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Inflammatory Response of Cardiomyocytes after Experimental Polytrauma and Haemorrhagic Shock

DISSERTATION

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For my parents.

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Abbreviations

Abbreviations of technical terms

2ME	2-mercaptoethanol
2pOH	isopropyl-alcohol/ 2-propanol
AD	aqua destillata/ distilled water
ANOVA	one-way analysis of variances
AP	alternative pathway (of the complement system)
APS	ammoniumperoxisulphate
BCA	Pierce bicinchoninic acid
BSA	bovine serum albumin
C3aR	C3a receptor
C5aR1	C5a receptor 1
C5aR2	C5a receptor 2
CC3	cleaved caspase 3
CD59	cluster of differentiation 59
CGJ	cardiac gap junction
CLP	cecal ligation and puncture
CM	cardiomyocyte(s)
CO_2	carbon dioxide
CP	classical pathway (of the complement system)
cTnI	cardiac troponin I
CTRL	control group (as an experimental group)
Cx43	connexin 43
DAMP(s)	damage-associated molecular pattern(s)
DAPI	4’,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DSR	densitometric sum red
EDTA	ethylenediamine tetraacetic-acid
ELISA	enzyme-linked immunosorbent assay
EP	electrophoresis

ETOH	ethyl-alcohol
G	gravitational force
H_2O	water (chemical formula)
H_2SO_4	sulfuric acid (chemical formula)
HCl	hydrochloric acid
HE	haematoxylin and eosin
HMGB1	high mobility group box nuclear protein 1
HR	heart rate
HRP	horseradish peroxidase
HS	haemorrhagic shock
HtCM	human transdifferentiated cardiomyocytes
IF	immunofluorescence
IgG	immunoglobulin, typ G
IHC	immunohistochemistry
IL	interleukin (e.g. 1β, 6, 8, 10, etc.)
ISS	injury severity score
KCl	potassium-chloride
KH_2PO_4	potassium dihydrogenphosphate (chemical formula)
LP	lectin pathway (of the complement system)
LPS	lipopolysaccharide
MAC	membrane attack complex
MAP	mean arterial pressure
MASP	MBL-associated serine proteases
MAX	maximum value
MBL	mannose-binding lectin
MI/R	myocardial ischemia and reperfusion
MIN	minimum value
mmHg	millimetres of mercury
MOF	multiple organ failure
mRNA	messenger ribonucleic acid
Na_2HPO_4	sodium hydrogen phosphate (chemical formula)
NaCl	sodium-chloride

NETs	neutrophil extracellular traps
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	nitric oxide
PAMP(s)	pathogen associated molecular pattern(s)
PBS	phosphate-buffered saline
pH	negative of the decadic logarithm of the molar concentration of hydrogen ions; measure of acidity
PI	proteinase inhibitor
PMN	polymorphonuclear neutrophils
PMSF	phenyl-methane-sulphonyl
Prod.-No.	product/ catalogue number
PT	polytrauma
PTHS	polytrauma and haemorrhagic shock (experimental group)
QRT	quartile(s)
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RT	room temperature
SDS	sodium-dodecyl-sulphate
SE	standard error (of the mean)
SIRS	systemic inflammatory response syndrome
SOV	sodium-orthovanadate
TBS	tris-buffered saline
TBST	tris-buffered saline plus tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNFα	tumor necrosis factor α
WB	western blot

SI-Units and derived units of measurement

°C	degree(s) of Celsius
A	ampere(s)
g	gram(s)
h	hour(s)
L	litre(s)
M	molar (concentration)
min	minute(s)
s	second(s)
V	volt(s)
W	watt(s)

1 Introduction

The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.

Avicenna

1.1 The significance of traumatic injury and its impact on the global health burden

Early recorded descriptions of traumatic injuries on the battlefield and their archaic medical and surgical treatment date back to Homer's Iliad [36]. In the centuries to come, trauma management was tended to by many respected physicians from the ancient Greeks Hippocrates and Galen, the Arabic Ibn Sina (Avicenna) to the military surgeons of the renaissance and the enlightenment such as John of Arderne, Ambroise Paré and John Hunter. These surgeons were always in a race with scientific innovations, increasing mobility and modern warfare [48, 51, 118, 122]. Today, traumatic injury from traffic accidents is still the leading cause of death world-wide in the adolescent population [104]. Although a recent report from the United States' Centre for Disease Control stated a decline in motor vehicle related deaths, deadly outcomes from falls in the elder population increased over the past decade [71]. These developments may very well be expected to gain in significance as the population grows older. And with the death toll rising from new threats as terrorist attacks [2] and civil gun violence [100], one can be tempted to predict an even gloomier future.

1.2 The definition and clinical course of severe trauma and haemorrhagic shock

Among patients admitted to the hospital with severe trauma, the majority presents with injuries to more than one anatomical body region [115]. In the current literature exists a variety of terms for this kind of trauma, inter alia "polytrauma" and "multiple injuries". In

German-speaking countries, polytrauma (PT) was originally defined as multiple injuries, where at least one of them or the combination of all injuries is life-threatening [22, 41]. In modern literature and for research purposes, severe trauma is commonly classified by applying the injury severity score (ISS) [21]. This score was developed in 1974 [10] and various modified versions have been proposed since [28, 114]. However, its validity in predicting the outcome of severely injured patients remains uncertain [115, 129]. For calculation of the original ISS, six anatomical areas of the body are each scored from 1 to 5 (with 1 meaning only minor injury and 5 meaning severe life-threatening damage). Subsequently, the squared scores of the three most severely injured areas are summed up to provide total score values between 0 and 75 [10]. Most clinical studies define severe trauma as an ISS $\geq$ 16.

The mortality of severely injured patients ranges from 10% in the younger population to up to 50% in older patients [4, 52]. Massive bleeding and consecutive haemorrhagic shock (HS) significantly contribute to these fatal cases [11, 121] mostly by deranging the coagulation system, by driving impairment of the blood-organ-barriers and enhancing organ dysfunction after trauma [15, 57, 92]. Further, due to a weakened immune system [154], patients may be prone to local infection and sepsis. Sepsis occurs in about one in ten patients, with an even greater incidence in patients with higher ISS scores [155]. Both trauma and sepsis may lead to multiple organ failure (MOF) [128, 150] with a reported lethality of just over forty percent within the first thirty days of hospitalisation [46]. For the purpose of investigating the pathophysiological processes of trauma and consecutively developing new strategies in treatment, it is neither feasible nor useful to strictly separate these clinical entities as they notably overlap in their clinical and pathophysiological qualities. Although first clinical evidence for novel treatment strategies for trauma patients emerges [66, 94, 140], the strive for better understanding of the pathophysiological mechanisms of the systemic inflammation that lead to the development of MOF and the resulting therapeutic consequences continues.

1.3 The systemic inflammatory response following severe trauma and haemorrhagic shock

Hallmark of the development of organ dysfunction after severe trauma is a sterile systemic inflammatory response syndrome (SIRS) [94, 150], mainly triggered by two mechanisms: First, by release of damage-associated molecular patterns (DAMPs) from injured cells and their mitochondria and subsequent stimulation of the cellular immune system [5, 67, 133, 147, 167]. Second, by activation of the fluid phase of the innate immune system, mainly represented by the complement system [20, 78], part of whose activated components act as chemotactic agents [7, 78, 111].

Among the diverse and numerous molecules recognized as DAMPs, the high mobility group box nuclear protein 1 (HMGB1) is of special importance. HMGB1 is a nuclear protein binding to deoxyribonucleic acid (DNA), that upon release acts mainly by an interaction with the toll-like receptor 4 (TLR4) [90, 162] which at least in part is regulated by activated components of the complement system [126]. Serum levels of HMGB1 rise as early as thirty minutes after severe trauma [27] and have been shown to be even further enhanced after additional HS [62]. After binding to TLRs on the surface of immunocompetent cells, HMGB1 triggers the secretion of interleukin 8 (IL-8) and tumor necrosis factor α (TNFα) in vitro [162]. Moreover, in a rodent model of HS, blocking of HMGB1 with a specific antibody ameliorated shock-induced gut-barrier-dysfunction by means of a lower translocation of bacteria and preserved tissue levels of tight junction proteins [159].

Other important mediators of sterile inflammation after trauma are histones. Like HMGB1, histones are multimer nuclear proteins comprised of six types (termed H1, H2A and B, H3, H4, H5) which, due to their chemical structure form complexes with DNA (termed nucleosomes) to provide structural stability of chromosomes [26]. To date, two main mechanisms have been described for the release of histones in trauma: first, the secretion of neutrophils in the course of formation of so called “neutrophil extracellular traps” (NETs) [19] and second the release from apoptotic and necrotic cells [6, 64]. Histones have been detected in the blood of both septic and severely injured patients. Plasma levels of histones correlated with an impairment of the coagulation system and were associated with the development of organ dysfunction and a higher mortality rate [1, 40, 86]. Similar to

HMGB1, histones act via interaction with TLR4 [40], but have also been reported to bind to TLR9 [64] and to activate the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome resulting in the secretion of interleukin 1β (IL-1β) [5].

The complement system acts as an important humoral cascade of the innate immune system, especially as a first line of defence against hostile microbes [111]. Over the past years, it has been assigned to numerous protective and detrimental effects upon activation in critically ill patients [124]. The complement system can be activated by three different pathways (Fig. 1): the classical pathway (CP) is activated by binding of immunoglobulin-antigen complexes to C1 launching a protease cascade that ultimately results in the formation of the C3-convertase C4b2a [39, 107, 127]. The same C3-convertase C4b2a can be activated by the lectin pathway (LP). Recognition of mannose residues on the surface of microbes by the mannose-binding lectin (MBL) and consecutive formation of a complex of MBL and two MBL-associated serine proteases (MASP 1 and 2) enhances the cleavage of C2 and C4, yielding the components of the C3-convertase [39]. Finally, there is the alternative pathway (AP), allegedly the oldest pathway in the course of evolution [110, 134]. The AP is, unlike the CP and LP, constitutively activated by a process called “tick-over” [146], by which a water-bound C3 is formed that binds factor B [39]. This complex, termed $C3(H_20)B$ is then cleaved by another enzyme, factor D, resulting in the AP’s C3-convertase $C3(H_20)Bb$ (Bb for factor B-fragment “b”). The resulting complement fragment C3b also binds factor B, therefore amplifying the generation of downstream complement products, a process further enhanced by the protein properdin [146]. The AP has recently been proposed to be the driving force of complement activation in the early phase after trauma, with its activity correlating with clinical outcome [47]. Activation of either pathway results in the common terminal path of C3a activation and formation of the C5-convertase, which cleaves C5 in its cleavage products C5a and C5b. While C5a, together with C3a acts as the central mediator of downstream inflammatory and interactive pathways, C5b then starts off the formation of the membrane attack complex (MAC) [127]. The MAC functions as an important downstream instrument of defence against microbes. Beside the three canonical pathways of complement activation, various additional modes of activation have been proposed, namely by cleavage through enzymes belonging to the coagulation cascade [7] or by c-reactive protein and serum amyloid P [50].

Although formation of the MAC is the endpoint of the complement cascade and increased plasma levels of soluble MAC have been reported directly after critical injury [20], the role of the MAC in trauma is not well characterised. Notably more attention has been paid to the diverse roles of the complement anaphylatoxins C3a and C5a. C5a mediates its cellular effects by interaction with two surface receptors, C5a-receptor 1 (C5aR1) and C5a-receptor 2 (C5aR2). For C3a only one receptor (C3aR) has been described so far [39, 107, 157]. Deleterious roles of C3a and C5a in organ dysfunction following systemic inflammation have been described for a variety of different organs including lungs [16], blood [151], kidneys [169] and the heart [108].

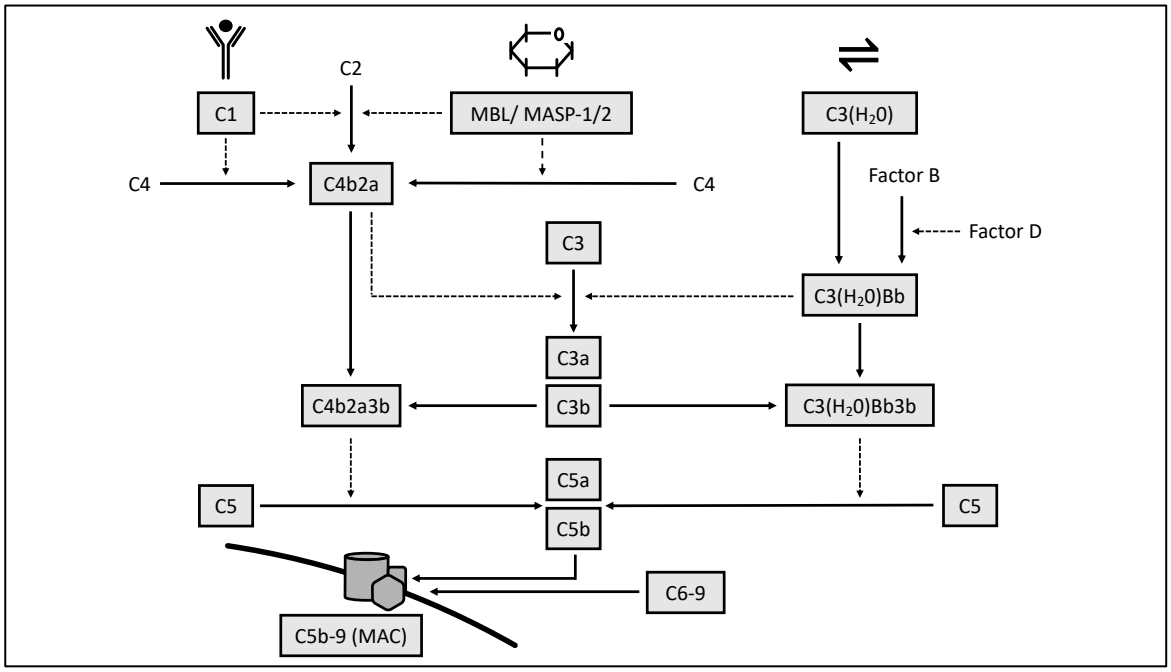

Figure 1 – The complement system

Schematic and simplified depiction of the three pathways of activation of the complement system. C1-9: complement components 1-9. C3a/ b: cleavage product a/ b of complement component 3. C5a/ b: cleavage product a/ b of complement component 5. MBL: mannose-binding lectin. MASP-1/2: MBL-associated serine proteases 1 and 2. C3(H_2O): water-bound C3. C4b2a: C3 convertase of the classical and the lectin pathway. C4b2a3b: C5 convertase of the classical and the lectin pathway. C3(H_2O)Bb: C3 convertase of the alternative pathway. C3(H_2O)Bb3b: C5 convertase of the alternative pathway. MAC: membrane attack complex. Solid arrows implicate formation of protein derivates or cleavage products. Dashed arrows implicate protease activity.

1.4 Mechanisms of cardiac injury in the setting of acute systemic inflammation

In sepsis, various mechanisms have been proposed to impair the cardiac function [25, 61, 69]. Whether these effects occur in a comparable way during the inflammatory phase after

trauma is not well known (see Fig. 2 for an overview of the proposed mechanisms). Pathologically detectable myocardial lesions have been observed in fatally injured trauma patients [49, 77]. In a recent study, the specific serum marker for cardiac damage, troponin, was found to be elevated in about forty percent of a trauma patient cohort and serum levels were significantly increased in patients with demand of catecholamine therapy and fatal outcome [77]. Furthermore, troponin I can predict arrhythmia and left ventricular dysfunction after blunt chest injury [123].

