discussed in this chapter.

The incidence of coagulation abnormalities in TBI is 15–100%, depending on the definition and patient selection [43]. In a recent meta-analysis a pooled incidence of acute traumatic coagulopathy (ATC) was calculated, resulting in a figure of 35%. Notably, nineteen different ATC definitions were used in the studies included in this meta-analysis [44]. Common definitions of coagulopathy in cases of TBI include an abnormal International Normalized ratio (INR), a prolonged activated partial thromboplastin time (APTT) or prothrombin time (PT), an elevated DIC score (1–5), a low venous platelet count and low plasma fibrinogen. In some instances, viscoelastic methods such as ROTEM or TEG are used to define or “diagnose” coagulopathy [45, 46]. In our own studies performed on TBI patients, we investigated possible associations between some traditional coagulation tests and bleeding tendency. The latter was subjectively estimated by the surgeon or intensivist using a scale of 0–1–2. We believe the scoring system is relevant, since the term “inadequate haemostasis” indicates subjectivity. Inadequate haemostasis can also be characterized by the capacity of blood to cause thrombotic and/or bleeding complications, but this feature can only be observed retrospectively.

The development of coagulopathy seems to be very rapid: 23% of patients with isolated TBI presented with acute coagulopathy as early as in the emergency department. Importantly, such findings were associated with increased morbidity and mortality [47] (see below). Notably, coagulopathy is recognized as a major contributor to complications in TBI [48] and is one of the most important independent predictors of poor outcome [49]. Development of coagulopathy in TBI is associated with higher transfusion rates, longer hospital- and ICU-stays, a higher incidence of multiple organ failure, more disability at discharge [44], and last but not least a three- to tenfold increased risk of death [50-53]. Among the many routine laboratory tests available, venous platelet count and perhaps prothrombin time (PT/INR)

seem to be the most reliable coagulation-related markers of TBI outcome. This conclusion is based on a large amount of data from 1613 rigorously monitored patients [54]. Plasma fibrinogen turnover (D-dimer levels) or ongoing hyperfibrinolysis diagnosed by viscoelastic methods have also been reported to be predictors of outcome [49, 55-58].



**Fig. 1** Haemorrhagic progress of contusion between the first examination at admittance and the second examination six hours later.

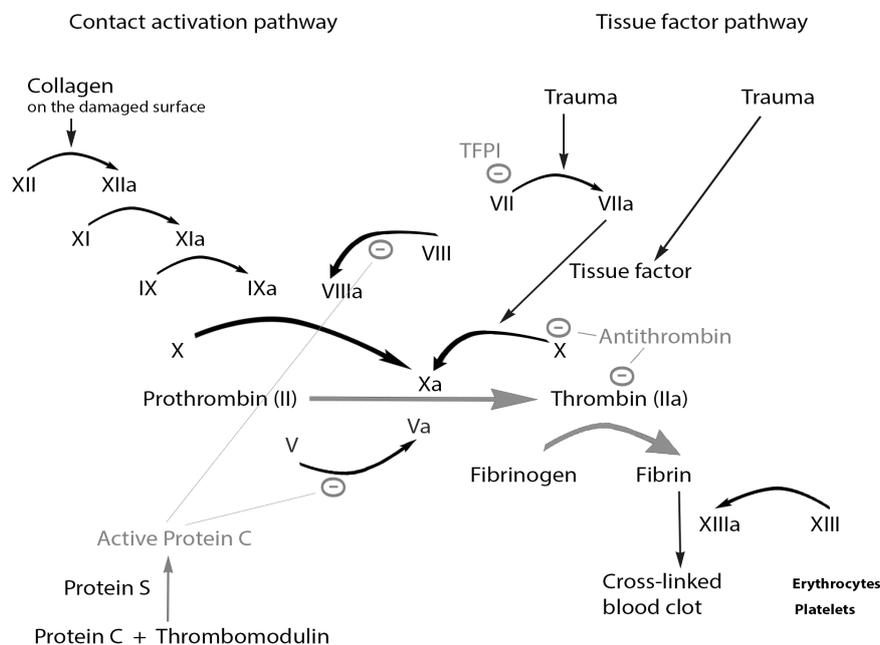
Approximately half of the patients suffering from haemorrhagic contusions demonstrate progress in the volume of bleeding in subsequent computed tomographic examination [59, 60]. Importantly, the main risk factor of progressive bleeding in a follow-up CT scan is the presence of coagulopathy [52]. At the same time, microthrombosis is also reported to be a universal response in TBI and an important cause of secondary insults [61]. Coagulation disturbances can also elicit thrombotic complications in the peripheral circulation, including, for example, deep vein thrombosis (DVT) [62], pulmonary embolism [63], myocardial infarction [64], and gastrointestinal bleeding [65].

A subject of controversy is the question of whether coagulation abnormalities after TBI are substantially different from those in other types of trauma. The predominant “classic” view is that TBI coagulopathy has the same pathophysiology as other types of trauma [66]. This opinion was, however, challenged for many years by researchers claiming that the essence of TBI-related coagulopathy is different [56, 67]. The volume of damaged tissue is smaller and blood loss is less profuse. Despite this, coagulopathy is much more common in TBI and it is also frequently associated with severe complications. An important fact is that the brain is extremely rich in tissue factor (TF). It is believed that disruption of the BBB following

traumatic impact will lead to release of TF into the circulation, which in turn will trigger coagulation [68], as well as inflammation (see above). Frequent and well-known causes of coagulopathy in general polytrauma such as hypothermia, haemodilution and acidosis are less important in TBI [67, 69].

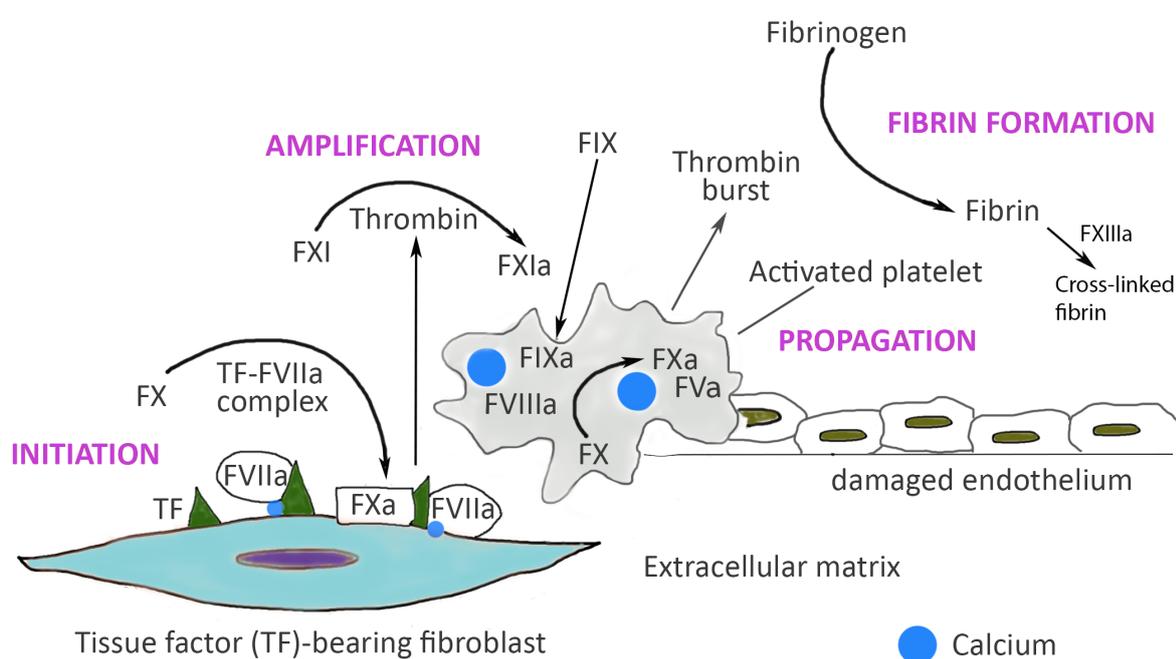
## 2.1 The coagulation cascade

The coagulation system was first discovered in the late 19<sup>th</sup> century, and traditionally described as a cascade of events, leading from initial activation to production of fibrin and building of a blood clot. Historically, the final stages of this process were described first; thus Factor I (fibrinogen, described by Rudolf Virchow in 1860s) is the final product before clot formation (**Fig. 2**). The frequently used term “Virchow’s triad” emphasizes three major conditions for the thrombotic process: hypercoagulation of blood, rheological changes such as stasis or turbulent blood flow, and endothelial dysfunction/changes in the vascular wall [70]. This clearly remains true for different types of arterial, venous and microcirculatory thrombotic events such as acute myocardial infarction, acute ischaemic stroke, DVT and DIC.



**Fig. 2** The coagulation cascade.

Within the coagulation cascade, according to the classical definition, two “arms” or pathways can be separated. The contact activation pathway (“intrinsic pathway” in older literature), triggered by activation of FXII on the collagen surface of a damaged vessel, and the tissue factor-induced pathway (“extrinsic pathway”) that starts with activation of FVII by TF when the TF/FVII complex is formed [71]. To monitor activation of the TF-induced pathway, prothrombin time (PT) and INR are used, whereas the partial thromboplastin time (PPT) or activated PPT (APPT) are used to monitor activation of the contact activation pathway [72].



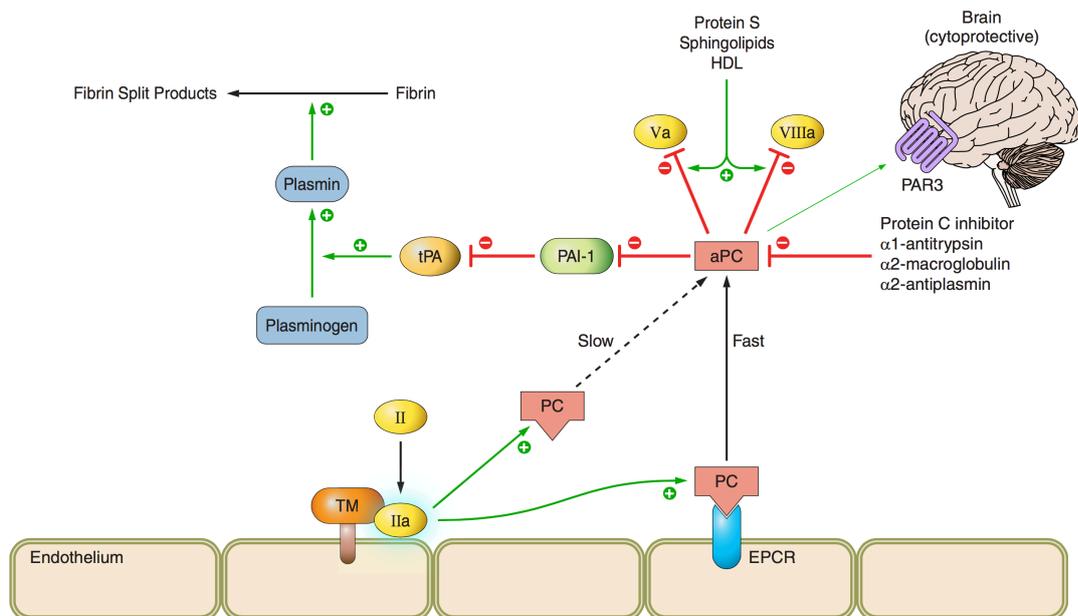
**Fig. 3** The cellular model of coagulation activation.

A novel approach to describe coagulation, however, is to separate it into three stages of “activation”: initiation, amplification and propagation. This modern view is physiologically more relevant and understandable. During the initiation phase, platelets, von Willebrand factor (VWF) and collagen play a major role: VWF binds to collagen fibres at the site of the vessel injury, and connects to a platelet. This “connection” is receptor-dependent and binding between the ligand (VWF) and platelet receptor for VWF will trigger intracellular activation of the platelet. Thus, the activated platelet will change its shape (swell, become irregular and throw out pseudopodia) and release the contents of its  $\alpha$ -granules (e.g. VWF, fibrinogen, FV and FXIII) and dense granules (ADP, serotonin and Ca<sup>++</sup>). Some granule contents, such as

ADP and serotonin, as well as thromboxane A<sub>2</sub> (which is generated and secreted from the activated platelet) will activate surrounding platelets. This will lead to the next phase – the amplification phase. Of note, the most important amplifying factor is thrombin, which is the most potent platelet-activating factor known. Thrombin is produced from prothrombin under the influence of activated factors FVa/FXa in a rapid and “violent” fashion, also called the thrombin pulse. Thus, in the next stage, i.e. propagation, there is an excess of thrombin, which in addition to platelet activation converts fibrinogen into soluble fibrin and activates FXIII into FXIIIa, which binds soluble fibrin molecules into larger fibres, and builds up a tight fibrin network with intermingled platelet aggregates (blood clot) [73, 74].

In fact, the coagulation system involves both procoagulative and anticoagulant molecules. The latter include Antithrombin (AT), Tissue Factor Pathway Inhibitor (TFPI), protein C and thrombomodulin (TM), where anticoagulation dominates in normal conditions [75].

Antithrombin is a protease inhibitor, acting on enzymes of the coagulation system and preventing generalized intravascular coagulation, thus limiting the process to the vicinity of injury. AT activity is stimulated by heparin and also by heparin-like molecules on the surface of endothelial cells [76]. Another important anticoagulant molecule is TFPI, which modulates the initial steps of coagulation involving FVIIa and TF. A lack of TFPI is incompatible with life [77]. The protein C is a vitamin K-dependent proenzyme, which in an activated state together with protein S inhibits FVIIIa and FVa. Protein C is activated by a complex of thrombin together with thrombomodulin on the surface of endothelial cells, which means that thrombin can exert both thrombotic and antithrombotic actions. The procoagulative effects dominate at sites of vascular lesion, whereas anticoagulant properties are seen in intact vessels. Deficiencies in protein C or TM are described as lethal conditions [78]. The protein C anticoagulant pathway is especially important in hypoperfusion situations such as traumatic shock [79].



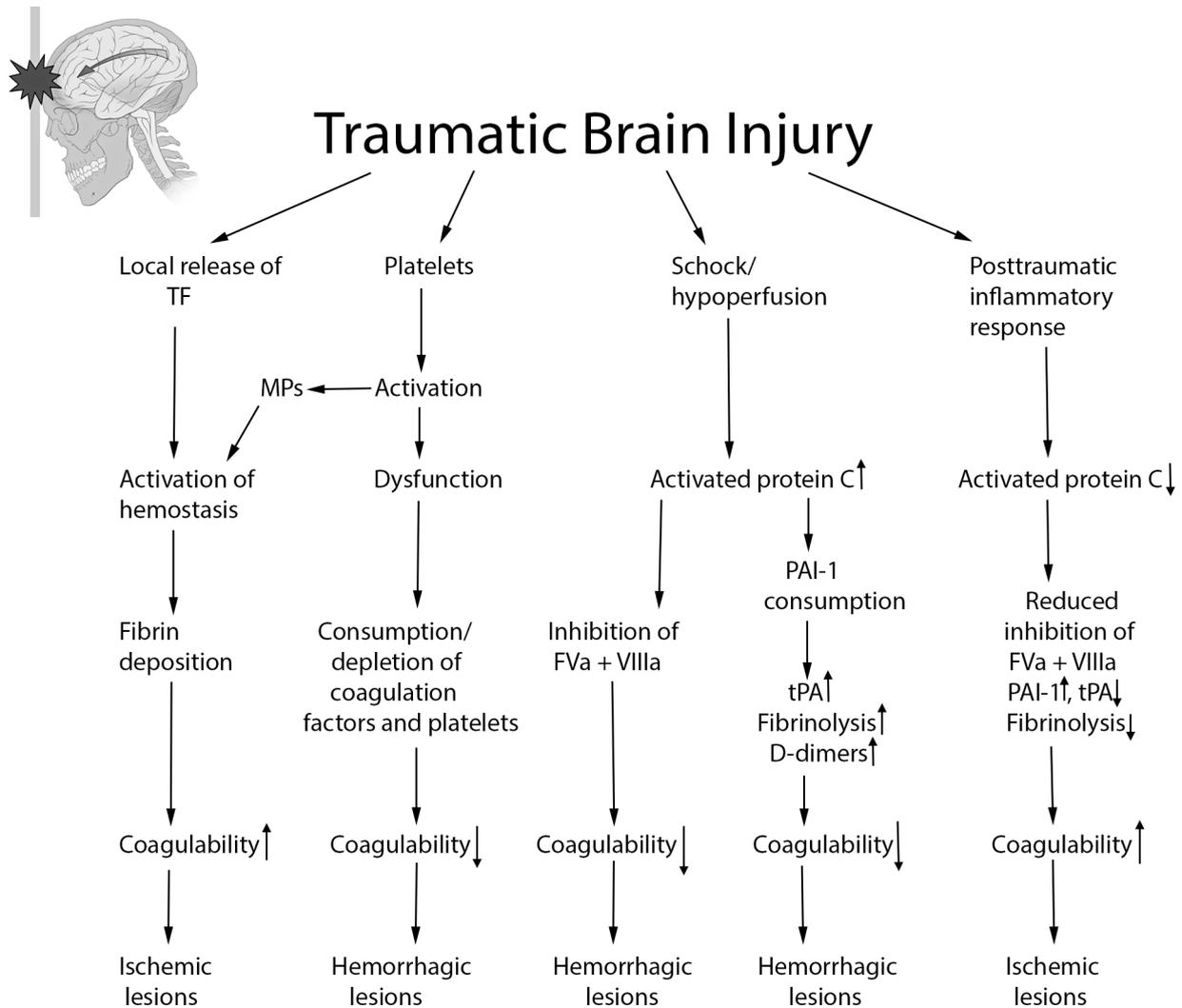
**Fig. 4** Anticoagulant and cytoprotective properties of activated protein C (aPC). Protein C is activated after binding to endothelial protein C receptor (EPCR) at the endothelial surface via cleavage by thrombin. This cleavage happens in the presence of thrombin-thrombomodulin (TM) complexes. aPC exerts anticoagulant effect mainly by cleaving coagulation factors Va and VIIIa, and also by inhibiting plasminogen activator inhibitor 1 (PAI-1). aPC activity is inhibited by protein C inhibitor,  $\alpha_1$ -antitrypsin,  $\alpha_2$ -macroglobulin and  $\alpha_2$ -antiplasmin. aPC induces cytoprotective effects for the brain via the PAR-3 receptor. (Reproduced from Christiaans et al., 2013 with editors' permission.)

