Early changes in immune, coagulation, and organ function after clinical and experimental polytrauma

Dissertation submitted in partial fulfillment of the requirements for the degree of ”Doctor rerum naturalium” (Dr. rer. nat.) of the International Graduate School in Molecular Medicine Ulm

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List of abbreviations

AIS  Abbreviated Injury Score
AKI  Acute kidney injury
aPTT Abbreviated partial thromboplastin time
ANOVA Analysis of variance
BSA  Bovine serum albumin
C3a  Complement activation product 3a
C5a  Complement activation product 5a
C5aR Complement activation product 5a receptor
CC16 Clara cell secretory protein
CD   Cluster of differentiation
cDNA Complementary DNA
CRP  C-reactive protein
Ctrl Control
DAMP Damage-associated molecular pattern
DNase Deoxyribonuclease
EDTA Ethylenediaminetetraacetic acid
ELISA Enzyme-linked immunosorbent assay
FGF basic Basic fibroblast growth factor
FITC Fluorescein isothiocyanate
G-CSF Granulocyte colony-stimulating factor
GCS  Glasgow Coma Scale
GM-CSF Granulocyte-macrophage colony-stimulating factor
Healthy Healthy volunteers
HR   Heart rate
HMGB1 High mobility group box 1
HS   Hemorrhagic shock
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-FABP</td>
<td>Intestinal fatty acid-binding protein</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL1-RA</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>Interleukin-12 heterodimer</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>ISS</td>
<td>Injury Severity Score</td>
</tr>
<tr>
<td>L-FABP</td>
<td>Liver-type fatty acid-binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MIP-1</td>
<td>Macrophage inflammatory protein-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiple organ failure</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MV</td>
<td>Microvesicle</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen-activator inhibitor-1</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCT</td>
<td>Procalcitonin</td>
</tr>
<tr>
<td>PDGF-bb</td>
<td>Platelet-derived growth factor subunit B</td>
</tr>
</tbody>
</table>
Persistent low-grade inflammation, immunosuppression, and high protein catabolism syndrome

Phorbol 12-myristate 13-acetate

Pattern recognition receptor

Polytrauma

Polytrauma cocktail

(Real-time) quantitative polymerase chain reaction

Pearson correlation coefficient

Receptor for advanced glycation end products

Red blood cell

Revised Injury Severity Classification

Rotational thromboelastometry

Roswell Park Memorial Institute medium

Reactive oxygen species

Succinate dehydrogenase complex, subunit A, flavoprotein variant

Systemic inflammatory response syndrome

Sepsis-related or Sequential Organ Failure Assessment score

Sphingosine-1-phosphate

Trauma Associated Severe Hemorrhage score

Traumatic brain injury

Terminal complement complex

Trauma-induced coagulopathy

Toll-like receptor

Thrombomodulin

Tumor necrosis factor

Triggering receptor expressed on myeloid cells

Thoracic trauma
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>uPA</td>
<td>Urokinase (plasminogen activator)</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase receptor</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1 Introduction

Physical injuries cause more than 5 million deaths worldwide every year, representing the major cause of death in the population below the age of 44 years and accounting for 10% of global mortality [89,123]. Despite improved public safety measures, rescue chains, as well as emergency and intensive care, trauma remains a major burden of disease in all societies [58]. In 2016, 33,000 people sustained injuries that required primary care in the emergency room and a subsequent stay at the intensive care unit (ICU) in Germany alone. Patients were predominantly male, mostly suffered from blunt trauma and stayed in hospital an average of 16 d. Furthermore, 20% developed multiple organ failure and 11.3% died in the hospital [56]. Polytrauma (PT), defined by a complex injury pattern affecting several physical regions or organ systems and including the severity of the injuries so that at least one injury or the combination of several injuries are life-threatening (Injury Severity Score (ISS) ≥ 16) [3,130,131,169], was present in 55% of patients [56].

1.1 Acute posttraumatic inflammatory response

After severe injury, an immediate systemic response occurs to the traumatic insult. Breakdown of cellular structures and barriers results in the concomitant release of damage-, but also pathogen-associated molecular patterns (DAMPs and PAMPs, respectively). These molecules trigger the activation of different humoral serine protease systems, including the coagulation and the complement system, with the aim of sealing off injured areas to stop bleeding and recognizing invading microbes to stop their growth by transmitting the danger signal to phagocytic leukocytes [27,35,53,85]. Leukocytes can also directly recognize DAMPs and PAMPs via pattern recognition receptors [5,24,120]; complement and leukocyte activation induces the secretion of pro-inflammatory cytokines and initiates a systemic inflammatory response which can culminate in development of the systemic inflammatory response syndrome (SIRS). Besides clinical parameters [8], SIRS is characterized by local and systemic production and release of acute-phase proteins, components of the contact phase and coagulation systems, complement factors, pro- and anti-inflammatory cytokines, as well as an accumulation of immune cells at the site of tissue damage [54,69,81]. An excessive immune reaction can reinforce dysfunction of cellular barriers [86], allowing the entry and generation of more PAMPs and DAMPs, thereby amplifying a vicious cycle of tissue injury and harmful immune processes [5,54,81] (Fig. 1).

However, in parallel to the pro-inflammatory response, there is also a marked induction of suppressive processes immediately after injury which may render patients susceptible to
infection [62,118,166]. It is therefore considered essential that the initial immune reaction contains both pro- and anti-inflammatory aspects to establish an immune balance facilitating clearance of the damaged tissues and induction of an effective regeneration [69] (Fig. 1).

**Fig. 1: Posttraumatic immune response.** Injury induces the release of molecular danger signals, so-called pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively) after cell damage and destruction of local barriers. These are sensed by activation of the complement and the coagulation systems which may be reinforced by local hypoxia and acidosis. The danger signal is transmitted to leukocytes via pattern recognition receptors (PRRs), inducing chemotaxis towards the inflammatory stimulus, release of cytokines, reactive oxygen species (ROS), and microparticles (MPs), neutrophil extracellular trap (NET) generation, and phagocytosis of opsonized particles. Effective immune control can aid to establish a balanced reaction, allowing timely and effective clearance of damaged tissue, repair and regeneration. Adapted and personalized trauma care may support the avoidance of an escalation of the danger response due to hemorrhage, extended surgical interventions or nosocomial infection. Influenced by individual risk factors, the innate immune response can become dysbalanced, with patients developing coagulopathy and barrier dysfunction, resulting in edema formation, invasion of microorganisms, development of sepsis and multiple organ dysfunction syndrome (MODS). From Huber-Lang et al. [69].

Apart from pre-existing physical conditions, exogenous factors such as the severity of the injury itself (“first hit”) and surgical interventions (“second hit”) play an important role in SIRS development and posttraumatic outcome [149]. Therefore, in the clinical setting, additional tissue injury should be minimized during life-saving procedures and primary
surgery immediately after trauma; secondary reconstructive surgery is recommended to be
delayed until after a stabilization phase ranging from a few days up to three weeks [4,54].

1.2 Mechanisms of acute trauma-induced coagulopathy
Tissue damage is almost always associated with rupture of blood vessels, and several studies
have reported that approximately 25% of PT patients arrive at the emergency department
with hemodynamic instability and acute traumatic coagulopathy, more recently termed
trauma-induced coagulopathy (TIC) [16,97-99]. About 40% of deaths after injury are caused
by bleeding [80]. Disintegration of macro- (e.g. the skin) and microbarriers such as
endothelial cell membranes immediately activates multiple hemostatic pathways of the
coagulation system, including fibrinolysis, with rapid consumption of coagulation factors
and thrombocytes [21]. This hypocoagulable state is mainly caused by tissue hypoperfusion
and predominantly involves protein C activation [13]. Moreover, platelets have been shown
to become hyporesponsive to pro-coagulatory stimuli with impaired clot assembly and
stability as early as 30 min after injury [90,180]. The risk of bleeding is reinforced by
hypoxia, acidosis, and hypothermia, also termed the “lethal triad” [106]. Furthermore,
increased secretion of catecholamines contributes to the activation of endothelial cells and
results in downregulation of thrombomodulin and secretion of plasminogen-activator
inhibitor-1 (PAI-1) [40,70,105,183]. Increased adrenaline levels and hypoperfusion also lead
to shedding of endothelial glycocalyx components and thereby amplify endothelial
dysfunction and coagulopathy [76]. Additionally, hyperfibrinolysis was demonstrated to be
a central risk factor for posttraumatic mortality [73,79,153]. Conversely, other studies
indicated that shutdown of fibrinolysis may be even more prominent than hyperfibrinolysis,
especially in severely injured patients, in regard to incidence and mortality [113,114]. As a
further driver of coagulation disturbances, fluid resuscitation especially with crystalloids
seems to increase the severity of TIC since the replaced volume was demonstrated to be
proportional to the degree of coagulopathy [98]. In other studies, however, TIC was also
present in patients who had received little or no fluids [13,14,34,51], suggesting that the
injury pattern and severity may also play a significant role. Currently, apart from addressing
hypoxia, acidosis, and hypothermia, treatment strategies such as balanced resuscitation,
antifibrinolytics, patient-tailored recombinant coagulation factors as well as fibrinogen
concentrates are employed. Guided by conventional coagulation assays or viscoelastic
functional testing, all of these can significantly improve the outcome [27].
1.3 Systemic response to severe blood loss

Deaths due to bleeding occur for different reasons, ranging from delayed admission to surgery to detrimental effects of prolonged shock periods despite successful control of bleeding [15]. Upon arrival at the emergency room, 31% of patients with severe trauma as defined by Paffrath et al. [130] and Pape et al. [131] present with shock (systolic blood pressure ≤ 90 mmHg) and 24% with acidosis (base excess ≤ –6 mmol/l) as characteristics of significant hemorrhage. Of note, in the presence of shock, mortality was increased to 37% [56].

Severe blood loss and hypovolemia due to blunt or penetrating injury initiate a compensatory response via sympathetic reflexes, inducing peripheral vasoconstriction in order to maintain blood pressure. Blood flow in internal organs such as the liver, pancreas, kidney, and gastrointestinal tract can be reduced (centralized) to such an extent that patients remain normotensive despite severe shock [115]. Only after the loss of more than 30% of the blood volume, decreases in cardiac output, blood pressure, and organ perfusion may become evident [179]. The reduction in oxygen supply and transport of metabolites quickly results in tissue hypoxia and an increase in lactate levels. In the following, sustained mitochondrial anaerobic metabolism of glucose can lead to cellular destruction and metabolic acidosis [19]. If rapid and effective restoration of normotension and removal of the source of bleeding is not achieved, the accumulation of cell damage leads to disseminated intravascular coagulopathy, microvascular damage, and SIRS with a mostly unfavorable outcome [115].

Recent studies have furthermore suggested hemorrhagic shock (HS) as a major driver of systemic inflammation and disintegration of the blood-organ barrier. In rat models of hemorrhage, a loss in intestinal tight-junction proteins and increased amounts of mitochondrial DAMPs in the mesenteric lymph were detected, suggesting cellular breakdown and a deterioration in barrier function [165,187]. These processes seem to be mainly dependent on complement activation [49]. Shock has also been demonstrated to be a potent inducer of mitochondrial DAMP release which in turn increases secretion of pro-inflammatory mediators in neutrophils and other tissues [192]. Furthermore, posttraumatic bleeding is also considered to play a central role in development of coagulopathy and complementopathy [18,50,51,74] as well as organ failure [78]. These are presumed to be especially driven by pathological activation of the endothelium, termed endotheliopathy. In this regard, disturbances in the endothelial glycocalyx layer after trauma may depend on shock severity: in severely injured patients, higher catecholamine levels were shown to lead
to an increase in glycocalyx shedding from the endothelial surface [75,125,126] which is likely to advance barrier failure and organ damage.

1.4 Development of trauma-induced organ dysfunction and failure

In all patients admitted to emergency departments in Germany, early mortality during the first 24 h accounted for 5.1% of deaths while another 5.3% of patients died later during the first 30 d after injury. On the ICU, 34% developed organ failure which culminated in multiple organ failure (MOF) in 20% of patients [56]. After injury, the aforementioned activation of serine protease cascades (such as the complement system) and release of cytokines and other pro-inflammatory mediators from immune cells and tissues, especially from the gut, prime and mobilize neutrophil granulocytes and other leukocytes [100,112,122,155]. Neutrophil priming can be augmented by ischemia/reperfusion conditions. Further iatrogenic contributors are represented by blood products which are deemed to be immunoactive and contain considerable amounts of pro-inflammatory coagulation and complement factors as well as cytokines and lipids [41,63]. The risk of MOF is additionally increased in patients after severe hemorrhage due to tissue hypoperfusion during the initial shock phase [167,175,178]. Early neutrophilia often turns into neutropenia, suggesting organ sequestration based on increased adhesion molecules on neutrophils and endothelium and transmigration into damaged tissues [12,26]. This is accompanied by increased endothelial and epithelial permeability facilitating electrolyte and protein shifts and subsequent edema formation. Degranulation of neutrophils in inflamed tissues releases further pro-inflammatory cytokines, but also proteases and reactive oxygen species which can damage host tissues and promote bystander injury [163,170]. These processes are reinforced by ischemia and reperfusion; when substantial amounts of oxygen reenter the tissue, accumulation of superoxide anions, hydrogen peroxide, and hydroxyl radicals causes the peroxidation of cell membranes, inducing apoptosis and necrosis and stimulating the local generation of inflammatory mediators [144,151,172]. In the context of the proceeding compensatory anti-inflammatory response in parallel to SIRS development, Gentile et al. established the concept of the persistent low-grade inflammation, immunosuppression, and high protein catabolism syndrome (PICS). PICS is characterized by defects in innate and adaptive immunity with decreased activity of dendritic cells, macrophages, and T effector cells mediated by myeloid-derived suppressor cells (MDSCs), culminating in an increased risk of late MOF [55].
1.5 Lack of reliable immune and organ monitoring in trauma care

Despite considerable advances in trauma care in the last decades, some issues remain which continue to complicate treatment and may impede further improvements in patient management. There are several well-established techniques in order to monitor organ function on the ICU, including clinical parameters and organ performance scores such as the Sepsis-related or Sequential Organ Failure Assessment (SOFA) score [177]. Considering the deleterious effects of extensive surgical interventions with substantial DAMP and PAMP generation when the patient is currently at high risk of overwhelming inflammation or immunosuppression, there is an obvious need for parallel monitoring of the immune function during the time course after injury. Nevertheless, to date, no tests exist to provide the clinician with reliable data on the remaining functional capacity of the immune system and support decision-making in order to optimize timing of necessary interventions or definitive surgical care. In this context, there is also a lack of information on the alterations in peripheral blood leukocytes regarding their responsiveness to inflammatory or pathogenic stimuli in order to implement an immune reaction after injury. Furthermore, not much is known on how leukocytes may be involved in disturbed coagulation after trauma and hemorrhage.