Whereas in sepsis, the effects of inflammatory mediators, activated complement components and interactions of pathogen-associated molecular patterns (PAMPs) and DAMPs with their respective receptors are held responsible for cardiomyocyte dysfunction [42, 61, 74, 93, 108], in trauma, the situation is far from clear. Case studies often demonstrate gross structural damage like valve impairment, laceration or muscular rupture as a cause of posttraumatic heart failure [98, 136, 145]. On the other hand, recent clinical [65, 137] and experimental studies on multiple trauma [72, 73] show cardiac injury in the absence of such morphological mutilations. To which extent this impairment is attributable to cardiac contusion or to the consecutive inflammatory response to injury remains to be investigated. The premise of a significant depression of cardiac function by sterile inflammation is further underpinned by recent findings, where cardiomyocyte shortening and relaxation was impaired in-vitro after in-vivo exposure to burn-injury [60, 96]. These impairments occurred in the absence of lipopolysaccharide (LPS) and could be aggravated by preceding incubation with LPS [60], suggesting that sepsis or endotoxemia are contributing to but are not necessary for cardiac depression in the setting of acute systemic inflammation.

The interactions of cardiomyocytes (CM) and the immune system in a state of systemic inflammation like sepsis and trauma are manifold. Pro-inflammatory cytokines, namely IL-1β, interleukin 6 (IL-6) and TNFα diminish velocity and extent of cardiomyocyte contraction in-vitro [84, 85, 96, 117]. Reversely, CM also secret TNFα via activation of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathway, IL-1β and IL-6, and may therefore locally enhance the pro-inflammatory milieu [95, 109]. The detrimental effects of these important cytokines are at least in part triggered by action of reactive oxygen species (ROS) like nitric oxide (NO) and downstream activation of the guanylate

cyclase [83, 85]. In line with these early findings, recent studies have shown a local increase of ROS in the cardiac muscle in models of trauma and septic shock [112, 144]. Additionally, other interleukins such as interleukin-17A (IL-17A) have been proposed to act harmfully on CM [8].

In the last decade, the crucial function of the complement system in various heart diseases (including sepsis) has been unveiled [43, 89]. Particularly, the activated complement component C5a is suggested to enact a pivotal role in septic patients. C5aR1 is expressed on the surface of rat CM and binding C5a decreased the cardiac function by means of sarcomere shortening for more than forty-eight hours in vivo [108]. Consequently, blocking of C5a in an animal model of scalded burn injury ameliorated these effects [60]. But the significance of the complement system seems to go far beyond simple ligand-receptor interactions. A striking linkage between C5aR1 and C5aR2 signalling, plasma levels of histone and cardiac dysfunction has recently been stated [76]. Additionally, binding of LPS to TLR4 resulted in increased expression of complement factor B, a crucial player in the activation of the alternative pathway in the heart and in increased deposition of C3 [169]. These results suggest a strong interaction between the complement system and various PAMPs and DAMPs. However, while most of the literature addressing these interactions investigated sepsis and burn models, only few focused on the connection of the complement system and the heart after severe trauma. Recent works on PT in pigs and rats have hinted to a relevancy of the complement system after traumatic injury by demonstrating alterations in surface expression levels of complement receptors on CM [72, 73]. Yet, the functional and structural consequences of systemic and local complement activation on cardiac cells still needs to be determined.

Besides pro-inflammatory cytokines and the complement system, DAMPs have recently emerged to greater relevance in the pathophysiology of inflammatory cardiomyopathy as current studies demonstrated direct links between those molecules and CM impairment. Among them, HMGB1 and histones, both of nuclear origin have been shown to aggravate cardiac injury after sepsis and haemorrhagic shock [76, 168]. HMGB1 contributes to CM apoptosis and diminishes CM contractility [34, 158, 165]. Histone plasma levels correlate with troponin T levels and cardiac arrhythmias in septic patients [3]. Further, in experimental sepsis, the administration of deactivating antibodies against histones

significantly reduced cardiac dysfunction, leading to the assumption, that histones may play an important role in the development of septic cardiomyopathy [3]. Whether these interrelations hold true for traumatic injury is not known, but elevated histone plasma levels have been described in patients after traumatic injury [86] as well as after experimental trauma [72, 73]. It is tempting to speculate that, after trauma, histones act out their detrimental role in a similar manner as in sepsis.

Apart from intracellular signalling upon ligand-receptor activation, morphological alterations of the CM seem to contribute to cardiac impairment after sepsis and trauma. Of particular importance in this context are spatial and functional changes of the cardiac gap junction (CGJ) and its related structural proteins. The CGJ consists of hexamer connexin proteins, of which connexin 43 (Cx43) and connexin 45 are the most predominant in the ventricular myocardium [30]. Connexins play a vital role in the electric coupling of adjacent CM allowing synchronized beating of the cardiac muscle [102]. During states of hypoxia, internalisation of Cx43 occurs [29] and expression levels decrease with a subsequent reduction in conductive properties of the gap junction [163]. Such disruptions of the CGJ with internalisation of Connexin 43 have been demonstrated for sepsis [143] and recently for severe trauma [72, 73]. As newly occurring cardiac arrhythmias can be observed in clinical patients and in the experimental setting [3], such pathologies of the electric conductance system of the heart suggest a promising link between morphological changes and functional impairment.

1.5 Objectives of the thesis

The aim of this thesis was to define the impact of severe trauma and the consecutive systemic inflammatory reaction of the organism on the integrity of cardiac cells. In the first part, a well characterised rodent model of PT and HS was employed to investigate the early influence of various traumatic injuries and consecutive pressure-controlled haemorrhagic shock on morphological and molecular properties of CM (cf. Fig. 2). In the second part, the effects of various pro-inflammatory stimulants on the release of both CM specific and non-specific damage markers were studied in-vitro on a human cell line of transdifferentiated heart-muscle cells.

The addressed hypotheses were:

1) Severe trauma and HS cause cellular damage of mouse CM in-vivo early after injury. This cellular injury is perceivable either by histomorphological evaluation and/ or by detection of specific and non-specific surrogate markers of cardiomyocyte damage.
2) Severe trauma and HS drive the activation of the complement system, leading to an increase in plasma concentrations of the activated complement components C5a and C3a.
3) Severe trauma and HS cause secondary quantitative and/ or spatial alterations of the surface expression of complement receptors C5aR1 and C3aR and the surface complement regulator CD59 (cluster of differentiation 59).
4) Local or systemic activation of the complement system upon PT and HS propagate formation of the MAC in cardiac tissue as part of the pathophysiological response of the organism to trauma.
5) PT and HS trigger quantitative and/ or spatial changes of the proteins of the cardiac gap junction, namely of Cx43.
6) Exposure of transdifferentiated human cardiomyocytes to anaphylatoxins and histones in-vitro triggers passive release or active secretion of both the specific marker of cardiac cell damage troponin I and the unspecific cell damage marker HMGB1.

By addressing these hypotheses, this study aims to contribute to a better understanding of the cellular reaction and impairment in the early phase after trauma and haemorrhagic shock. The transfer of insights from in-vivo studies to a controlled, human cell based in-vitro model by isolating single agents that are physiologically acting systemically and interdependently in-vivo aims to better characterise the impact of those agents. The results may theoretically contribute to new diagnostic and/ or therapeutic strategies in the future.

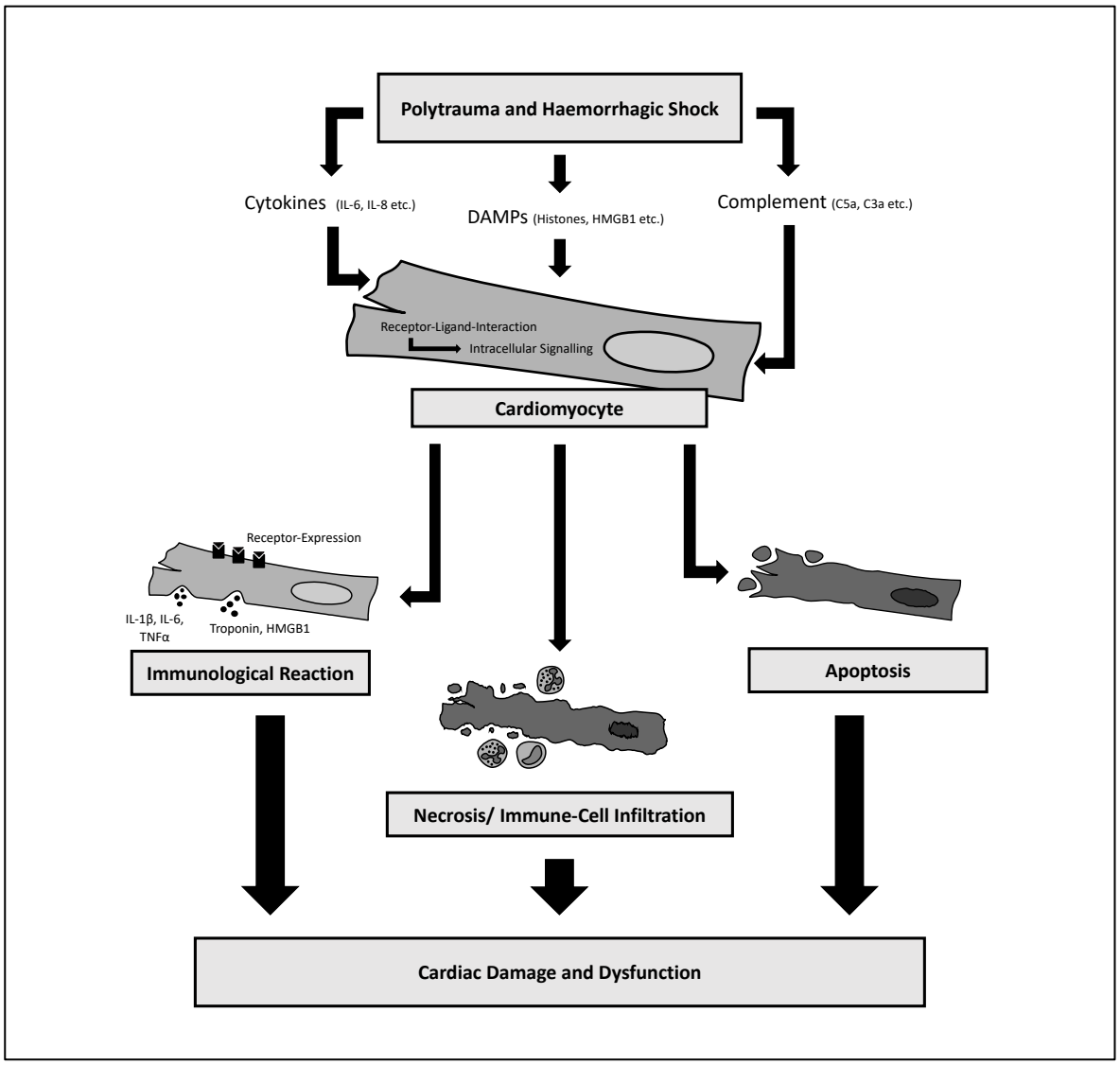

Figure 2 – Mechanisms of cardiac damage and dysfunction after trauma and haemorrhagic shock

Polytrauma and haemorrhagic shock lead to an increase in levels of systemically acting cytokines, damage-associated molecular patterns (DAMPs) and activated complement components which act on cardiomyocytes via binding to their respective receptors. These ligand-receptor interactions mediate an immunological reaction of the cardiomyocytes and the activation of pro-apoptotic signalling pathways. Additionally, direct damage and/ or infiltrating immune cells cause necrosis of cardiomyocytes which in turn enhances the local pro-inflammatory reactions in the tissue. IL-6: Interleukin 6. IL-8: Interleukin 8. HMGB1: high mobility group box nuclear protein 1. TNFα: tumor necrosis factor α. C3a: activated complement component 3. C5a: activated complement component 5.

2 Materials and Methods

2.1 Devices and reagents

2.1.1 General reference of material

The following chapter comprises an assortment of devices and reacting agents repeatedly used for the various protocols and their preparation receipts in alphabetical order. Reagents specific to single protocols, antibodies, software and ready-to-use (assay) Kits are referred to in the respective sections. Trademark and patent reference was omitted for simplicity. All trademarks, protected names and patent rights are property of their respective owners.

2.1.2 Devices

All devices were regularly maintained and standardised by the laboratory staff or responsible service representatives.

Accu-Jet	Brand, Germany
Balance CP2202 S	Sartorius, Germany
Blotting Device Transblot-Turbo	Bio-Rad, USA
Cell-Culture Microscope AE 2000	Motic, China
Centrifuge 5417R	Eppendorf, Germany
ChemiDoc XRS+ Gel Imaging System	Bio-Rad, USA
EP Chamber Mini PROTEAN Tetra Cell	Bio-Rad, USA
Flow MSC Advantage	Thermo Fisher Scientific, USA
Fridge (-20°)	Bosch, Germany
Fridge (-80°C) HERAfreeze	Thermo Fisher Scientific
Fridge (4°)	Bosch, Germany
Heat bath SW22	Julabo, Germany
Heating plate/ magnetic stirrer MR 3001 K	Heidolph, Germany
Incubator	Heraeus, Germany
Incubator HERAcell 150i	Thermo Fisher Scientific, USA
Liquid nitrogen storage Arpege 55	Air Liquide, France

Microtome Cut 60620	Slee, Germany
Microwave NN-K101WM	Panasonic, Japan
Multifuge 3SR+	Thermo Fisher Scientific, USA
Multipette stream	Eppendorf, Germany
pH-meter	Sartorius, Germany
Pipettes/ Tips	Brand, Germany; Greiner Bio-One, Germany; Biozym Scientific, Germany; Sigma-Aldrich, USA
PowerPac HC High-Current Power Supply	Bio-Rad, USA
Roller Mixer SRT1	Stuart Scientific, UK
Tecan sunrise absorbance reader	Tecan, Switzerland
Thermostat	Grant/Dunn, Germany
Tubes	Falcon/ Corning, USA; Eppendorf, Germany
Ultra Turrax T15 basic	IKA Werke, Germany
Vortex Genie-2	Scientific Industries, USA

2.1.3 Reagents

Aqua destillata/ distilled water (AD): Provided by the pharmacy of the university hospital of Ulm.

Citrate buffer (pH 6.0): First 1.47 mg of sodium citrate dehydrated dry powder (Prod.-No.: W302600-1KG-K; Sigma-Aldrich, Germany) was solved in 480 mL of AD and subsequently pH was adjusted to 6.0 by titration with 37% hydrochloric acid (HCl; Prod.-No.: 20525.290; VWR Chemicals, Germany).

Diluent solution for immunohistochemistry (IHC): 30 µL of tween 20 (Prod.-No.: P-1379; Sigma-Aldrich, USA) and 10 µL of goat serum (Prod.-No.: 005-000-121; Jackson Laboratories, USA) were diluted in tris-buffered saline (TBS) for IHC.