An intriguing question is whether mechanisms in normal haemostasis are different from those in thrombosis. According to a review by Geddings and Mackman [80], mechanisms of interest in this respect involve factor FXII, inorganic polyphosphates, TF-positive MPs and neutrophil extracellular traps (NETs) [80].

## 2.2 The coagulation system in TBI

The main mechanisms involved in TBI-induced coagulopathy are shown in **Fig. 5**. These include release of TF, hyperfibrinolysis, hypoperfusion with subsequent activation of the protein C pathway, DIC and TBI-induced platelet dysfunction [56]. Mechanisms involving TF release, platelet activation and the inflammatory response are described in separate chapters of this thesis (see below). Looking at the pathogenic picture from a “helicopter perspective” it becomes clear that TBI-induced coagulopathy incorporates both *hypercoagulative* and *hypocoagulative* states. Secondary injuries may then be due to either

microthrombosis or haemorrhage, or both [56, 81]. This seemingly controversial situation arises as a result of the complicated pathophysiology of coagulopathy and its progress through different phases. Thus, TBI-induced coagulopathy may be considered as a dynamic process with an initial hypercoagulative state and later on development of bleeding diathesis [81-85].

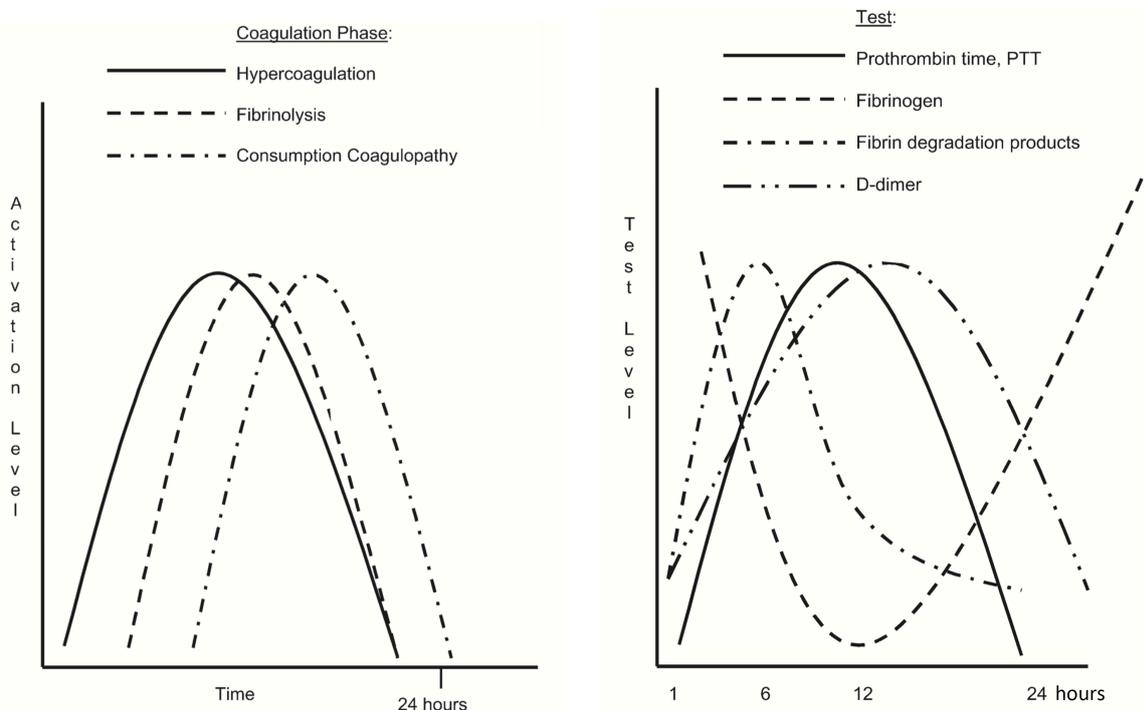


**Fig. 5** Mechanisms of coagulopathy following TBI. The illustration is based on a review by Maegele, [56]. Reproduced with editor's permission.

The time course of coagulopathy has been discussed since the 1970s [56, 86]. In the classic publication by Stein and Smith (2004), a thorough description of the coagulopathy phases from the moment of trauma to 24 hours thereafter is given (**Fig. 6**). Levels of fibrin degradation products and D-dimers rise quickly in the plasma and show pathological values

within minutes after trauma. PT and APPT are usually normal during the first hour after injury, but start to rise and peak within 6 hours. In cases of a favourable course of trauma, these parameters then return to normal within 24 hours [43]. Other researchers, however, have reported somewhat different temporal patterns of changes of coagulation variables; e.g. PT peaks in 1–6 hours with normalization in 6–12 hours [87]; PT peaks in 6–36 hours; venous platelet count reaches its lowest value in 24 hours [88]; D-dimer peaks in 4 hours [89]; plasma fibrinogen decreases in 0–3 hours with stabilization in 3–6 hours, with a return to normal or increased values in 6–12 hours [57]. In the majority of studies, TBI-induced coagulopathy reaches its maximum within 24 hours after trauma and then starts to normalize. However, late in the course of trauma, a prolonged hypercoagulative state may be present and if not treated this may lead to peripheral thrombotic complications such as DVT [62, 81].

Early onset of coagulopathy and its fast development are factors strongly related to complications. Patients with coagulopathy within 24 hours of injury have been shown to have a mortality rate of 55% compared with a rate of 23% in patients developing coagulation abnormalities later than the first 24 hours [88].



**Fig. 6** Phases of coagulation disturbances after TBI. Reproduced from Stein and Smith, Neurocritical Care, 2004, with the editor’s permission.

Another mechanism is triggered by shock and hypoperfusion, which promotes endothelial thrombomodulin, binding to thrombin and building thrombin-TM complexes, which then activate protein C into activated protein C (aPC), blocking plasminogen activator inhibitor (PAI-1), resulting in hyperfibrinolysis and promoting coagulation factors Va and VIIIa [90]. At a later stage of TBI, posttraumatic neuroinflammatory responses may result in depletion of protein C, causing a hypercoagulative state [81]. Hypoperfusion is certainly important in the pathogenesis of TBI. However, its role has been challenged by Lustenberger et al., who concluded that hypoperfusion is a significant but not essential factor for development of coagulation abnormalities [91].

It is possible that certain types of TBI result in specific changes in coagulation variables. For example, in one study, serum levels of TM originating from damaged cerebral endothelial cells were higher in localized than in diffuse brain injury [92]. The same pattern is seen in fibrinolysis markers D-dimer/FDP, which is fairly logical since fibrinolytic changes usually follow those of TM [57, 93]. Comparing different types of haematoma, traumatic epidural hematoma (tEDH) is not often associated with parenchymal injury and thus results in minimal activation of the coagulation system. This is true only in cases of isolated tEDH. However, the majority of cases have combined injuries, with subdural haematoma, haemorrhagic contusions etc.[57, 94]

Another intriguing parallel between the clinical picture and coagulation laboratory variables concerns the “talk-and-deteriorate” (T&D) phenomenon, i.e. a rapid drop in patient level of consciousness, which can be due to progression of intracranial haematoma or other poorly understood pathophysiological events [95]. The T&D phenomenon is often associated with hyperfibrinolysis measured by  $\alpha_2$ -plasmin inhibitor or D-dimer in plasma [96], which may be interpreted as a reflection of microcirculatory disturbances.

An interesting approach in the detection of molecular events in the CNS following TBI is to biochemically analyse cerebrovenous blood. This can be possible through the use of a jugular bulb catheter, which is used in neurointensive care for monitoring of oxygen saturation (SjO<sub>2</sub>) as an indicator of adequate cerebral perfusion [97] [98]. While the majority of brain trauma studies have been performed using conventional sampling of peripheral venous blood, some projects have involved assessment of cerebrovenous blood and arteriovenous (transcranial) gradients of substances of interest. Blood sampling from a peripheral vein is simple and prompt, readily available directly after patient arrival at the hospital, or even at the site of accident. In contrast, inserting a jugular bulb catheter is demanding and time-consuming, and this approach is only available in specialized neurointensive care units (NICUs) [99].

In summary, laboratory findings reflecting defibrination, thrombocytopenia and hyperfibrinolysis are signs of TBI-induced coagulopathy and also predictors of mortality.

### **2.3 Tissue factor in TBI**

Tissue factor (TF) or CD142 according to the CD (cluster of differentiation) nomenclature, or thromboplastin (in older literature) is a transmembrane glycoprotein related to the cytokine receptor family. It is expressed on perivascular smooth muscle cells, pericytes and fibroblasts [100-102]. Importantly, the brain is exceptionally rich in TF, where it is also present on astrocytes [103, 104]. TF is known to be a potent coagulation activator [105, 106], triggering the TF-induced pathway of coagulation (see above). The abundance of TF in the brain and its powerful procoagulant properties resulted in the popular hypothesis that TF, if released into the circulation, triggers activation of the coagulation cascade, thus causing a hypercoagulative state following TBI [81]. This idea constitutes the prevailing model of the pathophysiological mechanism responsible for triggering coagulopathy in TBI. This point of view is widely described in textbooks, review articles and original papers [41, 43, 86, 87, 107-110].

However, none of the studies referred to involved measurement of the release of TF into the circulation, to prove that this concept is true. This is probably because of methodological difficulties in measuring circulating TF. Attempts have been made to use TF as a marker of brain damage, an indicator of coagulopathy or a possible prognostic factor in TBI [49, 82, 96], but again such attempts have been limited by methodological problems. Thus, the focus has shifted towards monitoring TF effects indirectly, and to assess fibrinolysis or the development of DIC [56, 96, 111]. In addition to the methodological issues, the presence of soluble TF in plasma is a subject of great debate. Since TF is a large transmembrane protein, it has been argued that it should not be present in the circulation. However, there are data in support of the existence of soluble TF in an “encrypted form” [112-114]. Alternative sources of circulating TF may be leukocytes, platelets and microparticles from platelets, leukocytes and endothelial cells. Indeed, circulating monocytes have shown increased TF expression and interaction with platelets following TBI [115]. It should also be noted that circulating MPs carry TF and that such MPs have pronounced procoagulant properties and may amplify the thrombotic process [113, 116]. Circulating MPs expressing TF have been found in high concentrations in many pathological conditions such as cancer patients with DVT, liver disease, urinary tract infections and endotoxaemia [117].

## 2.4 Fibrinogen in TBI and general trauma

Fibrinogen is one of the most central and important parts of the coagulation process, but it is also an acute-phase reactant, and its plasma levels may rise up to tenfold during trauma, infection or SIRS [118]. Reduced plasma levels of fibrinogen or impairment in its functionality may lead to serious consequences in situations where haemostasis is needed. Low levels of fibrinogen have been reported in severe general trauma, resulting from profuse blood loss and haemodilution [119], or hepatic insufficiency with impaired fibrinogen production and functionality [120]. The ultimate case of hepatic insufficiency is liver transplantation, where the anaesthesiologist needs to pay close attention to monitor and replace low levels of fibrinogen and other coagulation factors [121, 122].

Target levels of fibrinogen substitution have been progressively raised from 0.8–1 g/L, which was the recommendations 30 years ago [123] to the current level of 2–2.5 g/L [124, 125], and in certain clinical situations even > 3 g/L [126]. A level of 1 g/L is nowadays considered to be a critical threshold below which sufficient haemostasis cannot be achieved. Substitution therapy includes plasma or preferably fibrinogen concentrate [118] (e.g. RiaSTAP™, where a dose of 2g increases fibrinogen plasma levels by approximately 1 g/L in adults).

The “TBI fibrinogen story” began in 1974, with a classic piece of work by S.H.Goodnight, who took coagulation samples shortly after trauma directly at the site of accident and found grave hypofibrinogenemia in TBI cases [86]. This excellent publication needs to be cited literally: *“Defibrination was not observed in 13 patients in whom trauma did not destroy brain tissue. In contrast, evidence of defibrination and low platelets was found in 9 of 13 patients in whom brain-tissue destruction was established by direct inspection. All nine patients had a strongly positive protamine test, suggesting that intravascular coagulation has occurred. ... The potentially salvageable patient with acute TBI may require emergency replacement of hemostatic factors”*. (A protamine test was used as a marker of DIC, since coagulation occurs more rapidly in blood samples from patients with DIC in the presence of protamine [127]). The authors proposed early replacement therapy, admitting that it would result in some overuse of blood products, with associated risks. This publication started a debate, which is still ongoing, regarding guidelines for procoagulative therapies in TBI. Up to now, there are more than 1670 publications in a PubMed search using terms “coagulation” and “head injury”.

## 2.5 Microthrombosis vs. bleeding in TBI

Comparing the dangers of thrombosis vs. bleeding reminds one of the ancient figures of Scylla and Charybdis, both equally lethal. However, the risk of bleeding is more immediate and visible, and raises most concern among neurosurgeons [52, 128, 129]. The patient case shown in **Fig.1** illustrates the progress of intracranial haemorrhage between the first and second CT scans (a follow-up scan is carried out six hours later). Interestingly, this patient demonstrated an overt bleeding tendency despite seemingly normal standard coagulation test results, which might be a result of dysfunctional platelets (see next chapter).

This phenomenon of progressive bleeding is well known among neurosurgeons and is described in the literature as “progressive haemorrhagic injury (PHI)” [60, 130], “delayed intracerebral haematoma” [131] or “haemorrhagic progression of a contusion” [128]. Notably, about one half of TBI patients demonstrate haemorrhagic progress on follow-up CT scans [60, 132]. Haemorrhagic progression occurs within 12 hours in most cases, but may be observed up to 3–4 days after the injury [133]. Development of PHI is associated with an unfavourable outcome [134] and a nearly fivefold increase in mortality [52]. Extravasated blood is toxic to the brain tissue, causing necrosis of neurons and formation of perifocal oedema, which contributes to further extravasation of blood and local ischaemia [135, 136]. The pathophysiological origin of PHI is a subject of debate. According to the traditional view, microvessels in the border zones are damaged at the instant of traumatic impact. The damage is, however, not visible at the initial CT scan. Recent findings indicate, however, that vessels are intact immediately after the trauma but they become disrupted and open a few hours later, following a series of specific pathophysiological events [128]. The brain vasculature contains mechanosensitive structures and signalling pathways, involving integrins, ion channels and transcription factors. The event sequence begins when the mechanosensitive transcription factors specificity protein 1 (Sp1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) are triggered by the kinetic energy [137, 138]. These two are linked to transcription of sulphonylurea receptor 1 (Sur1), which in turn upregulates the Ca-ATP channels [139]. This process takes a few hours to develop and it promotes oncotic cell swelling and necrosis in neurons, astrocytes and endothelial cells [140]. Damage to endothelial cells leads subsequently to vessel disruption, sometimes called “capillary fragmentation” [141]. This intriguing mechanism is a recent discovery, but an attempt to intervene pharmacologically has already been undertaken using glibenclamide (Sur1 inhibitor), resulting in blockage of PHI development in an experimental animal setting [141].