Although bleeding is a frequent concomitant condition in trauma patients, there is a lack of studies assessing the influence of HS on the posttraumatic inflammatory response and especially its impact on organ damage. For this purpose, reliable and specific organ damage markers might provide better insight into pathophysiological processes particularly before the onset of organ dysfunction. Given the central role that a loss in integrity of the endothelial glycocalyx layer seems to play in posttraumatic coagulopathy and mortality, assessing the extent of damage inflicted on the glycocalyx after traumatic hemorrhage could also support an early recognition of barrier breakdown preceding loss of function with improved and personalized treatment options for the severely injured. However, immune and organ monitoring during the early response after severe trauma is not yet established neither scientifically nor clinically.
1.6 Aims of the study

The following hypotheses were tested in the course of this thesis:

- Leukocyte surface molecules interacting with complement, DAMPs, and coagulation are altered during the time course after injury.
- Using a clinically applicable approach, the cellular immune response to an inflammatory stimulus can be monitored.
- The posttraumatic development of coagulopathy can be observed reliably using functional thromboelastometry.
- The organ injury after severe trauma is aggravated by an additional HS, and this damage can be assessed by specific serum markers.
- HS plays a significant role in the loss in endothelial glycocalyx and barrier function after PT.
2 Materials and methods

2.1 Consumables

- BD FACS Clean: BD Biosciences, Heidelberg, Germany
- BD FACS Flow: BD Biosciences, Heidelberg, Germany
- BD FACS Shutdown Solution: BD Biosciences, Heidelberg, Germany
- Cannula, 25 G: B. Braun Melsungen AG, Melsungen, Germany
- Centrifugation tube, 15 ml and 50 ml: BD Biosciences, Heidelberg, Germany
- Cup & Pin mini: tem, Munich, Germany
- Filter (0.2 µm pore size): GE Healthcare, Solingen, Germany
- Nunc™ MicroWell™ 96-Well plate: NUNC A/S, Roskilde, Denmark
- Nunc™ MaxiSorp™ 96-Well ELISA plate: NUNC A/S, Roskilde, Denmark
- Mylar® polyester film, 50 µm: Du Pont de Nemur, Bad Homburg, Germany
- Polyethylene catheter (0.62 mm): Föhr Medical Instruments GmbH, Seeheim/Ober-Beerbach, Germany
- Polystyrene tube, round bottom, 5 ml: BD Biosciences, Heidelberg, Germany
- Reaction tube, 0.5 ml, 1.5 ml and 2 ml: Eppendorf, Hamburg, Germany
- TruCulture® tube (Null, LPS): HOT Screen GmbH, Germany
- S-Monovette® 7.5 ml, Clotting Activator/Serum: Sarstedt, Nümbrecht, Germany
- S-Monovette® 9 ml K3E (EDTA): Sarstedt, Nümbrecht, Germany
- S-Monovette® 10 ml 9NC, Citrate 3.2% (1:10): Sarstedt, Nümbrecht, Germany
- Safety Multifly cannula, 18 G: Sarstedt, Nümbrecht, Germany
- Silkam® 5/0 suture: B. Braun Melsungen AG, Melsungen, Germany
- Syringe, 1 ml: BD Biosciences, Heidelberg, Germany

2.2 Buffers and media

- Aqua dest.: Fresenius Kabi, Bad Homburg, Germany
Materials and methods

DPBS\textsuperscript{−/−} (Dulbecco’s Phosphate-Buffered Saline without Ca\textsuperscript{2+}/Mg\textsuperscript{2+})
Gibco BRL, Grand Island, NY, USA

HBSS\textsuperscript{+/+} (Hank’s Balanced Salt Solution with Ca\textsuperscript{2+}/Mg\textsuperscript{2+})
Gibco BRL, Grand Island, NY, USA

Jonosteril
Fresenius Kabi, Bad Homburg, Germany

Sodium chloride solution (0.9%)
Fresenius Kabi, Bad Homburg, Germany

RPMI 1640 (Roswell Park Memorial Institute-Medium)
Gibco BRL, Grand Island, NY, USA

2.3 Reagents and chemicals

AffinityScript QPCR cDNA Synthesis Kit
Agilent, Santa Clara, CA, USA

Annexin V-Alexa Fluor 647
Invitrogen, Carlsbad, CA, USA

Annexin V binding buffer (10x)
BD Biosciences, Heidelberg, Germany

Bovine serum albumin
Sigma-Aldrich, Steinheim, Germany

Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix
Agilent, Santa Clara, CA, USA

Buprenorphine (Temgesic\textsuperscript{®})
Boehringer, Mannheim, Germany

C5a from human serum
Complement Technology, Inc., Tyler, TX, USA

CellFIX (10x, concentrate)
BD Biosciences, Heidelberg, Germany

Chloroform (≥ 99%)
Sigma-Aldrich, Steinheim, Germany

CountBright™ Absolute Counting Beads
Invitrogen, Carlsbad, CA, USA

DEPC-treated water (RNase-free)
Invitrogen, Carlsbad, CA, USA

DNase I
Invitrogen, Carlsbad, CA, USA

Ethanol (pure)
Sigma-Aldrich, Steinheim, Germany

Ethylenediaminetetraacetic acid (EDTA)
Sigma-Aldrich, Steinheim, Germany

Ex-tem
Tem, Munich, Germany

FACS Lysing Solution
BD Biosciences, Heidelberg, Germany

Fib-tem
Tem, Munich, Germany

Ficoll-Paque
GE Healthcare, Freiburg, Germany
Heparan sulfate: Amsbio, Abingdon, UK
Human recombinant C3a: Calbiochem Inc., San Diego, USA
Human recombinant interleukin-1β: PeproTech, Rocky Hill, NJ, USA
Human recombinant interleukin-6: Biomol, Hamburg, Germany
Human recombinant interleukin-8: Biomol, Hamburg, Germany
Human recombinant syndecan-1: R&D, Wiesbaden-Nordenstadt, Germany
In-tem: Tem, Munich, Germany
Isopropanol: Sigma-Aldrich, Steinheim, Germany
Latex beads, amine-modified polystyrene, fluorescent red (1.0 μm): Sigma-Aldrich, Steinheim, Germany
Latex beads, carboxylate-modified polystyrene, fluorescent red, (0.5 μm): Sigma-Aldrich, Steinheim, Germany
Latex beads, polystyrene (0.3 μm): Sigma-Aldrich, Steinheim, Germany
Lipopolysaccharides from Escherichia coli O55:B5: Sigma-Aldrich, Steinheim, Germany
Mucin from porcine stomach Type II: Sigma-Aldrich, Steinheim, Germany
Norepinephrine: Sanofi, Frankfurt am Main, Germany
Phorbol 12-myristate 13-acetate: Sigma-Aldrich, Steinheim, Germany
Sevoflurane (Sevorane™): Abbott, Wiesbaden, Germany
TRIzol™ Reagent: Thermo Fisher Scientific, Waltham, MA, USA

2.4 Primers
Human PLAUR QuantiTect® Primer Assay: QIAGEN, Hilden, Germany
Human SDHA QuantiTect® Primer Assay: QIAGEN, Hilden, Germany
Human SERPINE1 QuantiTect® Primer Assay: QIAGEN, Hilden, Germany
Human TBP QuantiTect® Primer Assay: QIAGEN, Hilden, Germany

2.5 Antibodies and isotype controls
Mouse α-human C3aR, PE: BioRad, Kidlington, UK
Materials and methods

Mouse α-human TREM-1, PE
Mouse IgG1 Negative Control, PE
Mouse α-human C5L2, PE
Mouse IgG2a, κ Isotype Control, PE
Mouse α-human CD66b, FITC
Mouse IgM, κ Isotype Control, FITC
Mouse α-human CD88, FITC
Mouse IgG2a Negative Control, FITC
Mouse α-human CD142/Tissue factor, FITC
Mouse IgG1 Negative Control, FITC
Mouse α-human CD282 (TLR2), PE
Mouse α-human CD284 (TLR4), PE
Mouse IgG2a Negative Control, PE
Mouse α-human PAR-2, PE
Mouse IgG2a κ Isotype Control, PE
Mouse α-human TCC, FITC
Mouse IgG2a Isotype control, FITC
Mouse α-human Thrombomodulin, FITC
Mouse IgG1 Isotype Control, FITC
Rabbit α-human PAI-1, FITC
Rabbit α-human RAGE/AGER, FITC
Rabbit IgG Isotype Control, FITC

Mouse IgG1 Negative Control, PE
BioRad, Kidlington, UK
Mouse IgG2a, κ Isotype Control, PE
BioLegend, San Diego, CA, USA
Mouse α-human CD66b, FITC
BD Biosciences, Heidelberg, Germany
Mouse IgM, κ Isotype Control, FITC
BD Biosciences, Heidelberg, Germany
Mouse α-human CD88, FITC
BioRad, Kidlington, UK
Mouse IgG2a Negative Control, FITC
BioRad, Kidlington, UK
Mouse α-human CD142/Tissue factor, FITC
BioRad, Kidlington, UK
Mouse IgG1 Negative Control, FITC
BioRad, Kidlington, UK
Mouse α-human CD282 (TLR2), PE
BioRad, Kidlington, UK
Mouse α-human CD284 (TLR4), PE
BioRad, Kidlington, UK
Mouse IgG2a Negative Control, PE
BioRad, Kidlington, UK
Mouse α-human PAR-2, PE
Santa Cruz Biotechnology, Heidelberg, Germany
Mouse IgG2a κ Isotype Control, PE
Santa Cruz Biotechnology, Heidelberg, Germany
Mouse α-human TCC, FITC
Hycultec, Beutelsbach, Germany
Mouse IgG2a Isotype control, FITC
Hycultec, Beutelsbach, Germany
Mouse α-human Thrombomodulin, FITC
Abcam, Cambridge, UK
Mouse IgG1 Isotype Control, FITC
Abcam, Cambridge, UK
Rabbit α-human PAI-1, FITC
Bioss, Woburn, MA, USA
Rabbit α-human RAGE/AGER, FITC
Bioss, Woburn, MA, USA
Rabbit IgG Isotype Control, FITC
Bioss, Woburn, MA, USA

2.6 Immunoassays

2.6.1 Multiplex immunoassay
Bio-Plex Pro™ Human Cytokine 27-plex Assay
BioRad, Hercules, CA, USA
2.6.2 ELISA kits for human proteins

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Manufacturer and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM / Adrenomedullin ELISA Kit</td>
<td>LifeSpan BioSciences, Seattle, WA, USA</td>
</tr>
<tr>
<td>Angiopoietin-2 DuoSet ELISA</td>
<td>R&amp;D, Wiesbaden-Nordenstadt, Germany</td>
</tr>
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<td>C-Reactive Protein/CRP Quantikine ELISA Kit</td>
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<tr>
<td>CC16 (Clara Cell 16kD protein) ELISA Kit</td>
<td>Elabscience, Bethesda, MD, USA</td>
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<tr>
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<td>Cusabio, College Park, MD, USA</td>
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<td>FABP2/I-FABP DuoSet ELISA</td>
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<td>HS (Heparan Sulfate) ELISA Kit</td>
<td>Elabscience Biotechnology Co., Houston, TX, USA</td>
</tr>
<tr>
<td>IL-6 ELISA Set</td>
<td>BD Biosciences, Heidelberg, Germany</td>
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<tr>
<td>L-FABP ELISA kit</td>
<td>Hycultec, Beutelsbach, Germany</td>
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<td>Lipocalin-2/NGAL DuoSet ELISA</td>
<td>R&amp;D, Wiesbaden-Nordenstadt, Germany</td>
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<td>MUC2 ELISA Kit (Human)</td>
<td>Aviva Systems Biology, San Diego, CA, USA</td>
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<td>Serpin E1/PAI-1 DuoSet ELISA</td>
<td>R&amp;D, Wiesbaden-Nordenstadt, Germany</td>
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<td>Serum Albumin DuoSet ELISA</td>
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<td>Sphingosine 1 Phosphate ELISA Kit</td>
<td>MyBiosource, San Diego, CA, USA</td>
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<td>Syndecan-1 DuoSet ELISA</td>
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<td>Total MMP-9 DuoSet ELISA</td>
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<td>Total MMP-13 DuoSet ELISA</td>
<td>R&amp;D, Wiesbaden-Nordenstadt, Germany</td>
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2.6.3 ELISA kits for murine proteins

<table>
<thead>
<tr>
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<tr>
<td>ALB/Serum Albumin ELISA Kit</td>
<td>LifeSpan BioSciences, Seattle, WA, USA</td>
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<tr>
<td>Claudin-5 (CLDN5) ELISA kit</td>
<td>Cusabio, College Park, MD, USA</td>
</tr>
<tr>
<td>Hspg2 (Basement membrane-specific heparan sulfate proteoglycan core protein) ELISA Kit</td>
<td>Amsbio, Abingdon, UK</td>
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<tr>
<td>MMP9/Gelatinase B ELISA Kit</td>
<td>LifeSpan BioSciences, Seattle, WA, USA</td>
</tr>
<tr>
<td>MMP13 ELISA Kit</td>
<td>LifeSpan BioSciences, Seattle, WA, USA</td>
</tr>
</tbody>
</table>
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SDC1/Syndecan 1/CD138 ELISA Kit  
LifeSpan BioSciences, Seattle, WA, USA

THBD/CD141/Thrombomodulin ELISA Kit  
LifeSpan BioSciences, Seattle, WA, USA

2.7 Devices

Anesthesia device  
Vapor Drägerwerk AG, Lübeck, Germany

Bio-Plex® 200 platform  
BioRad, Hercules, CA, USA

Blood pressure analyzer  
Data Sciences International, New Brighton, MN, USA

FACSCanto II  
BD Biosciences, Heidelberg, Germany

Mx3000P QPCR System  
Agilent, Santa Clara, CA, USA

Vial Program 1 syringe pump  
BD Medical, Heidelberg, Germany

Qubit 2.0  
Thermo Fisher Scientific, Waltham, MA, USA

ROTEM® delta  
Tem, Munich, Germany

Tecan Sunrise Reader  
Tecan, Crailsheim, Germany

Temperature plate  
Föhr Medical Instruments, Seeheim/Ober-Beerbach, Germany

Test tube rotator  
Snijders, Tilburg, Netherlands

ThermoMixer C  
Eppendorf, Hamburg, Germany

2.8 Software

BD FACSDiva™ Software (Version 6.1.2)  
Becton Dickinson, Heidelberg, Germany

FlowJo (Version 10.2)  
FlowJo, LLC, Ashland, OR, USA

Ponemah (Version 5.0)  
Data Sciences International, New Brighton, MN, USA

SAS (Version 9.3)  
SAS, Cary, NC, USA

SigmaPlot (Version 11.0)  
Systat Software, Erkrath, Germany

SigmaStat (Version 3.5)  
Systat Software, Erkrath, Germany

XFluor4 (Version 4.51)  
Tecan, Crailsheim, Germany
2.9 PT study design at Ulm University Hospital

A prospective clinical study was conducted in 12 patients after severe PT (ISS ≥ 32) that were admitted to the University Hospital Ulm between December 2013 and May 2015. The study protocol was approved by the Independent Local Ethics Committee of the University of Ulm (approval numbers 244/11 and 94/14). The study was registered on ClinicalTrials.gov, identifiers NCT00710411 and NCT02682550, and was performed in accordance with the Declaration of Helsinki and its recent modifications. Exclusion criteria were age < 18 years, pregnancy, infection with the human immunodeficiency virus, cardiogenic shock as the primary underlying disease, underlying hematologic disease, cytotoxic therapy given within the previous 6 months, and the presence of rapidly progressing underlying disease anticipating death within the next 24 h. Whole venous blood was drawn at the time of admission (0 h) and 4 h, 12 h, 24 h, 48 h, 5 d and 10 d after trauma with a maximum divergence from time points of ± 10%. Seven healthy volunteers served as a control group. Before inclusion, written informed consent was obtained from all patients and volunteers; if the patient was incapable of making decisions because of intubation, sedation or altered mental status, informed consent was obtained directly after recovery or from the next of kin.