Dulbecco's phosphate-buffered saline (PBS): Prod.-No. 14190144; Thermo Fisher Scientific, USA.

Ethyl-alcohol (ETOH) dilutions: 99.8% desaturated ETOH was diluted in AD to provide concentrations of 70% and 90% respectively.

Ethyl-alcohol/ Ethanol (ETOH): Provided by the pharmacy of the university hospital of Ulm.

Gelatine 0.1%: 500 mg of powdered, type A gelatine (cell culture tested; Prod.-No.: G-1890; Sigma-Aldrich, USA) were dissolved in 500 mL of AD. Gelatine was sterilised by autoclaving and/ or filtered prior to usage and stored at room temperature (RT).

Isopropyl-alcohol/ 2-propanol (2pOH): Prod.-No. 20842.330; VWR Chemicals, France.

Methyl-alcohol/ methanol: Provided by the pharmacy of the university hospital of Ulm.

TBS for IHC: One pack of powdered tris-buffered saline (Prod.-No.: T-6664; Sigma-Aldrich, USA) was diluted in 1000 mL of AD.

Tween 10%: 10 mL of tween 20 (Prod.-No.: P-1379; Sigma-Aldrich, USA) was diluted in 90 mL of AD.

Xylene: Provided by the pharmacy of the university hospital of Ulm.

2.2 Assessment of cardiomyopathy in a rodent in-vivo model of polytrauma and haemorrhagic shock

2.2.1 Experimental animal model

2.2.1.1 Animals

Healthy C57BL/6 wild-type mice (aged 8-9 weeks; Jackson Laboratories, USA) were housed in a germ-free environment under strict hygiene standards with free access to food (Altromin 1314 Forti total pathogen free; Altromin International, Germany) and water. To limit the stress factor induced by relocation, the animals rested in the animal care facility for 14 days post arrival.

2.2.1.2 Protocol

Animal handling and experiments were carried out strictly adhering to the National Institutes of Health Guidelines for good practice in the use of laboratory animals. The protocol for the induction of haemorrhagic shock and polytrauma in rodents was submitted

to the University Animal Care Committee and the Federal Authorities of Baden-Württemberg (Tübingen) and approved prior to experiments (Document-Number.: 1194).

To investigate the impact of PT and HS on the heart, specimens from two experimental subgroups were analysed from the original study (published 2018: [32]): animals receiving both polytrauma and haemorrhagic shock and healthy, untreated control. All animals of the PT and HS receiving group received 0.03 mg/ kg buprenorphine (Movianto, Germany) subcutaneously for analgesia and were anaesthetised with 0.025 sevoflurane fraction (Abbott, USA) in pure oxygen prior to experimental trauma and HS and were in deep narcosis throughout the whole procedure. To simulate polytrauma, air-blast-induced blunt chest trauma, weight-induced traumatic brain injury and a weight-induced closed femoral fracture with soft tissue injury were inflicted on anaesthetised animals randomly assigned to the experimental group (PTHS). After the infliction of polytrauma, catheters were placed in the jugular vein and the femoral artery for the simulation of haemorrhage, volume resuscitation, application of catecholamines and monitoring of mean arterial blood pressure (MAP) and heart rate (HR). Also, body-temperature was measured continuously with a rectal probe. Following instrumentation, blood was drawn to reach a final MAP of 30 ± 5 mmHg (40 ± 6.6 mbar) which was maintained over 60 min. Subsequently, animals were reperfused with the fourfold of the drawn blood volume over 30 min, using a crystalloid solution (Jonosteril; Fresenius Kabi, Germany) to reach a minimum MAP of 50 mmHg (66.6 mbar). MAP was maintained over a period of 120 min by volume infusion and catecholamine therapy (norepinephrine, 0.01–0.12 µg/ kg/ min; Sanofi, Germany) and finally the animals were sacrificed (4 h after infliction of injuries). Healthy animals, which did not receive any injury or monitoring procedure served as controls (CTRL).

2.2.1.3 Organ explantation and asservation

Animals were sacrificed in deep narcosis by cardiac exsanguination. Hearts were explanted and either immediately placed in formalin for histological fixation or atria were removed cautiously and ventricular specimens were shock-frozen in liquid nitrogen and then stored at -80°C. Blood mixed with ethylenediamine tetraacetic-acid (EDTA) was centrifuged with 500 G for 5 min at RT to yield plasma for further processing. Organs from animals with lethal cardiac trauma (cardiac tamponade, aortic rupture) were excluded from assessment.

2.2.2 Enzyme-linked immunosorbent assay (ELISA) from plasma

2.2.2.1 Reagents

Assay diluent: 1 g of bovine serum albumin (BSA; Prod.-No.: A-8022; Sigma-Aldrich, USA), 0.05 g of tween 20, and 2 mL of 0.5 M EDTA (Prod.-No.: 318892, Honeywell Fluka, USA) were added to 100 mL of PBS.

Stop solution: 20 mL of 5 normal (N) solution of sulfuric acid (H_2SO_4; Prod.-No. 109912, Merck Millipore, USA) are added to 30 mL of AD to yield 50 mL of 2 N H_2SO_4.

Tetramethylbenzidine reagent (TMB): 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide (both: Prod.-No.: 2642KK, Pharmingen, USA) were mixed at equal measures directly before use.

Washing buffer: 8 g of sodium-chloride (NaCl; Prod.-No.: 71380; Sigma-Aldrich, USA), 0.2 g of potassium-chloride (KCl; Prod.-No.: 4936; Merck Millipore, USA), 1.5 g of sodium hydrogen phosphate dehydrate (Na_2HPO_4 x $2H_2O$; Prod.-No.: 6580; Merck Millipore, USA), 0.4 g of potassium dihydrogenphosphate (KH_2PO_4; Prod.-No.: 4873; Merck Millipore, USA) and 0.5 g of tween 20 (Prod.-No.: P-1379; Sigma-Aldrich, USA) were diluted in 1000 mL of AD and pH was adjusted to 7.0. Washing buffer was stored at 4°C for further use.

2.2.2.2 ELISA for analysis of plasma concentrations of C5a and cardiac troponin I

To assess the plasma concentrations of C5a and cardiac troponin I (cTnI) in mouse plasma the following ELISA kits were used: 1) Mouse Complement Component C5a DuoSet ELISA (Prod.-No.: DY2150; R&D Systems, Germany); 2) Ultra-sensitive Mouse cardiac Troponin-I ELISA (Prod.-No.: CTNI-1-US; Life Diagnostics, USA). All assays were performed strictly adhering to the manufacturer's manual.

2.2.2.3 ELISA for analysis of plasma concentrations of C3a

For analysis of mouse C3a concentrations in plasma, ELISA was performed as follows: 100 µL per well of a monoclonal rat anti-mouse anti-C3a antibody (Prod.-No.: 558250; BD Biosciences, USA) diluted 1:250 in 0.2 M phosphate buffer were incubated on 96-well plates over night at 4°C. Before proceeding, plates were washed three times with washing buffer and 200 µl of assay diluent were added per well and incubated for 1 h at RT. Then, plates were washed, as described before. 100 µL per well of plasma samples and a dilution

series of recombinant mouse complement component C3a (Prod.-No.: 8085-C3; R&D, USA) as reference standard were then pipetted and incubated over night at 4°C. The next day, plates were washed five times with washing buffer and subsequently incubated for 2 h at RT with 100 µL per well of a biotin-linked rat anti-mouse anti-C3a antibody (Prod.-No.: 558251; BD Biosciences, USA) diluted 1:500 in assay diluent for antigen detection. After a washing step as described before, 100 µL of streptavidin linked to horseradish peroxidase (HRP; Prod.-No.: 554066; BD Biosciences, USA) were added per well, incubated for 30 min at RT before continuing with another seven rounds of washing. For colour development, 100 µL of TMB were added and plates were incubated for 30 min at RT in the dark. Before measurement of the colorimetric reaction, 50 µL of stop solution were added.

2.2.2.4 Absorbance measurements and calculations of values relative to total plasma protein quantity

The colorimetric reaction from ELISA assays was measured using an absorbance reader (Tecan, Switzerland). Absorbance was measured at 450 nm wavelength. Absolute amount of protein was calculated derived from measurements of standard dilution series using Excel 2013 and Excel 2016 (Microsoft, USA). Levels of respective components of interest were normalised to the total amount of protein in the plasma to unmask clinical diluting effects of volume resuscitation.

To measure the total amount of protein of each plasma sample a Pierce bicinchoninic acid (BCA) assay (Prod.-No.: 23225; Thermo Fisher Scientific, USA) was used for protein quantification. The following components were included in the kit: BSA standard solution (2 mg/ mL) in 0.9% saline and 0.05% sodium azide; Reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide; Reagent B: 4% cupric sulfate. In brief, this assay is based on a reduction of Cu^{+} by proteins in an alkaline medium (“Biuret reaction”) and a subsequent colorimetric measurement. First, specimens were diluted in AD (1:20) and a series of decreasing concentrations of BSA for reference was prepared. Preparations of standard series and specimen were then transferred in duplicates to a 96-well plate (Nunc Microwell 96-Well Plates, untreated; Prod.No.: 243656; Thermo Fisher Scientific, USA). Reagent solution comprised of Reagent A and Reagent B (mixing ration 50:1) was added to each well and the plate was incubated at 37°C for 30 min. After incubation, colour-intensity was measured at 562 nm with an absorbance reader

(Tecan, Switzerland). Standard curves were calculated and absolute values were derived from comparison with standards using Excel 2013 (Microsoft, USA)

2.2.3 Western blotting

2.2.3.1 Reagents

Ammoniumperoxisulphate (APS) 10%: 0.5 g of powdered APS (Prod.-No.: A-3678; Sigma-Aldrich, USA) were diluted in 5 mL of AD.

Milk 5%: 5 g of skim milk powder (Prod.-No.: T145.2; Roth, Germany) were diluted in 100 mL of tris-buffered saline plus tween (TBST).

RIPA buffer solution: 0.05g sodium-deoxycholate (Prod.-No.: D-6750; Sigma-Aldrich, USA) plus 0.01 g sodium-dodecyl-sulphate (SDS; Prod.-No.: L-3771; Sigma-Aldrich, USA) were diluted in 10 mL of PBS and mixed well. Then, 0.1 mL of 1% Igepal CA 630 (Prod.-No.: J-7771; Sigma-Aldrich, USA) were added slowly and the reagent was stored at 4°C.

Radioimmunoprecipitation assay buffer (RIPA) and proteinase inhibitor (PI) solution (RIPA+PI): To yield 1% phenyl-methane-sulphonyl (PMSF) solution, 10 mg of PMSF (Prod.-No.: P-7626; Sigma-Aldrich, USA) were dissolved in 1 mL of 2pOH. 18,3 mg of sodium-orthovanadate (SOV; Prod.-No.: S-6508-10G; Sigma-Aldrich, USA) were dissolved in 1 mL of AD. For final RIPA+PI stock solution, 930 µL of RIPA solution were mixed with 10 µL 1% PMSF, 10 µL of SOV and 50 µL of PI (Prod.-No.: P-8340; Sigma-Aldrich, USA).

Running buffer 5x: 15.15 g of tris-base (Prod.-No.: T-6066; Sigma-Aldrich, USA) and 72 g of glycine (Prod.-No.: A1377; Applichem, Germany) and 5 g of SDS were diluted in 1000 mL of AD. For electrophoresis, Running Buffer was diluted 1:5 in AD.

Sample buffer solution: 0.5 mL of 2-mercaptoethanol (2ME; Prod.-No.: M-3148; Sigma-Aldrich, USA) was mixed with 9.5 mL of Laemmli sample buffer (Prod.-No.: 161-0737; Bio-Rad, USA) and stored at 4°C.

Transfer buffer: 2.93 g of glycine, 5.81 g of tris-base and 0.375 g of SDS were diluted in methanol and filled up to 1000 mL with AD.

TBS 10x for western blotting: 24.2 g of tris-base (Prod.-No.: T-6066; Sigma-Aldrich, USA), 80 g of NaCl (Prod.-No.: 71380; Sigma-Aldrich, USA) were diluted in 950 mL of AD, then

pH was adjusted to 7.6 by titrating 37% HCl and stored at 4°C. For washing procedures TBS was diluted 1:10 in AD.

TBST: 100 mL of 10x TBS and 10 mL of 10% tween were diluted in 890 mL of AD.

Tween 10%: 10 mL of tween 20 (Prod.-No.: P-1379; Sigma-Aldrich, USA) was diluted in 90 mL of AD.

2.2.3.2 Preparation of tissue homogenates

To yield tissue homogenates for western blotting, ventricular tissue samples were thawed in RIPA+PI buffer solution and crushed repeatedly with an Ultra-Turrax T15 homogenizer (IKA Werke, Germany) repeatedly. The device was cleaned from tissue residues with PBS and 70% ETOH after every specimen. After lysis, specimens were centrifuged for 15 min at 16,000 G and 4°C and supernatant was stored at -80°C for further processing.

2.2.3.3 Quantification of total protein amount in tissue homogenates

To quantify the total amount of protein per specimen a BCA assay was performed, as described above (2.2.2.4).

2.2.3.4 Preparation of gels

For electrophoresis, 10% polyacrylamide gels were used that were casted freshly before electrophoresis and blotting. For gels a TGX Stain-Free FastCast Acrylamide Kit (Prod.-No.:161-0183; Bio-Rad, USA) was used. The following components were included: Resolver Reagent A; Resolver Reagent B; Stacker Reagent A; Stacker Reagent B. Resolver solution was prepared by mixing 3 mL each of Resolver Reagent A and Resolver Reagent B and adding 3 μL of N,N,N',N'-tetramethylethylenediamine (TEMED; Prod.-No.: T-9281; Sigma-Aldrich, USA) and 30 μL of fresh APS 10%. Stacker solution was prepared by mixing 1 mL each of Stacker Reagent A and Stacker Reagent B in equal parts and adding 2 μL of TEMED and 10 μL of fresh APS 10%. Both were filled between prepared glass plates for polymerisation and incubated for 45 min at RT.

2.2.3.5 Electrophoresis

Sample volumes were calculated for the exact amount of protein (30 µg per specimen) and mixed at equal measures with sample buffer. As reference for molecular weight, both stained (Precision Plus Protein WesternC Standard; Prod.-No.: 161-0376; Bio-Rad, USA) and unstained markers (Precision Plus Protein Unstained Standard; Prod.-No.: 161-0363; Bio-Rad, USA) were used for each blot, mixed at equal measures with sample buffer. After that, mixtures were heated to 96°C for 7 min and then loaded to gel wells. Electrophoretic separation of proteins was performed with 200 V for 60 min using an electrophoresis (EP) chamber (Bio-Rad, USA) filled with Running Buffer. After EP, gels were kept in transfer buffer and activated using the ChemiDoc Imaging System (Bio-Rad, USA).

2.2.3.6 Blotting to membranes

Blotting was performed using nitrocellulose membranes (Trans-Blot Turbo Transfer Pack 0.2 µm PVDF; Prod.-No.: 170-4156; Bio-Rad, USA) pre-treated with methanol for 15 min and a blotting device (Bio-Rad, USA). Currents of 1-3 A at 25 V were applied for 5-10 min depending on number of blots and the protein of interest. Blotted membranes were kept in transfer buffer and imaged using the ChemiDoc Imaging System for later reference of total protein.