Microcirculatory events following trauma play a central role in TBI pathophysiology, not

only contributing to development of PHI but also to other dynamic changes of cerebral perfusion following TBI, oedema generation and microthrombosis.

Cerebral perfusion goes through several phases including an immediate decrease in cerebral blood flow (CBF) and metabolism [142], followed by vasodilation with hyperaemia [143], which is partly a nitric oxide-dependent mechanism. Approximately one week following injury, another phase may develop, characterized by spasm and hypoperfusion, especially when traumatic subarachnoid haemorrhage is present [143]. Mechanisms behind hypoperfusion are still a subject of debate, but brain oedema and vessel compression [144], as well as vasoconstriction and microthrombosis have been proposed as underlying mechanisms [145]. Microcirculatory changes following experimental TBI in mice have been described in detail in a study by Schwarzmaier et al. [146]. Using *in-vivo* fluorescent microscopy, the authors demonstrated formation of platelet–leukocyte aggregates and microthrombi in 77% of the venules and 40% of the arterioles examined. These findings were observed as soon as 30 minutes after trauma and reached a maximum at 60–90 min. Microthrombosis has also been detected in histopathological studies of deceased TBI patients [48, 147] and in other experimental studies in animals [84, 85]. In a recent study Thelin et al. showed that 39% of patients suffering from TBI developed secondary increases in serum levels of the brain injury marker S100b, mostly due to cerebral infarctions [148]. Based on a fairly large amount of research data, it can be concluded that microthrombosis is probably the principal mechanism responsible for hypoperfusion in the border zone following TBI [146]. However, this pathophysiological phenomenon probably also has a “protective” role, as it will seal damaged vessels and prevent bleeding and plasma leakage, decreasing the risk of brain oedema development. In support of this idea, it has been shown that blocking microthrombus formation through platelet inhibition results in increased oedema formation [149].

It is conceivable that a better understanding of the microcirculatory disturbances associated with TBI will provide keys to solving the “microthrombosis vs. bleeding” problem.



### 3 PLATELETS

Platelets, or thrombocytes, are nucleus-free “cells” present in blood at levels of 150–350  $\times 10^9/L$  in healthy persons. Platelets are discoid in the resting state, have a diameter of 2–3  $\mu\text{m}$ , and originate from megakaryocytes in the bone marrow. They circulate in blood for 9–11 days and their primary function is to stop bleeding in case of vessel injury by building a blood clot and initiating healing [150-152]. Beside this “emergency” function, platelets also participate in vitally important interactions with endothelium, leukocytes and inflammatory processes, playing a wider role in maintenance of homeostasis in normal functioning healthy organisms. Thus, platelets are sensitive “health markers”, prone to activation due to trauma or other coagulative disturbance, inflammatory diseases, infection or cancer [153, 154].

Platelets are important in maintaining vessel integrity, not only in trauma but also in normal physiological conditions. For instance, in situations with profound thrombocytopenia, disruption and fenestration of the endothelial barrier is seen, resulting in leakage of red blood cells into the tissue (extravasation of blood, observed as petechiae) [155, 156]. The mechanism of this protective action is not entirely known, but pathways involving the trophic effect of platelets upon endothelial cells, release of mitogens, recruitment of bone marrow progenitor cells and direct adhesion of platelets to gaps in the endothelial lining are discussed in the literature [155, 157, 158]. Recent studies show that platelets can also interact with neutrophils, promoting the formation of so-called neutrophil extracellular traps (NETs). These are network-like structures composed of released DNA and various highly reactive proteins including histones [159-161]. These NETs have an antimicrobial action, but they may also contribute to thrombus formation, e.g. in DVT or in ischaemic stroke [160, 162].

#### 3.1 Platelet activation

Platelets normally circulate in a “resting state” under the influence of platelet-inhibiting endothelial-derived substances such as NO and  $\text{PGI}_2$ . Platelet activation starts upon vessel injury, when molecules present in the subendothelial layer come into direct contact with blood. Von Willebrand factor plays an important role linking platelets to the site of injury, especially under high-flow conditions [163, 164]. Collagen, which is also present in the subintimal layer of vessels, is a strong platelet agonist and is also responsible for early platelet activation. Schematically, this activation process involves the following steps: adhesion and shape change, aggregation, secretion and vesiculation (i.e. generation of

platelet-derived microparticles [PMPs]). Upon activation, a rapid process of change in shape of the platelet starts. The platelet thus transforms from a discus-like shape into a dendritic shape. The surface area of the platelet is grossly increased and its ability to stick firmly to various surfaces is enhanced. Subsequently, activated platelets secrete what is stored in cytoplasmic  $\alpha$ -granules and dense granules. Dense granules contain smaller molecules such as ADP, calcium and serotonin, while  $\alpha$ -granules contain more complex molecules such as coagulation factors (fibrinogen, FV, FXIII), P-selectin, VWF, and growth factors such as PDGF [165-169]. Vesiculation involves generation of membrane microvesicles, or microparticles, which are tiny circular fragments of cell membranes with a size range of 100–1000 nm. The MP membrane exposes the same antigens as the mother cell, thus reflecting its cellular origin. MPs may also reflect the physiological state of the mother cell by exposing various “activation molecules”. For example, platelet microparticles (PMPs) that expose P-selectin indicate that the “mother platelet” has degranulated. Notably, MPs should not only be viewed as markers of cellular activation, but also active mediators of various biological processes such as thrombosis or inflammation. For more details about the role of MPs, see Chapter 5.

### 3.2 Platelet receptors and platelet inhibitors

The platelet cell membrane carries several types of receptors, which can be targets of pharmacological intervention. The most important receptors in this respect are shown in the left column below. In the right column pharmacological agents that inhibit the corresponding receptors are shown:

- |                                       |                                    |
|---------------------------------------|------------------------------------|
| - Glycoprotein (GP) IIb/IIIa receptor | abciximab, tirofiban, eptifibatide |
| - ADP receptor P2Y <sub>12</sub>      | clopidogrel, ticagrelor, prasugrel |
| - TxA <sub>2</sub> receptor           | ifetroban, terutroban              |
| - Protease-activated receptors (PARs) | vorapaxar                          |

GPIIb/IIIa is a transmembrane spanning receptor, which mainly binds fibrinogen but also other molecules such as VWF and vitronectin. The GPIIb/IIIa receptor can be considered as the most important receptor in platelet aggregation, as it is mainly through divalent fibrinogen-GPIIb/IIIa binding that activated platelets are “glued together” in aggregates. A defect in the gene coding for the GPII/b/IIIa receptor results in Glanzmann’s thrombastenia, a condition with severely impaired platelet aggregation, which mechanistically is due to a defect in or low levels of the GPII/b/IIIa receptor [170]. There are, however, also acquired

forms of this disease [171].

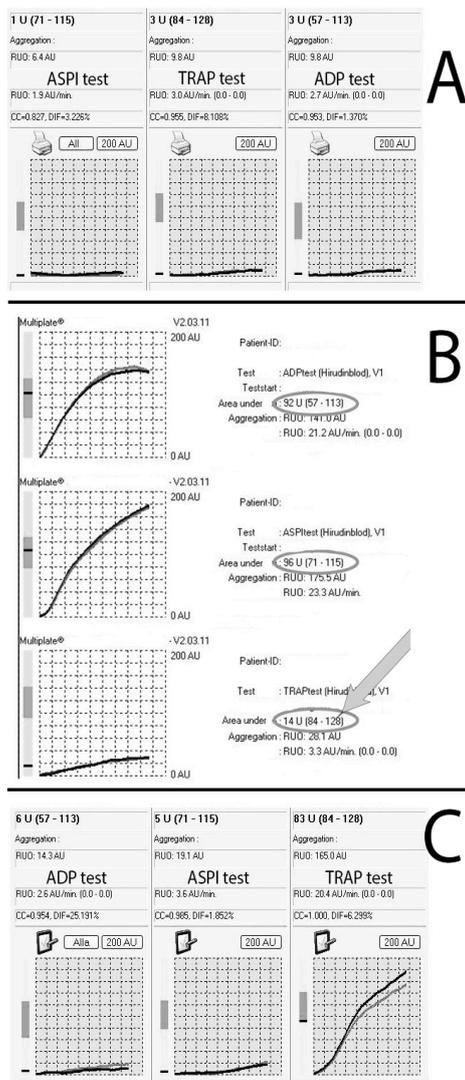
Stimulation of platelets with various agonists, such as ADP or thrombin, leads to clustering and conformational changes of the GPIIb/IIIa receptors. This increases fibrinogen binding several-fold and induces aggregation. Consequently, platelet aggregation can be inhibited through blockage of the GPIIb/IIIa receptors, e.g. by agents such as abciximab or tirofiban (see above), leading to powerful and irreversible platelet inhibition, sometimes down to zero responsiveness (**Fig. 7A**).

The physiological role of ADP receptors emerges from the fact that platelets, and also damaged endothelial cells release ADP, which activates platelets. Recent research has shown that there are at least two types of ADP receptor present on platelets, i.e. P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors [172]. Stimulation of the P2Y<sub>1</sub> receptor leads to platelet shape change and reversible platelet aggregation, while stimulation the P2Y<sub>12</sub> receptor amplifies the platelet aggregation response [172]. Inhibition of the P2Y<sub>12</sub> receptor is today widely used as anti-platelet therapy in coronary artery disease, ischaemic stroke and peripheral arterial disease, and in invasive procedures with stent implantation of stenotic arteries [173].

Inhibition of the platelet-activating compound thromboxane A<sub>2</sub> (TxA<sub>2</sub>) is probably the oldest known principle to influence platelet function since the introduction of ASA by Felix Hoffmann in 1897 [174]. It is now well known that ASA inhibits the formation of TxA<sub>2</sub> through blocking the enzyme cyclooxygenase (COX), which catalyses the synthesis of endoperoxides (including thromboxane) from arachidonic acid (AA). More specifically it has been shown that ASA blocks COX irreversibly by acetylating the catalytic centre of the enzyme [175]. Thromboxane is a potent platelet agonist and released TxA<sub>2</sub> activates platelets via binding to the Tx receptor, a G-protein-coupled receptor present on the platelet cell membrane. Insufficient formation of TxA<sub>2</sub> results in attenuated platelet aggregation in response to various platelet agonists, resulting in prolonged bleeding time and arterial thrombus instability [176].

Thrombin is the most potent platelet agonist known, and it acts via protease-activated receptors (PARs). There are two types of PAR receptor on platelets, PAR-1, which responds to low thrombin concentrations, and PAR-4, which responds to high thrombin concentrations [177]. In addition to PARs, the platelet GPIb receptor also possesses a high-affinity site for thrombin and is involved in thrombin signalling [178]. A PAR-1 receptor antagonist, vorapaxar, has been developed and tested in clinical trials concerning cardiovascular disease. However, due to bleeding problems the drug has not been taken to the market [179]. In our

own practice, we treated a patient who participated in this study and was admitted to a NICU because of intracerebral bleeding. The patient suffered from an overt bleeding tendency, which was confirmed by a low platelet response in multiple electrode aggregometry (MEA)(see below). Despite various pharmacological treatment attempts and infusion of platelet concentrate it was impossible to reverse the platelet inhibition by vorapaxar (**Fig. 7B**).



**Fig. 7** Analysis of platelet function using multiple electrode aggregometry (MEA).

**Panel A:** Absence of platelet responses to ADP, AA and TRAP in a patient receiving an abciximab infusion. **Panel B:** Low platelet response to thrombin receptor activating peptide-6 (TRAP) in a patient medicated with vorapaxar. **Panel C:** Reduced platelet responsiveness to ADP and AA in a patient with TBI.

### 3.3 Measurement of platelet function

The standard “coagulation test battery” includes platelet count, but examination of platelet function demands other methods. One aspect of platelet function is platelet aggregation, which can be assessed by different methods. The most traditional method is light-transmission aggregometry (LTA) as described by Born as early as in 1962 [180]. This assay is based on an increase in light transmission through platelet-rich plasma when platelets aggregate upon the addition of a platelet agonist, e.g. ADP [180]. There have been substantial developments in platelet methodology since then, and nowadays it is also possible to assess changes in light transmission in whole blood. The VerifyNow® (Accumetrics, Ca) system is a rapid and simple point-of-care method [181-183] performed using whole blood samples, which assesses changes in light transmission when platelets adhere and aggregate to fibrin-coated beads. Disadvantages of this method are limited haematocrit and platelet count range, and high cost. Another point-of-care system that measures platelet aggregation is Plateletworks® (Helena Laboratories, Tx). The principle of the analysis is based on platelet counting in a whole blood sample to which a certain amount of a platelet agonist has been added. Formation of platelet aggregates will yield fewer platelet counts in the sample. This method is very sensitive to platelet activation, but it requires rapid handling of the sample, and the method is less well evaluated in clinical trials than, for example, VerifyNow® or whole blood impedance aggregometry [184, 185]. The latter method utilizes the change in electrical impedance when the platelet plug starts to grow on the surface of an electrode placed in a whole blood sample. Activation of platelets occurs under more “physiological” conditions in whole blood compared with platelet-rich plasma, which makes the results more relevant for the clinical situation. Impedance aggregometry is the principle in the Multiplate® analyzer (ROCHE, Switzerland)(i.e. MEA), a relatively well-documented method used for monitoring of long-term antiplatelet therapy, within vascular surgery, neuroradiology and other applications [186-189]. Platelet function can also be studied by thromboelastography (TEG), for example using the modified TEG-platelet mapping™ (TEG-PM) assay, (Haemoscope Corporation, IL). This assay compares clot firmness in four parallel channels, containing whole blood with and without agonists. The method can be used for monitoring antiplatelet therapy and also to detect TBI-induced platelet dysfunction [190]. Being relatively laborious and expensive, the method has not become widely popular in point-of-care settings.

The Platelet Function Analyzer (PFA-100, Siemens Healthcare Diagnostics, IL) represents an attempt to create an “injured blood vessel-like” situation with continuous blood flow through

a capillary coated with platelet-activating compounds like collagen, ADP and adrenaline, under a relevant shear rate. Based on this unique setting, the analysis is also called “*in-vitro* bleeding time” [191, 192]. This method is influenced by various disturbances and confounding factors, such as variations in haematocrit and levels of VWF, which may limit its use. Another disadvantage is inability to detect the effect of clopidogrel [193, 194].

Despite the relative simplicity and availability of the methods described above, flow cytometry is considered to remain “the gold standard” of platelet function studies. Flow cytometry can, in principal, be used in connection with all cellular types in fluid suspension. It can also be used to detect and study blood microparticles of various cellular origins. The technique is based on how the particles in flow reflect a laser beam, and to what extent fluorescently labelled markers are present on the surface of the particles. Knowing the antigen profile, the researcher is able to identify the origin of the cell and its functional state of activation or transformation, and possible interactions with other cells or MPs [195, 196]. Analyses of platelet-derived MPs (PMPs) are practical methods to assess platelet function in stored plasma samples, as it is possible to measure MPs in frozen-thawed plasma samples. Through assessment of binding to phalloidin, a fungal toxin that binds to actin with high specificity, it is possible to discriminate PMPs from cell membrane fragments which may occur in the flow cytometry gate [197]. In this way the quality of the plasma sample can be assessed, excluding samples that, for example, have been insufficiently pre-analytically handled [197, 198]. In order to study platelet activation through assessment of circulating PMPs, CD42a (also called GPIX, a member of the platelet GPIb-IX-V receptor complex) is used to identify platelet origin. Then CD62P (P-selectin), which is exposed on the surface of activated platelets, is measured on the CD42a-positive particles, since it will indicate that the “mother platelets” have undergone activation/degranulation [197, 199, 200].

### **3.4 Platelets in general trauma and TBI**

A low platelet count or abnormal platelet function can be observed in both general trauma and in TBI, and they are important aspects of coagulopathy. However, in general trauma coagulopathy has been considered to originate mostly from haemodilution, whereas in TBI it may mostly be due to consumption (DIC) [56, 67, 201].