2.10 Plasma and serum collection

At all seven time points after injury, blood was drawn into serum, ethylenediaminetetraacetic acid (EDTA) and sodium citrate tubes and transported on ice. Tubes with EDTA-chelated and citrated blood were centrifuged immediately at 800 x g and 4°C for 10 min; supernatants were centrifuged again at 16,000 x g at 4°C for 2 min to remove platelets. For serum collection, blood was allowed to coagulate at 4°C for 30–120 min; tubes were centrifuged at 1,560 x g at 4°C for 10 min. All supernatants were aliquoted on ice and stored at −80°C until further analysis.

2.11 Flow cytometric analysis of whole blood

To assess changes in surface molecules related to complement activation, modulation of coagulatory processes and sensing of DAMPs and PAMPs on leukocytes, leukocytes in whole blood were analyzed by flow cytometry during the course after trauma and compared to healthy volunteers. 100 µl of EDTA-anticoagulated blood were stained for the cellular receptors for the complement component 5a (C5a, C5aR1 and C5aR2), the terminal complement complex (TCC), plasminogen activator inhibitor-1 (PAI-1), thrombomodulin, receptor for advanced glycation end products (RAGE), toll-like receptors 2 and 4.
(TLR-2/-4), and triggering receptor expressed on myeloid cells (TREM) using fluorescence-labeled antibodies. Respective IgG isotype controls were used to correct for unspecific binding. After staining at room temperature in the dark for 20 min, erythrocyte lysis was performed for 12 min using FACS Lysing Solution. Cells were pelleted at 340 \( x \) \( g \) for 5 min, washed with phosphate-buffered saline (PBS), resuspended in 100 \( \mu l \) CellFix and stored at 4°C for no more than 8 h until the analysis by flow cytometry. Light scatter characteristics were used to distinguish granulocytes, lymphocytes, and monocytes, and at least 2,000 cells of each population were recorded. The mean fluorescence intensity (MFI) for the cell populations was analyzed using FlowJo (version 10.2, FlowJo, LLC, Ashland, OR, USA).

### 2.12 TruCulture® assay

Whole blood \textit{ex vivo} stimulation was performed using TruCulture® tubes with blood taken 4 h, 24 h and 5 d after injury. Tubes were prefilled by the manufacturer under standardized conditions with 2 ml culture medium, and contained unfractionated heparin as an anticoagulant at a final concentration of 50 IU/ml. To assess the immune reaction to a bacterial stimulus, tubes with or without 100 ng/ml lipopolysaccharide (LPS, from \textit{Escherichia coli}, O55:B5, or Null) were used. The TruCulture® system minimizes the risk of contamination, but also reduces intra-individual variation by direct blood withdrawal without additional pipetting steps [117]. Tubes were stored at –20°C and thawed in a water bath at 37°C immediately before use. 1 ml of blood was drawn into blood collection tubes from healthy volunteers and PT patients at indicated time points. After incubation at 37°C for 24 h, sedimented cells were separated from the supernatant using a valve, and supernatants were stored at –80°C until analysis. In order to increase clinical applicability, a second set of tubes ± LPS was taken at the second time point (24 h after trauma), and incubation at 37°C was reduced to 4 h before supernatant collection. Samples were analyzed using a multiplexed sandwich immunoassay on a multiplex platform according to the manufacturer’s recommendations. Since many samples were out of range for the pro-inflammatory marker interleukin (IL)-6, they were re-assessed using the IL-6 Quantikine kit (BD Biosciences, Germany). Concentrations of cytokines after LPS stimulus in supernatants from PT patients were compared with those from healthy volunteers. To evaluate the shorter incubation period, the differences in released mediators in stimulated and control samples after 4 h incubation were compared to those after 24 h incubation.
2.13 PAI-1 plasma detection
PAI-1 concentrations in EDTA-plasma of PT patients and healthy volunteers were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit strictly in accordance to the manufacturer’s protocol (R&D Systems and BD Biosciences, respectively).

2.14 Neutrophil isolation
Venous blood from healthy volunteers was drawn into tubes containing 3.2% sodium citrate from the antecubital vein. After mixing the blood with an equal volume of isotonic saline (0.9% NaCl), 20 ml were layered carefully over 10 ml of Ficoll in a 50 ml centrifugation tube and cells were separated at 340 x g for 30 min with slow brakes. The supernatant containing plasma, mononuclear cells and Ficoll was removed, and the remaining pellet was mixed with dextran in 0.9% NaCl at a final concentration of 1%. After sedimentation of erythrocytes for 30 min, the supernatant was collected and neutrophils were pelleted by centrifugation at 340 x g for 5 min. The remaining erythrocytes were lysed with distilled water, followed by addition of 2.7% NaCl to restore isotonic conditions. Neutrophils were pelleted and resuspended in medium at 5–10*10⁶/ml.

2.15 PAI-1 surface detection on stimulated neutrophils
For surface staining, freshly isolated neutrophils were resuspended in RPMI 1640 + 0.1% bovine serum albumin (BSA) at 5*10⁶/ml. After addition of LPS (5 µg/ml) or the so-called polytrauma cocktail (PTC) [65], containing complement component 3a (C3a, 500 ng/ml), C5a (10 ng/ml), IL-1β (200 pg/ml), IL-6 (500 pg/ml), and IL-8 (150 pg/ml), cells were incubated at 37°C for 1 h while rotating at 80 rpm to simulate blood circulation. After centrifugation, 5*10⁵ cells were stained with anti-PAI-1 antibody or the isotype control at 4°C for 20 min, followed by washing and fixation. Measurement of PAI-1 surface binding on at least 10,000 cells was performed by flow cytometry and analyzed using FlowJo.

2.16 Gene expression analysis in neutrophils
For messenger RNA (mRNA) isolation, neutrophils isolated from citrated blood were adjusted to 10⁷/ml in Roswell Park Memorial Institute (RPMI) medium + 0.1% BSA and stimulated as described in 2.15. After pelleting at 800 x g for 5 min at 4°C, 2*10⁷/ml cells per sample were lysed using Trizol, and mRNA was isolated following the manufacturer’s protocol. In brief, the cell lysate was mixed with chloroform and centrifuged to separate the aqueous phase containing RNA from the interphase and the phenol-chlorophorm phase containing DNA and cellular proteins. RNA was precipitated with isopropanol, pelleted,
washed with 75% ethanol, dried and solubilized in ribonuclease-free water by incubation at 60°C for 10 min. Until further analysis, RNA was stored at –80°C.

RNA concentrations were determined using the Qubit fluorometer and the respective quantitation assay, employing a dye that only produces a fluorescent signal when bound to RNA. Genomic DNA was removed by deoxyribonuclease (DNase) I digestion of 1 µg RNA per sample. RNA was mixed with 10x DNase I Reaction Buffer, DNase and diethyl pyrocarbonate water and incubated for 15 min at room temperature. To inactivate the DNase, EDTA was added to a concentration of 2.5 mM before heating the sample at 65°C for 10 min. For reverse transcription of 550 ng RNA per sample, the AffinityScript QPCR cDNA Synthesis Kit was used. After mixing RNA, first strand master mix, oligo(DT) primers and AffinityScript RT/RNase Block enzyme mixture, samples were incubated at 25°C for 5 min to allow primer annealing. Complementary DNA (cDNA) synthesis was performed at 42°C for 15 min and the reaction was terminated at 95°C for 5 min. cDNA was diluted 1:5 with water and stored at –20°C until further analysis.

Gene expression was measured using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix and commercially available primers in reaction volumes of 20 µl in duplicates on the Mx3000P real-time quantitative PCR (qPCR) system. Expression was compared to the neutrophil house-keeping genes succinate dehydrogenase complex, subunit A, flavoprotein variant (SDHA) and TATA-binding protein [91] employing the 2⁻ΔΔCT method [95]. For final analyses, SDHA was used as a reference.

2.17 Detection of surface PAI-1 and microvesicles after whole blood stimulation

In order to analyze membrane-bound molecules on leukocytes in a more physiological setting, EDTA-anticoagulated blood was drawn from healthy volunteers. Blood was stimulated with PTC, 5 µg/ml LPS or 20 ng/ml of the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) [23] at 37°C and rotating at 80 rpm to simulate blood circulation. Whole blood was stained for PAI-1 for 20 min at room temperature. Following erythrocyte lysis, cells were washed and fixed and fluorescence was detected by flow cytometry.

To assess microvesicles released by neutrophils during stimulation, plasma was obtained by centrifugation at 2,200 x g for 15 min, and stored at –20°C. Plasma was mixed with Alexa Fluor 647-labeled Annexin V, fluorescein isothiocyanate (FITC)-labeled anti-cluster of differentiation (CD)66b antibody and CountBright™ Absolute Counting Beads in Annexin V binding buffer (filtered with 0.2 µm pore size). 20 mM EDTA was added as a control to inhibit Annexin V binding. After incubation for 15 min in the dark, samples were
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washed with binding buffer and measured using the FACSCanto II flow cytometer. For compensation, single stain controls were used. Gates were set using latex beads with defined sizes (0.3 µm, 0.5 µm and 1 µm) and Annexin V-positive microvesicles with a diameter of up to 1 µm were analyzed. The gating strategy is shown in Fig. 2. For volume control, measurement was stopped after detecting 1000 counting beads corresponding to 2 µl of plasma.

Fig. 2: Representative gating strategy for detection of microvesicles in plasma after whole-blood stimulation. After gating for size according to light scatter characteristics (side scatter area, SSC-A, and forward scatter area, FSC-A), annexin-V-positive vesicles were selected and mean fluorescence intensity as well as percentage of fluorescein isothiocyanate (FITC)-positive microvesicles were analyzed. Counting beads were used for volume control. Here, samples after control (Ctrl) or phorbol 12-myristate 13-acetate (PMA) stimulus are shown.

2.18 PT study design at Zurich University Hospital

Since the low number of patients in the study Ulm PT group did not allow stratification for the presence or absence of HS, a randomly selected subcohort of 30 patients from a monocentered, observational, prospective study at the University Hospital Zurich (Trauma Level I Center) including a total of 104 patients (ClinicalTrials.gov identifier: NCT02508272,
Materials and methods

[146,147]) was used. The study was approved by the Cantonal Ethic Commission Zurich (StV 26–2007) and performed in accordance with local and international guidelines. Patients with an ISS ≥ 18, age ≥ 18 y, and time passed since the injury < 6 h were included under informed consent. Blood was drawn upon arrival at the emergency room (day 0) and daily during the next 21 d.

The subcohort of 30 patients was stratified for signs of manifest HS [88,108,190] on day 0; the presence of HS was assumed when at least one of the following criteria was fulfilled: a Trauma Associated Severe Hemorrhage (TASH) score ≥ 10, base excess < −6 mmol/l, lactate ≥ 2.5 mmol/l, and/or red blood cell (RBC) transfusion during the first 24 h > 2 U. Venous whole blood was drawn into serum or sodium citrate collection tubes and centrifuged at 2000 x g at 4°C for 10 min. Serum concentrations of IL-6, the organ damage markers Clara cell secretory protein (CC16), neutrophil gelatinase-associated lipocalin (NGAL), intestinal fatty acid-binding protein (I-FABP), and liver-type fatty acid-binding protein (L-FABP), albumin, sphingosine-1-phosphate, thrombomodulin, angiopoietin-2, matrix metalloproteinases (MMP)-9 and -13, claudin-5, vascular endothelial (VE-) cadherin, syndecan-1, heparan sulfate and mucin-2 were determined serially using commercially available sandwich ELISA kits. After determining optimal dilution conditions, samples were incubated on ELISA plates over night at 4°C; afterwards, samples were transferred to the next plates and ELISAs were performed according to the manufacturers’ recommendations, allowing measurement of up to 4 parameters in one sample. According to a previous publication, serial ELISA measurements produce valid and reliable data [127]. Concentrations of fibrinogen, D-dimer, soluble TCC and C5a in citrated plasma were determined by respective commercial sandwich ELISA kits. To correct for dilution effects after mass transfusion, absolute serum protein was determined using a bicinchonic acid assay (Pierce). Measurements in patients with signs of HS were compared to those without shock; six healthy volunteers served as controls.

2.19 Rotational thromboelastometry analysis

Due to the low sample volume available from PT patients and a required volume of 310 µl for common rotational thromboelastometry (ROTEM®) analyses, small-volume ROTEM® was established in collaboration with Prof. van Griensven, Munich; employing smaller cups and pins allowed a reduction of the sample volume to 105 µl per analysis. To avoid variations in measurements due to long time periods between patient inclusions, citrate plasma from patients included in the Ulm PT study was stored at −80°C until analysis. For analysis,
plasma was pipetted into the cups, recalcified and mixed with thromboplastin as inducer of coagulation via the extrinsic pathway. A pin was inserted into the cup and rotated while detecting resistance by clot formation (Fig. 3). Reagents for extrinsic activation of coagulation without (extem) and with (fibtem) inhibition of thromocyte activation by cytochalasin D were used. Measurements were performed on a ROTEM® delta device. Results were compared to ROTEM® measurements of plasma from healthy volunteers.

Fig. 3: Exemplary depiction of a rotational thromboelastometry (ROTEM®) measurement with central readout parameters. By courtesy of Instrumentation Laboratory, Bedford.

2.20 Testing of endothelial glycocalyx and intestinal mucus components in rotational thromboelastometry

To assess whether dilution by volume replacement therapy after HS increases the anticoagulatory potency of glycocalyx and mucus components, blood was drawn from healthy volunteers and the respective hematocrit was determined by centrifugation at 340 x g for 5 min. The hematocrit was adjusted to 33% or 25% with Jonosteril, and blood samples with different hematocrit were incubated with heparan sulfate, human recombinant syndecan-1 and mucin-2 for 30 min at room temperature. The activity of the intrinsic pathway (in-tem test) was analyzed using a ROTEM® delta device.