2.2.3.7 Blockade of unspecific binding sites and antibody incubation

Membranes were blocked with 5% milk for 1.5 h and afterwards directly incubated with primary antibody (anti-Cx43, Cell Signaling Technologies; anti-NOS2, Santa Cruz; see Table 3) diluted 1:1000 in 5% milk over night at 4°C on a shaker. After incubation with the primary antibody, the membranes were washed three times in TBST for 15 min each and subsequently incubated with a secondary antibody (anti-rabbit IgG, HRP-linked; Prod.-No.: 7074; Cell Signaling, USA) diluted 1:1500 in 5% milk. Streptactin-HRP diluted 1:15000 (Precision Protein StrepTactin-HRP Conjugate; Prod.-No.: 161-0381; Bio-Rad, USA) was added for the last 15 min of incubation for signal enhancement. After that, membranes were washed three times in TBST and once in TBS for 15 min each. Directly before imaging, membranes were incubated with development substrate (Clarity Western ECL Kit; Prod.-No.: 170-5060; Bio-Rad, USA) mixed adhering to the manufacturer's manual for 1 min.

2.2.3.8 Imaging and density analysis

Images of blots were taken using the ChemiDoc Imaging System. Bands were analysed for pixel density employing the Image Lab 5.2 Software (Bio-Rad, USA) and normalized to total protein amount per lane from the native images of blotted membranes. Therefore, no loading controls were needed. Results are depicted as normalized mean density of all bands for each group.

2.2.4 Histomorphology and immunohistochemical assays

2.2.4.1 Paraffin-embedding, preparation and slicing

After explantation, hearts were fixed in 3.7% formaldehyde (Prod.-No.: 27242; Fischar, Germany) for 48 h and afterwards hydrated by traversing them through baths of ETOH dilutions and xylene. Then, the tissue was embedded in paraffin (Paraplast Plus; Prod.-No.: 39502004; McCormick Scientific, USA) and casted in cubical blocks for slicing. Sections of 4 µm were sliced using a microtome, placed on microscope slides (SuperFrost Plus; Prod.-No.: 631-0108; VWR, Belgium) and dried for 1 h at 50°C and afterwards stored at 37°C over night.

2.2.4.2 Paraffin-elimination, rehydration and long-term preservation

To rid the sections from paraffin wax and rehydrate them after fixation, slides were incubated in pure xylene four times and afterwards rinsed four times in ETOH baths of descending concentrations (Table 1). After paraffin-washout and rehydration, the slides were either stained with chemical dyes for morphological assessment or processed for IHC/ immunofluorescence (IF) respectively (see below). To preserve and dehydrate specimens and clean them from dye residues, the slides were then traversed through baths of ascending ETOH concentrations for 3 min each, two baths of pure xylene for 5 min each and finally were mounted with a conserving medium (NeoMount; Prod.-No.: HX54867116; Merck Millipore, Germany) and covered with a Menzel glass-coverslip (Thermo Fisher Scientific, USA) for long term preservation (Table 1) and further assessment.

Table 1 – Steps of paraffin-elimination, rehydration, dehydration and long-term preservation

Different steps and durations of paraffin-elimination, rehydration, dehydration and long-term preservation of the histological preparation protocols. ETOH: ethyl-alcohol; min: minute(s).

Step	Compound	Concentration	Incubation
Paraffin-elimination and rehydration			
1	Xylene	pure	2 min
2	Xylene	pure	3 min
3	Xylene	pure	4 min
4	Xylene	pure	5 min
5	ETOH	100%	1 min
6	ETOH	100%	5 min
7	ETOH	90%	2 min
8	ETOH	70%	5 min
Dehydration and long-term preservation			
1	ETOH	70%	3 min
2	ETOH	90%	3 min
3	ETOH	100%	3 min
4	ETOH	100%	3 min
5	Xylene	pure	5 min
6	Xylene	pure	5 min
7	NeoMount	pure	-

2.2.4.3 Haematoxylin and eosin staining

Haematoxylin and eosin staining was performed for histomorphological evaluation using a staining kit (Prod.-No.:13139; Morphisto, Germany) comprised of Gill's haematoxylin liquid dye-solution and 1% eosin liquid dye-solution. Following the above described cleansing and rehydration steps the slides were shortly washed in AD to clean away any ETOH residues. Subsequently the slides were incubated in Gill's haematoxylin, in 0.1% HCl and 1% eosin consecutively (see Table 2). After staining procedure, the slides were washed again in 99.8% ETOH twice, incubated in 2pOH for 2 min, washed twice in pure xylene for 5 min each and mounted for preservation as described above.

2.2.4.4 Indirect enzyme-linked IHC

For Immunohistochemical preparation, sections were proceeded as follows: after paraffin-elimination, and rehydration steps (see above) sections were shortly rinsed in AD and then cooked in citrate buffer for retrieval of epitopes. Slides were placed in a citrate buffer bath and heated in a microwave at 600 W for 4 min. The amount of buffer vaporized through this first cooking step was replaced by AD and a second cooking step followed again at 600 W for 3 min. Ensuing this second cooking step the slides cooled down in the citrate buffer

bath for about 10 min and then were washed in TBS for 2 min. For further processing, tissue sections on slides were circled with a liquid film (Super PAP-Pen Liquid Blocker; Prod.-No.: N71310; Science Services, Germany) to retain treatment solutions on slides.

Table 2 – Steps for staining with haematoxylin and eosin

Steps of haematoxylin and eosin staining of tissue sections. ETOH: ethyl-alcohol; min: minute(s); s: second(s).

Step	Compound	Concentration	Incubation
1	Gill's haematoxylin III	Pure	6 min
2	Running tap water	-	10 s
3	Hydrochloric acid	0.1%	10 s
4	Tap water	-	6 min
5	Eosin	1%	30 s
6	Tap water	-	30 s

To prevent unspecific antibody binding to random structures, all sections were then blocked with 10% goat serum (Prod.-No.: 005-000-121; Jackson Laboratories, USA) in diluent for 30 min. After blocking, slides were washed in TBS for 2 min and then incubated with specific antibodies recognizing the epitopes of interest either for 1 h at RT or overnight at 4°C (see Table 3 for dilution and incubation time of various antibodies). Following washing steps in TBS for 1 min, 10% Tween in TBS for 3 min and again TBS for 1 min slides then were incubated with an alkaline-phosphatase conjugated goat anti-rabbit antibody (Prod.-No.: 111-055-144; Jackson Laboratories, USA) diluted 1:25 in diluent solution for 30 min. Afterwards slides were washed in TBS, TBST and again TBS as described above and finally incubated with a detection solution (Dako REAL Detection System, Alkaline Phosphatase/RED; Prod.-No.: K5005; Agilent Technologies, USA). Incubation length was determined individually for different primary antibodies (Table 3). Slides were washed in TBS for 1 min, TBST for 3 min and shortly rinsed in AD. Then, for counterstaining of nuclei, slides were incubated in Mayer's haematoxylin (Prod.No.: 51275-1L; c) for 1 min and then shortly rinsed twice in fresh tap water and once in AD before dehydration, mounting and long-term preservation as described above.

For each antibody, controls were prepared by using non-specific IgG (Prod.-No.: X0936; Agilent Technologies, USA) and by replacing primary, secondary antibodies and detection solution with TBS respectively (Fig. 3).

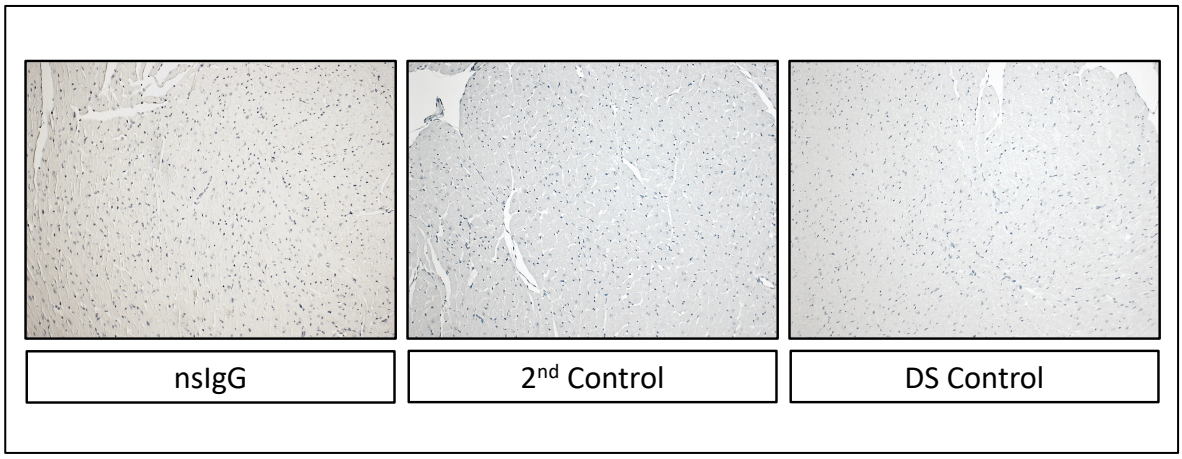

Figure 3 – Representative images of control preparations for immunohistochemistry

Representative images of immunohistochemical preparations to control for unspecific staining, by either using non-specific immunoglobulin G (nsIgG) or replacing the secondary antibodies (2nd Control) and the detection solution (DS Control) respectively with TBS. Magnifications of all images: 100x.

2.2.4.5 Indirect enzyme-linked IF

For IF preparation, slides were washed from paraffin, rehydrated, cooked in citrate buffer and washed exactly as described for IHC. Blocking was proceeded for 2 h, and afterwards slides were incubated with the primary antibody (anti-Cx43; Cell Signaling Technologies; see Table 3) for 1 h at RT and subsequently incubated with an Alexa Flour 568 conjugated goat anti-rabbit secondary antibody (Prod.-No.: A11036; Thermo Fisher Scientific, USA) for an additional hour. In between steps, slides were washed as described for IHC. After incubation with the secondary antibody, slides were mounted with ProLong Gold Antifade containing 4',6-diamidino-2-phenylindole (DAPI) for detection of nuclei (Prod.-No.: P36931; Thermo Fisher Scientific, USA) and covered with coverslips for preservation.

2.2.5 Histomorphological evaluation and assessment of a new histological damage score of the heart

2.2.5.1 Protocol for evaluation

All sections were examined for direct cardiac injury from experimental chest trauma or post-mortem trauma from preparation such as exsanguination or explantation. For every item, the whole specimen was analysed, and the right and left ventricle were each scored individually.

Table 3 – Antibodies for immunohistochemistry and western blot

A list of antibodies used in this study. C5b-9: complement-components 5b-9; CD59: cluster of differentiation 59; C3aR: receptor for activated complement component 3; C5aR: receptor for activated complement component 5; Asp: Asparagine; HMGB1: high-mobility group box 1; Prod.-No.: product number/ catalogue number of distributer/manufacturer; O/N: incubation over night at 4°C. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

Designation	Prod.-No./ Manufacturer	Dilution	Development
Anti-C5b-9 Antibody	ab55811; Abcam, USA	1:100	12 min
Anti-CD59 Antibody (N-Terminus)	LS-B10081; LS-Bio, USA	1:50	10 min
Anti-Nitrotyrosine	AB5411; Merck Millipore, USA	1:150	15 min
C3aR Polyclonal Antibody	bs-2955R; Bioss, USA	1:175	10 min
C5aR Antibody	21316-1-AP; Proteintech, USA	1:100	10 min
Cleaved Caspase 3 (Asp175) Antibody	#9661; Cell Signaling Technologies, USA	1:100 (O/N)	20 min
Connexin 43 Antibody	#3512S; Cell Signaling Technologies, USA	1:100	10 min
HMGB1 (D3E5)	#6893; Cell Signaling Technologies, USA	1:200	12 min
TLR4 Polyclonal Antibody	PA5-23124; Invitrogen, USA	1:50	15 min

2.2.5.2 Establishment of a new histological damage score for posttraumatic assessment of heart specimen

To establish a novel score for assessing cardiac muscle cell damage after PT and HS, the following items were scored after careful evaluation of the published literature on histological signs of heart tissue damage (see Table 4): 1) apoptosis, 2) contraction band necrosis, 3) neutrophilic infiltration, 4) intramuscular bleeding, 5) rupture and disintegration of CM, 6) oedema and 7) ischemia.

2.2.6 Densitometric analysis of IHC preparations and imaging

2.2.6.1 Densitometric analysis

For quantification of epitope expression in IHC preparations, signal intensity was measured using the Zeiss Axio Vision Software (Edition 4.9; Zeiss, Germany). Images of five distinct and representative fields of view were taken at a 100x magnification using a Zeiss Axio Imager.M1 microscope (10x magnification), Zeiss Axio EC Plan-NEOFLUAR objective (10x magnification) and a Zeiss AxioCam MRc camera (all Zeiss, Germany). For each experiment, a measuring-algorithm was programmed using the software to detect positively reacting cells in the tissue samples and to quantify the colorimetric density of the stained area.

Table 4 – Histological damage score

Items scored for assessment of microscopical damage and the morphological criteria that were applied. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

Item	Criteria	Score	Reference
Apoptosis	Shrinkage of cell and nucleus, karyorrhexis/ nuclear condensation, fragmentation, vacuolisation detachment from cell-formation	0, not present, single cell events 1, groups of cells, unifocal 2, groups of cells, several foci 3, wide spread apoptosis	[31, 87, 132, 133]
Contraction band necrosis	Hypercontraction, cell lysis, basophilia due to mineralisation	0, not present 1, single focus 2, multiple foci	[12, 78, 105]
Neutrophilic infiltration	Marked areas of neutrophil accumulation	0, not present 1, single focus 2, multiple foci 3, wide spread infiltration areas	[56]
Intramuscular bleeding	Marked erythrocyte accumulation between cardiomyocytes, visibly ruptured vessels	0, not present 1, single focus, occasional lesions 2, multiple lesions 3, extensive rupture/ bleeding, destruction of myocardium	[31, 72]
Rupture	Detachment of myofibers	0, not present 1, small areas of detachment 2, spread areas of detachment 3, extensive rupture bleeding, destruction of myocardium	[31]
Oedema	Interstitial	0, not present 1, single focus, occasional lesions 2, multiple foci	[56]
Ischemia	Eosinophilisation	0, not present 1, single focus, occasional lesion 2, multiple lesions	[56]

Detection was standardised to a positive sample from the same experiment and the programmed measuring-algorithm was evaluated comparing positive and negative controls prior to analysis. For statistical comparison of groups, means from all five measured fields of view were calculated for each animal. Measured density is depicted as densitometric sum red (DSR; arbitrary units).

2.2.6.2 Preparations of images for publication

Scales and annotations were added using the Zeiss Axio Vision Software (Edition 4.9; Zeiss, Germany). The respective magnifications can be drawn from figure captions. After

measurements, the representative images depicted in this thesis were modified for aesthetic purposes regarding contrast, shading and exposure with Zeiss Axio Vision and Photos 2.0 (Apple, USA).