Thrombocytopenia is a well-recognized prognostic factor in TBI, indicating high risk and poor prognosis [202-204], while platelet function is less well studied in TBI, despite the

importance of cellular haemostasis in this kind of trauma. In some TBI cases, an obvious bleeding tendency cannot be explained by any of the standard coagulation measures such as venous platelet count, PT, INR, APTT, and D-dimer or fibrinogen levels in plasma [52, 205]. This represents an important argument to perform additional functional tests such as viscoelastic procedures or various platelet function tests.

Kutcher et al. [206] undertook a thorough study of platelet function in 101 trauma patients, demonstrating an abnormal platelet response in 45% of patients at admission and 91% at some time point during their ICU stay. Platelet dysfunction was defined as a test value below normal (i.e. below the 5<sup>th</sup> percentile of the manufacturer's reference value) as regards one or more agonists tested. The patients with dysfunctional platelets at admission had lower GCS scores and were more acidotic compared with the group with normal platelet function. Importantly, their mortality rate was almost tenfold higher. Low platelet responsiveness to arachidonic acid, to thrombin receptor-activating peptide-6 (TRAP) and to collagen at admission, but not thrombocytopenia, were associated with death in this unselected trauma population (general trauma and TBI) [206]. In other trauma material, low responsiveness to ADP and to TRAP has been found in those who did not survive [189].

Interestingly, platelets seem to behave in different ways in TBI and in non-head injury. This was first observed by Jacoby et al. in 2001 [207] and later confirmed in several studies carried out in different neurotrauma centres. In the study by Jacoby et al., TBI patients had increased platelet activation measured by flow cytometry (CD62P and PAC-1 antibodies), but decreased functionality, defined as prolonged closure times using the PFA100 device (see section 3.3, Measurement of platelet function) [207]. In more recent studies, platelet function has been systematically tested in TBI patients. In our patients (see below), decreased reactivity to AA was the most prominent finding, while other researchers have found deficiency in the ADP response [208] and Davis et al. noted decreases in both AA and ADP responses [209]. **(Fig. 7C)**

A hypothetical mechanism of trauma-induced platelet dysfunction was, however, proposed as early as 1980 [210]. According to this early hypothesis, in the trauma situation platelets are exposed to TF and platelet activating factor (PAF), activating them to the point of exhaustion and subsequent anergy. This theory is still a predominant view of the problem [56, 211, 212]. An alternative explanation could be an unknown endogenous platelet inhibitor acting in brain trauma patients. In fact, some specific pathways capable of inhibiting platelets are described in the literature, such as degradation of COX by thrombin and consequent non-responsiveness to AA [213]. However, the relevance of such a mechanism in TBI is

unknown.

Substance abuse, predominantly involving alcohol, is common in cases of TBI. Around 35–50% of TBI patients have alcohol in the blood at the moment of injury [214]. Alcohol has platelet-stabilizing effects and when present in blood in sufficient amounts it may attenuate platelet reactivity [215]. Thus, although alcohol is an obvious risk factor for attaining brain injury, it may have some protective effects once TBI occurs, probably due to suppression of coagulopathy development through platelet-inhibiting and perhaps anticoagulant effects [216]

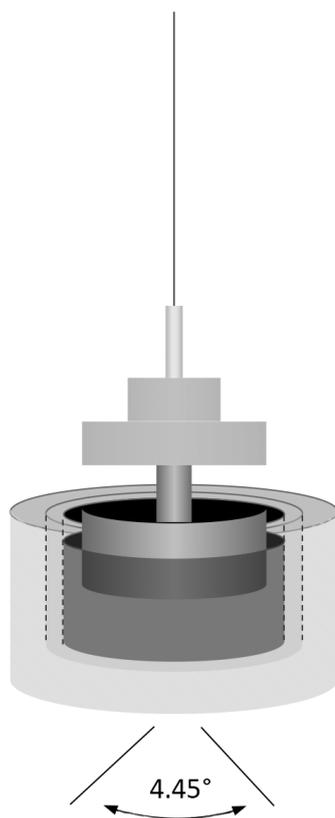
## 4 VISCOELASTIC METHODS FOR THE MONITORING OF COAGULATION

Viscoelastic coagulation tests such as TEG® and ROTEM® have become increasingly popular in recent years. Traditional coagulation tests involve analysis of specific factors in plasma, which are isolated fragments of a complex system. In contrast, viscoelastic methods provide a “global” view of haemostasis, taking into account interactions between pro and anticoagulation factors, platelets, fibrinolysis and sometimes also pharmacological substances that may be present in the blood sample [217]. Thromboelastography (TEG) was introduced in the 1940s [218], but until recently its clinical application was limited by technical imperfections and inconvenience of use. The method is based on measurement of the viscoelastic changes in a clotting whole blood sample placed in a test cartridge. In TEG analysis the speed of the coagulation process (initialization), its progress, strength of clot and, finally, fibrinolysis are evaluated. The whole analysis including fibrinolysis takes about 90 minutes, but the initiation phase can be evaluated as soon as after 5–10 min and maximum strength after 20–30 min. The method is relatively simple and can be used in bedside settings.

Several studies have dealt with TEG applications in various perioperative situations, in trauma and massive transfusion [219, 220], liver transplantation [121], cardiac surgery and in the diagnosis of heparin-induced thrombocytopenia [221]. Viscoelastic methods such as TEG and ROTEM have been considered to outperform traditional tests in detecting coagulopathy due to haemorrhagic shock and hypothermia [222, 223]. TEG-based algorithms provide guidance in massive transfusion situations in trauma, resulting in lower overall transfusion requirements [224, 225]. However, the general opinion among the majority of authors is that TEG-guided protocols are not better than traditional ones in reducing mortality [220, 226].

Detection of abnormal fibrinolysis by means of viscoelastic methods seems to be superior compared with traditional tests such as D-dimer assay, plasma fibrinogen level measurements and euglobulin lysis time. ROTEM has been put forward as the “gold standard” in diagnosis of hyperfibrinolysis [227]. This condition is common in trauma patients and strongly correlated to mortality. In a study by Schöchl et al. [228] patients were divided into three groups (fulminant, intermediate and late fibrinolysis), and those with fulminant hyperfibrinolysis showed 100% mortality.

Studies carried out with viscoelastic methods have also been performed in TBI patients since the 1980s [229, 230] and until recently [231], demonstrating prolongation of clotting time, reduced speed of clot formation and clot strength, and also increased fibrinolysis. Looking at patient outcome, the survivors had faster clot formation and better clot firmness compared with non-survivors. Both the TF-induced pathway (EXTEM), contact activation (INTEM) pathway and fibrin clot formation (FIBTEM) were tested, with similar results, but clot firmness in FIBTEM showed the strongest association with outcome [231]. However, clinicians and researchers should keep in mind that interpretation of viscoelastic test data is different from the interpretation of traditional tests, and thus demands certain training. For instance, sensitivity in detecting hyperfibrinolysis seems to be very different: based on D-dimer, increased fibrinolysis was shown in nearly all TBI patients [57], while studies using viscoelastic methods give a much lower incidence [228, 232, 233], in one study as low as 14% among non-survivors [231].

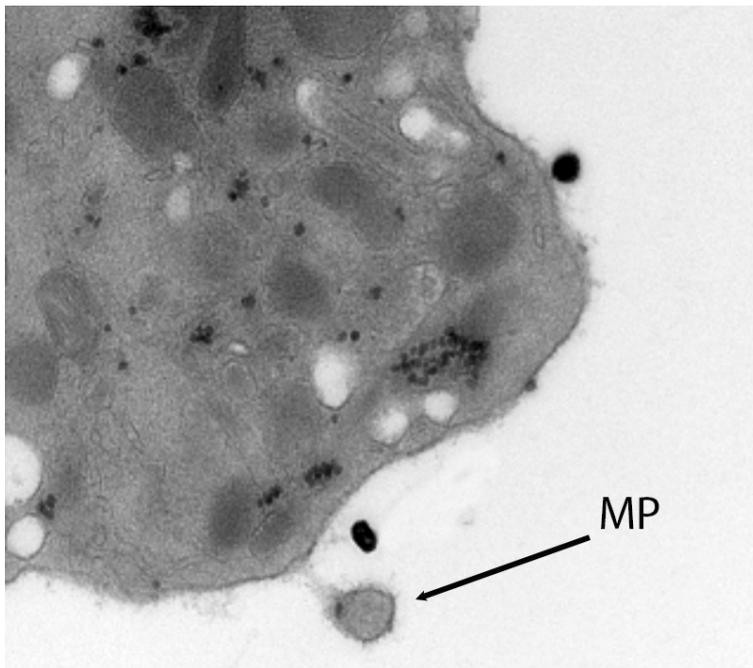


**Fig. 8** Working principle of viscoelastic coagulation tests.

## 5 MICROPARTICLES

Microparticles, also called microvesicles, were investigated as markers of platelet and endothelial activation in Study 3, and as potential biomarkers of brain injury in Study 4. These tiny structures have attracted great interest in various areas of medicine. Microparticles are small fragments of cell membrane, 0.1–1  $\mu\text{m}$  in size, and released from mother cells in certain conditions such as cell activation, various cellular interactions or cell death [234]. Thus, MPs may provide valuable information on functional status of mother cells and underlying physiological events [235]. However, MPs are not only markers of physiological processes, but may also be active triggers in processes such as inflammation, thrombosis and cancer [205, 236-241]. Over the years there has been great interest in the possible role of MPs as messengers of biochemical signals in the CNS. Thus, MPs may be involved in a variety of processes such as neuron development, synaptic activity and nerve repair [242-244]. These aspects of MP function are beneficial for the organism. However, detrimental effects of MPs may also prevail in neuroinflammatory diseases [245], causing prothrombotic effects, neuronal apoptosis and microglial activation. Elevated levels of MPs have not only been reported in neuroinflammatory disease but also following TBI [113]. Indeed, virtually all types of cell are able to produce MPs, but platelet-derived MPs were the first ones to be discovered, as early as in the 1960s, then described as “platelet dust” [246]. The mechanisms of their generation and significance, however, remained obscure for decades until modern research revived interest in them [247]. Several different types of vesicles are described in the literature and different words are used to define the vesicles investigated, such as exosomes, microvesicles, ectosomes, membrane particles, apoptotic vesicles and microparticles. In the present work we studied MPs sized 100–1000 nm; these types of MPs are also called microvesicles in publications by other researchers.

Analyses of MPs are usually performed by means of flow cytometry, using fluorescent dyes conjugated to cell-specific antibodies for the identification of subpopulations of MPs. For instance, CD42a is an antigen highly specific for platelets, CD144 an antigen on endothelial cells and CD45 is a pan leukocyte antigen used to identify leukocytes. It is possible to combine antibodies directed against two or more antigens, thus selecting even more specific subgroups, e.g. endothelial-derived MPs expressing TF (CD144 and CD142), platelet-derived MPs expressing P-selectin (CD42a and CD62P) [197, 248].



**Fig. 9** Platelet releasing a microparticle. Electron microscopy photograph by Fariborz Mobarrez and Kjell Hultenby.

## **6 POTENTIAL TREATMENT STRATEGIES IN TBI-INDUCED COAGULATION DISORDERS**

Despite the fact that coagulation-related problems in TBI patients were recognized long ago, there are no guidelines available for the monitoring and/or correction of TBI-associated coagulopathy. The reason is probably the lack of knowledge in this field, or the contradictory evidence regarding the use of various therapies. For instance, many aspects of management of TBI patients are compiled in the guidelines issued by the Brain Trauma Foundation [249]. However, the only notification of a coagulation-related problem in this voluminous document deals with deep vein thrombosis (DVT): “*Level III evidence supports the use of prophylaxis with low-dose heparin or LMWH for prevention of DVT in patients with severe TBI. However, no reliable data can support a recommendation regarding when it is safe to begin pharmacological prophylaxis. Moreover, no recommendations can be made regarding medication choice or optimal dosing regimen*”. These latest guidelines were issued in 2007. Fortunately, scientific debates within this field have been intense during the last decade, and a lot of new knowledge was added recently. Hopefully, the next

version of the guidelines will be more extensive and specific.

### *Plasma, erythrocytes*

Transfusions of plasma and red blood cells are routinely used in severe bleeding situations. There is convincing evidence that treatment protocols based on transfusion of blood components are superior to infusions of colloids and synthetic colloids [250]. The recommended target values for transfusion, providing the optimal haemostatic environment, are as follows: Hb > 90 g/L, fibrinogen >2–2.5 g/L, Ca<sup>++</sup> > 1 mmol/L, pH > 7.2. However, TBI patients might behave differently from the general trauma population. The level for “optimal Hb” is a subject of discussion, but levels of Hb above 115 g/L are advocated within the “Lund concept” [251].

Experimental data on large animals with TBI and haemorrhagic shock have shown a beneficial effect of plasma resuscitation on mortality, lesion size and long-term outcome [252]. In humans, the use of plasma in the general trauma population has been associated with an increased risk of developing multi-organ failure (MOF), transfusion-related acute lung injury (TRALI) and acute respiratory distress syndrome (ARDS) [253]. However, these data do not change the consensus of opinion on the use of blood products in massive bleeding situations (see above). In patients with TBI the available data are contradictory: a study by Etemadrezaie et al. demonstrated that infusion of fresh frozen plasma (FFP) in patients with severe TBI was associated with adverse effects and increased mortality (63% vs. 35%) [254]. Unfortunately, an unselected patient cohort was studied, both with and without coagulopathy. Other researchers found that use of two units of FFP for reversal of coagulopathy reduced mortality [255]. In a recent review by Reddy et al., a cautious use of FFP and red blood concentrate (RBC) is recommended, with target levels of Hb > 70 g/L for transfusion [256].

### *Low molecular weight heparin (LMWH)*

Patients with TBI are prone to the development of deep vein thrombosis (DVT). This complication occurs in 54% of TBI patients not given DVT prophylaxis [62] and the use of LMWH in prophylaxis dose is common in TBI. Apart from its role in DVT prophylaxis, LMWH administered early in TBI could have other important beneficial effects [257]. As shown in animal studies, LMWH exerts neuroprotective effects by improving the microcirculation of the brain, and it can reduce adverse neuroinflammatory responses and oedema formation within the damaged area [258]. Furthermore, cognitive outcome in rats subjected to TBI improved in the treated group [258,

259]. Unfortunately, there are no solid data available on LMWH as a neuroprotective agent in TBI in humans. The reason for this knowledge gap is probably fear of bleeding complications or ethical problems associated with controlled clinical trials on the matter.

#### *Antithrombin*

Administration of antithrombin has been tested in a small clinical trial, resulting in a marginal reduction of hypercoagulative state after TBI, but no obvious effect on the brain injury or clinical outcome [260].

#### *Acetylsalicylic acid (ASA)*

Treatment with ASA is used for secondary prophylaxis of myocardial infarction and stroke, although the beneficial effects in primary prophylaxis are controversial [261, 262]. Furthermore, low dose ASA (75 mg daily) has a protective effect against postoperative stroke following carotid surgery [263]. ASA has been put forward as a neuroprotective agent due to its anti-inflammatory effect and by its potentially suppressive effect on neurotoxicity exerted by the excitatory neurotransmitter glutamate. This latter effect has been considered to be mediated by nuclear factor kappa B (NF- $\kappa$ B) [264]. Although there is a potential risk of bleeding complications in a TBI situation when using ASA [265], the results of two studies showed no hazardous effects of ASA intake prior to injury [266, 267].

#### *Platelet concentrate*

It is a logical assumption that thrombocytopenia and platelet dysfunction should be corrected by infusion of stored platelets. However, it is not certain that stored platelets perform as well as native ones, since storage causes metabolic and structural changes in platelets [268]. The functional activity of stored platelets drops rapidly with time: it has been shown that after 72 hours only 2% of platelets respond adequately towards agonist stimulation [269]. A systematic review indicated a benefit of prophylactic platelet transfusion only in patients with haematological disorders undergoing chemotherapy or stem cell transplantation. The results in other conditions were inconclusive [270]. However, the efficacy of platelet transfusion in patients with acute bleeding has been verified in multiple studies, and platelet concentrates are routinely used as a part of standardized transfusion protocols in cases of massive bleeding [250]. Another situation in which infusion of platelet concentrates might be needed is reversal of antiplatelet therapy in patients with trauma or spontaneous intracerebral haematoma. These problems are likely to increase, since the number of patients medicated with anticoagulants and platelet inhibitors is growing steadily [271-273]. *In-vitro* experiments have shown that the function of pharmacologically inhibited platelets

can be restored following mixing the blood sample with drug-naïve platelet concentrates. However, relatively high doses of platelet concentrate have been used to obtain a reversing effect [274].