2.21 Murine model of PT and HS

The murine PT model with and without HS published by Denk et al. [36] was performed in adherence to national and international guidelines for the use of laboratory animals. Animals were housed in groups of five in individually ventilated cages Type II long, with unrestricted access to food and water. The study protocol was approved by the Federal Authorities for Animal Research (Tübingen, Germany) under approval number 1194. 32 male C57BL/6

mice at the age of 8–9 weeks (Charles River WIGA GmbH, Sulzfeld, Germany) with a mean body weight of 25 g (± 2.5 g) were randomly assigned to sham treatment, PT, HS, or combined PT with HS. Anesthesia was induced by inhalation of 2.5% sevoflurane in oxygen in a tube for narcosis induction and maintained during the experiment after transferring animals to a face mask. Mice received 0.03 mg/kg body weight buprenorphine subcutaneously for analgesia. Animals were placed on a feed-back loop temperature control device with connected rectal probe to maintain a core temperature of 37°C. Chest and upper abdomen, legs and head were shaved. Sham animals were catheterized and monitored, but did not undergo trauma or HS. An overview of the study protocol is shown in Fig. 4.

**Fig. 4: Overview of the study protocol.** Animals in deep anesthesia underwent polytrauma including thoracic trauma (TXT), traumatic brain injury (TBI), a closed fracture and soft tissue injury. After instrumentation, HS was applied to achieve a mean arterial pressure (MAP) of 30 mmHg. MAP, heart rate (HR) and temperature were monitored until termination of the experiment 240 min after trauma. From Denk et al. [36].

### 2.21.1 Trauma application

PT was induced by combining blunt bilateral chest trauma, traumatic brain injury and femur fracture/soft tissue trauma. Animals were fixated in a supine position, and blunt chest trauma was induced using a blast wave generator as established by Knöferl et al. [84]. Mice were positioned 1.6 cm below the generator’s nozzle with its lower edge in alignment with the costal margin. The upper section of the nozzle served as pressure reservoir which was connected to a storage tank of compressed air and separated from the lower opening by a 50-µm polyester film. The film would burst when the pressure in the upper part, created by opening the storage tank, exceeded 17 bar, and generate a reproducible single blast wave with a duration of 1–2 ms. The described protocol induces local and systemic inflammation, but does not cause injuries of the bony thorax or of abdominal organs [83,84,94,133,134].
For induction of traumatic brain injury, animals were positioned on the abdomen and fixated. The left galea aponeurotica frontoparietal was incised with a scalpel and the scalp was exposed. A weight of 333 g was dropped on the cranial vault of the left hemisphere from a height of 2.0 cm, inducing a contusion of the underlying brain tissue without injuring the bony lamina interna [157].

A closed fracture was applied on the center of the right femur using a weight-drop device modified from Bonnarens and Einhorn [10]. After positioning of the limb on the device, a weight of 50 g was dropped from 120 cm. The resulting dynamic impulse induced a dynamic three-point bending, leading to a transverse diaphyseal fracture and injury of the surrounding soft tissue.

2.2.1.2 Induction of pressure-controlled HS

After trauma application, mice were instrumented aseptically by insertion of an arterial polyethylene catheter (diameter 0.62 mm) in the distal left femoral artery, using a minimally invasive dissection technique. The arterial catheter was connected to a device for blood pressure monitoring. The same technique was employed to insert a catheter into the left jugular vein for volume replacement and continuous infusion of norepinephrine regulated by a perfusor. Hemorrhage was induced by controlled bleeding via the arterial catheter until a mean arterial pressure of 30 mmHg ± 5 mmHg was reached; this was maintained over a period of 60 min. Afterwards, animals were reperfused over the venous catheter with a bolus of 400 µl Jonosteril over 5 min and slower infusion of additional Jonosteril until the fourfold volume of the blood drawn during hemorrhage had been given. Animals in the sham or PT groups only received the bolus of 400 µl. After HS, mice were monitored for 120 min after a standardized protocol, maintaining a mean arterial pressure of 50 mmHg by reducing the depth of anesthesia or by norepinephrine support (0.01–0.12 µg/kg per min) via the venous catheter. 240 min after PT or sham treatment, animals were exsanguinated by thoracotomy and cardiac puncture. Blood was collected in EDTA tubes; plasma was obtained by centrifugation at 800 x g and 4°C for 5 min; supernatants were centrifuged at 13,000 x g and 4°C for 2 min to remove platelets and stored at −80°C for further analyses.

2.2.1.3 Plasma analysis

Mouse plasma was analyzed using commercially available sandwich ELISA kits for serum albumin, claudin-5, heparan sulfate proteoglycan core protein, MMP-9 and -13, syndecan-1 and thrombomodulin following the manufacturers’ recommendations.
2.22 Statistical analyses

Experimental results were compared using paired t-test for two groups or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test for three or more groups. Correlation analyses were performed using Pearson Product Moment Correlation. Differences in leukocyte cytokine secretion after endotoxin stimulus were compared by Student’s t-test. Clinical parameters of patients with and without HS were compared using the Chi-square test in case of categorical variables and Student’s t-test in case of continuous parameters. These statistical analyses were performed using SigmaPlot. To evaluate whether the presence or absence of HS significantly changed overall plasma values of barrier and organ molecules during the time course after trauma, repeated-measures ANOVA was used employing SAS. For all ANOVA testing, no formal statistical test on normality was applied due to its limited validity regarding the available sample size [140]. Results are presented as mean ± standard error of the mean. A p-value < 0.05 was considered statistically significant.
Results

3 Results

3.1 Alterations in leukocyte surface molecules after trauma and correlation to shock parameters

In order to analyze whether a severe trauma was reflected by changes in the surface expression patterns of central receptors and molecules interacting with DAMPs/PAMPs, complement, and coagulation, whole blood was stained during the course after injury and the surface expression was measured by flow cytometry. Expression levels were then correlated to clinical parameters with focus on hemorrhagic shock.

3.1.1 Complement receptors and terminal complement complex

C5aR1 expression was significantly lower on neutrophils and monocytes of injured patients especially during the late phase after trauma as detected by flow cytometry (Fig. 5).

Fig. 5: Complement component 5a receptor 1 (C5aR1) on leukocytes after trauma. C5aR1 mean fluorescence intensity (MFI) on A, neutrophils, B, lymphocytes, and C, monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). *, p < 0.05; #, p < 0.05 compared to Healthy; â, p < 0.05 compared to 0 h after PT; n = 7-12.
Similarly, C5aR2 was reduced on neutrophils 10 d after injury (Fig. 6 A), and demonstrated a significant decrease already after 4 h on monocytes when compared to controls (Fig. 6 B). Of note, expression of both C5a receptors was considerably lower on lymphocytes compared to granulocytes and monocytes (Fig. 6 C).

**Fig. 6**: Complement component 5a receptor 2 (C5aR2) on leukocytes after trauma. C5aR2 mean fluorescence intensity (MFI) on A, neutrophils, B, lymphocytes, and C, monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). *, p < 0.05 compared to Healthy; &, p < 0.05 compared to 0 h after PT; n = 4-7.
On neutrophils and lymphocytes, TCC could not be detected on the cell surface neither in healthy volunteers nor in trauma patients. In contrast, on monocytes, considerable membrane formation of the TCC was measured, which, after an early slight reduction, increased significantly in PT patients 24 h and 48 h after injury, and returned to baseline levels on days 5 and 10 (Fig. 7).

![Fig. 7: Terminal complement complex (TCC) on monocytes.](image)

The TCC formation at 4 h after injury was significantly associated with the number of applied erythrocyte concentrates during the first day (Fig. 8).

![Fig. 8: Pearson correlation between transfused blood products and terminal complement complex (TCC) expression on monocytes.](image)
3.1.2 DAMP and PAMP receptors

While RAGE expression was detectable, but not significantly increased on neutrophils and monocytes in patients during the later course after PT, there were no alterations in its surface levels on lymphocytes (Fig. 9).

Fig. 9: Receptor for advanced glycation end products (RAGE) on peripheral leukocytes after trauma. RAGE mean fluorescence intensity (MFI) on A, neutrophils, B, lymphocytes, and C, monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). N = 7-11.
However, RAGE expression on lymphocytes measured at the early time points after injury correlated significantly with the base excess and the number of transfused erythrocyte concentrates (Fig. 10 A–C). Furthermore, expression on monocytes 48 h and 5 d post trauma correlated negatively with blood lactate at admission and positively with erythrocyte concentrates given during the first day (Fig. 10 D, E).

Fig. 10: Pearson correlation between receptor for advanced glycation end products (RAGE) expression on leukocytes and clinical parameters. Shown are correlations between initial base excess, the number of applied erythrocyte concentrates during the first day, and blood lactate concentrations upon admission and RAGE expression on lymphocytes 0 h (A), 4 h (B), and 12 h (C) after trauma and on monocytes 48 h (D) and 5 d (E) after injury as determined by mean fluorescence intensity (MFI). R, Pearson correlation coefficient.
The expression of TLR-2 and TLR-4 was notably decreased on all leukocyte types to around 50% especially during the early time course after trauma, but alterations were not significant compared to healthy volunteers (Fig. 11).

**Fig. 11:** Toll-like receptor (TLR) expression on leukocytes after trauma. Mean fluorescence intensity (MFI) of TLR-2 (A, C, E) and TLR-4 (B, D, F) on granulocytes (A, B), lymphocytes (C, D) and monocytes (E, F) of healthy volunteers (Healthy) and polytrauma patients (PT). N = 4-12.
Nevertheless, TLR-2 on monocytes 4 h and 12 h after injury revealed a significant positive correlation with the patients’ TASH score upon admission (Fig. 12 A, B). Similarly, TLR-4 on monocytes 12 h after trauma correlated positively with the TASH score (Fig. 12 C). Several negative correlations were found for TLR-4 expression: at 24 h on granulocytes and monocytes with base excess, and on lymphocytes with erythrocyte concentrates (Fig. 12 D-F).

**Fig. 12:** Toll-like receptor (TLR) expression in correlation to clinical parameters. Pearson correlation between Trauma Associated Severe Hemorrhage (TASH) score, base excess, and the number of applied erythrocyte concentrates during the first day with TLR-2 (A, B) and TLR-4 (C-F) on monocytes 4 h (A) and 12 h (B, C) after trauma, and on granulocytes (D), monocytes (E), and lymphocytes (F) 24 h after injury as determined by mean fluorescence intensity (MFI). R, Pearson correlation coefficient.
While TREM-1 expression on granulocytes and lymphocytes was unaltered over the time course after PT (Fig. 13 A, B), monocytes demonstrated some increase in surface TREM-1 already at the time of admission which was significant after 12 h compared to healthy controls. After 10 d, TREM-1 values had returned to baseline levels (Fig. 13 C).

Fig. 13: Triggering receptor expressed on myeloid cells-1 (TREM-1) expression on peripheral leukocytes during the time course after trauma. TREM-1 detected as mean fluorescence intensity (MFI) on A, neutrophils, B, lymphocytes, and C, monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). *, p < 0.05; #, p < 0.05 compared to Healthy; &, p < 0.05 compared to 0 h after PT; n = 4-12.
In line with these results, monocyte TREM-1 expression at 4 h post injury correlated significantly with lactate concentration and the number of transfused erythrocyte concentrates at admission (Fig. 14).

Fig. 14: Triggering receptor expressed on myeloid cells-1 (TREM-1) correlated to clinical parameters. Pearson correlation between initial lactate concentration (A) and the number of applied erythrocyte concentrates during the first day (B) with TREM-1 expression as detected by mean fluorescence intensity (MFI) on monocytes 4 h after trauma. R, Pearson correlation coefficient.

3.1.3 Molecules interacting with the coagulation system
Thrombomodulin was detectable only on monocytes, and surface expression was significantly increased 24 h and 48 h after trauma compared to healthy volunteers and values upon admission (Fig. 15). There were no significant correlations between thrombomodulin and clinical parameters of shock.

Fig. 15: Thrombomodulin (TM) expression on monocytes after trauma. TM mean fluorescence intensity (MFI) on monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). *, p < 0.05 compared to Healthy; #, p < 0.05 compared to 0 h after PT; n = 7-12.
On the surface of all leukocytes, PAI-1 was detected in increased amounts after trauma compared to healthy volunteers. While neutrophils and monocytes demonstrated a significant increase only 5 d after trauma (Fig. 16 A, C), binding on lymphocytes was significantly increased 24 h and 48 h compared to emergency room values, and days 5 and 10 only showed some increase compared to controls (Fig. 16 B).

Fig. 16: Surface plasminogen activator inhibitor-1 (PAI-1) on leukocytes during the time course after trauma. PAI-1 mean fluorescence intensity (MFI) on A, neutrophils, B, lymphocytes, and C, monocytes in whole blood from healthy volunteers (Healthy) or patients after polytrauma (PT). *, p < 0.05; #, p < 0.05 compared to Healthy; &, p < 0.05 compared to 0 h after PT; n = 7-12.
Results

PAI-1 on granulocytes (Fig. 17 A) and on lymphocytes (Fig. 17 B, C) correlated significantly with the initial number of needed erythrocyte concentrates.

Fig. 17: Plasminogen activator inhibitor-1 (PAI-1) in correlation to clinical parameters. Pearson correlation between the number of applied erythrocyte concentrates during the first day with PAI-1 expression as detected by mean fluorescence intensity (MFI) on granulocytes 4 h (A) and lymphocytes 4 h (B) and 12 h (C) after injury. R, Pearson correlation coefficient.

3.2 PAI-1 in PT plasma and *ex vivo* on the granulocyte surface

Since PAI-1 was detected in increased amounts on all leukocytes especially on day 5 after injury, soluble PAI-1 was measured in plasma samples to check whether higher plasma concentrations could induce an increased surface binding. However, plasma PAI-1 was only increased during the initial days post trauma (Fig. 18), therefore presumably not accounting for increased surface binding on leukocytes.
Fig. 18: Soluble plasminogen activator inhibitor-1 (PAI-1) increase early after trauma. PAI-1 in plasma from healthy volunteers (Healthy) and from patients after polytrauma (PT). N = 7-12.

To analyze whether in vitro incubation with DAMPs/PAMPs stimuli related to trauma and/or sepsis could alter the PAI-1 surface pattern similar to the clinical setting, freshly isolated neutrophils from healthy volunteers were stimulated with PTC or LPS for 1 h and PAI-1 was determined using flow cytometry. The amount of protein present on the cellular surface was significantly increased after LPS and only slightly higher after PTC stimulus compared to the control (Fig. 19 A). The percentage of PAI-1-positive cells was significantly increased in both stimulated groups (Fig. 19 B).

Fig. 19: Surface plasminogen activator inhibitor-1 (PAI-1) on neutrophils ex vivo. PAI-1 on isolated neutrophils after stimulation with polytrauma cocktail (PTC), lipopolysaccharide (LPS) or control treatment (Ctrl) for 1 h. A, mean fluorescence intensity (MFI), and B, percentage of cells positive for PAI-1 as detected by flow cytometry. *, p < 0.05; n=5-8.
Results

Since isolated neutrophils demonstrate a considerable apoptosis rate after longer incubation periods (own unpublished data), whole blood anticoagulated with EDTA was used for a longer incubation protocol. As a positive control for cell stimulation, the protein kinase C activator PMA was used. In contrast to the results for isolated neutrophils, stimulation of whole blood by LPS or PTC resulted in no increase in surface PAI-1 expression. Only PMA was able to induce a significant increase, and this was true for both 1 h (Fig. 20 A, B) and the prolonged 24 h incubation time (Fig. 20 C, D).