2.3 In-vitro assessment of cardiomyocyte damage after stimulation with DAMPs and cytokines

2.3.1 Cell culture

2.3.1.1 Cells

For a transitional assessment of trauma-induced cardiomyopathy, human cardiomyocytes, transdifferentiated from induced human pluripotent stem cells (HtCM; Prod.-No.: CMC-100-010-001; Cellular Dynamics, USA) were cultured. Storage, thawing, seeding and maintenance of HtCM were performed strictly adhering to the manufacturer's instructions. HtCM were shipped on dry ice and stored in liquid nitrogen directly upon receipt. 99.9% purity of cells was guaranteed by the manufacturer.

2.3.1.2 Coating of cell-culture plates prior to seeding

Before thawing, 24-well plates were coated with 600 µL of 0.1% gelatine under sterile conditions and incubated at 37°C for 1 h. After incubation, gelatine solution was aspirated and discarded.

2.3.1.3 Thawing, seeding and culturing

Cryovials were then thawed in a 37°C heat bath for 4 min exactly and cell solution was transferred to a 50 mL centrifuge tube. To recover any remnant cells, the cryovial was then rinsed with 1 mL of plating medium (Prod.-No.: CMM-100-110-001; Cellular Dynamics, USA) and then carefully transferred drop-wise to the centrifuge tube containing the thawed cell suspension to prevent osmotic shock and cell lysis.

For calculation of cell density, live cells were counted using a Neubauer chamber (0.0025 mm^2; Laboroptik Ltd., United Kingdom). 20 µL of the cell suspension were mixed with 20 µL of 0.4% trypan blue (Prod.-No.: T-8154; Sigma-Aldrich, USA) and two drops of the mixture were added to the Neubauer chamber. Viable (non-stained) cells were counted and total cell density was calculated. After cell counting, an additional 8 mL of plating

medium were added to the suspension.

For plating, cell density was adjusted for seeding of 63,000 cells/ cm^2 and a suspension volume of 600 μL was added to 24-well plates resulting in an approximate total cell count of 120,000 cells per well.

After plating, plates were incubated at 37°C and 7% carbon dioxide (CO_2) for 48 h continuously. After the initial 48 h, wells were washed several times with warmed PBS to wash off inadvertent cells and debris and fresh maintenance medium (Prod.-No.: CMM-100-120-001; Cellular Dynamics, USA) was added to wells. Medium subsequently was changed every 24 h under strictly sterile conditions. No experiments were performed before the seventh day in culture to determine at least 70% density and synchronised beating of the cell layer. No experiments were performed after the fourteenth day in culture to avoid contamination or loss of cell line heterogeneity by expansion of non-CM cells.

2.3.2 Experimental protocol

2.3.2.1 Preparation of stock solutions

Histones: Purified bovine histones (Prod.-No.: H-9250; Sigma-Aldrich, USA) were solved in PBS (20 mg/ mL) and stored in aliquots at -80°C. After thawing, aliquots were diluted with fresh Maintenance Medium to reach final concentration of 100 μg/ mL.

C3a: C3a (Prod.-No.: 204881; Merck Millipore, USA) was solved in PBS (0.5 mg/ mL) and stored in aliquots at -80°C. After thawing, aliquots were diluted with fresh Maintenance Medium to reach final concentration of 1000 ng/ mL

C5a: C5a (Prod.-No.: A144; Complement Technology, USA) was solved in PBS (0.5 mg/ mL) and stored in aliquots at -80°C. After thawing, aliquots were diluted with fresh Maintenance Medium to reach final concentration of 100 ng/ mL

Control: Controls were treated with 10% PBS in fresh Maintenance Medium.

2.3.2.2 Stimulation protocol of HtCM

To examine markers of cardiac cell damage, cells were stimulated with histones, C5a and C3a respectively, then HMGB1 and cTnI concentrations were measured at three time points

(6 h, 12 h and 24 h) from the supernatant via ELISA. For each stimulant and time point, five equally seeded and cultured wells were chosen plus two wells that were treated with PBS and that served as controls. For stimulation, old medium was aspirated and discarded and 150 µL of freshly prepared stock solutions were immediately added to the corresponding wells. Then wells were placed in a sterile cabinet at 37°C and 7% CO_2 and incubated for the respective time-period. After incubation, the supernatant was aspirated and transferred to a 2 mL tube with 1.5 µL of PI (see above). Then, the tubes were centrifuged with 16,000 G at 4°C for 5 min. The resulting supernatant was stored at -80°C.

2.3.2.3 ELISA from supernatants

To assess levels of cTnI and HMGB1 in supernatants, the following ELISA kits were used adhering to the manufacturer's manual (see 2.2.2 for protocol): Cardiac Troponin I Human ELISA (Prod.-No.: ab200016; Abcam, UK), HMGB1 (Prod.-No.: ST51011; IBL, Germany). Plain Maintenance Medium was additionally loaded for control of native absorbance.

2.4 Statistical analysis

2.4.1 Software and protocol for statistical testing

Statistical analyses were carried out using Sigma Plot (Edition 11.0; Systat Software, Germany). In case of multiple measurements per specimen, means were calculated for each specimen. Data sets were analysed for descriptive statistics and distribution pattern prior to testing. Statistical tests included testing for equality of variance. Values for $p<0.05$ were considered statistically significant. Data sets were not tested for outlier-values. The statistical data analysis in this study is to be considered exploratory, not confirmatory.

2.4.2 Statistics

2.4.2.1 Statistical testing for experiments with two experimental groups

For parametrical distribution and equality of variances, differences among group means were tested for statistical significance using the t-test. Respective data is presented as mean ± standard error of the mean (SE).

For non-parametrical distribution and/ or inequality of variances, differences among group medians were tested for statistical significance using the Mann-Whitney-U-test. Respective data is presented as median, quartiles (QRT) and minimum (MIN)/maximum (MAX) values.

2.4.2.2 Statistical testing for experiments with more than two experimental groups

For parametrical distribution and equality of variances, differences among group means were tested for statistical significance using one-way analysis of variances (ANOVA) with either Tukey's test or Holms-Sidak test as post-hoc tests for multiple adjusted comparisons of group pairs. Respective data is presented as mean $\pm$ standard error of the mean (SE).
For non-parametrical distribution and/ or inequality of variances, differences among group medians were tested for statistical significance using one-way ANOVA on ranks with Dunn's test as a post-hoc test for multiple adjusted comparisons of group pairs. Respective data is presented as median, QRT and MIN/ MAX values.

3 Results

3.1 Rodent in-vivo model of PT and HS

3.1.1 PT and HS do not cause histomorphological alteration of CM four hours after injury

To assess histomorphological changes in the myocardium early after infliction of multiple injuries and hypovolaemia a newly developed damage score was applied on whole transversal sections of the heart (CTRL: n=5; PTHS: n=5). All analysed histological specimens comprised both ventricles and parts of the muscular septum. The cardiomyocytes in all evaluated sections were orderly arranged with no signs of chronic cardiac pathology like fibrosis, dilatation or hypertrophy. Also, striation of the muscle fibres was well detectible. No examined specimen showed signs of direct cardiac injury from experimental chest trauma and no section had to be excluded due to post-mortem disintegration.

There were no wide-spread signs of cellular damage identifiable in both experimental groups. Average scores for the items assessed failed to show any significant differences between both groups (Table 5). Signs of neutrophil infiltration were never found, nor were signs of wide-spread cell necrosis. Oedema, bleeding and muscle rupture as well as hypereosinophilisation, an early sign of cell damage, were scarce and equally distributed among animals of the PTHS group and controls (Table 5; see Fig. 4 for representative examples). Furthermore, these findings did not occur in any pattern, such as in a sub-endothelial or in a coup-contrecoup distribution.

3.1.2 Structural and molecular changes indicate CM damage early after PT and HS

As a humoral marker of cell damage, cTnI was quantified in plasma via ELISA. This specific indicator of CM damage (Fig. 5A; CTRL: n=4; PTHS: n=8) was significantly enhanced 4 h after PT and HS. Results are presented as picograms (pg) of protein quantity per mg of total protein in plasma to compensate for potential diluting effects due to volume resuscitation in the PTHS group.

Immunohistochemical assessment of CC3 (Fig. 5B; CTRL: n=5; PTHS: n=5), a sensitive marker for apoptosis, did not show any positive staining in any section. These findings are consistent with the investigations on HE stained sections (see above). Densitometric analysis of HMGB1, an unspecific damage-associated molecule, in immunohistochemical tissue preparations (Fig. 5C; CTRL: n=5; PTHS: n=5) showed significantly enhanced staining signals in sections of PTHS animals.

Table 5 – Histomorphological assessment of cardiomyocyte damage after polytrauma and haemorrhagic shock

Median score (1. quartile; 3. quartile) of the left (LV) and right (RV) ventricle for each group (CTRL: n=5; PTHS: n=5). For each animal, the whole transversal section was examined and each ventricle was scored separately. Data was analysed for normality using the Shapiro-Wilk test and differences in medians were analysed using a one-way analysis of variances (ANOVA) on ranks with Dunn's test for multiple adjusted comparisons of group pairs. CTRL: control group. PTHS: polytrauma and haemorrhagic shock group. CBN: contraction band necrosis. NS: Statistically not significant. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

	CTRL		PTHS		p-value
	LV	RV	LV	RV	
Apoptosis	1 (0.5; 1)	0 (0; 1)	0.5 (0; 1.75)	0.5 (0; 1)	NS
CBN	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	NS
Infiltration	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	NS
Bleeding	0 (0; 0)	0 (0; 0)	0.5 (0; 1)	0 (0; 0)	NS
Rupture	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	NS
Oedema	1 (0.5; 1)	0 (0; 0.5)	1.5 (0.25; 2)	0.5(0; 1)	NS
Eosinophilisation	1 (0.5; 1)	0 (0; 1)	1.5 (1; 2)	1 (0.25; 1)	NS

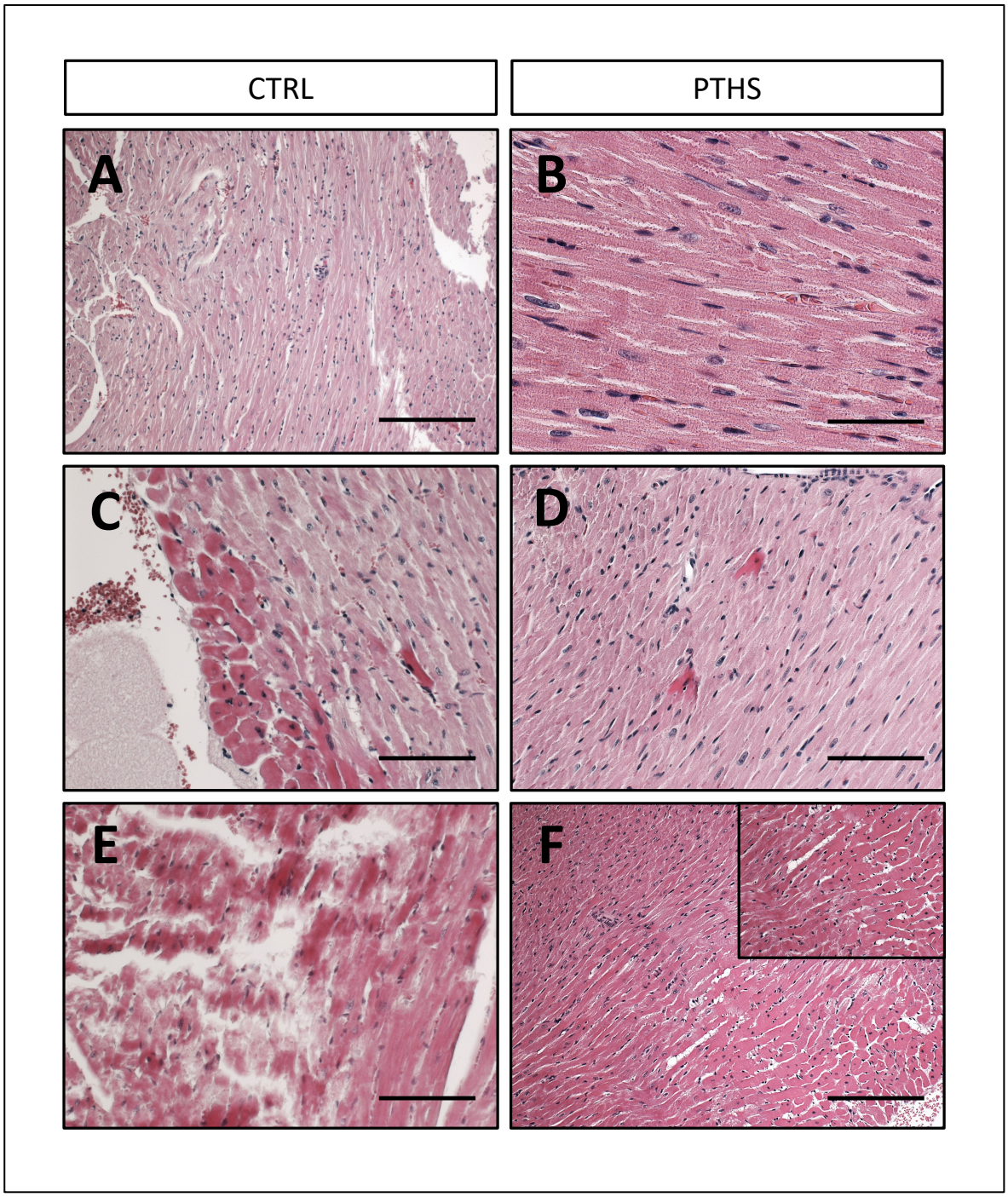

Figure 4 – Histomorphological findings in haematoxylin and eosin stained sections

Representative haematoxylin and eosin stained transversal sections of whole heart preparations. Both (A) and (B) depict healthy cardiac muscle cells (CM). Regular cross-striations of CM are visible in (B). Note fixation-artefact in right upper corner of (A). (A): control animal (CTRL; No. 51), 100x magnification, length-bar: 200 µm. (B): animal of polytrauma and haemorrhagic shock group (PTHS; No. 47), 400x magnification, length-bar: 50 µm. (C) Eosinophilisation in sub-endocardial region of the left ventricle of a control animal (No. 33). Magnification: 200x, length-bar: 100 µm. (D) Single cells showing signs of apoptosis like karyorrhexis and hypereosinophilisation (PTHS; No. 47). Magnification: 200x, length-bar: 100 µm. (E) Fixation artefact mimicking rupture of ventricle (CTRL; No. 33). Magnification: 100x, length-bar: 200 µm. (F) Contraction bands in an animal of the PTHS group (No. 59). Inset: CM presenting eosinophilisation, condensation of nuclei and intercellular oedema. Magnification: 100x, length-bar: 200 µm. Inset: identical region, 200x magnification. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