Regarding the use of platelets in neurosurgical practice, information is much more scarce compared with general trauma, and there is no consensus of opinion concerning how to use platelet concentrates in this setting. A large prospective randomized clinical study on 780 patients with hypertensive intracerebral haematoma demonstrated improved outcome in those who received intravenous infusion of stored platelets: postoperative haematoma volumes were smaller in the treated group and the mortality rate lower compared with controls (16% vs. 34%) [275]. There are four retrospective studies dealing with the effect of stored platelets in patients with TBI, treated with antiplatelet drugs prior to trauma [276-279]. These four studies have been summarized in a meta-analysis by Leong and David [280], overall showing no treatment benefits. On the contrary, infusion of platelet concentrates resulted in higher mortality in the treated group (odds ratio 1.77, 95% CI 1.0–3.1). However, the studies included in this review have several limitations, such as lack of a standardized protocol regarding indications, timing of the intervention and dosage of platelet transfusion. Furthermore, platelet function was tested only in a few cases. These limitations make it difficult to draw any general conclusions from this data. However, it appears that one should be rather cautious as regards the use of stored platelets in TBI patients. There is a need to define and identify certain subgroups of TBI patients benefiting from platelet transfusion. This will, however, require additional clinical studies.

### *Desmopressin*

Desmopressin has been documented to reverse the effects caused by some platelet inhibitors [281]. The effects of desmopressin have also been investigated in a retrospective study on 408 patients with TBI. The patients were given stored platelets together with desmopressin but no benefit of this treatment could be demonstrated [282]. However, based on experimental data and limited clinical reports, Beynon et al. in their review recommend use of desmopressin in TBI patients with an apparent bleeding tendency [271].

### *Tranexamic acid (TXA)*

Tranexamic acid is a protease inhibitor used as a haemostatic agent, as it inhibits fibrinolysis. Administration of TXA has resulted in clearly improved survival of patients with general trauma and massive bleeding, with few side-effects [283] [284]. It has, however, been claimed that it is important to administer TXA early, within three hours of injury [285]. Despite some reports of adverse effects [232], administration of TXA can result in survival benefits even in a TBI population, as shown in two recent meta-analyses [286] [287].

### *Factor VIIa*

This is a powerful haemostatic agent, used in the treatment of various bleeding disorders [288]. Treatment with recombinant factor VIIa reduced haematoma growth in patients with spontaneous intracerebral haemorrhage (ICH), but did not affect the functional outcome [289]. In general trauma patients, administration of factor VIIa resulted in immediate reduction of coagulopathic haemorrhage [290]. This treatment has also been reported to be useful in correction of coagulopathy caused by platelet inhibitors such as clopidogrel [291]. One of the TBI patients in our material, a young male with severe TBI and poor radiological prognostic signs, was successfully treated with factor VIIa, and showed good recovery (GOS 5). In this particular case, factor VIIa was administered very early – about 1.5 hours after the incident. However, there are also drawbacks with recombinant factor VIIa, as this agent has been shown to increase the risk of thrombosis [292, 293], and its use should, according to most guidelines, be restricted to cases with very severe bleeding.

## 7 AIMS

The overarching aim of this work has been to acquire information about the pathophysiology of TBI, with a focus on coagulopathy and platelet function. More specifically, our aims have been to:

- Study coagulation and platelet function when blood passes through the injured brain,
- Use different methods to monitor coagulation and platelet function in TBI, and to relate data obtained with these methods to bleeding complications,
- Investigate if blood microparticles reflect the brain injury, and may be used as biomarkers of severity and bleeding/thrombotic complications in TBI.

The present work is a part of ongoing research aiming to develop clinical routines for monitoring and early diagnosis of coagulation abnormalities in TBI patients. This would, in turn, allow timely correction of coagulation disorders and possibilities to monitor effects of treatment, minimizing complications and improving outcome.

## 8 MATERIALS AND METHODS

All the studies presented in this work were performed at the neurointensive care unit (NICU), Karolinska University Hospital, a Level One trauma centre with a catchment area of approximately 2.5 million inhabitants. The patients included in the studies suffered from severe isolated TBI (GCS < 8, non-head AIS  $\leq$  3) and were included after obtaining informed consent from relatives. The local ethics committee at Karolinska Institutet approved the designs of the studies. Eleven patients participated in Study #1, 20 TBI patients and 10 patients with general trauma in Study #2, 16 patients in Study #3 and Study #4. Beside these four studies, unpublished data presented at international congresses are also included in the thesis, e.g. data from “Multiplate registry” 2009–2015 concerning 744 patients, and a small study on platelet transfusion in TBI patients with signs of platelet dysfunction.

All TBI patients were treated and monitored according to local guidelines at the NICU, based on Brain Trauma Foundation recommendations [11, 294].

### *Blood sampling techniques.*

In Study #1, #3 and #4 blood was drawn from an arterial line and from a jugular bulb catheter (cerebrovenous blood). Arterial and cerebrovenous samples were collected simultaneously into syringes containing citrate solution or EDTA and centrifuged. The supernatant was pipetted into smaller tubes and frozen before analysis. This allowed comparison of arterial and cerebrovenous blood; a “transcranial gradient” of the investigated substances or MPs can thus be calculated. Analysis of cerebrovenous blood is, therefore, advantageous as it makes it possible to investigate pathophysiological events within the brain. The method, however, demands certain skills from the intensivist inserting the jugular bulb catheter and the intervention is time-consuming. Thus, this approach is relatively rarely presented in the literature. We have been able to find only a few publications in the literature devoted to coagulation studies involving cerebrovenous samples in cases of TBI [99, 295-300].

### *Blood chemistry*

Standard coagulation tests, i.e. for INR, APTT, fibrinogen, platelet count, D-dimer and the brain damage marker S100b, were all performed at the Dpt. of Clinical Chemistry, Karolinska University Hospital, using routine methods. Commercially available immunoassays (ELISAs) were used to analyse IL-6 and C5b-9, TAT and F1+2 in plasma (for details, see the relevant publication).

### *Viscoelastic and platelet function methods*

TEG® and ROTEM® were used as bedside methods of monitoring coagulation disturbances.

*Platelet function* was evaluated by using modified TEG (TEG-PM), MEA and Ivy bleeding time. Ivy bleeding time is one of the oldest methods of assessing haemostasis, introduced as early as in the 1850s [301] and is still in use. The method is often criticized for low reproducibility, but it is the only existing method for evaluating interplay between platelets and the blood vessel wall that we know of.

### *Microparticles*

Blood microparticles were assessed by means of flow cytometry on a Beckman Gallios instrument (Beckman Coulter, CA) [197, 302].

### *Miscellaneous*

Radiological examinations (CT scans) were performed on the basis of clinical indications by an experienced specialist at the Dpt. of Neuroradiology, Karolinska University Hospital.

The patients' bleeding tendencies were evaluated by neurosurgeons and intensivists using a scale of "no bleeding tendency =0", "moderate bleeding tendency =1", "overt bleeding tendency =2".

Procoagulant treatments given were divided into three levels: "red blood cell concentrate (RBC) given alone, plasma given alone or no coagulative treatment given = 0", "2–4 modalities of coagulative treatment given = 1", "more than 4 modalities of coagulative treatment given = 2". The following modalities were included: RBC, plasma, tranexamic acid, desmopressin, fibrinogen, FVIIa and platelet concentrate.

### *Statistics*

Statistical analyses were performed using SPSS (IBM Software, NY), Statistica (StatSoft Inc., OK) and GraphPad (GraphPad Software Inc., CA) software. We performed analyses to obtain descriptive statistics of data, to compare median and mean values, to determine possible time trends in the measured variables and to examine possible correlations between the coagulation tests and severity/outcome variables. Post-hoc tests were performed using Sidak's correction for multiple comparisons. In Studies #3 and #4 we used "mixed model" statistical analysis, in order to compensate for missing data; the mixed model procedure accommodates the missing data, assuming that data is missing at random.

### *Ethical aspects*

The research in this work was performed in connection with unconscious patients with severe TBI; thus it was not possible to obtain informed consent from the patients themselves. Instead, consent was given by the patient's next of kin, which is a standard procedure in medical research within Intensive Care in Sweden. In the research presented, we performed blood sampling in order to describe and understand the pathophysiological changes after TBI. In addition, in a small unpublished study on five patients we investigated the effects of infusion of platelet concentrate, which was given in order to reverse trauma-induced platelet dysfunction (**Fig. 13**). A physician not involved in the study administered infusion of platelet concentrate as a clinical decision. The reason for administering platelet infusion was the presence of an overt bleeding tendency and pronounced

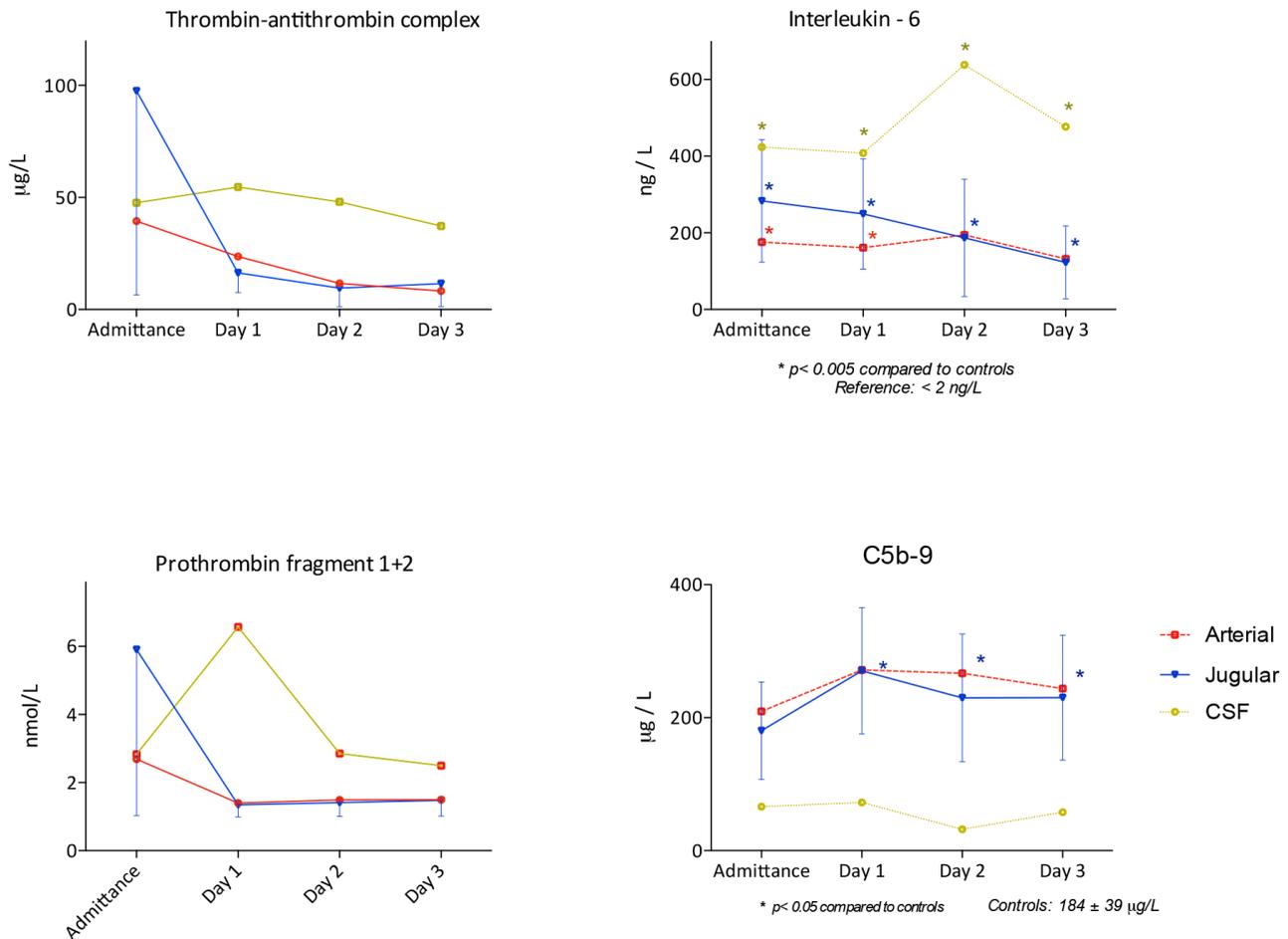
platelet dysfunction as shown by TEG-PM. All the other studies in this work were observational. However, even blood sampling itself may be harmful if the volumes taken are significant. Thus, sample volumes were kept to a minimum. (An ethics permit was obtained for up to 23 mL × 6 in Studies #3 and #4, but smaller volumes were taken in these studies). According to our own assessment and considering the ethics committee approval, the scientific value of our work outweighs the minimal harm to patients resulting from the small volumes of blood drawn. Sampling of cerebrovenous blood from the jugular bulb catheter also involves risks related to insertion of the catheter. However, we performed sampling of cerebrovenous blood only in patients having jugular bulb catheters due to a clinical indication. The decision to insert a catheter was taken by a clinician not involved in the research project. The clinical management of patients followed the local guidelines at our NICU, which are based on recommendations issued by the Brain Trauma Foundation. All the patient data were stored, processed and presented anonymously, according to the rules for protection of a patient's integrity.

All the research presented in this thesis, both published and unpublished data was approved by the Ethics Committee at Karolinska Institutet.

## 9 RESULTS AND CONCLUSIONS

The research presented in this thesis deals with a clinically important issue within neurosurgery and critical care. The design of our studies is based on previous research and clinical observations at our NICU. The results of this research have partly been implemented in clinical praxis at the NICU (since 2007).

*Study #1* was designed to investigate the triggering mechanisms of coagulopathy after TBI, and also the possible connections to inflammation and complement activation. We hypothesized that TF released into the circulation from the damaged brain was a triggering mechanism, but we could not confirm or reject this hypothesis because of methodological difficulties. The TF data obtained showed great variance, with no difference compared with healthy controls (unpublished data). We concluded that either the commercial ELISA-based TF analysis kit did not work satisfactorily in our setting, or TF was not present in plasma in a soluble form after TBI, or both. Thus, we chose to focus on coagulation activation parameters TAT, F1+2 and D-dimer, the inflammation marker IL-6 and the complement marker C5b-9, measured in arterial blood, cerebrovenous blood and CSF. The measurements were performed at four time points: at admission, day 1, day 2 and day 3.



**Fig. 10.** Time profile of the coagulation and inflammatory variables in arterial blood, cerebrovenous blood and CSF.

The results (**Fig. 10**) clearly showed increases in TAT, F1+2, D-dimer, IL-6 and C5b-9 concentrations in plasma shortly after TBI, compared with healthy controls. Interestingly, IL-6 levels were (on average) more than a hundred-fold higher in TBI patients compared with healthy controls, in both blood and CSF samples. Over time TAT, F1+2 and D-dimer plasma levels declined from admittance to the NICU (highest concentrations) to day 3 (lowest concentrations). Since there was a decline in concentrations at all three time points after admission it is likely that the coagulation variables had already passed their peak values before the patient’s admission. The response of the coagulation system to injury is rapid and earlier sampling would be required to determine peak timing and magnitude. Notably, the elapsed time between trauma to first blood sampling was 11–19 hours.

A transcranial concentration gradient was seen at admittance as regards TAT, F1+2 and IL-6, but not D-dimer and C5b-9. We interpret the presence of concentration gradients of TAT and F1+2 as evidence of coagulation activation during blood passage through the damaged brain. This conclusion might seem obvious, but at the time of our publication there was a vivid debate between the holders of two alternative points of view: local vs. systemic activation of coagulation in TBI patients. According to the latter concept, there is systemic activation of the coagulation system, due to release of TF into systemic circulation [66] and other systemic factors such as a powerful catecholamine release seen in both TBI and subarachnoid haemorrhage [66, 303, 304]. Our results, however, in agreement with previous report by Murshid and Gader [300], strongly support the theory of local activation of coagulation at the site of injury, but do not exclude “secondary” systemic activation of coagulation.

The reason for absence of a transcranial gradient of D-dimer is not clear. D-dimer may be cleared rapidly from the “cerebral compartment” because of its relatively low molecular weight [305]. Another explanation may be that activation of fibrinolysis in systemic circulation could “conceal” the cerebral findings. Differences in half-life in the circulation between the markers may perhaps also be involved. D-dimer has the longest half-life in blood and therefore it may be a less sensitive marker of dynamic changes in coagulability after injury. Notably, the half-life of TAT is about 15 minutes, that of F1+2 around 90 minutes, and that of D-dimer up to 8 hours [306, 307].