**Fig. 20: Surface plasminogen activator inhibitor-1 (PAI-1) on neutrophils after whole-blood stimulation.** Blood was incubated with polytrauma cocktail (PTC), lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) or without stimulant (Ctrl) for 1 h (A, B) and 24 h (C, D). A, C, mean fluorescence intensity (MFI) on neutrophils and B, D, percentage of neutrophils positive for PAI-1 as assessed by flow cytometry. ***, p < 0.001; n = 3.

To analyze whether this increase in surface PAI-1 on neutrophils stimulated in whole blood was due to a reduced shedding of microvesicles from the cell membrane, microvesicle number and origin in plasma were determined using flow cytometry. After 1 h, there were no differences in the total microvesicle number (Fig. 21 A). Similarly, nor the absolute count nor the percentage of CD66b-positive microvesicles was altered after either stimulus (Fig. 21 C, E). After 24 h, there was some increase in total microvesicle numbers (Fig. 21 B) and
Results

a significant elevation in total and relative numbers of CD66b-positive microvesicles after PMA incubation, but again, there were no changes in samples with PTC or LPS (Fig. 21 D, F).

Fig. 21: Microvesicles (MV) in supernatant after whole-blood stimulation. Blood was incubated with polytrauma cocktail (PTC), lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) or without stimulant (Ctrl) for 1 h (A, C, E) and 24 h (B, D, F). A, B, total microvesicles per µl plasma, C, D, number of CD66b+ MV per µl plasma, and E, F, percentage of CD66b+ MV as assessed by flow cytometry. ***, p < 0.001; n = 3.
3.3 Gene expression of PAI-1 and urokinase receptor in stimulated neutrophils

To test the hypothesis that the increased PAI-1 detected on isolated neutrophils after inflammatory stimuli was due to an increase in gene transcription, mRNA was isolated from stimulated isolated neutrophils and expression of SERPINE1 (coding for PAI-1) and PLAUR (coding for the urokinase receptor, uPAR) was analyzed using qPCR. There were no changes in PAI-1 expression in neutrophils after PTC stimulation for 1 h and 4 h; in contrast to the surface protein findings, incubation with LPS for 4 h lead to a significant downregulation of PAI-1 expression (Fig. 22 A). When analyzing the cellular anchor for PAI-1, uPAR, which is a receptor for urokinase (uPA) and thus facilitates PAI-1 binding, a slight, but nonsignificant increase in uPAR expression was detected after 1 h in PTC and LPS samples compared to unstimulated controls. However, the expression was downregulated in cells after 4 h incubation (Fig. 22 B).

**Fig. 22:** Expression of plasminogen-activator inhibitor-1 (PAI-1) and urokinase receptor (uPAR) in isolated neutrophils. A, SERPINE1 (coding for PAI-1) and B, PLAUR (coding for uPAR) in neutrophils 1 h and 4 h after polytrauma cocktail (PTC) or lipopolysaccharide (LPS) stimulation as fold-expression compared to control treatment (Ctrl) and normalized to the housekeeping gene. ***, p = 0.001; ***, p < 0.001; n=2-8.
3.4 Thromboelastic alterations in blood after PT

Citrated plasma was used to detect alterations in coagulation in PT patients compared to healthy volunteers by performing ROTEM® analysis. When comparing measurements from activation via the extrinsic pathway with (fibtem) and without (extem) inhibition of thrombocyte activation, results were highly similar (Fig. 23), presumably due to the removal of thrombocytes during centrifugation steps. For this reason, only results from extem measurements are shown in the following.

![Thromboelastic alterations in blood after PT](image)

Fig. 23: Analysis of platelet function in plasma samples. Comparison between representative thrombelastograms from extem (without platelet inhibition, left panel) and fibtem (with platelet inhibition, right panel) measurements of corresponding plasma samples from a patient 0 h, 4 h, 24 h, and 48 h after trauma.
3.4.1 Coagulation activation and clot polymerization

During the first 10 d after PT, clotting time in patients was significantly higher than in healthy controls, reaching a peak 48 h post injury (Fig. 24 A). Clot formation time was higher in tendency, but comparison of results was difficult with a partly low n-size due to several samples not reaching a 20 mm amplitude (Fig. 24 B). The $\alpha$-angle as the angle between the baseline and the tangent to the clotting curve through the 2-mm point was significantly reduced in trauma patients during the first 48 h, but then normalized on days 5 and 10 (Fig. 24 C). In line, the clot formation rate as the angle between the baseline and the tangent at maximum slope was significantly lower at the early time point after trauma and returned to normal values after 5 d (Fig. 24 D).

Fig. 24: Coagulation activation and clot polymerization after severe injury. Clotting time (A), clot formation time (B), $\alpha$-angle (C), and clot formation rate (D) in citrated plasma from healthy volunteers (white bars) and polytrauma patients (grey bars) at indicated time points after trauma. *, p < 0.05; #, p < 0.05 compared to Healthy; §§, p < 0.05 compared to 5 d; $\$, p < 0.05 compared to 10 d; n = 6-11 (A, C, D), n = 1-8 (B).
3.4.2 Clot firmness

Besides analyzing the onset of coagulation, ROTEM® also allows to determine the clot firmness during the course of measurement. The maximal clot firmness during the 90-min assay was significantly reduced in patients upon admission to the emergency room compared to healthy volunteers. The reduction remained present until 48 h after injury, and disappeared on day 5 (Fig. 25 A). This pattern was already apparent 10 min and 30 min after starting the assay (Fig. 25 B, C). Time to maximal clot firmness, however, was only slightly increased in patients 48 h after injury (Fig. 25 D).

**Fig. 25: Parameters of clot firmness after polytrauma.** Maximal clot firmness (A), clot firmness after 10 min (B) and 30 min (C), and time to maximal clot firmness (D) in citrated plasma from healthy volunteers (white bars) and polytrauma patients (grey bars) at indicated time points after trauma. *, p < 0.05; #, p < 0.05 compared to Healthy; §, p < 0.05 compared to 5 d; $, p < 0.05 compared to 10 d; n = 6-11.
3.4.3 Clot characteristics

As could be concluded from the lysis index at 30 min, there was no detectable clot lysis in any of the samples (Fig. 26 A). Maximum velocity as the calculated maximum of the curve’s first derivative was reduced in patient samples at all time points compared to healthy volunteers (Fig. 26 B). In line with the results for clot firmness, the shear elastic modulus strength was significantly reduced as early as upon hospital admission and remained low until 24 h after trauma, returning to baseline levels after 48 h and even surpassing healthy values on days 5 and 10 (Fig. 26 C).

![Clot characteristics in polytrauma patients.](image)

Fig. 26: Clot characteristics in polytrauma patients. Lysis index after 30 min (A), maximum velocity (B), and shear elastic modulus strength (C) in citrated plasma from healthy volunteers (white bars) and polytrauma patients (grey bars) at indicated time points after trauma. *, p < 0.05; #, p < 0.05 compared to Healthy; §, p < 0.05 compared to 5 d; $, p < 0.05 compared to 10 d; n = 6-11.
3.5 **Cytokine response to bacterial stimulus in an *ex-vivo* whole blood model**

Whole blood from healthy volunteers and from patients at different time points after PT was incubated with LPS for 24 h and cytokines in the supernatant were analyzed. For all cytokines, only results in stimulated samples were compared; unstimulated values are shown as reference.

IL-6 generation was lower by tendency, but not significantly, in PT patients compared to healthy volunteers (Fig. 27 A). TNF release was significantly reduced in patients 4 h and 5 d after injury (Fig. 27 B). IL-1β production and the IL-1β/IL-RA ratio were significantly decreased in stimulated patient groups at all time points (Fig. 27 C, D).

![Graphs showing cytokine response to bacterial stimulus](image)

**Fig. 27:** Release of pro-inflammatory cytokines in whole blood after lipopolysaccharide (LPS) stimulus. Interleukin (IL)-6 (A), tumor necrosis factor (TNF, B), and IL-1β (C) concentrations, and ratio between IL-1β and IL-1 receptor antagonist (IL1-RA) concentrations (D) in supernatants after whole blood stimulation with (+) and without (-) LPS for 24 h. Healthy, healthy volunteers; PT, blood samples taken from polytrauma patients at indicated time points after trauma. *, p < 0.05 compared to Healthy for analysis of stimulated samples; unstimulated values are shown as reference. N = 5-7.
Fig. 28: Cytokine release in whole blood after lipopolysaccharide (LPS) stimulus. Interferon-γ (IFN-γ, A), interleukin (IL)-10 (B), IL-12 (C), IL-17 (D), eotaxin (E), basic fibroblast growth factor (FGF basic, F), granulocyte colony-stimulating factor (G-CSF, G), and granulocyte-macrophage colony-stimulating factor (GM-CSF, H) in supernatants after whole blood stimulation with (+) and without (-) LPS for 24 h. Healthy, healthy volunteers; PT, blood samples taken from polytrauma patients at indicated time points after trauma. Analysis was performed for stimulated samples; unstimulated values are shown as reference. N = 5-7.
Although there were some alterations in the unstimulated samples after trauma, there were no significant differences in the production of interferon-\(\gamma\) (IFN-\(\gamma\), Fig. 28 A), IL-10 (Fig. 28 B), IL-12 (Fig. 28 C) or IL-17 (Fig. 28 D). Similarly, trauma also did not induce any significant changes in the secretion of eotaxin (Fig. 28 E), basic fibroblast growth factor (Fig. 28 F), granulocyte-colony stimulating factor (Fig. 28 G), or granulocyte-macrophage colony-stimulating factor (Fig. 28 H).

In contrast, 24 h after trauma, there was a significantly increased release of IL-2 (Fig. 29 A), IL-4 (Fig. 29 B), and IL-5 (Fig. 29 C). IL-7 production after LPS stimulus was also significantly higher in patients 24 h and 5 d after trauma compared to healthy volunteers (Fig. 29 D). IL-9 was slightly increased in patient samples 4 h and 5 d after trauma, and significantly higher 24 h after injury (Fig. 29 E). IL-13 was elevated in trauma patients only by tendency (Fig. 29 F). IL-15 secretion was significantly increased in patients at all time points compared to healthy volunteers (Fig. 29 G). Monocyte chemoattractant protein 1 release after LPS stimulus was significantly higher in blood samples from patients 4 h and 24 h after injury (Fig. 29 H).
Fig. 29: Release of inflammatory mediators in whole blood after lipopolysaccharide (LPS) stimulus. Interleukin (IL)-2 (A), IL-4 (B), IL-5 (C), IL-7 (D), IL-9 (E), IL-13 (F), IL-15 (G), and monocyte chemoattractant protein 1 (MCP-1, H) in supernatants after whole blood stimulation with (+) and without (-) LPS for 24 h. Healthy, healthy volunteers; PT, blood samples taken from polytrauma patients at indicated time points after trauma. *, p < 0.05 compared to Healthy for analysis of stimulated samples; unstimulated values are shown as reference. N = 5-7.
When comparing a shortened incubation time of 4 h in samples taken 24 h after trauma, there was a highly similar pattern of cytokine release (Fig. 30).

**Fig. 30:** Comparison of differences in cytokine concentrations in stimulated and unstimulated samples after 4 h and 24 h incubation. Data are shown as mean in pg/ml. FGF-basic, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1RA, IL-1 receptor antagonist; IL-12 (p70), IL-12 heterodimer; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein-1α; PDGF-bb, platelet-derived growth factor subunit B; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
A comparison of p-values for the difference between stimulated and unstimulated samples for the two incubation periods is shown in Table 1.

**Table 1: Comparison between sample incubation for 4 h or 24 h.** Shown are p-values for differences between stimulated and unstimulated samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p-value LPS vs. Ctrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h incubation</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.018</td>
</tr>
<tr>
<td>IL1-RA</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.005</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-7</td>
<td>0.532</td>
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<tr>
<td>IL-9</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.015</td>
</tr>
<tr>
<td>IL-15</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-17</td>
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</tr>
<tr>
<td>Eotaxin</td>
<td>0.001</td>
</tr>
<tr>
<td>FGF basic</td>
<td>0.002</td>
</tr>
<tr>
<td>G-CSF</td>
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</tr>
<tr>
<td>GM-CSF</td>
<td>0.021</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.001</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.069</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>0.083</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>0.006</td>
</tr>
<tr>
<td>PDGF-bb</td>
<td>0.615</td>
</tr>
<tr>
<td>TNF</td>
<td>0.009</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Abbreviations: FGF-basic, basic fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL1-RA, IL-1 receptor antagonist; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1; MIP-1α/β, macrophage inflammatory protein-1α/β; PDGF-bb, platelet-derived growth factor subunit B; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
3.6 Impact of HS on barrier disturbance and organ dysfunction after PT

Since there were several significant correlations between surface molecules on leukocytes in our PT cohort and clinical parameters related to the presence or absence of HS (see 3.1), the influence of an additional HS after severe trauma, especially focusing on inflammation, vascular barrier and organ function, was analyzed. For this purpose, a PT patient cohort and a murine model of PT and/or HS were studied.

3.6.1 Parameters of inflammation, barrier and organ dysfunction, and coagulation in a human PT study and correlation with clinical data

A patient cohort from Zurich University Hospital was stratified for the presence or absence of HS according to established parameters. The strategy identified 10 patients without and 20 patients with HS. The cohorts had similar injury patterns except for the prevalence of abdominal trauma. Despite similar ISS values, patients with HS displayed a higher maximal SOFA score during the course of observation and required a three-fold higher ICU and two-fold increased in-hospital length of stay. Stratification criteria such as the TASH score, numbers of packed RBC transfusion, and initial lactate levels were also significantly different in the two groups. Further clinical parameters and a statistical comparison are shown in Table 2.

Several inflammatory mediators and parameters related to barrier and organ (dys-)function were analyzed over the course of the first 5 days after injury employing sandwich ELISAs. P-values < 0.05 represent a significant difference between patients with compared to those without HS over the 5-day period.
Table 2: Descriptive statistics and comparison of demographic parameters in stratified groups. Statistical comparison of stratified groups is shown as p-values. Modified from Hallgebauer et al. [60].