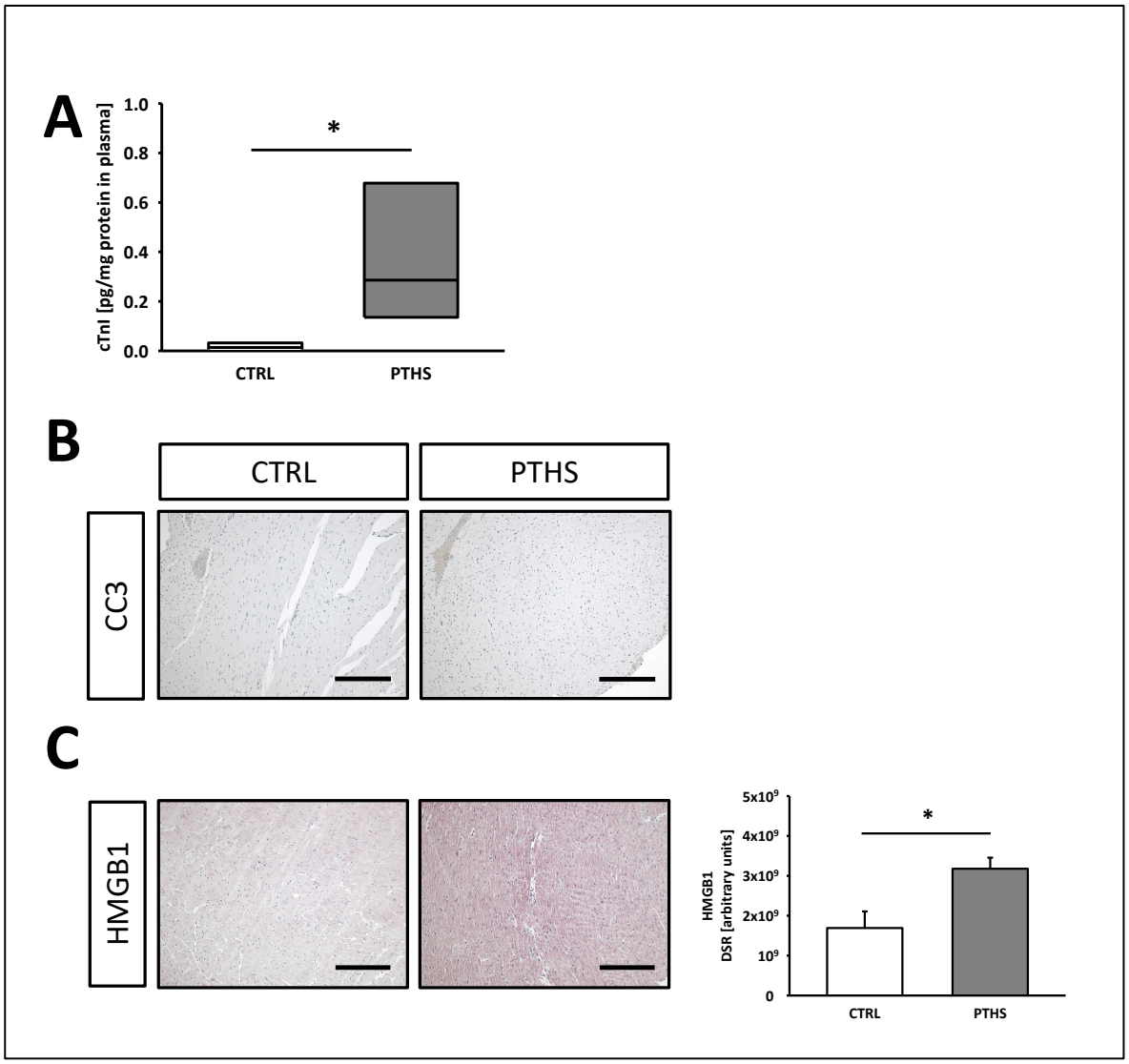

Figure 5. Tissue resident and humoral markers of cardiac cell damage

(A) Cardiac troponin I (cTnI; group median and quartiles), evaluated via enzyme-linked immunosorbent assay (ELISA) from plasma (CTRL: n=4; PTHS: n=8), was significantly enhanced after exposure to polytrauma and haemorrhagic shock (p=0.004). Results are presented as pg protein quantity per mg of total protein in plasma to compensate for potential diluting effects due to volume resuscitation in the PTHS group. (B) Sections immunohistochemically stained for cleaved caspase 3 (CC3) did not show any ongoing apoptosis, neither in control animals nor early after polytrauma and haemorrhagic shock. Shown are representative images from a control animal (CTRL; No. 51; Magnification: 100x, length-bar: 200 µm) and an animal after infliction of polytrauma and haemorrhagic shock (PTHS; No. 64; Magnification: 100x, length-bar: 200 µm). (C) Immunohistochemical assessment of tissue-bound high mobility group box 1 (HMGB1; group mean + standard error of the mean). Densitometric analysis of staining signal showed marked enhancement in PTHS animals (p=0.018). Shown are representative images from the CTRL group (No. 63; Magnification: 100x, length-bar: 200 µm) and the PTHS group (No. 37; Magnification: 100x, length-bar: 200 µm). For statistical comparison of experimental group medians/ means the Mann-Whitney rank sum test (A) and the unpaired t-Test (C) were used. DSR: densitometric sum red. CTRL: control group. PTHS: polytrauma and haemorrhagic shock group. *: p<0.05. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

3.1.3 Surface expression of complement receptors and CD59 and deposition of MAC on CM are altered early after PT and HS

In the interest of assessing activation of the complement system in the blood, plasma concentrations of activated complement component C3a (Fig. 6A; CTRL: n=5; PTHS: n=8) and activated complement component C5a (Fig. 6B; CTRL: n=5; PTHS: n=8) were analysed via ELISA. There was no statistically significant increase of plasma levels of both components of interest. However, there was a slight, although insignificant increase in plasma C3a levels of trauma animals compared to controls.

For analysis of tissue expression of the related complement component receptors and regulating molecules, IHC and densitometric quantification was performed. Median intensity of the signal for C5aR1 (Fig. 7A; CTRL: n=5; PTHS: n=6) was significantly increased in PTHS group compared to control group. Median intensities of signal for both C3aR (Fig. 7B; CTRL: n=5; PTHS: n=6) and CD59 (Fig. 7D; CTRL: n=5; PTHS: n=6) were decreased in the PTHS group compared to controls. Densitometric analysis of tissue formation of MAC (Fig. 7C; CTRL: n=5; PTHS: n=6) did not show distinct results, although mean densitometric sum was increased in PTHS animals. In neither of the analysed sections a distinctive distribution pattern of assessed molecular antigens was observable.

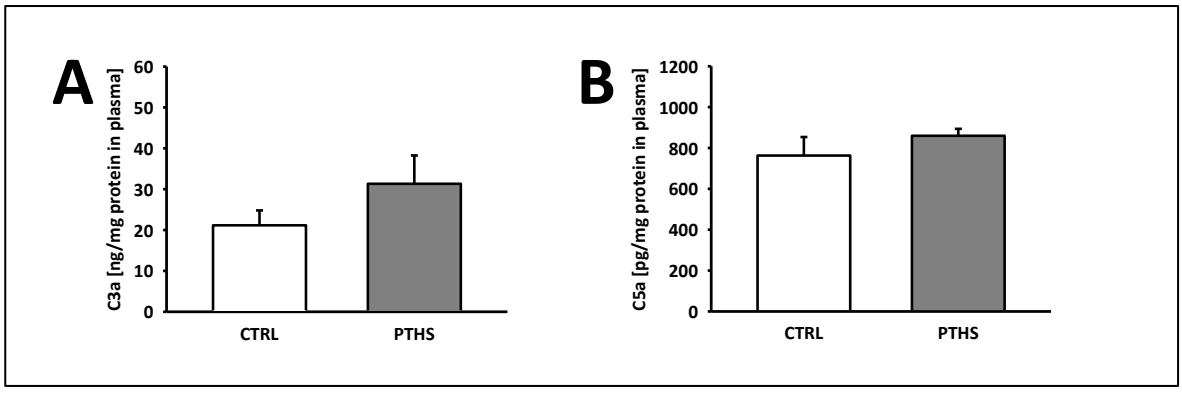

Figure 6 – Plasma concentrations of activated complement components.

Plasma levels of activated complement components C3a and C5a obtained via enzyme-linked immunosorbent assay (ELISA). Plasma levels of (A) C3a (CTRL: n=5; PTHS: n=8) and of (B) C5a (CTRL: n=5; PTHS: n=8) did fail to present statistically significant differences between group means. Results presented as group mean + standard error of the mean. For statistical comparison of experimental group means the unpaired t-Test was used. DSR: densitometric sum red. CTRL: control group. PTHS: polytrauma and haemorrhagic shock group. (A) modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

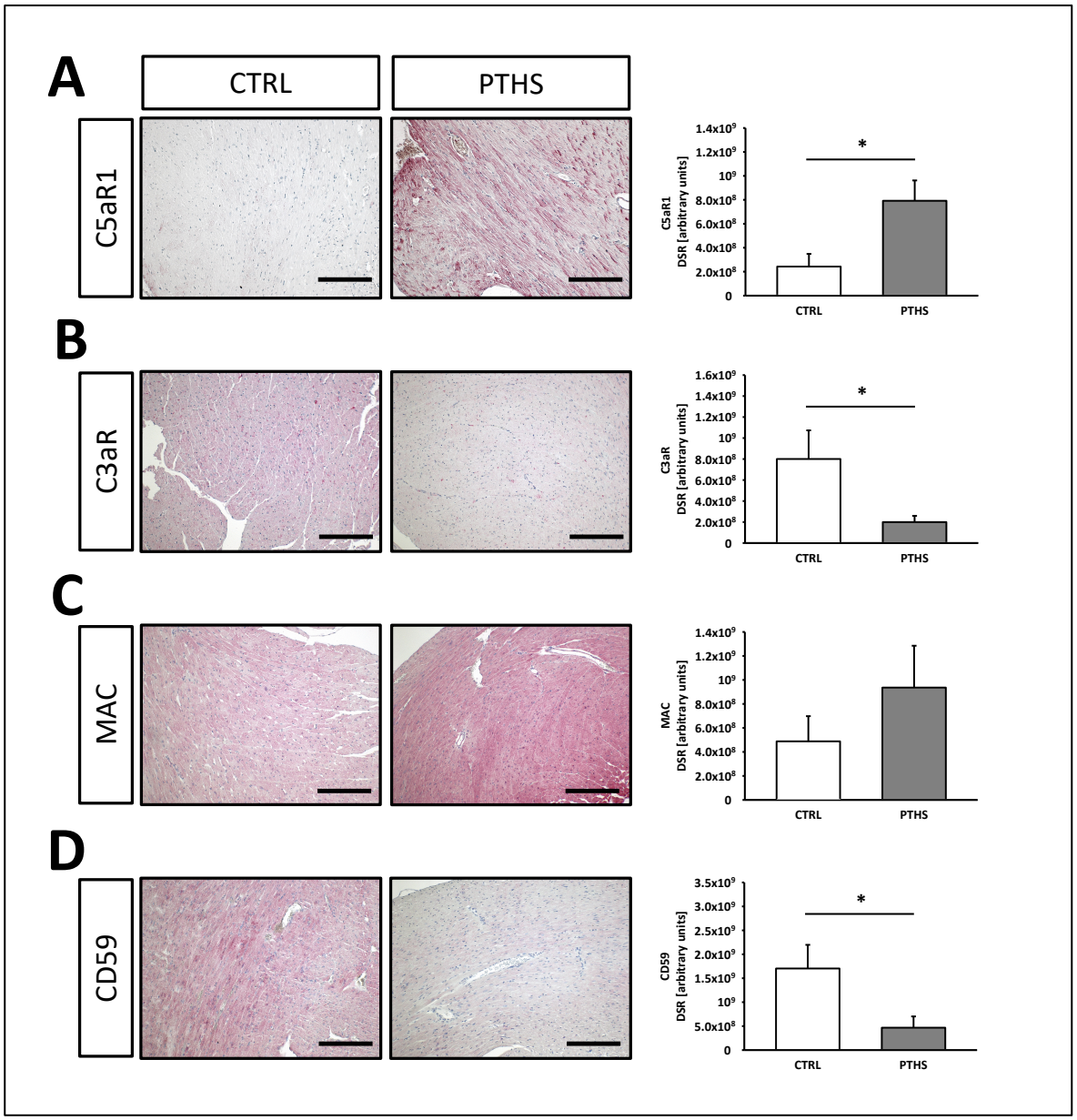

Figure 7 – Tissue levels of complement receptors, complement-associated regulating proteins and MAC.

Densitometric analysis of immunohistochemical staining signal of complement receptors C5aR1 and C3aR, membrane attack complex (MAC) and cluster of differentiation 59 (CD59) (A) Staining signal of C5aR1 was significantly increased in the PTHS group compared to native controls. Shown are representative images from the CTRL group (No. 63; Magnification: 100x, length-bar: 200 µm) and the PTHS group (No. 59; Magnification: 100x, length-bar: 200 µm). (B) Staining signal of C3aR was significantly decreased after polytrauma and haemorrhagic shock compared to controls. Shown are representative images from the CTRL group (No. 51; Magnification: 100x, length-bar: 200 µm) and the PTHS group (No. 64; Magnification: 100x, length-bar: 200 µm). (C) Staining signal of MAC did not show distinct differences between the polytrauma and haemorrhagic shock group (PTHS) and the native controls (CTRL). Shown are representative images from the CTRL group (No. 33; Magnification: 100x, length-bar: 200 µm) and the PTHS group (No. 54; Magnification: 100x, length-bar: 200 µm). (D) Staining signal of CD59 was significantly decreased in animals of the PTHS group compared to the CTRL group. Shown are representative images from the CTRL group (No. 56; Magnification: 100x, length-bar: 200 µm) and the PTHS group (No. 47; Magnification: 100x, length-bar: 200 µm). Results presented as group mean + standard error of the mean. For statistical comparison of experimental group means the unpaired t-Test was used. DSR: densitometric sum red. CTRL: control group. PTHS: polytrauma and haemorrhagic shock group. C5aR1: receptor 1 for the activated complement component 5. C3aR: receptor for the activated complement component 3. *: $p<0.05$.

3.1.4 PT and HS cause spatial alterations of the gap junction protein Cx43

After PT and HS, disintegration of gap junctions and lateralisation of Cx43 on the cell surface could be detected in immunohistochemically stained tissue preparations (Fig. 8A; CTRL: n=5; PTHS: n=6). These findings were consistent among all animals exposed to traumatic injury and HS and could be reproduced in immunofluorescence assays (Fig. 8A; CTRL: n=3; PTHS: n=3). Sections of control animals rarely showed signs of CGJ disintegration.

Densitometric analysis of the IHC signal could not reveal significant differences in means between control and trauma animals (Fig. 8B). A western blot assay using the same antibody did show a slight increase in amount of total Cx43 for PTHS group, although the difference failed to be statistically significant (Fig. 8C).