Absence of a transcranial gradient in C5b-9 plasma levels may be due to the fact that complement activation in the blood compartment is greater than in brain tissue and/or that components of the complement system are less prone to leak from brain tissue into the circulation. This explanation is in line with our findings of lower C5b-9 concentrations in CSF compared with plasma. However, we know from histopathological studies that the complement system is profoundly activated within human brain tissue after TBI, and that complement activation plays an important role in the pathogenesis of secondary insults [33, 34]. In this context, it would be of interest to compare the concentrations of C5b-9 in the three compartments: cerebral blood, CSF and brain tissue. The CSF levels were  $60 \pm 70$   $\mu\text{g/L}$  in our study, compared with  $190 \pm 90$   $\mu\text{g/L}$  in a study by Stahel et al. (different analytical methods were used in these two studies) [308]. Levels of C5b-9 in blood were  $270 \pm 110$  in our study (Stahel et al. did not perform measurements in blood). Bellander et al. elegantly showed the presence of C5b-9 in human brain tissue by using immunohistochemical staining of biopsy samples collected perioperatively from TBI patients [33]. Unfortunately, it is not possible to calculate the concentrations of C5b-9 in these samples. The question of which compartment, blood or brain, is the main source of C5b-9 in TBI remains open.

CSF levels of TAT and F1+2 were increased on day 1 *vs.* those observed in plasma (**Fig. 10**), reflecting accumulation of these coagulation products within the CNS and possible involvement of thrombin-related mechanisms in TBI pathogenesis. There are indications that thrombin is involved in events outside the blood compartment, and also the main inhibitor of thrombin, antithrombin, has been shown to be present in the extravascular space. Its molecular targets are largely unknown, although inhibition of transmembrane serine proteases involved in oncogenesis has been reported [309]. Levels of D-dimer, on the other hand, were relatively low in the CSF in early samples, with a slight increase later on, matching plasma concentrations at day 2 and day 3.

CSF levels of IL-6 were 2–3 times higher than in plasma, and showed an increasing trend from day 0 to day 3. We interpret this finding as an indicator of secondary neuroinflammatory processes that tend to increase during the first days after TBI. CSF levels of C5b-9 were lower than in blood, with no detectable changes over time. This marker of complement activation is a large conglomerate of several protein subunits with a very high total molecular weight (approximately one million Daltons)[310] and its release into CSF appears to be slow.

**Table 1.** Coagulation, inflammation and complement variables at admittance and during the first three days after injury (means  $\pm$  standard deviations). N = 11.

<i>Variable</i>	<i>At admittance</i>	<i>Day 1</i>	<i>Day 2</i>	<i>Day 3</i>	<i>Reference values</i>
Plt $\times 10^9$ /L	218 $\pm$ 125	175 $\pm$ 56	142 $\pm$ 10 *	146 $\pm$ 49	145–350
Bleeding time	578 $\pm$ 269 *	-	585 $\pm$ 250 *	840 $\pm$ 85 *	< 410
Antithrombin, IU/mL	0.53 $\pm$ 0.2 *	0.78 $\pm$ 0.1*	0.87 $\pm$ 0.2 *	0.9 $\pm$ 0.4	0.85–1.25
Fibrinogen, g/L	1.8 $\pm$ 0.8 *	2.8 $\pm$ 0.5	4.7 $\pm$ 0.4 *	5.4 $\pm$ 0.7 *	2–4.2
TAT <sub>jugbulb</sub> , $\mu$ g/L	87 $\pm$ 65 *	17 $\pm$ 6 *	12 $\pm$ 4 *	14 $\pm$ 7 *	< 4.0
F1+2 <sub>jugbulb</sub> , nmol/L	5.9 $\pm$ 6.5 *	1.3 $\pm$ 0.4 *	1.3 $\pm$ 0.5 *	1.5 $\pm$ 0.6 *	0.4–1.5
D-dimer <sub>jugbulb</sub> , mg/L	3.7 $\pm$ 2.6 *	1.2 $\pm$ 0.9 *	1 $\pm$ 0.5 *	1.2 $\pm$ 0.7 *	< 0.25
IL-6 <sub>jugbulb</sub> , ng/L	283 $\pm$ 179 *	250 $\pm$ 176 *	187 $\pm$ 203 *	123 $\pm$ 117 *	< 2
C5b-9 <sub>jugbulb</sub> , $\mu$ g/L	181 $\pm$ 82	270 $\pm$ 106 *	230 $\pm$ 118 *	241 $\pm$ 123 *	184 $\pm$ 39

Plt = Platelet count in venous blood, Bleeding time according to Ivy, Antithrombin in venous blood, Fibrinogen in venous blood, TAT<sub>jugbulb</sub> = Thrombin–Antithrombin complex in cerebrovenous blood, F1+2<sub>jugbulb</sub> = Prothrombin fragment 1+2 in cerebrovenous blood, D-dimer<sub>jugbulb</sub> = D-dimer in cerebrovenous blood, IL-6<sub>jugbulb</sub> = Interleukin-6 in cerebrovenous blood, C5b-9<sub>jugbulb</sub> = Complement marker C5b-9 in cerebrovenous blood. \* = Pathological value *vs.* reference

It is also of interest to study the results of standard coagulation tests, presented in **Table 1**. Similar findings were observed in all four presented studies, with some variations regarding peak values and timing of coagulopathy development.

We note low fibrinogen with increased D-dimer and antithrombin at admittance, thrombocytopenia most prominent at day 2, and prolongation of bleeding time reaching its maximum at day 3. We interpret these findings as reflecting thrombin activation (elevated levels of TAT and F1+2) with consumption of coagulation factors such as fibrinogen and antithrombin, and somewhat later consumption of platelets (reduced platelet count) and development of platelet dysfunction and/or dysfunctional endothelium (manifested as prolonged bleeding time) [88, 128].

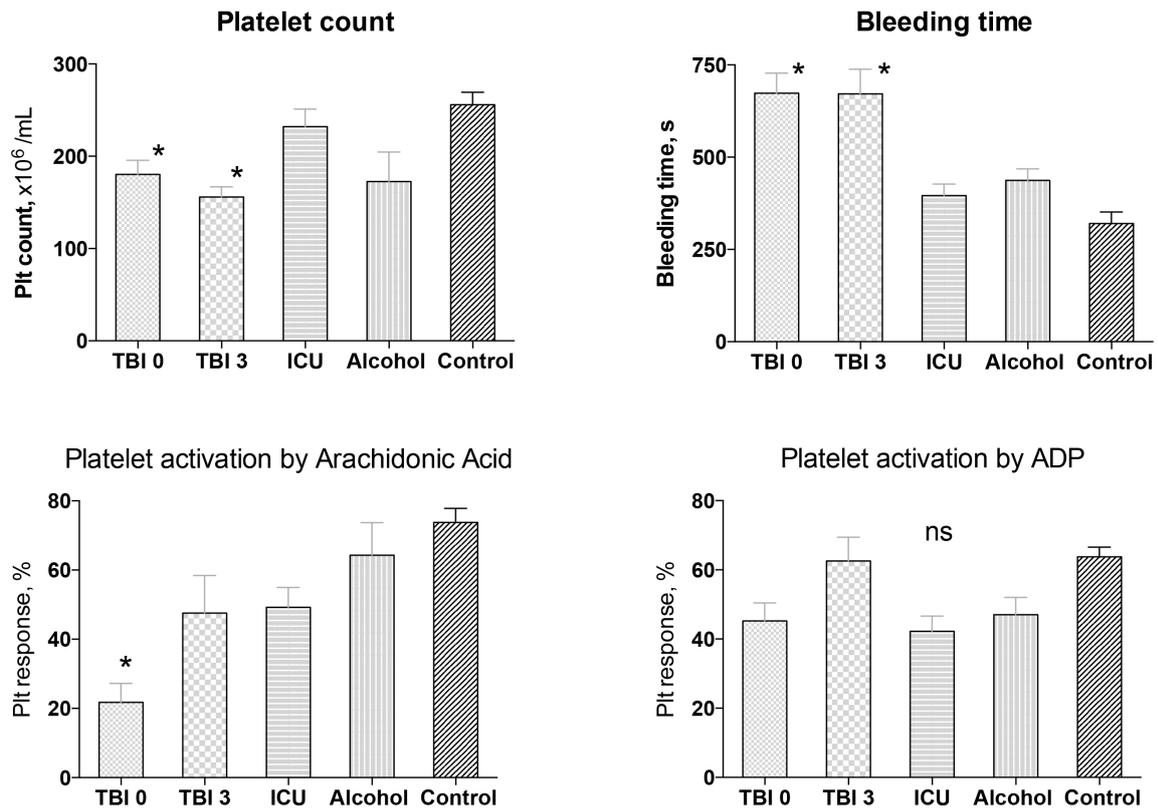
Based on these findings, it was logical to investigate platelet function after TBI more in more detail in our next study.

#### *Conclusions Study #1:*

- Activation of the coagulation system takes place during the passage of blood through the damaged brain. This in turn may lead to the development of consumption coagulopathy.
- Somewhat later platelets are consumed and bleeding time is increased. This is likely to be a response to previously strong *in-vivo* activation of platelets, which may partly be thrombin-dependent.
- IL-6 and activation of the complement system (C5b-9), show co-variation with haemostatic parameters, indicating potential interplay between haemostasis and inflammation in TBI patients.

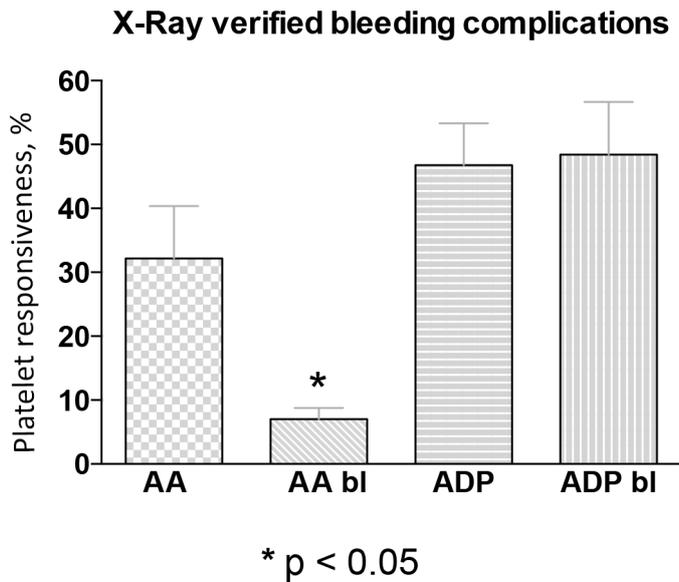
*Study #2* was designed to investigate platelet function after TBI compared with that in patients after general multiple trauma without TBI. In addition, two more “control groups” were added: healthy volunteers and patients without TBI treated for alcohol abuse. This latter group was included because about 50% of TBI patients have alcohol in their blood at the moment of trauma, and also because alcohol influences platelet function [215, 311]. Blood samples were obtained at admittance to the NICU and at day 3. We also compared platelet function before and after giving procoagulative agents such as plasma, fibrinogen, desmopressin, tranexamic acid, factor VIIa and platelet concentrate. Platelet function was measured by means of a modified TEG method, i.e. Platelet Mapping (TEG-PM).

The main findings are shown in **Figs. 11** and **12**.



**Fig. 11.** Venous platelets count, Ivy bleeding time, and platelet responsiveness to AA and ADP (TEG-PM).

TBI patients had lower platelet counts and prolonged bleeding times compared with both the healthy controls and the general trauma group. The platelet count in the alcohol abuse group was comparable to that in brain trauma patients. Platelet function as measured by platelet responsiveness to AA was impaired in TBI patients in the acute phase, compared with other study groups. Platelet responsiveness to ADP was, however, not significantly different between the study groups in our material. This is contrast to results published by other researchers, who have found reduced platelet responsiveness to ADP in TBI patients [208] or a reduction in responsiveness to both ADP and AA [209]. Some discrepancies between studies may depend on differences in patient populations and perhaps also on the fact that different methods were used for measurement of platelet function.



**Fig. 12.** Platelet responses to AA and ADP in patients who subsequently developed bleeding complications (AAbl; n=8) and those who did not (n=12). Blood samples were taken at admittance to the NICU, i.e. before bleeding complications developed.

Platelet dysfunction at admittance, defined as reduced responsiveness to AA, was associated with development of bleeding complications during the first week of stay at the NICU (**Fig. 12**).

On the third day at the NICU, platelet responsiveness to AA and to ADP in the TBI patients had improved, but the bleeding time was still prolonged. It could be hypothesized that the prolonged bleeding time reflects capillary oozing due to altered vascular reactivity, a “dysfunctional” mechanism that may be relevant in terms of bleeding complications in TBI. It should be noted that Ivy bleeding time has been criticized for unsatisfactory reproducibility and poor predictive value, so its “popularity” has declined in recent years. However, the method may still be of value in neurotrauma research, since a defect in primary haemostasis may be responsible for development of capillary bleeding in contusion areas and in the postoperative setting. Several potential sources of error of Ivy bleeding time should, however, be remembered, such as the location of skin puncture, variations in stasis pressure and in skin temperature [312, 313]. Our research group has plans to investigate Ivy bleeding time further, by eliminating the error sources (by using an automatic pressure cuff, pre-warming to 37 °C and using a standardized puncture location), but we have not yet completed these studies. More studies of primary haemostasis and microcirculatory events in TBI patients are needed in future.

Regarding the alcohol abusers, we observed that they did not have a reduced response to AA, but tended to have a lower platelet count ( $p < 0.05$ ) and a slightly decreased (but not statistically

significant) responsiveness to ADP compared with healthy controls. These data do not support the idea that an influence of alcohol is a mechanism behind reduced responsiveness to AA.

Another important aspect is the effect of procoagulative treatment on platelet function. Unfortunately, we could not carry out blood sampling immediately before and after the given procoagulative treatments, so we simply compared platelet function measurements at admittance with those performed at day 3, in six patients who received massive procoagulative therapy (defined as more than three procoagulative substances given, which were plasma, fibrinogen, TXA, desmopressin, platelet concentrate and recombinant FVIIa). Platelet responsiveness to AA improved in all but one patient (responsiveness unchanged at a very low level). This patient was tested again more than two weeks later, at day 18, then showing a normalized platelet response to AA. In patients with prolonged bleeding times at day 3, we continued to measure bleeding time for a few more days; the values remained unchanged or tended to normalize.

#### *Conclusions Study #2:*

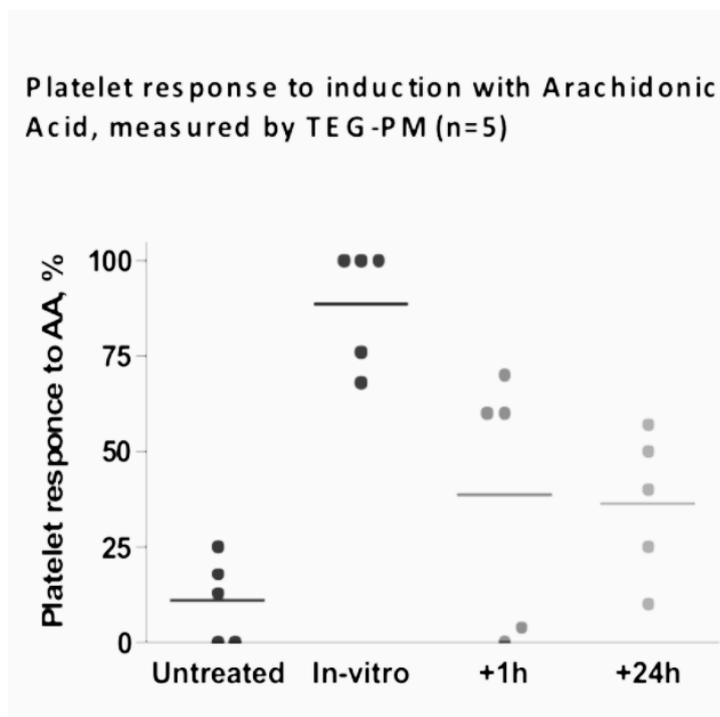
- In response to brain trauma a significant proportion of patients develop a transient hyporesponsiveness to AA. This “platelet dysfunction” is associated with bleeding complications.
- Monitoring of coagulation and platelet function in cases of TBI gives additional information that can be used to identify high-risk patients and optimize treatment.