<table>
<thead>
<tr>
<th>Stratification of hemorrhagic shock</th>
<th>Polytrauma without HS (n=10)</th>
<th>Polytrauma with HS (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All criteria fulfilled:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>base excess ≥ −6 mmol/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate &lt; 2.5 mmol/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRBC ≤ 2 units on day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TASH score &lt; 10 points</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
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<tr>
<td>One or more criteria fulfilled:</td>
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<tr>
<td>base excess &lt; −6 mmol/l</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>lactate ≥ 2.5 mmol/l</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pRBC &gt; 2 units on day 0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TASH score ≥ 10 points</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Demographics
- **Age [y]**
  - Polytrauma without HS: 44.4 ± 6.3
  - Polytrauma with HS: 37.6 ± 3.8
  - P-value: 0.45
- **Sex [male/total]**
  - Polytrauma without HS: 4/10 (40%)
  - Polytrauma with HS: 17/20 (85%)
  - P-value: 0.011
- **GCS**
  - Polytrauma without HS: 11.5 ± 1.5
  - Polytrauma with HS: 12 ± 0.9
  - P-value: 0.93
- **Traumatic brain injury (GCS ≤ 12)**
  - Polytrauma without HS: 4/10 (40%)
  - Polytrauma with HS: 7/20 (35%)
  - P-value: 0.789
- **AIS max.**
  - Polytrauma without HS: 4.6 ± 0.9
  - Polytrauma with HS: 4.5 ± 0.8
  - P-value: 0.7
- **AIS head/neck/cervical spine**
  - Polytrauma without HS: 2.9 ± 0.9
  - Polytrauma with HS: 2.25 ± 0.4
  - P-value: 0.45
- **AIS face**
  - Polytrauma without HS: 0.3 ± 0.3
  - Polytrauma with HS: 0.8 ± 0.2
  - P-value: 0.2
- **AIS thorax/thoracic spine**
  - Polytrauma without HS: 3.3 ± 0.6
  - Polytrauma with HS: 3.7 ± 0.3
  - P-value: 0.49
- **AIS abdomen/lumbar spine**
  - Polytrauma without HS: 1.1 ± 0.4
  - Polytrauma with HS: 2.8 ± 0.3
  - P-value: 0.01
- **AIS upper/lower extremity**
  - Polytrauma without HS: 2.3 ± 0.4
  - Polytrauma with HS: 2.8 ± 0.2
  - P-value: 0.23
- **ISS**
  - Polytrauma without HS: 36.2 ± 6.3
  - Polytrauma with HS: 38.7 ± 2.8
  - P-value: 0.54
- **SOFA score initial**
  - Polytrauma without HS: 4.6 ± 1.2
  - Polytrauma with HS: 5.7 ± 0.7
  - P-value: 0.4
- **SOFA score max.**
  - Polytrauma without HS: 6 ± 1.1
  - Polytrauma with HS: 10.5 ± 1
  - P-value: 0.009

Outcomes
- **RISC [% survival]**
  - Polytrauma without HS: 79.1 ± 10.2
  - Polytrauma with HS: 80 ± 5.7
  - P-value: 0.52
- **Survival**
  - Polytrauma without HS: 3/10 (30%)
  - Polytrauma with HS: 4/20 (20%)
  - P-value: 0.542
- **Hospital length of stay [d]**
  - Polytrauma without HS: 17.2 ± 3.2
  - Polytrauma with HS: 34 ± 4.1
  - P-value: 0.01
- **Intensive care unit length of stay [d]**
  - Polytrauma without HS: 5.9 ± 1.3
  - Polytrauma with HS: 19.2 ± 2.8
  - P-value: 0.001

Allogenic blood transfusions
- **TASH score [points]**
  - Polytrauma without HS: 4.6 ± 0.9
  - Polytrauma with HS: 10.5 ± 1.2
  - P-value: 0.004
- **Initial (d0) RBC transfusion [units]**
  - Polytrauma without HS: 1.1 ± 0.4
  - Polytrauma with HS: 7.9 ± 1.5
  - P-value: 0.002
- **Total RBC transfusion [units]**
  - Polytrauma without HS: 3.8 ± 0.5
  - Polytrauma with HS: 18.2 ± 3.5
  - P-value: < 0.001
- **Massive transfusion rate**
  - Polytrauma without HS: 0/10
  - Polytrauma with HS: 6/20 (30%)
  - P-value: 0.053

Infectious complications
- **Nosocomial infections**
  - Polytrauma without HS: 4/10 (40%)
  - Polytrauma with HS: 15/20 (75%)
  - P-value: 0.872
- **Sepsis**
  - Polytrauma without HS: 0/10
  - Polytrauma with HS: 6/20 (20%)
  - P-value: 0.053

Hemostasis and blood gas analysis
- **Initial base excess [mmol/l]**
  - Polytrauma without HS: −2.2 ± 0.4
  - Polytrauma with HS: −3.6 ± 0.7
  - P-value: 0.38
- **Initial lactate [mmol/l]**
  - Polytrauma without HS: 1.5 ± 0.2
  - Polytrauma with HS: 2.7 ± 0.3
  - P-value: 0.007
- **Initial Quick [%]**
  - Polytrauma without HS: 73.5 ± 5.7
  - Polytrauma with HS: 53.4 ± 4.1
  - P-value: 0.008
- **Initial aPTT [s]**
  - Polytrauma without HS: 29.7 ± 1.5
  - Polytrauma with HS: 46.1 ± 6.4
  - P-value: 0.04
- **Initial hematocrit [%]**
  - Polytrauma without HS: 33.5 ± 1.5
  - Polytrauma with HS: 26.6 ± 1.7
  - P-value: 0.013
- **Initial temperature [°C]**
  - Polytrauma without HS: 35.1 ± 0.2
  - Polytrauma with HS: 35.4 ± 0.3
  - P-value: 0.58

P-values < 0.05 are bold.

Abbreviations: AIS, Abbreviated Injury Score; aPTT, abbreviated partial thromboplastin time; GCS, Glasgow Coma Scale; HS, hemorrhagic shock; ISS, Injury Severity Score; RBC, red blood cells; PT, polytrauma; RISC, Revised Injury Severity Classification; SOFA score, Sequential Organ Failure Assessment score; TASH score, trauma associated severe hemorrhage score.
IL-6 was higher in PT patients with HS as soon as they arrived at the hospital and further increased until day 1; while PT only patients almost returned to baseline concentrations after 5 d, IL-6 after PT+HS remained elevated over the entire course of observation (Fig. 31 A). In contrast, C-reactive protein (CRP) was initially low in both groups, then increased on days 1 and 2. PT+HS patients showed a further elevation of serum CRP while those without HS had slightly decreased concentrations on day 5 (Fig. 31 B). Complement activation as reflected by systemic C5a and TCC concentrations was initially low in both cohorts and gradually increased over the days following trauma. Both C5a and TCC were slightly higher in trauma patients with HS compared to those without (Fig. 31 C, D).

**Fig. 31: Inflammatory markers during the early time course after severe trauma.** Concentrations of A, interleukin-6 (IL-6), B, C-reactive protein (CRP), C, complement activation product 5a (C5a), and D, terminal complement complex (TCC) in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 17-20). Where \( p < 0.05 \), plasma values in the HS group were significantly altered overall compared to PT alone. Modified from Halbgebauer et al. [60].
To assess the extent of organ dysfunction, markers specific for damage of lungs (CC16), kidneys (NGAL), intestine (I-FABP) and liver (L-FABP) were analyzed. All showed significantly higher concentrations in PT+HS patients compared to those with PT only, but with a very distinct temporal pattern. CC16 was increased starting on day 1 after trauma and gradually returned to healthy values until day 5 while PT patients demonstrated slightly elevated concentrations only on day 0 (Fig. 32 A). In contrast, NGAL was initially low in both groups, but then increased strongly in the PT+HS cohort over the course of observation and remained high until day 5 (Fig. 32 B). I-FABP was initially high in PT+HS patients and only slightly elevated in PT only; during the course of observation, the values approached baseline which was reached on day 3 (Fig. 32 C). L-FABP was initially increased in both groups; while after PT alone, concentrations returned to levels found in healthy volunteers already on day 2, they remained elevated until day 5 in PT+HS (Fig. 32 D).

**Fig. 32:** Organ-damage markers during the early time course after severe trauma. Concentrations of A, Clara cell secretory protein (CC16), B, neutrophil gelatinase-associated lipocalin (NGAL), C, intestinal fatty acid-binding protein (I-FABP), and D, liver-type fatty acid-binding protein (L-FABP) in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 17-20). When p < 0.05, overall plasma values in the HS group were significantly altered compared to PT alone. Modified from Halbgebauer et al. [60].
In order to determine whether certain clinical parameters revealed an influence on organ damage, correlation analyses were performed between damage markers and clinical data. CC16 on day 0 significantly correlated with the total number of transfused erythrocyte concentrates during the first 21 d (Fig. 33 A), and on day 1, higher plasma concentrations were associated with a higher fluid balance on day 2 (Fig. 33 B). NGAL on day 1 correlated significantly with the AIS of the abdomen (Fig. 33 C), and on day 3 with concurrent procalcitonin concentrations (Fig. 33 D). Upon arrival, I-FABP serum concentrations correlated with lactate (Fig. 33 E) and with the fluid balance (Fig. 33 F). L-FABP on day 1 correlated negatively with Quick values (Fig. 33 G), and positively with procalcitonin two days later (Fig. 33 H).
Fig. 33: Pearson Product Moment Correlation of plasma concentrations of organ damage markers to clinical parameters. Correlation analyses were performed for polytrauma patients without (PT) and with hemorrhagic shock (PT+HS) on indicated days after injury. Significant correlations were found for clara cell secretory protein (CC16) with A, total applied red blood cell (RBC) concentrates and B, the fluid balance, neutrophil gelatinase-associated lipocalin (NGAL) with C, the abbreviated injury score (AIS) of the abdomen and D, procalcitonin (PCT). Intestinal fatty acid-binding protein (I-FABP) concentrations correlated with E, Lactate d0 [mmol/l] with F, the fluid balance, Quick d1 [%] with G, the fluid balance, and PCT d3 [µg/l] with H.
blood lactate and \( F \), the fluid balance; liver-type fatty acid-binding protein (L-FABP) was significantly correlated with \( G \), Quick values and \( H \), PCT concentrations. \( R \), Pearson correlation coefficient. Modified from Halbgebauer et al. [60].

As could be expected from hemodilution conditions by resuscitation protocols, albumin concentrations were lower in all patients compared to healthy volunteers, but there was no significant difference between the patient cohorts (Fig. 34 A). Thrombomodulin as endothelial damage marker was initially lower in patients, but then increased until day 5 especially in PT+HS patients (Fig. 34 B). The lipid mediator sphingosine-1-phosphate was decreased in all patients compared to controls, but there was no significant difference between the cohorts (Fig. 34 C). After lower concentrations in patients upon hospital admission compared to healthy individuals, the vascular growth factor angiopoietin-2 normalized in both groups on day 1. Starting on day 2, there was an increase in serum concentrations in PT+HS patients, and concentrations remained elevated until day 5 (Fig. 34 D). The vasodilator adrenomedullin was lower in PT only patients compared to controls during the course of observation. In contrast, PT+HS patients revealed slightly increased serum concentrations on day 0 which further increased until highest levels were reached on day 2, then slowly decreasing until day 5 (Fig. 34 E).
Fig. 34: Mediators of vascular permeability during the early time course after severe trauma. Concentrations of A, albumin, B, thrombomodulin, C, sphingosine-1-phosphate, D, angiopoietin-2, and E, adrenomedullin in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 17-20). Where p < 0.05, overall plasma values in the HS group were significantly altered compared to PT alone. Modified from Halbgebauer et al. [60].
In both patient cohorts, MMP-9 was strongly increased upon arrival at the emergency room; concentrations dropped already on day 1, but remained elevated until day 5 compared to healthy volunteers (Fig. 35 A). MMP-13, however, showed a very different pattern; PT patients had concentrations comparable to those in controls which only increased slightly on day 5; levels in PT+HS patients were strongly increased on day 0 and slowly decreased after that (Fig. 35 B). Claudin-5 as an important tight-junction molecule was initially increased in both patient cohorts, although more pronounced in PT+HS patients; while concentrations returned to healthy values in PT patients on day 5, they remained high in PT+HS patients (Fig. 35 C). VE-cadherin was decreased in both patient cohorts over the observation period compared to controls, but there was no difference between the cohorts (Fig. 35 D).

**Fig. 35:** Mediators and components of endothelial intercellular junctions during the early time course after trauma. Concentrations of A, matrix metalloproteinase (MMP)-9, B, MMP-13, C, claudin-5, and D, vascular endothelial (VE-) cadherin in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 17-20). Where p < 0.05, overall plasma values in the HS group were significantly altered compared to PT alone. Modified from Halbgebauer et al. [60].
Angiopoietin on day 3 correlated significantly with the number of transfused packed erythrocyte units on day 0 (Fig. 36 A); similarly, higher claudin-5 concentrations on day 2 were associated with a higher number of erythrocyte concentrates (Fig. 36 B). Serum claudin-5 over the entire observation period significantly correlated with blood lactate levels (Fig. 36 C).

**Fig. 36: Correlation between markers of barrier dysfunction with clinical parameters of shock.** Pearson Product Moment Correlation of angiopoietin and claudin-5 with clinical parameters in polytrauma patients without (PT) and with hemorrhagic shock (PT+HS) on indicated days after injury. Significant correlations were found for A, angiopoietin with applied red blood cell (RBC) concentrates, and between claudin-5 plasma concentrations with B, applied RBC concentrates and C, blood lactate. R, Pearson correlation coefficient. Modified from Halbgäubner et al. [60].

Syndecan-1 as a key marker for endotheliopathy was significantly higher in PT+HS patients compared to the PT only cohort beginning on the day of admission, and concentrations remained strongly elevated. In contrast, PT alone did only lead to increased syndecan-1 on day 5 compared to healthy volunteers (Fig. 37 A). This result was confirmed when normalizing to serum protein in order to correct for dilution effects after fluid resuscitation (Fig. 37 B). The glycocalyx component heparan sulfate was slightly lower, but not significantly altered in any patient cohort (Fig. 37 C). However, when normalized to serum protein, PT+HS patients had significantly higher values compared to PT alone which only
slowly normalized until day 5 (Fig. 37 D). Mucin-2 as major component of the intestinal mucosa was slightly, but not significantly lower in the PT cohort compared to PT+HS (Fig. 37 E); in contrast, normalization to total protein revealed significantly higher mucin-2 concentrations in PT+HS although levels were similar on day 5 (Fig. 37 F).

Fig. 37: Components of the endothelial glycocalyx and intestinal mucus after trauma. Plasma concentrations of A, syndecan-1, C, heparan sulfate, and E, mucin-2 in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 17-20). B, syndecan-1, D, heparan sulfate, and F, mucin-2 concentrations after normalization to total plasma protein. For p < 0.05, overall plasma values in the HS group were significantly altered compared to PT alone. Modified from Halbgebauer et al. [60].
On day 1, higher MMP-13 concentrations correlated significantly with serum heparan sulfate (Fig. 38 A), and syndecan-1 was significantly associated with the measured maximal SOFA score (Fig. 38 B). Furthermore, over the entire observation period, syndecan-1 serum concentrations correlated significantly with L-FABP (Fig. 38 C), NGAL concentrations (Fig. 38 D), and the abbreviated prothrombin time (Fig. 38 E).