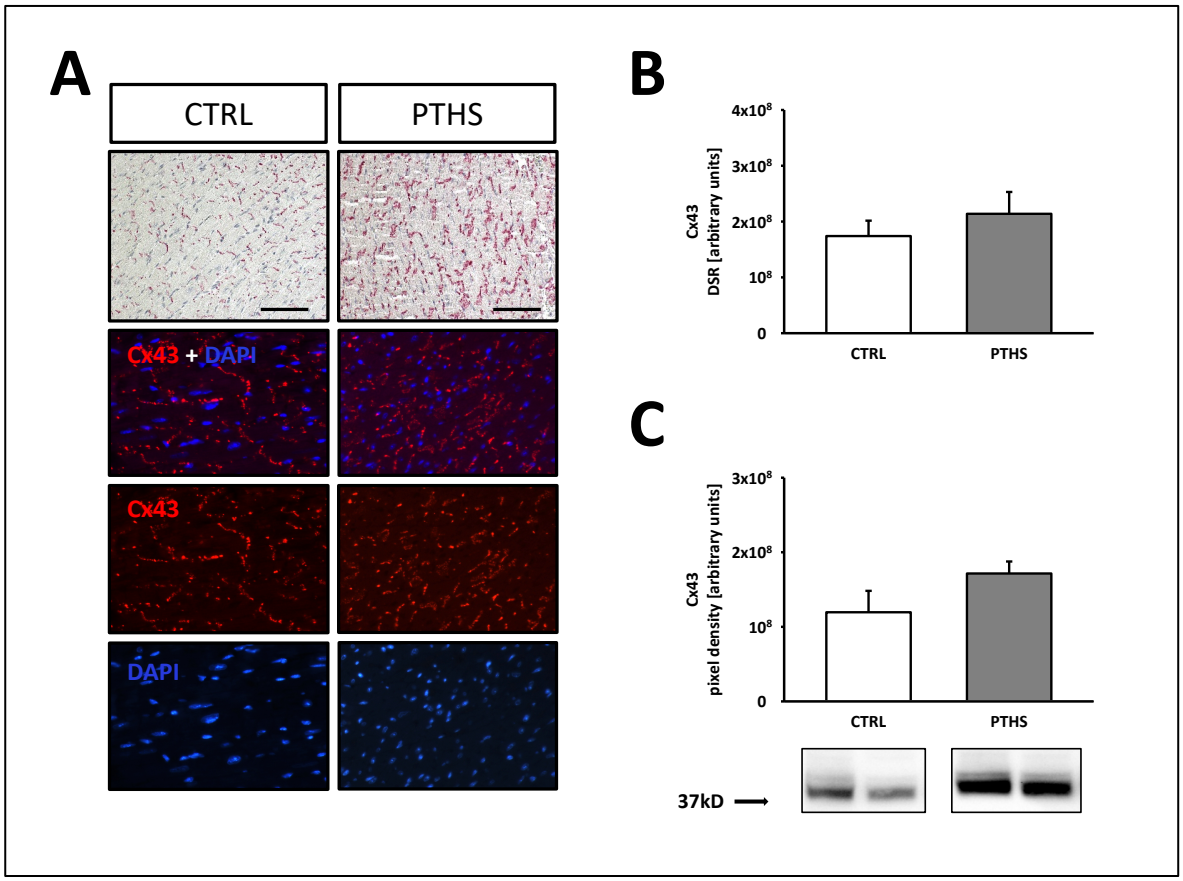

Figure 8 – Disruption of cardiac gap junctions and spatial alterations of the gap junction protein connexin 43

(A) Representative images of immunohistochemical (IHC; upper panel; CTRL: n=5; PTHS: n=6) and immunofluorescence (IF; lower panels; CTRL: n=3; PTHS: n=3) assessment of the cardiac gap junction (CGJ) protein connexion 43 (Cx43). In the right panel orderly arranged lean gap junction proteins can be detected. The left panel showing coarsely spread signal clusters, demonstrating disruption and changes of spatial distribution of Cx43. CTRL IHC (upper panel): No. 63; Magnification: 100x, length-bar: 200 µm. PTHS IHC (upper panel): No. 59; Magnification: 100x, length-bar: 200 µm. CTRL IF (lower panels): No. 63; Magnification: 400x. PTHS IF (lower panels): No. 59; Magnification: 400x. Counterstaining of nuclei with 4',6-diamidino-2-phenylindole (DAPI). (B) Densitometric analysis (CTRL: n=5; PTHS: n=6) of staining signal of Cx43 failed to demonstrate any significant differences between groups. (C) Western blotting of total Cx43 protein (CTRL: n=4; PTHS: n=6) in left ventricle homogenates showed a slight increase of protein amount after polytrauma and haemorrhagic shock, however not statistically significant. Results presented as group mean + standard error of the mean. For statistical comparison of experimental group means the unpaired t-test was used. DSR: densitometric sum red. CTRL: control group. PTHS: polytrauma and haemorrhagic shock group. kD: kilo Dalton. *: $p<0.05$. Modified from [18] (CC BY 4.0, https://creativecommons.org/licenses/by/4.0/).

3.2. In-vitro modelling of posttraumatic cardiac damage

3.2.1 Exposure of cardiac muscle cells to histones cause the release of cellular damage markers.

3.2.1.1 Release of cTnI

For evaluation of the cellular response to DAMPs and activated complement factors in-vitro, cultured HtCM were grown 7 days until cell culture formed a beating syncytium (approximately 50 beats per min) and then stimulated with 100 μg/ mL histones (HIS+), 1000 ng/ mL C3a (C3A+) and 100 ng/ mL C5a (C5A+) for 6 h, 12 h and 24 h respectively (HIS+/ C3a+/ C5a+: n=5 for each point of time, CTRL: n=6 for 6 h and 12 h, n=5 for 24 h). To assess levels of released cTnI and HMGB1, ELISA from supernatant were performed. After stimulation with histones, cTnI in supernatant was significantly increased at all time points compared to controls (Fig. 9). On the other hand, neither C3a nor C5a did enhance levels of cTnI in the supernatant. Moreover, absolute cTnI levels differed significantly between the time points after stimulation with histones, peaking after an exposure for 24 h (Fig. 9).

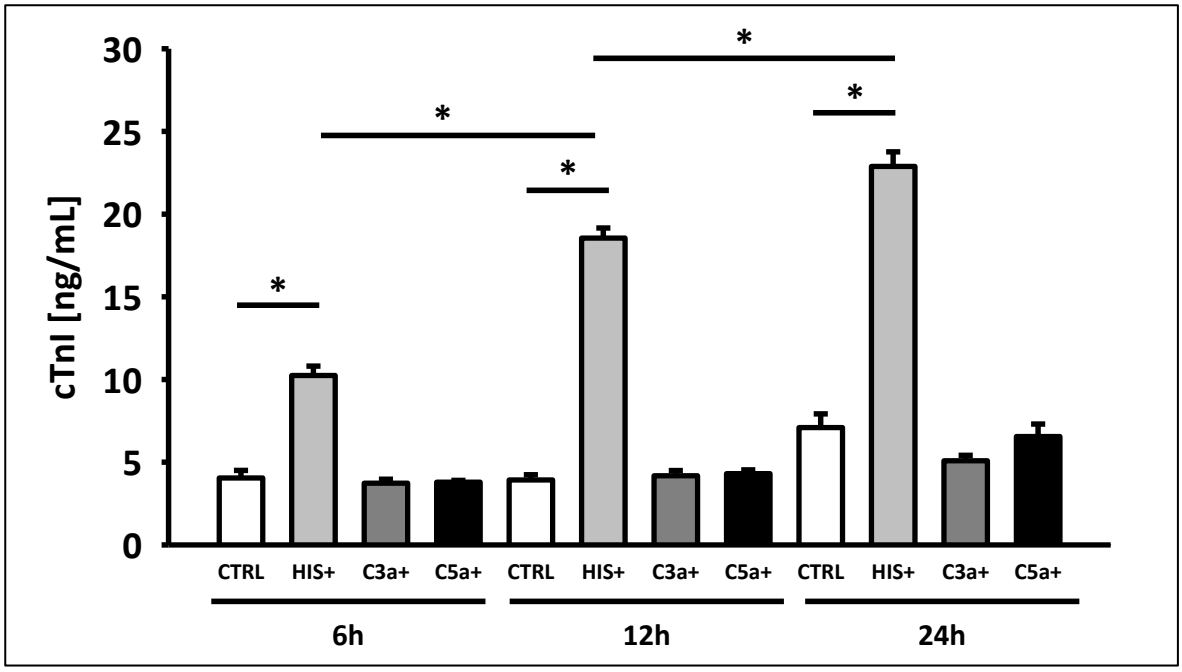

Figure 9 – Levels of cTnI in supernatant of cultured cardiomyocytes after exposure to histones, C3a and C5a

Cultured human cardiomyocytes were treated with 100 μg/mL bovine histones (HIS+), 1000 ng/ mL human C3a (C3a+) and 100 ng/ mL human C5a (C5a+) respectively for 6 h, 12 h and 24 h (HIS+/ C3a+/ C5a+: n=5 for each point of time, CTRL: n=6 for 6 h and 12 h, n=5 for 24 h). Supernatant was collected afterwards and cardiac troponin I (cTnI) was quantified via enzyme-linked immunosorbent assay (ELISA). Shown are measured absolute concentrations of cTnI after 6 h, 12 h and 24 h with respective stimulation. Only treatment with histones significantly increased cTnI concentrations in supernatant. Results presented as triplet mean + standard error of the mean. For statistical comparison of the experimental group means one-way ANOVA and the Holm-Sidak method as a post-hoc test were performed. CTRL: controls. h: hour(s). C5a/C3a: activated complement components 3/ 5. *: $p<0.05$.

3.2.1.2 Release of HMGB1

Simulation with histones also drove the release of HMGB 1 with significant enhancement at each respective time point compared to respective controls. The maximum of concentrations of HMGB1 in the supernatant was found at 12 h. As was for cTnI, stimulation with C3a and C5a respectively did not increase supernatant levels of HMGB1 (Fig. 10).

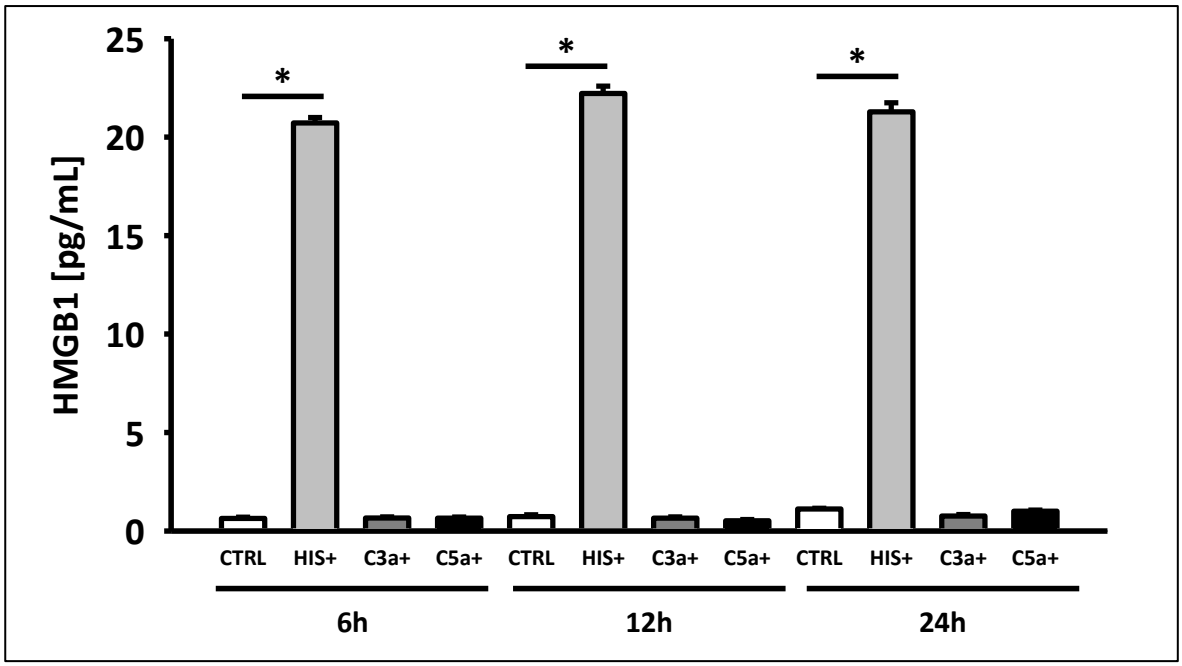

Figure 10 – HMGB1 levels in supernatants of cultured cardiomyocytes after exposure to histones, C3a and C5a.

Cultured human cardiomyocytes (HtCMs) were treated with 100 µg/mL bovine histones (HIS+), 1000 ng/mL human C3a (C3a+) and 100 ng/ mL human C5a (C5a+) respectively for 6 h, 12 h and 24 h (HIS+/ C3a+/ C5a+: n=5 for each time point, CTRL: n=6 for 6 h and 12 h, n=5 for 24 h). Supernatant was collected afterwards and high mobility group box 1 (HMGB1) was quantified via enzyme-linked immunosorbent assay (ELISA). Shown are absolute measured concentrations of HMGB1 after 6 h, 12 h and 24 h incubation with respective stimulating agent. Incubation with histones significantly increased HMGB1 concentrations in supernatant for each respective incubation period. Incubation with C3a or C5a failed to stimulate the release of HMGB1. Results presented as triplet mean + standard error of the mean. For statistical comparison of the experimental group means one-way ANOVA and the Holm-Sidak method as a post-hoc test were performed. CTRL: controls. h: hour(s). C5a/C3a: activated complement components 3/ 5. *: $p<0.05$.

4 Discussion

4.1. Discussion of results

4.1.1 Cellular damage of CM after PT and HS

4.1.1.1 Histopathological findings in HE stained sections and evaluation of the applied damage score

Recently, hypoxic myocardial lesions were described in patients after severe non-cardiac trauma and in the absence of coronary pathologies [49]. Lesions in HE-stained heart sections could also be detected in trauma models using rodents and pigs [72, 73]. However, these studies lack a systematic and quantitative assessment of the histological attributes of cell and organ damage. Also, all studies conducted in animals so far, evaluated certain end points at a much later time point. This study represents the first analysis of histological changes in the heart as early as four hours after multiple trauma and HS.

To establish a histopathological scoring system for further use in animal trauma models, whole transversal sections of mouse hearts were scored according to precedingly described typical microscopical properties of cardiac cell damage. Signs of ongoing apoptosis (see Table 5) were only rarely detectable, which is in concurrence with IHC evaluation of CC3, that was negative in both the control group and the PTHS group. CC3 was shown to be a sensitive marker of apoptosis [58, 87] and the few single cell events that could be identified in HE-stained sections by means of attributes like eosinophilisation, vacuolisation and nuclear shrinkage are most likely to be coincidental or artificial. Contraction band necrosis, the predominate appearance of CM necrosis [12, 105] was only found twice (once in the PTHS group and once in the control group; see Fig. 4) and thus was assumed to be artificial. Immigration of blood immune cells, namely of polymorphonuclear neutrophils (PMN) was never detected, ruling out marked early tissue inflammation. In rodent myocardial infarction by coronary occlusion and subsequent reperfusion (MI/R), marked PMN infiltration has been described as early as six hours after injury with a peak after twenty-four hours [138] and was associated with significant cell loss and disintegration of the tissue [58, 138]. These findings may lead to the assumption that the time point of four hours of this study is too early to detect PMN infiltration in the heart tissue. On the other hand,

MI/R injury with subsequent local inflammation is only limitedly comparable to the relatively “mild” cardiac injury in the course of trauma and posttraumatic systemic inflammation. Further studies with long-term histological follow-up will be needed to fully estimate the extent of immigration of immune cells into the heart tissue after direct or remote trauma. Intramuscular bleeding and muscular rupture were particularly difficult to assess, as these changes may very well be artificial due to post-mortem handling of the explanted organ. Posttraumatic intramuscular bleeding has been shown for rats after cardiac contusion (from a falling weight) [31] and after blunt chest trauma [72] and also for pigs in model of multiple injuries [73]. Occasional minor foci of bleeding were found in the left ventricles of 3 animals of the PTHS group and no bleeding was visible in the CTRL group. Marked rupture of cardiac myofibers with adjacent cell loss and tissue disintegration was only observed once and was confined to a few cells. These findings suggest an only marginal role of microscopical tissue damage for the evident injury to CM reflected by local and systemic markers of cardiac cell damage (cTnI and HMGB1, see 3.1.2 for results and 4.1.1.2/ 4.1.1.3 for discussion). Oedema and hypereosinophilisation are non-specific histological features seen after myocardial ischemia [58]. Interstitial oedema was also demonstrated in septic hearts of rabbits [53]. Although not statistically significant, score medians for both items were slightly higher four hours after PT and HS compared to controls. However, as these alterations were visible in both PTHS and CTRL animals these findings may as well represent fixation artefacts. For future studies, scoring of these items should be modified for more specificity and selectivity, e.g. by more differentiated criteria.