Procoagulative treatments may, however, have adverse effects. Recently the possible risks associated with platelet transfusions in TBI patients have been highlighted [280]. The probable explanation as to why platelet transfusions may be harmful is the microthrombosis that these transfusions may cause in the damaged brain area [146]. This necessitates restrictive use of transfusions, which should be limited to well-defined subgroups of TBI patients with an apparent risk of bleeding or ongoing bleeding.

The risk groups could perhaps be identified by using a combination of standard coagulation tests, viscoelastic methods and platelet function analyses, but a specific protocol for such a procedure is yet to be developed. Beside identification of risk groups, more specific procoagulative treatments may provide safer and more effective ways to treat and/or correct coagulopathy. The development of specific treatments requires, however, a better understanding of the pathophysiology.

The logical continuation of Study #2 was to investigate the possibility of reversing TBI-induced platelet dysfunction *in vivo* and *in vitro*. Thus, patients with manifest platelet dysfunction (responsiveness to AA < 25%) were identified by means of TEG-PM. Two units of stored platelet

concentrate (approx. 250 mL each) were given intravenously; meanwhile the *in-vitro* effectiveness of platelet concentrate was tested simultaneously. During the *in-vitro* experiment, the patients' blood was mixed with stored platelets (ratio 7:1) and tested by TEG-PM for responses to AA. Blood sampling was repeated one hour after platelet infusion was completed, and also the next day, i.e. 24 hours after platelet transfusion.



**Fig. 13** Reversal of TBI-induced platelet dysfunction *in vitro* and *in vivo*. The platelets' “non-responsiveness” to AA is partly corrected following infusion of stored platelet concentrates. The effect persists for at least 24 hours (unpublished data, presented at Neurotrauma Congress, Orlando, USA, 2008).

The effect of adding stored platelets *in vitro* is shown in **Fig. 13**. We used a blood/platelets mixing ratio of 7:1, which corresponds to transfusion of 700 mL of platelet concentrate to an adult weighing 70 kg. This dosage is somewhat higher than routinely used (two units, or approximately 500 mL). The effectiveness of this treatment *in vivo* varied considerably between the patients: two patients showed no response, whereas three responded with a clear increase in AA-induced platelet activation. This pilot study was small, with only five patients included. Control experiments should have been included to obtain more solid evidence and to adjust for normalization of non-responsiveness to AA over time. Owing to doctor's awareness of coagulation problems, which

increased at the time of the study (including more “liberal” administration of platelet concentrates on “clinical grounds”) we had, however, problems continuing our investigation. According to the present literature there is no strong evidence in favour of platelet transfusions in TBI patients [280]. More research is needed to provide evidence-based recommendations on the matter. This should be easier to obtain with more convenient and rapid methods such as MEA, which we currently use at our NICU.

*Conclusions* from the platelet concentrate transfusion study:

- TBI-induced platelet dysfunction, defined as poor responsiveness to AA, can be reversed by infusion of platelet concentrate in some patients.
- The effect of this treatment should be investigated in well-designed studies, using methods that assess platelet reactivity and with patient outcome as the primary variable.

Assessment of platelet function by means of TEG-PM or MEA was implemented at our NICU during 2008, not only as a research tool but also in order to make clinical decisions regarding procoagulative treatment. MEA replaced TEG-PM completely after 2010, having advantages in simplicity and reduced cost. Data from MEA readings have been saved for evaluation, and over a four year period (from May 2010 to May 2014) a total of 774 patients have been tested; 1380 measurements altogether (some cases have been tested repeatedly). Among all the tested patients, 177 suffered from severe TBI and in these patients 405 tests were performed.

During the same period, a total of 387 TBI patients were admitted to the NICU, i.e. 46% of all TBI patients were tested by MEA. The reasons for performing platelet function tests were as follows:

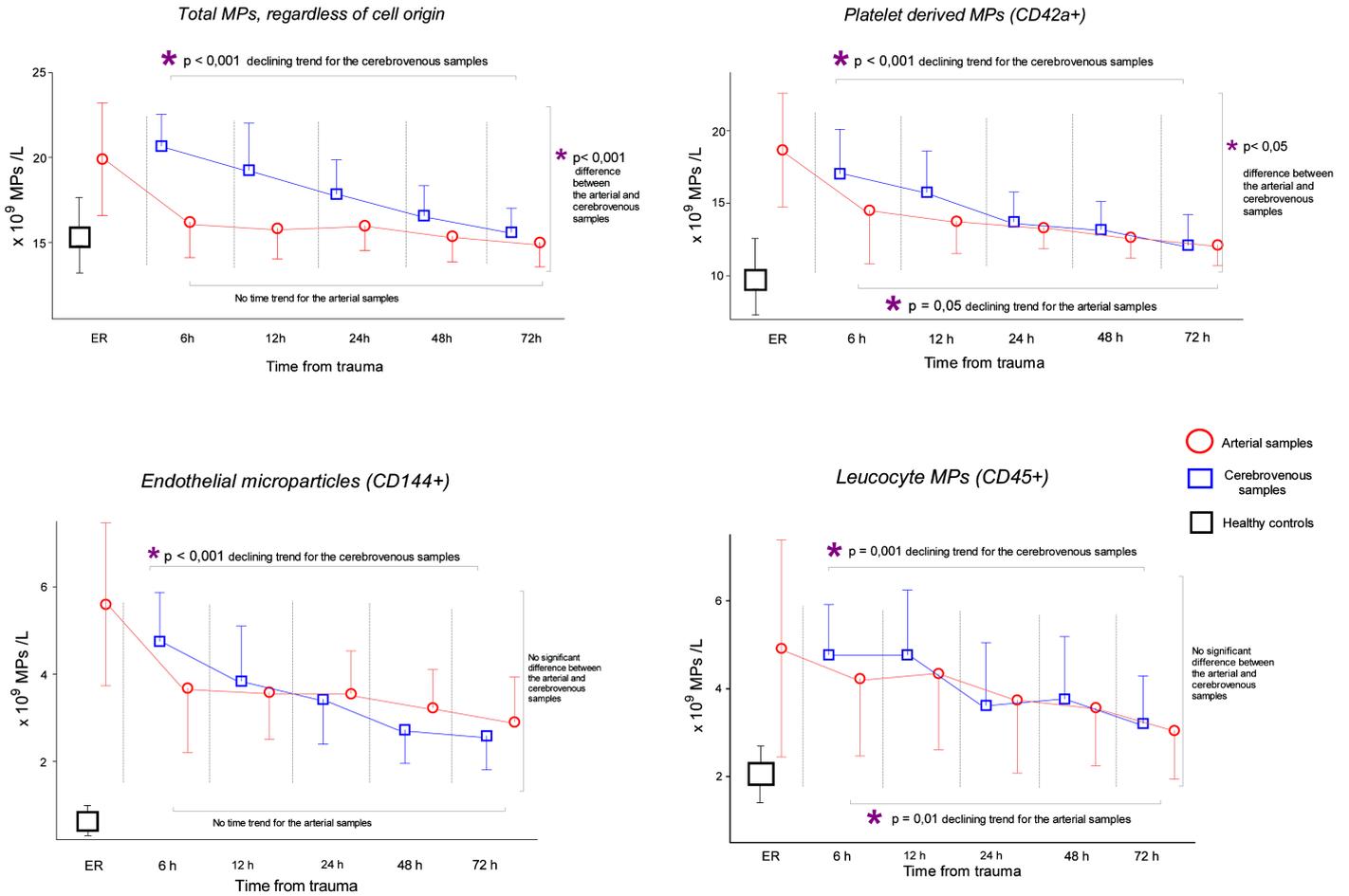
- Known antiplatelet therapy prior to trauma
- Bleeding tendency observed by surgeon or intensivist
- Pathological standard coagulation tests results (venous platelet count, INR, APTT)
- Recommendation from coagulation specialist on call
- Research
- Earlier pathological MEA measurement
- No reason for testing specified

Detailed analysis of the MEA database in relation to bleeding complications and treatments given will be undertaken in future studies.

In order to investigate the pathophysiology of TBI further, we performed *Study #3*, where we measured changes in circulating MPs of platelet origin (PMPs), endothelial origin (EMPs) and leukocyte origin (LMPs). The study design has similarities to Study #1, i.e. repeated blood sampling in arterial and cerebrovenous blood was performed, but with more frequent sampling. Altogether there were six time points for sampling: at admittance to the NICU and at approximately 6, 12, 24, 48 and 72 hours after the injury. Platelet-derived MPs were defined by the presence of the surface antigen CD42a, endothelial-derived MPs by CD144 and leukocyte-derived MPs by CD45. Expression of TF on the MPs was detected by using fluorescent antibodies to CD142, and the presence of P-selectin by antibodies to CD62P.

The study results are presented in **Fig. 14**.

Panel A

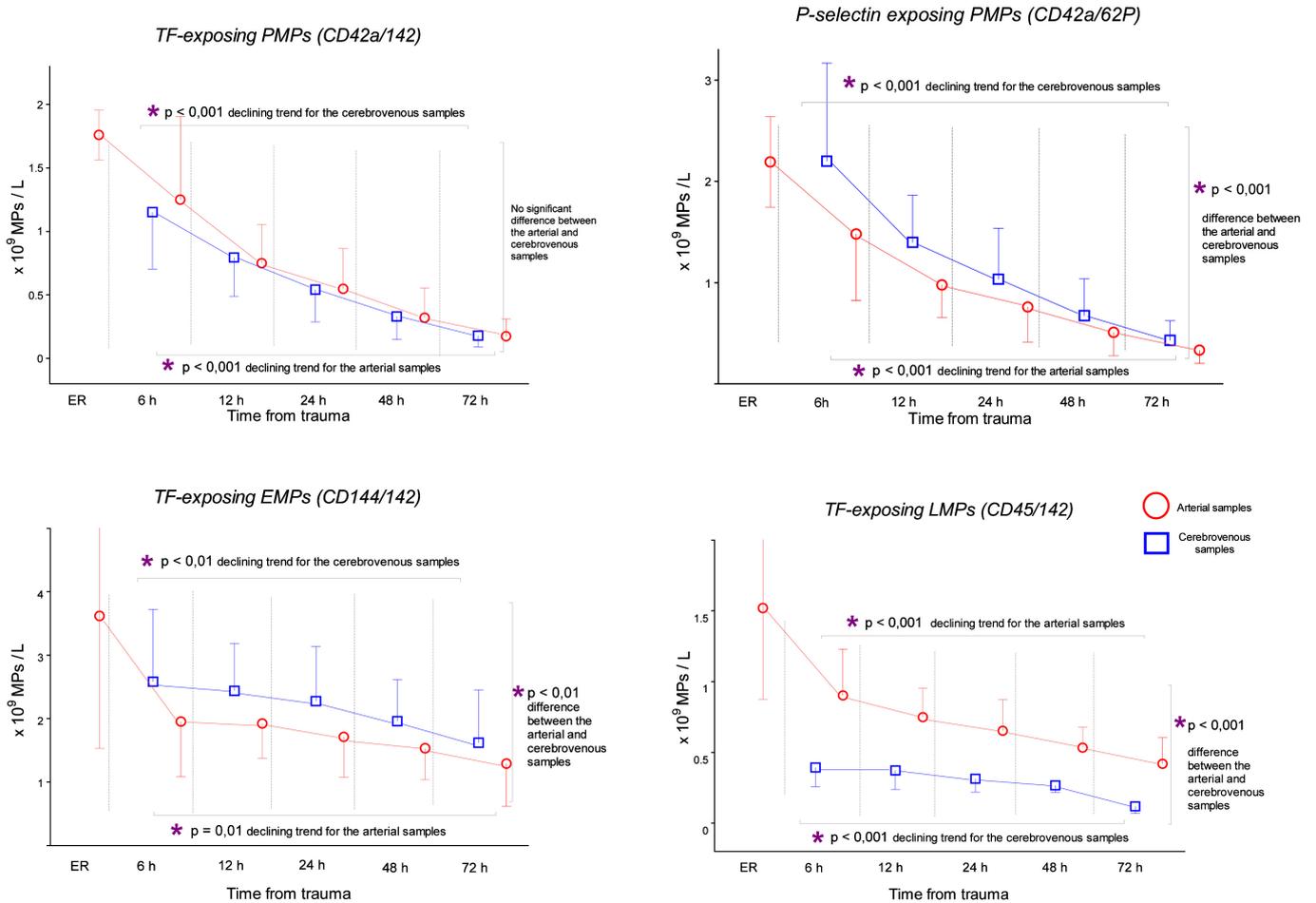


**Fig. 14A** Microparticle counts in arterial (red) and cerebrovenous (blue) plasma collected at the emergency room (ER) and 6–72 hours after trauma in 16 TBI patients. Data presented as mean values  $\pm$  SD.

Panel A: Microparticles irrespective of cellular origin (upper left), and originating from platelets (upper right) vascular endothelial cells (lower left) and leukocytes (lower right).

Data on microparticles from healthy controls are also shown for comparison (n=15; squares).

Panel B



**Fig. 14B** Microparticle counts in arterial (red) and cerebrovenous (blue) plasma collected at the emergency room (ER) and 6–72 hours after trauma in 16 TBI patients. Data presented as mean values  $\pm$  SD.

Panel B: platelet microparticles exposing TF (upper left) or P-selectin (upper right), endothelial microparticles (lower left) and leukocyte microparticles (lower right) exposing TF.

As can be seen in **Figure 14A**, the levels of circulating MPs were significantly higher in TBI patients compared with healthy controls. The temporal profile and the transcranial gradient of MPs were also analysed. MP levels were highest at admission, and then gradually declined to day 3, remaining however above the levels in blood samples from healthy persons. As in Study #1, we were probably not able to detect the true time point for peak values of MPs, which is likely to occur early after trauma; the patients were included 3–20 hours after trauma.

Interestingly, the MPs of endothelial origin were elevated the most, i.e. around sevenfold compared with controls, whereas LMPs were approximately twofold higher and PMPs around 1.4-fold higher

than controls, but with great variability between individuals. The finding that EMPs were the most elevated type was somewhat surprising to us. We would rather have expected that PMPs should have the greatest elevation, since platelets are such a reactive type of cell and would give rise to high concentrations of MPs upon tissue injury. It is possible, however, that the peak levels of PMPs were considerably higher closer to the trauma. To give a somewhat wider perspective, we can compare the absolute levels of MPs in TBI with those observed in some other pathological conditions. Thus, circulating PMPs in our TBI patients were around 35% lower than the peak count observed in patients with acute coronary syndrome [314]. TF-expressing EMPs were twice as numerous in TBI compared with numbers in the prothrombotic antiphospholipid syndrome [315]. However, these comparisons should be made with some caution, since the methodology of MP measurements varied somewhat between the different studies, and, again, there was great variability between individuals with respect to circulating MP counts.

The release of EMPs probably reflects vascular injury due to the brain trauma, but perhaps also endothelial damage secondary to microthrombotic events and ischaemia in the penumbra area. Of note, we defined EMPs as MPs exposing CD144 (VE-cadherin), which is a molecule specific to endothelial cells and located in the junctions between the cells. An increase in circulating MPs expressing CD144 would therefore reflect damage of the endothelial lining (perhaps “capillary fragmentation”, see p.18), rather than reflect endothelial cell activation in response to thrombin or various cytokines, for example.

The possibility to assess transcranial gradients was advantageous. Increased gradients in MPs, which we were able to show in our study, are probably due to microthrombotic and inflammatory events occurring in the injured brain. For example, TF as an important trigger of coagulation disturbances in TBI has been hypothesized for many decades. As far as we know, this study is the first one that *de facto* demonstrates the release of TF from brain injuries in humans into the cerebrovenous circulation, further supporting the idea that TF is important in TBI. However, it was only in MPs from endothelial cells that TF exposure was increased. Regarding TF-expressing PMPs there were no differences between counts in cerebrovenous and arterial samples, and regarding LMPs the transcranial gradient of TF-expressing LMPs was actually reversed, i.e. levels were higher in arterial blood than in cerebrovenous blood, suggesting accumulation of leukocytes in the injured area. Deeper insights into these findings would require new studies where the types and subtypes of leukocytes could be characterized. Animal experiments would probably be optimal in this respect as histopathological examinations could then also be performed.