Fig. 38: Correlation analysis of markers of endothelial activation and clinical parameters. Pearson Product Moment Correlation in polytrauma patients without (PT) and with hemorrhagic shock (PT+HS) on indicated days after injury. A, correlation of matrix metalloproteinase 13 (MMP-13) with heparan sulfate; syndecan plasma concentrations correlated to B, the maximal sequential organ failure score (SOFA max.) during the course of observation, C, liver-type fatty acid-binding protein (L-FABP), D, neutrophil gelatinase-associated lipocalin (NGAL), and E, the abbreviated prothrombin time (aPTT). R, Pearson correlation coefficient. Modified from Halbgebauer et al. [60].
Fibrinogen was significantly lower in PT+HS patients compared to the PT cohort, but concentrations increased in both groups during the observation period (Fig. 39 A). The fibrin degradation product D-dimer was lower in all patients compared to healthy controls, and concentrations were significantly lower after HS compared to PT alone (Fig. 39 B). In an experiment simulating hemodilution in patients after extensive fluid resuscitation, adjustment of the hematocrit in the blood from healthy volunteers lead to a reduction in D-dimer concentrations (Fig. 39 C).

Fig. 39: Fibrinogen and fibrin degradation after severe trauma and hemodilution. Plasma concentrations of A, fibrinogen and B, D-dimer in plasma of healthy volunteers (Healthy, n = 6) and polytrauma patients without (PT, n = 9-10) and with hemorrhagic shock (PT+HS, n = 13-16). P < 0.05 indicates a significant alteration of overall plasma values in the HS group compared to polytrauma alone. C, D-dimer concentrations in plasma from healthy volunteers after hematocrit adjustment.
In a similar experiment, dilution of whole blood from healthy volunteers induced a significant increase in clotting time (Fig. 40 A) and clot formation time (Fig. 40 B). Incubation of blood with normal hematocrit with heparan sulfate concentrations comparable to those detected in patients lead to a significant delay in coagulation. This was also visible, but not significant, in samples with lower hematocrit (Fig. 40 A). Addition of mucin-2 did not alter clot formation time in blood with a normal hematocrit, but had an even stronger effect on clot formation time in samples with the lowest hematocrit (Fig. 40 B).

![Fig. 40: Influence of glycocalyx components on coagulation.](image)

Clotting time (A) and clot formation time (B) in rotational thromboelastometry measurements of citrated blood from healthy volunteers with normal (45%, n = 3-8) or adjusted (33% and 25%, n = 3-5) hematocrit after incubation with heparan sulfate and mucin-2 at the indicated concentrations; *, p < 0.05 vs. hematocrit 45% without heparan sulfate/mucin-2; *, p=0.005 vs. hematocrit 25% without mucin-2. Modified from Halbgebauer et al. [60].
3.6.2 Barrier disturbances in a murine model of traumatic HS

In order to investigate the impact of HS in trauma in a highly standardized and clinically relevant model, mice underwent PT or HS or the combination of both; healthy animals served as controls. Plasma was analyzed 4 h after trauma.

Plasma albumin was increased only in HS animals, but was significantly lower after additional PT (Fig. 41 A). Thrombomodulin was significantly decreased in both groups which were exposed to HS (Fig. 41 B).

Fig. 41: Modulators of endothelial permeability in a murine trauma model. Plasma albumin (A) and thrombomodulin (B) in mice after polytrauma (PT), hemorrhagic shock (HS), combined PT+HS, or in control (Ctrl) animals. *, p < 0.05; n = 5-8.

MMP-9 concentrations in plasma were significantly higher in PT+HS animals compared to controls, but also increased to some extent after HS or PT alone (Fig. 42 A). MMP-13 showed the same pattern, but results were not significantly different (Fig. 42 B). Claudin-5 concentrations were similar to healthy values in HS animals and higher by tendency in both PT groups (Fig. 42 C). Heparan sulfate was slightly, but not significantly increased in all trauma groups compared to controls (Fig. 42 D). Syndecan-1 was elevated after HS or PT alone, but only the combination lead to a significant increase compared to healthy animals (Fig. 42 E).
Fig. 42: Mediators and components of endothelial intercellular junctions in a murine model of severe trauma. Plasma matrix metalloproteinase (MMP)-9 (A), MMP-13 (B), claudin-5 (C), heparan sulfate (D), and syndecan (E) in mice after polytrauma (PT), hemorrhagic shock (HS), combined PT+HS, or in control (Ctrl) animals. *, p < 0.05; n = 5-8.
4 Discussion

In the present thesis, the influence of a severe injury on the molecular and cellular “first line” danger response in peripheral blood and development of coagulopathy in PT patients was evaluated. Furthermore, the role of an additional HS on endothelial and organ barrier dysfunction after PT was investigated in a larger patient cohort and in a standardized murine model of multiple injury. Main results found during the course of this thesis are summarized in Fig. 43.

Fig. 43: Summary of main findings. Severe trauma leads to early changes in the surface expression of molecules interacting with coagulation, receptors for complement activation products as well as endogenous and exogenous danger signals on peripheral leukocytes, resulting in alterations in the intracellular response to danger signals. Increased inflammation, augmented by an additional hemorrhagic shock, induces the breakdown of tight junctions and of the endothelial glycocalyx, water efflux into extravascular tissues and causes organ damage with a distinct spatial and temporal onset pattern which can be monitored using plasma markers. Heparin-like structures released during cleavage of glycocalyx components can hinder coagulation, resulting in trauma-induced coagulopathy. C5a, complement component 5 a; C5αR, C5a receptor; CC16, clara cell secretory protein; IL-6, interleukin-6; I-FABP, intestinal fatty acid-binding protein; L-FABP, liver-type fatty acid-binding protein; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; PAI-1, plasminogen activator inhibitor-1; sphingosine-1-P, sphingosine-1-phosphate, TCC, terminal complement complex; TLR, toll-like receptor; TM, thrombomodulin; TREM, triggering receptor expressed on myeloid cells; uPA, urokinase plasminogen activator; uPAR, uPA receptor. Modified from Halbgebauer et al. [60].
4.1 Leukocyte surface profiling in polytraumatized patients

In the first part of this study, the role of leukocytes in the molecular danger response after severe trauma was investigated. Confirming a previous study in polytraumatized patients [1], there were significant reductions in the expression of complement receptors on leukocytes from PT patients, possibly due to microvesicle shedding [171], cleavage from the cell surface by neutrophil proteases [174], or internalization and subsequent degradation or recycling processes [160]. Furthermore, TLR-2 and TLR-4 as the main receptors for bacterial and fungal PAMPs [135,161], but also for endogenous DAMPs such as extracellular histones and high mobility group box 1 (HMGB-1) [46,186] were rather decreased on most leukocytes post trauma, possibly causing a reduced responsiveness to microbial or DAMP stimulus. TREM-1, an amplifier of TLR activation by microbial components [28], but also involved in sterile inflammation with potential ligands such as HMGB-1 and heat-shock protein-70 [43,162] was only significantly higher on monocytes, but mostly unaltered on granulocytes and lymphocytes in trauma patients. Strikingly, there were several significant correlations of expression levels of RAGE, a multi-ligand receptor for several endogenous molecules including advanced glycation end products [52,92], TLR-2, TLR-4, and TREM-1 with parameters related to the presence of a HS, but not with injury severity (data not shown), prompting the assumption that presence or absence of HS might alter receptor expression in peripheral leukocytes. However, the relatively small number of patients prevented us from a more detailed analysis in this regard.

All blood leukocytes were demonstrated to express complement regulatory proteins, including CD59 as an inhibitor of TCC formation on healthy cells, and to increase their surface levels in response to PT [1]. This may be the reason for a lack of detectable TCC on neutrophils and lymphocytes. However, despite considerable amounts of CD59, TCC was detectable on monocytes and there was a significant increase in patients compared to early time points. Furthermore, early TCC surface levels correlated strongly with the number of erythrocyte concentrates transfused during the first day after admittance, suggesting increased complement activation after severe blood loss and extensive reperfusion therapy. Sublytic amounts of TCC have been demonstrated to activate the inflammasome in endothelial cells [168]. Further in vitro experiments will clarify whether this mechanism is similar in monocytes and may induce a pro-inflammatory cellular phenotype.

Thrombomodulin is mainly expressed by endothelial cells; binding of thrombin strongly reduces cleavage of fibrinogen and thereby clot formation, inhibits activation of endothelial
thrombin receptors which may decrease vascular leakage, and enhances activation of protein C with several anti-inflammatory and anti-coagulatory features [45,72,173]. Detection of thrombomodulin on monocytes and especially the strongly increased surface expression in traumatized patients indicate a modulatory function of monocytes during excessive inflammation and coagulation.

PAI-1 as the most potent inhibitor of fibrinolysis was bound to the surface of all leukocytes and levels increased significantly especially during the later course of injury, suggesting that peripheral leukocytes might be involved in altered coagulation and an increased risk of thrombosis in severely injured patients [21]. In line, higher PAI-1 surface levels on granulocytes and lymphocytes correlated with the numbers of given erythrocyte concentrates, suggesting that patients with a higher blood loss showed higher binding of PAI-1 on the leukocyte surface. In order to evaluate several possible causes for the aforementioned increase in membrane-bound PAI-1 around day 5 after trauma, concentrations in plasma from patients and healthy volunteers were measured. There was a strong increase in circulating PAI-1 likely due to an increased expression in the liver [136] during the early phase, but not in the later course after trauma, therefore presumably not accounting for the increased surface detection on leukocytes. In a clinical study, a decrease in active PAI-1 upon admission was detected in the 10% of patients after trauma with signs of severe hyperfibrinolysis [22], which did not seem to be the case in our cohort. A more recent study suggests that excessive tissue plasminogen activator release and complexation of PAI-1 may be responsible for initial hyperfibrinolysis and trauma-induced coagulopathy [25]. Of note, these studies only analyzed clotting disturbances in the very early phase and did not monitor coagulation during the later time course after injury. As a next step, isolated neutrophils were stimulated with LPS or a combination of inflammatory mediators (C3a, C5a, IL-1β, IL-6, IL-8) in trauma-relevant concentrations [65] in order to simulate the pro-inflammatory conditions in severely injured patients, resulting in a fast increase in PAI-1 detectable on the cell surface. However, after stimulating cells in a more complex setting using whole blood, this result could only be confirmed after stimulation with a potent activator of the protein kinase C [23], but not with LPS or trauma-relevant mediators. Similarly, alterations in microvesicles shed from the neutrophil surface were only detectable after protein kinase C activation. As an additional approach, gene expression levels of PAI-1 and its cellular anchor uPAR [6] were analyzed by qPCR. However, PAI-1 expression levels were not increased after short-term exposure to inflammatory stimuli in contrast to previous studies [145]. UPAR gene expression was by tendency higher after a short
incubation with inflammatory cytokines or LPS, suggesting that a modest increase in uPAR surface expression may be partly causative for elevated detection of PAI-1 on the cell surface. Considering the observed changes especially in PAI-1, it is tempting to speculate that blood leukocytes are involved in regulation of coagulation and may partially contribute to thrombotic events, including venous thromboembolism, in patients after trauma [21] since several leukocytes have been demonstrated to switch to pro-coagulatory phenotypes in inflammatory (micro)environments [103,145,182]. Furthermore, several neutrophil components, such as their cellular surface, shed microvesicles, granule proteins, or NETs, have been established to be significantly involved in the modulation of coagulatory processes [33,71,150] and can be presumed to play a role in coagulopathy after trauma. Therefore, future experiments need to characterize in detail which factors in patient blood might be responsible for changes in the expression of coagulation factors on peripheral leukocytes. In this regard, a promising approach would also be the stimulation with endogenous DAMPs such as HMGB-1, histones, or mitochondrial DNA which are able to substantially activate pro-inflammatory processes in leukocytes.

4.2 Coagulopathy in polytraumatized patients
Disturbances in processes of coagulation represent a central risk factor for mortality in trauma patients [16,97] and optimized transfusion strategies seem to be essential in order to improve outcome [32,42,67]. To assess whether there was apparent coagulopathy in our PT cohort, thromboelastometric analyses were performed in stored plasma samples. Patients revealed impaired clotting as evident in delayed clot formation and a reduced clot stability especially during the early time course after trauma. As summarized recently [15,176], abnormal clot amplitude or MCF are indicators of coagulopathy and can be valuable tools in the prediction of needed blood transfusion and mortality. The data in the present study suggest substantial disturbances predominantly in initial activation of coagulation, although, surprisingly, no hyperfibrinolysis as typically present after trauma [79,153] was detected in this cohort. This may be due to a difficulty in diagnosing moderate fibrinolysis employing thromboelastometric approaches by which the percentage of patients with fibrinolytic events may be underestimated [139]. Since a differential analysis of the contribution of the cellular blood components, including, but not limited to thrombocytes, was not possible due to analysis of plasma samples, future coagulation analyses in whole blood from patients during the time course after injury and corresponding correlations with flow cytometric data could also shed more light on the earlier discussed role of leukocyte surface molecules in the regulation of coagulatory and fibrinolytic processes.
4.3 Functional immune monitoring in trauma

In a translational pilot study using a highly standardized ex vivo whole blood model, blood from healthy volunteers and from polytraumatized patients was stimulated with LPS in order to develop a tool for the diagnosis of remaining functional capacity of a patient’s immune system. This experiment was performed with two aims: defining the changes in the immediate immune reaction to a pathogenic stimulus after severe injury, and shortening the incubation time in order to render this application of functional immune monitoring more suitable for clinical use. Several studies have been performed focusing on the effects of trauma, shock, or sepsis on peripheral blood cells and the alterations in their response to bacterial stimuli [17,39,44,61,62,64,87,102,142,156,159]. However, those studies analyzed isolated cells, not including effects of soluble plasma mediators or intercellular actions, or used protocols with low standardization potential which are not suitable for testing in clinical routine.

In the present study, when whole blood was stimulated with LPS, the secretion of predominantly monocyte- and macrophage-, but also lymphocyte-derived cytokines was significantly reduced during the time course after trauma in line with the literature [11,68,81,129,156,158], although this effect was less distinct 24 h after injury. In contrast, T cell function as reflected by the secretion of mediators mainly involved in T cell activation after endotoxin incubation was not compromised, indicating that the observed alterations in PAMP/DAMP surface receptors did not influence the lymphocytic capability to react to a defined PAMP stimulus. As a major limitation, the small sample size, also due to the high cost/sample ratio, and large interindividual variation [117] prevented a broad and definitive assessment of immune functionality in this approach. Validation in a larger patient cohort might also facilitate the detection of more subtle changes in the immune response as e.g. mediated by MDSCs [31,119]. Furthermore, for some of the assessed cytokines (e.g. IL-9 and IL-12), there were no alterations in the secreted amounts upon stimulus since concentrations in unstimulated samples were already higher. Nevertheless, this approach provided a rather comprehensive analysis of the complex immune response in peripheral blood. In addition, highly significant differences in stimulated and unstimulated samples despite the markedly shortened incubation time demonstrated that this methodology could provide a highly useful diagnostic tool for the immune surveillance of trauma patients and may aid to optimize timing of surgical procedures during the posttraumatic immune response [55,185]. Moreover, shortening stimulation time even further and analyzing only a few
selected parameters relevant for the current functional immune status in clinical laboratories might enable a valid and reliable immune monitoring and maximize clinical usefulness.

4.4 HS as a major driver of barrier and organ dysfunction after PT

In the past years, there has been accumulating evidence that severe blood loss may be one of the central causes for posttraumatic barrier damage and organ dysfunction and failure [49,78,154,188]. In order to assess whether the presence or absence of a HS was predictive for organ damage during the early time course after injury, a larger PT patient cohort from a clinical study at the University Hospital Zurich was stratified into those patients with and those without HS according to established clinical parameters [88,108,190]. Despite equal severity of injury in the two cohorts as reflected by the ISS and similar values in most assessed inflammatory mediators except for IL-6, HS induced a spatially and temporally specific injury of the lung, kidney, the intestine, and the liver as reflected by distinct serum organ damage markers [7,143,184].

The present data did not confirm that CC16 correlates with the contused lung tissue volume after PT [184] since there was no association with the AIS of the thorax; however, the correlation of serum CC16 concentrations with transfused volume and positive fluid balance suggest an increase in the permeability of the air-blood barrier [66], possibly leading to fluid accumulation in the lung. A similar result was recently published in our murine trauma model where only a combination of PT and HS induced a significant increase in plasma CC16 [36].

The kidneys represent central organs in the pathophysiology after PT and HS as evidenced by a report from a cohort of more than 2000 injured patients [181]; of those patients with early acute kidney injury (AKI), 78% developed multiple organ failure and 27% died; both rates are higher than those in patients with early lung, heart, or liver injury. NGAL has been described to be produced by a number of tissues and cells, including the kidneys, neutrophils, lungs, and liver [30,82,107,110]. HS is a strong inducer of renal hypoperfusion and hypoxia [189] and release of NGAL from renal tubular cells was shown in response to ischemia-reperfusion injury [116], concurrent with an early increase in serum NGAL in the HS patient cohort in this study. This is indicative of an early remote kidney injury induced by prerenal shock pathophysiology. Interestingly, NGAL values were associated with the initial severity of abdominal injury although the kidneys are rarely affected by the trauma hit, suggesting that HS significantly increases the risk of trauma-caused AKI and multiple organ failure. This was also shown in our recent study in murine PT and HS [36]. Furthermore, correlation
of serum NGAL with procalcitonin may be due to infectious complications since septic AKI is often induced by high amounts of trauma-induced release of DAMPs and PAMPs. It is noteworthy that NGAL was established as a marker for kidney damage with promising results [7,109], but its specificity has ever since been discussed controversially [59,104]. Nevertheless, its validity in diagnosing AKI especially in combination with other markers or in the urine remains high [2].

Abdominal injury was assessed using L-FABP, expressed in liver, intestine, kidney and pancreas [38,93,96] as a reliable marker of abdominal and especially liver damage [20,111,143], and I-FABP which is specifically expressed in the small intestine [132]. L-FABP was higher in HS patients and correlated with coagulation parameters as well as procalcitonin, likely due to the high blood loss in patients with abdominal injury, also reflected by the association of HS with higher AIS of the abdomen, and a well-established connection between abdominal trauma and procalcitonin [101,152]. A disruption of the intestinal barrier was indicated by an immediate increase in I-FABP serum concentrations in HS patients, and levels correlated with lactate and the fluid balance, again suggesting a connection with the shock-induced loss in intraluminal blood volume. A recent study in burn patients with multiple organ dysfunction demonstrated a highly similar serum I-FABP profile, most likely due to injury-induced intestinal ischemia and barrier breakdown [128]. It can therefore be presumed that HS is detrimental in the course of posttraumatic abdominal ischemia and organ damage.

A breakdown in endothelial barrier function is likely to play a significant role in the development of posttraumatic organ pathology. Of note, the different organ damage markers showed very distinct time patterns, possible due to differential alterations in the respective blood-organ barriers. Barrier permeability can be regulated by a large number of molecules. The lipid mediator sphingosine-1-phosphate, besides its involvement in activation of the immune system, seems to play a significant role in this regulation especially when stabilized by binding to plasma albumin [124,191]. Therapeutically, a functional sphingosine-1-phosphate analogue has already been applied in the context of trauma/HS [9]. Lower serum albumin upon admission has recently been linked to endotheliopathy in severely injured patients [148]. Concentrations of both sphingosine-1-phosphate and albumin clearly decreased early in patients after trauma, although HS only seemed to play a minor role here. Interestingly, HS alone in the murine trauma setting rather induced an increase in serum albumin, while a reduction was only visible after combined PT+HS. Furthermore, a decrease
in plasma thrombomodulin, detected immediately after injury in the PT+HS cohort and in both mouse groups which had undergone HS, can indicate the activation of endothelial cells [70]. These data are suggestive of an initiation of endotheliopathy with disturbances in anticoagulatory surface properties and barrier function as soon as patients arrive at the emergency room. Angiopoietin-2 as a mediator involved in pro-inflammatory processes and vascular leakage [57], but also adrenomedullin with rather opposite effects [164] were increased only in the HS patient cohort and rather at the later time points after trauma. These findings and the correlation of angiopoietin with the number of initially transfused erythrocyte concentrates point to a strong activation of pathways regulating endothelial permeability exclusively in patients after HS. This is also supported by initial increases in MMP-9 (albeit in both patient cohorts) and MMP-13, matrix proteases which have been shown to be involved in cleaving components of the apical glycocalyx layer off endothelial cells [138,191]. In line, increased levels of syndecan-1 and heparan sulfate were detected especially in the HS cohort; similar results were obtained in the murine PT setting where an isolated HS already induced a clear enhancement of MMP-9, -13, and syndecan-1 plasma concentrations compared to controls, but animals with combined PT+HS had highest corresponding concentrations. Shedding of glycocalyx components is likely to be responsible for increased vascular permeability after trauma and HS [137]. In this context, syndecan-1 and its fragment heparan sulfate have been increasingly used as markers of endothelial glycocalyx degradation especially by the group around P.I. Johansson [75,76,125,148] and may induce a so-called “autoheparinization” effect due to their anticoagulatory features [126,141]. This was assessed in an ex vivo experiment in blood samples with artificially adjusted hematocrits similar to those in healthy volunteers (45%), PT (33%), and PT+HS (25%) patients where heparan sulfate significantly delayed clotting. Furthermore, correlations of syndecan-1 with serum markers of organ dysfunction indicate a causal relationship between endothelial damage and organ failure. Mechanistically, release of (nor-)epinephrine induced by sympathoadrenal activation may be a central driver of endotheliopathy [77,125]. As a limitation of the present study, catecholamine levels were not measured in this patient cohort. However, in addition to endogenous production, PT patients especially after HS are likely to have received catecholamine support during the initial phase after trauma as part of the standard emergency care protocols. Plasma fibrinogen was lower in HS patients throughout the entire observation period; D-dimers were initially decreased in both cohorts, but an additional effect of HS was not detectable, presumably due to plasma dilution after extensive fluid replacement with crystalloids.
Increased endothelial permeability was furthermore assessed by measurement of circulating intercellular tight-junction and adherens-junction molecules. As recently published, we found an early increase in plasma JAM-1, a central component of tight junctions, in humans and mice after PT [37]. Claudin-5 as another molecule involved in regulation of tight-junction permeability, especially of the blood-brain barrier, but also in other organs and tissues [29,47,121], was increased in PT+HS patients and correlated with markers of HS, suggesting that barrier disturbances after HS not only include the apical glycocalyx, but also intercellular junctions. Unexpectedly, we also detected increased amounts of mucin-2, a central component of intestinal mucus, in the circulation, indicating a disturbed blood-gut barrier possible due to a disruption of intestinal mucus layers by pancreatic enzymes post trauma [48]. Similarly to heparan sulfate, mucin-2 in concentrations measured in our patient cohorts (up to 6000 pg/ml) prolonged coagulation in blood samples with low hematocrit, suggesting that components of the intestine released by a direct or indirect gut barrier damage may interfere with coagulation and could be partly responsible for coagulopathy in severely injured patients.

4.5 Conclusion
Severe trauma induces several alterations in leukocyte surface molecules and is associated with cell type-specific changes in the immune response to bacterial stimuli. This study demonstrated that coagulation is significantly impaired predominantly during the early phase after trauma, and leukocyte surface molecules may play a detrimental role. Furthermore, a clinically relevant testing system was established to allow functional bed-side monitoring of the patient’s remaining immune capacity which revealed a deficit especially in monocyte function. Lastly, the differential analysis of a larger patient cohort revealed HS as a major driver of posttraumatic organ damage. In addition, several molecules associated with barrier (dys-)function were significantly altered by posttraumatic HS with very distinct time patterns, underlining the importance of effective management of bleeding and reliable monitoring of barrier function in order to treat latent and evident organ damage before onset of leakage syndrome and organ failure.
5 Summary

Despite the considerable progress made in public safety measures, emergency and intensive care in the last decades, trauma remains a major medical issue and is responsible for a large number of deaths worldwide particularly in young people. In regard to the posttraumatic immune response, development of disturbances in coagulation, and detection and monitoring of end organ damage, several issues remain unresolved. Therefore, this thesis focused on the alterations in peripheral blood leukocytes as “first line of defense” after severe injury employing static and functional analyses. Furthermore, disturbances in coagulation were observed over a 10-day time course after trauma. In a larger patient cohort, the impact of an additional hemorrhagic shock on inflammation, organ damage, and breakdown of the endothelial glycocalyx was assessed. These findings were re-translationally evaluated in a murine model of isolated and combined trauma and hemorrhage.

In patients after severe trauma, peripheral leukocytes rapidly changed their surface expression profile of several receptors for complement activation products as well as damage- and pathogen-associated molecular patterns, involved in regulating the immune response, but also modulators of coagulation. In a pilot study, a highly standardized ex vivo testing system revealed defects in production of early pro-inflammatory cytokines such as tumor-necrosis factor and interleukin-1β upon incubation with microbial endotoxin in monocytes, but not in the lymphocyte function after severe injury. A shortened standardized exposure time (4 h) resulted in a similar immune response compared to the established incubation protocol. After further validation and optimization, this method might offer a useful tool to monitor the immune system’s remaining capacity to react to inflammatory or pathogenic stimuli. In a similar approach, functional monitoring of coagulation over the time course after injury detected striking defects in coagulation immediately after trauma which partly lasted until 10 days later, but no hyperfibrinolysis was found.

In a larger patient cohort, hemorrhagic shock was demonstrated to significantly contribute to posttraumatic inflammation and organ damage. Despite comparable injury severity, patients after blood loss had increased serum concentrations of specific damage markers of the lung (Clara cell secretory protein), liver (liver-type fatty acid-binding protein), kidneys (neutrophil gelatinase-associated lipocalin) and the intestine (intestinal fatty acid-binding protein) in distinct spatial and temporal patterns. Furthermore, presumably by increased generation of matrix metalloproteinases-9 and -13, there was an elevation of circulating components of the glycocalyx such as syndecan-1 and heparan sulfate, suggesting
endothelial damage and increased permeability. Interestingly, increased concentrations of elements of the intestinal mucus (mucin-2) were detected after trauma/hemorrhage, implying a deterioration in intestinal barrier function. In vitro experiments revealed that the detected amounts of glycocalyx as well as intestinal mucus components were able to interfere with coagulation, indicating that the posttraumatic barrier breakdown might also impair clotting and increase the risk of bleeding. These findings were mostly confirmed in a murine model of PT and hemorrhage, where shock alone already demonstrated marked impact on barrier dysfunction and glycocalyx breakdown. The data is indicative of an early development of barrier and organ damage, presumably hours to days before manifestation of barrier breakdown and organ dysfunction. Monitoring the posttraumatic course using novel organ damage markers in addition to traditional functional scores may aid to detect subpar tendencies before onset of (multiple) organ dysfunction.

In conclusion, severe trauma leads to significant alterations in leukocyte and immune function, coagulation, and considerable barrier and organ dysfunction which is worsened in the presence of a hemorrhagic shock. Reliable monitoring of the clinical course using valid functional markers and assays as well as rapid implementation into clinical routine and advanced treatment strategies may be decisive for improved outcome.
6 List of references


List of references


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Declaration

I hereby declare that I wrote the present dissertation with the topic

**Early changes in immune, coagulation, and organ function after clinical and experimental polytrauma**

independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“ [Rules of the University of Ulm for Assuring Good Scientific Practice].

Ulm, 28.03.2018
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Oral presentations

Komplement-induzierte Veränderung der Neutrophilenmorphologie während der Entzündung
German Congress of Orthopaedics and Trauma Surgery (DKOU), Berlin, Germany

05/2017  Wiegner R, Denk S, Gebhard F, Huber-Lang M
Early Barrier Dysfunction in Polytrauma
Retreat of the Collaborative Research Centre 1149, Oberstdorf, Germany

Hämorrhagischer Schock im Polytrauma
Gathering of the Trauma Research Network (Netzwerk Traumaforschung, NTF), Frankfurt, Germany

Leukozyten als Interaktionsplattform zwischen Komplement- und Gerinnungssystem bei polytraumatisierten Patienten
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06/2016  Wiegner R, Denk S, Perl M, Huber-Lang M
Immune monitoring in trauma
IgradU Student Retreat 2016, Kleinwalsertal, Austria

Role of stem cells after polytrauma
Student Symposium on Molecular Medicine, Ulm, Germany

02/2016  Wiegner R, Lampl L, Weiss M, Brenner R, Huber-Lang M
Rolle von VSELs im Polytrauma
Gathering of the Trauma Research Network (Netzwerk Traumaforschung, NTF), Frankfurt, Germany
Leukocytes mediate the crosstalk between the complement and coagulation system in polytrauma patients
XVI. Congress of the European Shock Society, Cologne, Germany

Coagulation-complement crosstalk on leukocytes in polytrauma patients
Gathering of the Trauma Research Network (Netzwerk Traumaforschung, NTF), Frankfurt, Germany

03/2014  Wiegner R, Kovtun A, Ignatius A
Role of neutrophils in fracture healing
German Congress of Surgery (DGCH), Berlin, Germany

Poster presentations

Hemorrhagic shock drives organ dysfunction in polytrauma patients
XVI. Congress of the European Shock Society, Paris, France

Cellular crosstalk between the complement and coagulation system in polytrauma patients
15th European Meeting on Complement in Human Disease, Uppsala, Sweden
Awarde with a Travel Award

05/2015  Wiegner R, Denk S, Hönes F, Kalbitz M, Gebhard F, Huber-Lang M
Immunophenotyping of severely injured patients
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06/2014  Wiegner R, Perl M, Denk S, Braumüller S, Huber-Lang M
Important role of Hsp70 in complement component C5a-induced delayed apoptosis of neutrophil granulocytes
37th Annual Conference on Shock, Charlotte, NC, USA
Original articles


**Review articles**