We used flow cytometry to measure MPs. A drawback with this technique is that one often measures only a certain predetermined type of MP, and ignores other types of MP that may be

relevant to the pathophysiology. Thus, one may only be able to see a fragment of a bigger picture. However, the possibility to study a certain type of MP exposing a certain molecule may also provide advantages, e.g. in our study where we were able to show that TF leaks out of the damaged brain into the circulation, as postulated long ago but not shown previously. Indeed, flow cytometry can be a powerful and sensitive technique, and is well suited for smaller clinical studies like the present one [316]. The optimal strategy however, is to combine flow cytometry with functional assays such as thrombin generation assays, for example, to assess the procoagulant effect of MPs [317].

### *Conclusions Study #3:*

- Hours after TBI there is increased formation of circulating PMPs, LMPs and EMPs.
- There is a transcranial gradient in PMPs exposing the activation marker P-selectin, indicating platelet activation within the circulation of the injured brain.
- The transcranial gradient of EMPs exposing CD144 suggests that their origin is mainly the damaged brain.
- The presence of TF-expressing EMPs in cerebrovenous blood has been demonstrated in TBI patients. In contrast, LMPs exposing TF show a reversed transcranial gradient, with higher counts in arterial than in cerebrovenous samples, indicating accumulation of LMPs in the injured brain.
- Over time, the numbers of MPs decline in cerebrovenous and arterial blood but they still remain elevated compared with healthy controls around three days after the trauma. This may reflect the “diseased state” of TBI patients, with various complications such as infections or SIRS.

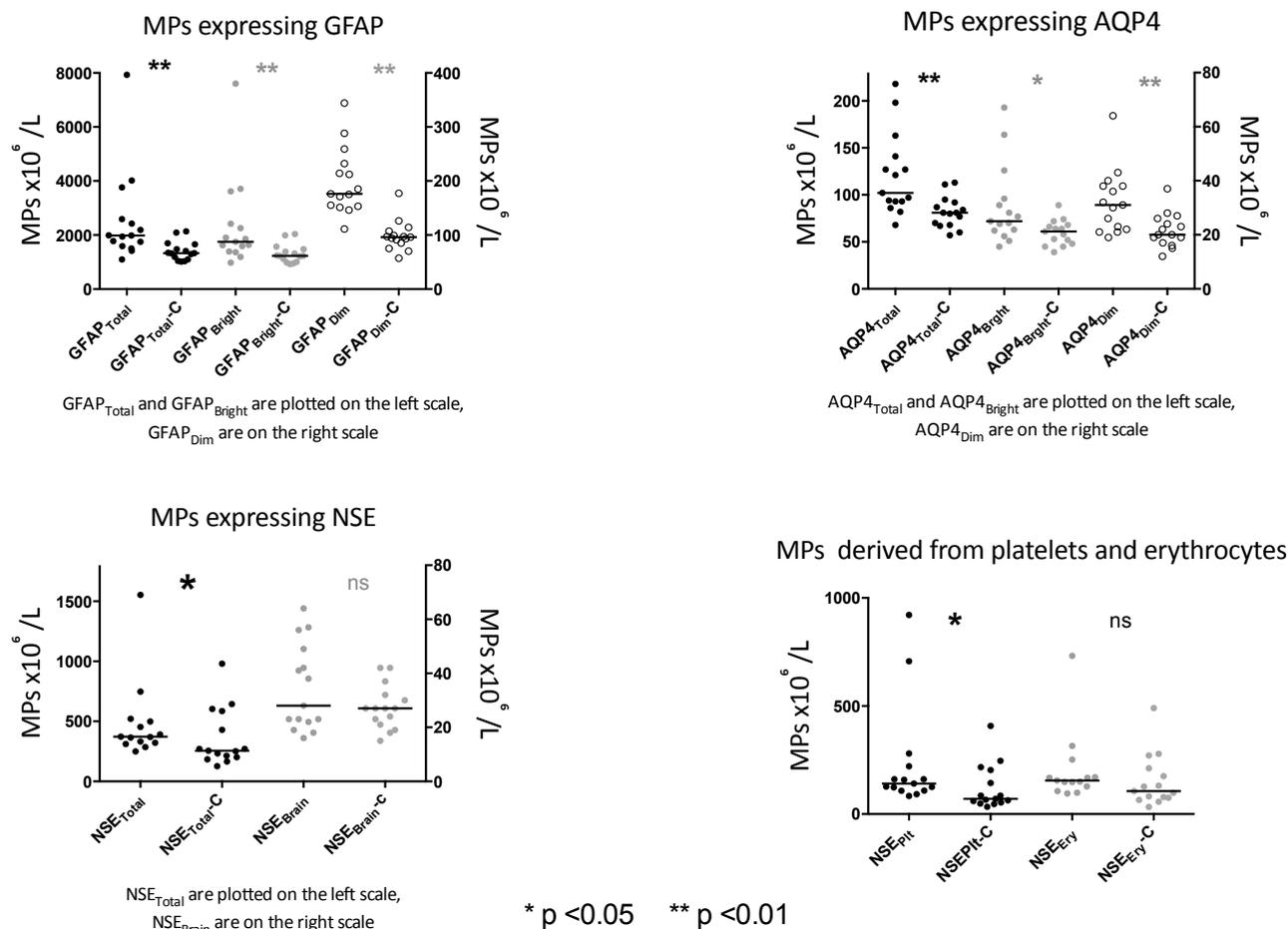
The MPs investigated in the previous study were not brain-specific markers, which is considered to be a limitation, and as pointed out in the review process of Study #3. Although some EMP and PMP phenotypes originated from or were generated in the brain circulation, the source was not the brain tissue itself. Our group has previously reported increased levels of MPs in cerebrospinal fluid in neuroinflammatory disease and schizophrenia, but as in our present project, these MPs were of blood cell or endothelial cell origin [245, 318]. Notably, there is a large amount of data showing that cells in the CNS release MPs to CSF in neurodegenerative and neuroinflammatory diseases [242]. Thus, *Study #4* was designed to search for MPs as brain-specific biomarkers of traumatic

brain injury. We analysed blood samples from the same patient group as in Study #3. Three main types of molecular marker were chosen as candidates: neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP) and aquaporin-4 (AQP4). NSE is used as a marker of neuronal origin of MPs, GFAP emanates from astrocytes, and AQP4 is an integral membrane protein in neurons, highly involved in TBI pathophysiology by controlling the generation of brain oedema [319, 320].

**Table 2.** Subpopulations of microparticles and their cellular origins.

<b>MPs subpopulations</b>	<b>Identification of MPs</b>	<b>Cellular origin</b>
NSE <sub>Platelet</sub>	NSE+, CD42a+	Platelets
NSE <sub>Ery</sub>	NSE+, CD235+	Erythrocytes
NSE <sub>Brain</sub>	NSE+, CD42a-, CD235-	Neurons
NSE <sub>Total</sub>	NSE <sub>Brain</sub> + NSE <sub>Platelet</sub> + NSE <sub>Ery</sub>	Neurons, Platelets, Erythrocytes
GFAP <sub>Bright</sub>	GFAP+ strong fluorescence	Astrocytes
GFAP <sub>Dim</sub>	GFAP+ weak fluorescence	Astrocytes
GFAP <sub>Total</sub>	GFAP <sub>Bright</sub> + GFAP <sub>Dim</sub>	Astrocytes
AQP4 <sub>Bright</sub>	AQP4+ strong fluorescence	Astrocytes, kidney
AQP4 <sub>Dim</sub>	AQP4+ weak fluorescence	Astrocytes, kidney
AQP4 <sub>Total</sub>	AQP4 <sub>Bright</sub> + AQP4 <sub>Dim</sub>	Astrocytes, kidney

TBI patients had significantly higher levels of GFAP-expressing and AQP4-expressing MPs compared with healthy controls (**Fig.15**).



**Fig 15.** Subpopulations of MPs in TBI patients and healthy controls.

In contrast to what was observed in blood cell-derived or endothelial derived MPs, there was no obvious time trend in brain-derived MP concentrations during the study period of 6–72 hours after the injury. Comparing MP concentrations in cerebrovenous and arterial blood samples (transcranial gradient), we observed only a weak tendency towards a gradient in GFAP-expressing MPs (arterial > cerebrovenous), and in NSE-expressing MPs (cerebrovenous > arterial). However, as shown in **Figure 15**, elevations in NSE-expressing MPs were mainly due to PMPs that exposed NSE, and these MPs cannot therefore be considered as “brain-specific”. We also investigated possible associations between brain-derived MP concentrations and clinical variables of severity of TBI such as presence of a bleeding tendency, procoagulant therapy and patient outcome (GOS), but we found no obvious associations. We found a positive association of borderline significance between MPs expressing AQP4 and bleeding tendency (evaluated by a physician using the score system 0–1–2), which may suggest a pathophysiological link between brain oedema and trauma-induced coagulopathy, but again the data are weak. In addition, we found a tendency towards a negative

association between ethanol in blood (i.e. serum concentration > 0.5 ‰) at the moment of injury and MPs expressing GFAP and AQP4. This observation would fit with the concept put forward by Lustenberger et al. [216], suggesting some protective effects of ethanol present in the blood of patients with TBI. However, all the above data are at best suggestive, and no conclusions regarding clinically relevant associations can be based on the results of Study #4. The sample size of the study is too small, and it is also obvious that we have to search for new and better brain-specific molecules than those used in the present study. Clearly much more research is needed before circulating MPs can be used as biomarkers in cases of TBI.

#### *Conclusions Study #4:*

- Levels of brain-derived MPs are elevated in TBI patients compared with healthy controls, but the difference is small, with considerable overlapping between patients and controls.
- Brain-derived MPs need to be studied more thoroughly to investigate their potential as biomarkers in cases of brain injury. The critical issue is to find CNS-specific molecules that are exposed on circulating MPs and are relevant to and reflect the pathophysiology of interest.

## 10 FUTURE PERSPECTIVES

Awareness of coagulation-related problems has dramatically increased among neurosurgeons and intensivists in recent years. Bedside methods of coagulation monitoring and testing of platelet function are nowadays often used in clinical praxis at larger hospitals. However, there is a knowledge gap between monitoring of haemostasis and identification of coagulopathy and its correction. More research is needed to define and document the optimal treatment of coagulopathy in TBI. This can be achieved by studying larger patient cohorts in well-designed trials. It is desirable to determine the efficacy and optimal dosage of commonly used drugs such as LMWH, which would protect patients from venous thromboembolic complications, and further document the effects of administration of platelet concentrates in cases of bleeding or if there is a high bleeding risk. However, these approaches are demanding and will probably be limited to a few of the most common conditions or treatments. In parallel we need to gather better knowledge of the pathophysiology of TBI, involving the rapidly changing phases of coagulopathy including hypercoagulation/platelet activation – consumption/platelet dysfunction – hyperfibrinolysis – and interactions between inflammation and coagulation.

There is a great demand to improve the diagnostic power and “resolution” of the monitoring methods used in NICUs. The ability of a method to detect rapidly shifting phases of coagulopathy can be called “resolution in time”, whereas “resolution in space” is the ability to distinguish local microcirculatory differences within the damaged brain. In some cases, a complicated mix of problems can be present, e.g. dysfunctional platelets resulting in a bleeding tendency with coexisting “humoral” activation of the coagulation system. In such cases, the patient could theoretically be treated with a “platelet boost” (platelet transfusion or perhaps a desmopressin analogue) together with an anticoagulant such as LMWH. An appropriate algorithm for such an intervention is yet to be developed.

Microcirculatory events play a central role in the pathophysiology of TBI, and monitoring of the microcirculation could provide the keys to understanding the pathology and control it.

Unfortunately, it is difficult to monitor microcirculation in a TBI patient *in vivo* online. Thus, some biomarkers of underlying events could perhaps be used, e.g. platelet-derived and/or endothelial-derived MPs. MP-based monitoring could be of value but requires introduction of novel and more specific antigens in order to identify the MP phenotypes that best reflect the ongoing pathophysiology in the injured brain. Animal experiments could be of significant value in determining the antigens on MPs that constitute the most informative biomarkers and learning how

to interpret the results. In a future perspective, the use of miniature fibre optics could theoretically make it possible to monitor microcirculatory events online. This technique is already used in long-term animal experiments, for monitoring neuroinflammatory and reparative processes in the brain [321, 322]. Tiny optic fibres, less than 1 mm in diameter, could theoretically be combined with microdialysis catheters and placed in penumbra areas in order to monitor the microcirculation. However, a great deal of technological development needs to be carried out before such methods become a reality.

Coagulation and platelet function disturbances following TBI constitute a demanding and exciting research area and significant improvements in this field would be of great clinical importance. Despite continuous attention to this problem since the 1970s and a lot of knowledge acquired so far, there are still plenty of challenges and problems to be solved.

## General summary:

- Activation of coagulation and platelet function takes place when blood passes through the injured brain. Intracerebral inflammation is also present in traumatic brain injury, and may modify coagulatory responses to injury.
- Microparticles from the vascular endothelium exposing tissue factor are released from the injured brain. This may enhance local and systemic coagulation. Leukocyte-derived microparticles exposing tissue factor seem, however, to accumulate in the injured brain and may enhance coagulation and inflammation locally.
- A bleeding tendency and signs of platelet dysfunction develop in some patients with traumatic brain injury, and is related to bleeding complications.
- Monitoring of haemostasis in traumatic brain injury may provide important information regarding the need for haemostatic (procoagulant) treatment in individual patients. Solid evidence that this improves patient outcome is, however, lacking at present.
- Future studies should aim at improving methods and techniques to monitor haemostasis and evaluate if decisions to use procoagulant treatments based on monitoring improve prognosis in patients with traumatic brain injury.

# 11 POPULÄRVETENSKAPLIG SAMMANFATTNING

## **Blödningsrubbningar hos patienter med svår traumatisk skallskada.**

Allvarlig skallskada beroende på olyckstillbud med hårt trauma mot huvudet är ett mycket allvarligt tillstånd med hög dödlighet. Skallskada utgör en av de vanligaste dödsorsakerna hos unga vuxna i Västeuropa och USA. De som överlever drabbas ofta av svåra handikapp. Under de senaste decennierna har behandlingsresultat vid många olika sjukdomstillstånd inklusive olyckstillbud med generella kroppsskador förbättrats avsevärt, tack vare framgångar inom den medicinska vetenskapen. Detta gäller dock inte isolerade allvarliga skallskador, där man tyvärr endast sett en marginell förbättring i behandlingsresultat.

Hjärnskador vid kraftigt våld mot skallen uppstår dels vid traumaögonblicket (s.k. primär hjärnskada) ofta med utveckling av hjärnblödningar, dels senare under vårdförloppet (s.k. sekundär hjärnskada). Det sistnämnda beror på flera olika sjukdomsprocesser vilka utvecklas under dagar efter den primära skadan. Den sekundära skadan beror på syrebrist och otillräcklig genomblödning i hjärnan, förhöjt tryck i hjärnan pga hjärnsvullnaden, inflammation, och död av skadade hjärnceller. En viktig bidragande orsak till dessa sekundära skador anses vara rubbningar i blodets proppbildande förmåga. Dels ses initialt en ökad blodproppsbildning, dels ses också – något senare i förloppet - en störning med utveckling av ökad blödningsbenägenhet, vilket kan leda till nya hjärnblödningar. Detta har varit känt sedan 1970-talet, men mekanismerna bakom dessa störningar i blodproppsbildningen, hur man kan mäta dem, och hur man skall behandla patienter med denna komplexa problematik har varit bristfälligt studerat.

I denna avhandling har vi undersökt patienter med svår isolerad skallskada med avseende på blodproppstendens och blödningsbenägenhet. Vi har kunnat visa att initialt i skadeförloppet så sker en kraftig aktivering lokalt i hjärnan av det s.k. koagulationssystemet – som är viktigt vid blodproppsbildning - men också aktivering av blodplättarna, vilka är celler som också medverkar i blodproppsbildningen. Över tiden utvecklar patienterna en störning även i blodplättarnas funktion, vilka börjar fungera sämre, och därigenom ökar patientens blödningsproblematik. Särskilt patienter med mer uttalad nedsättning i blodplättarnas funktion verkar vara de som åter får komplicerande blödningar i hjärnan. Vi har utvecklat tekniker för att mäta små cellmembranfragment i blodet vilka frisätts från blodplättar, men också från vita blodkroppar och celler från kärlväggen, s.k. endotelceller. Med dessa tekniker kan vi mäta vad som händer i hjärnan i samband med hjärnskadan. Teknikerna behöver dock utvecklas mer för att kunna användas i större omfattning. Flera studier behöver också genomföras för att utvärdera hur blödningskomplikationer i hjärnan skall behandlas.

## 12 ACKNOWLEDGEMENTS

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