Given the presented results, the question remains, whether a morphological approach to cardiac injury is practical. At this point, more studies in different species and with evaluation at later time points are needed, to make a definitive statement. An important argument in favour of conducting more morphological studies on cardiac cell damage after acute systemic inflammation is, that there is still no conclusive proof that cardiac impairment in this setting is fully reversible.

4.1.1.2 Elevated plasma levels of cTnI indicate subcellular cardiac damage

The present study showed significantly increased plasma concentrations of cTnI after PT and HS and also in-vitro after stimulation with histones. Its specificity for cardiac damage suggests injury of CM early after trauma. These findings concur with recent studies

demonstrating elevated troponin serum levels after trauma [77, 123]. However, the significance of elevated serum troponin levels after blunt chest trauma is controversially discussed [13, 37, 77, 123, 130]. In septic patients, a clear association between troponin I levels and left ventricular function was demonstrated [88, 139] and high troponin levels seem to predict a higher mortality [103]. Additionally, an association of increased incidence of cardiac arrhythmia and decreased left ventricular function with higher serum levels of troponin have been reported for septic patients [3]. Reduced cardiac ejection fraction was observed in a porcine model of multiple injuries [73]. Further, troponin elevation is predictive for poorer outcome after chest trauma [77].

It remains to be specified which impact cardiac contusion has on the release of damage markers and further studies on animal models and trauma patients deprived of chest impact need to answer the question where the apparent cardiac damage derives from. In this study, histological examination revealed no signs of marked cardiac contusion nor signs of rupture of the muscle. Also, by histological assessment, necrosis and wide-spread apoptosis could be ruled out, findings that propose a release of cTnI already at subcellular damage or upon ligand-receptor interaction with some consecutive downstream pathway activation. An unproven theory states, that early in the event of cardiac injury, cardiac cells are able to release cytosolic troponin, even in the absence of sustained cell damage [82]. Regarding the slight enhanced levels of MAC in IHC preparations from the experimental PT and HS study presented here, it is tempting to speculate, that formation of MAC on cardiac cells may contribute to this effect.

In-vitro, only stimulation with histones increased the release of cTnI by cultured HtCM in a time dependent manner and additionally seemed to desynchronize the beating syncytium in culture and to cause irregular beating (own non-quantified, unpublished observations). Though quantitative and qualitative evaluation of ongoing apoptosis or subcellular damage was not within the scope of this study, active secretion of troponin is perceivable. The finding, that isolated stimulation with histones in the absence of hypoxia, inflammation or other stimuli proposes that at least some kind of signalling is involved in the increase of cTnI.

4.1.1.3 Increased tissue and plasma levels of HMGB1 after trauma: cause or consequence?

Previous studies have shown increased serum levels of HMGB1 after trauma and established its role as an important driver of sterile inflammation [162, 168]. In the context of sepsis, it was demonstrated to be both actively secreted by blood cells upon stimulation and released from injured cells [33, 156]. In-vitro experiments in this study presented elevated HMGB1 supernatant levels after stimulation of CM with histones, another important DAMP released in inflammatory reactions. Whether this release resulted from actual cell loss, as shown before by another group [99] or from a physiological reaction triggered by intracellular signalling and secretion was beyond the scope of this study and has to be addressed in future research. Both histones [40] and HMGB1 [162] interact with TLRs, and those receptors are known to be expressed on cardiomyocytes [9, 17]. Therefore, further investigation should analyse alleged autosecretory effects of HMGB1, a conceivable resulting competition between histones and HMGB1 for the same receptor and a possible potentiation of both stimuli.

In concurrence with the described in-vitro findings, the signal obtained from IHC detection of HMGB1 in mouse hearts after PT and HS was significantly increased in trauma animals compared to controls. However, by means of IHC, it is not possible to conclusively state, whether the tissue-bound levels of HMGB1 increased by local secretion or by precipitation of plasma-derived molecules or a combination of both. By means of pathohistological evaluation that ruled out any major apoptotic or necrotic events, it is reasonable to assume that the detected HMGB1 is actively secreted and not released upon cell death.

Detrimental effects of HMGB1 have been described before. HMGB1 mediates apoptosis via interaction with TLR4 [34, 158] and by the same interaction depletes cytosolic Ca^{2+}-storage pools and reduces CM contractility in-vitro [165]. Thus, it can be perceived as a danger molecule, both acting as a marker with diagnostic and prognostic value and as a potentially harmful driver of cardiac dysfunction. In consequence, HMGB1 may initiate a vicious circle leading to further organ injury and ultimately to a greater threat for the trauma patient.

4.1.1.4 The role of apoptosis in posttraumatic cardiac injury

By means of pathohistological examination and IHC assessment of the pro-apoptotic CC3, there was no ongoing apoptosis evident in this study. In sepsis, CC3-induced apoptosis

supposedly plays a major role in the development of cardiac dysfunction [44]. A plethora of mechanisms have been proposed to prompt apoptosis of CM upon cardiac injury in sepsis [91, 149, 164] and other pathological processes such as myocardial ischemia [158]. In principle, these mechanisms may as well trigger apoptosis during a state of post-traumatic systemic inflammation. But whereas apoptotic events have been described in experimental models of trauma, their extent, time-course and consequences remain unknown. For instance, apoptosis in a trauma rodent model was reported to be significantly increased six hours after traumatic impact with a peak after twenty-four hours [144]. However, another study on rats with blunt chest trauma showed heightened levels of CC3 and an elevated rate of apoptosis after five days but not after twenty-four hours [72]. Interestingly, a study of CM apoptosis in patients with terminal heart failure, in which young victims from fatal traffic accidents served as a control group, no apoptosis could be observed after severe trauma [120]. By the current literature, the present data may suggest a more delayed induction of apoptosis in trauma, as opposed to sepsis, where the infusion of an anti-apoptotic agent failed to ameliorate apoptosis rates, when administered four hours after sepsis onset [44]. Also, as many studies on CM apoptosis were carried out in-vitro, the influence of protective effects in-vivo are not yet to be excluded. Nonetheless, no definite conclusion can be drawn from this study and further investigations are necessary, especially as CM apoptosis may theoretically pose a threat in the long-term outcome of patients. In paediatric patients, a slight depression of cardiac function has been described for the follow-up after several years [68, 81], but similar studies for adults are missing. Addressing apoptosis in future studies may not only link structural and functional impairment of the heart but also offer new therapeutic approaches.

4.1.2 Alteration of complement receptors and complement associated surface molecules after experimental trauma

4.1.2.1 Unaltered plasma levels of C3a and C5a after PT and HS: a question of timing?

One important purpose of this study was, to investigate the impact of severe trauma and HS on the pattern and quantity of expression of essential membrane-bound components of the complement system in heart tissue. Significant trauma in humans leads to a systemic activation of the complement cascade [20]. Furthermore, various animal models of isolated haemorrhage resulted in complement activation [38, 116, 141, 161]. In consequence,

inhibition of the complement system can mitigate organ dysfunction after HS [153]. Surprisingly, evaluation of plasma levels of C3a and C5a did fail to show significant alterations, although a slight increase of mean C3a levels was detectable in the PTHS group. Of note, for both complement components and more so for C5a, previous evaluation up to 10 days after trauma showed a dip in plasma levels after an initial rise around the time of the admission to the emergency room and up twenty-four hours after the impact [20]. Taken into consideration possible time differences in physiological reactions between species (i.e. humans and mice) in the present study, a "blind spot" of plasma kinetics of complement activation may exist. To further elucidate the complement kinetics, an analysis of the time course of plasma levels seems mandatory. However, due to the small blood volumes of mice, these data could not be obtained from this study. The pivotal role of the complement system in septic cardiomyopathy has been well established over the past years. For trauma, the available data is still deficient.

4.1.2.2 Alterations of tissue levels of C5aR1 and C3aR after severe trauma and shock

A notable study on sterile burn-injury could prove a linkage between functional impairment of CM and C5a-C5aR1 interactions [60]. Of note, this study also detected an enhanced expression of C5aR1 by IHC and western blot (WB) and increased levels of C5aR1 mRNA already at one hour after trauma. An upregulation of C5aR1 expression has also been demonstrated repeatedly after sepsis [108, 125] and myocardial ischemia [106, 166] and seems to constitute a hallmark of the complement related reaction of CM to systemic inflammation. However, recent studies on trauma in rats and pigs did controversially show decreased levels of C5aR1 levels after twenty-four and seventy-two hours respectively [72, 73]. Whether this discrepancy stems from the physiological differences in species (rats/ pigs versus mice), timing or confounding factors in experimental protocols is not clear and needs further investigation. As C5a-C5aR1 interactions can mediate the decrease of CM shortening [60, 108] and impair Ca^{2+}-homeostasis [75], the suggested consequence of increased C5aR1 levels on CM surfaces would be an aggravation of cardiac dysfunction. However, whether expression levels of C5aR1 correlate with functional impairment of CM remains to be elucidated.

The pathophysiological role of C3a after PT is even less defined. Enhanced of plasma concentrations of C3a may correlate with pro-inflammatory IL-6 release [54] and poor

outcome after severe trauma and sepsis [55, 59]. The role of C3aR interactions in the pathogenesis of organ injury was previously proposed [35, 119]. The base-line expression of C3aR in mouse hearts was demonstrated before [148], but there are no preceding studies on the changes of the expression and the role of C3aR on heart tissue, shortly after traumatic injury and haemorrhagic shock. In this study, a significant drop of C3aR-signal obtained by IHC was detected after four hours. Of interest, C3aR knock-out mice show a more enhanced inflammatory reaction to endotoxin challenge, and a lower expression of C3aR correlated with increased lethality of experimental endotoxemia [80]. It would be precipitous to deduce any conclusion from the present findings, but based on this publication, it seems tempting to hypothesize some detrimental effects of C3aR down-regulation on cardiac injury after trauma. There is a strong contrast between the proposed role of the anaphylatoxins C3a and C5a and their receptors derived from previous in-vitro studies on CM and the in-vitro findings presented in this thesis. Here, incubation of HtCM with anaphylatoxins did fail to increase cTnI and HMGB1 levels in the supernatant as in contrast to the significant elevation of both markers after stimulation with histones. These findings lead to the assumption that, at least in-vitro, activated complement components do not cause structural damage but rather solely compromise CM function. In-vivo, ischemic damage of the heart could be ameliorated by blocking the CP of the complement system via application of C1-esterase inhibitor [63]. However, given the physiological role of the anaphylatoxins in chemotaxis and in mediating the release of additional pro-inflammatory cytokines, the harmful structural effects of C3a and C5a may rely on the presence of further cellular and humoral actors. Their impact on the functionality of HtCM was not in the scope of this study and has to be addressed in future investigations.

4.1.2.3 Does the loss of CD59 render cardiomyocytes prone to MAC formation after trauma?

Investigation of CD59, a cell surface bound inhibitor of MAC formation, in heart tissue sections showed significantly lower levels of IHC signal in the PTHS group compared to controls. Further, a slight, although not statistically significant enhancement of the IHC signal of MAC in heart tissue sections was obtained after PT and HS. Considering the specific role of CD59, the lower expression levels may determine the increased MAC signal after PT and HS. Whether these changes occur as an unspecific pathophysiological reaction to cell injury or as a downstream effect of specific signalling cascades remains unclear.

No literature is available on the effects and role of MAC and its inhibitor CD59 in the course of traumatic cardiomyopathy. In the case of cardiac ischemia, acute myocardial infarction and heart failure, it has long been established, that injured CM present with increased levels of tissue-bound MAC and decreased levels of CD59 by IHC evaluation and these changes have been held responsible for aggravation of ischemic cell injury [70, 101, 113, 142, 152]. Early MAC formation was even proposed to detect areas of latent, microscopically imperceptible cell damage in autopsies [23]. Strikingly, neither MAC/ CD59 nor HMGB1 staining in IHC preparations showed any recognisable distribution pattern in the analysed sections. This may be conceived as an expression of diffuse inflammatory or ischemic injury rather than disintegration of the cardiac tissue by local force or tear stress. Either way, further studies are necessary for the analysis of related pathomechanisms and the actual co-dependency of CD59 and MAC formation on CM after severe injury.

4.1.2.4 Conclusion of the findings of the complement analyses

Despite the obvious similarities between sepsis and acute systemic inflammation after severe trauma and shock, original research on the role of the complement system in post-traumatic cardiac injury is imperative. The definite impact of complement activation on the heart, both locally and systemically, remains elusive. The present study has added new insights on the reactive expression of central complement-related surface proteins on heart muscle cells after trauma. Taken together, the complement system constitutes a promising link between the various pathophysiological mechanisms driving cardiac injury after polytrauma.

4.1.3. Alterations of the gap junction protein Cx34 after experimental trauma and haemorrhagic shock

4.1.3.1 Histological evaluations revealed spatial shifts of Cx43 localisation in heart muscle cells

Cx43 is a cell-cell-linking protein which acts as main conductor of electric charges across the membranes of adjacent CM thereby facilitating synchronized contraction [102]. In the present study similar alterations could be detected via IHC and IF. Although the described findings were only mild and appeared in a patchy pattern across the sections of PTHS animals, such disintegration was mostly absent in sections of the CTRL group. Previous

studies regarding immunohistochemical analyses of Cx43 in heart tissue after severe trauma disclosed changes in its spatial distribution, from strictly confined localisation within cell-cell contacts in control animals to a disrupted pattern with distribution of Cx43 across the CM [72, 73]. Such spatial shifts of Cx43 are also observed in histological specimens from autopsies of septic patients [143]. It has been pointed out, that hypoxia leads to internalisation of Cx43 in CM cultures [29].

However, striking as these morphological findings may be, their pathophysiological implication for the clinical setting remains to be elucidated. Septic patients are at risk of developing both ventricular and atrial arrhythmias [135] and the incidence of arrhythmia was shown to correlate with plasma levels of cardiac troponins and histones [3], but to date could not be linked to changes in Cx43 distribution or expression. In the setting of trauma, new-onset atrial fibrillation has been described as an independent risk factor for poor outcome [56], but no large studies on the incidence and pathophysiology of ventricular arrhythmias in trauma patients exist. Nonetheless it seems physiologically plausible, that disintegration of the cell-cell conduction system renders patients prone to the occurrence of cardiac arrhythmia which in turn may worsen functional impairment of the heart. Additionally, a recent study could demonstrate, that mice with a knocked-out allele of a regulatory domain of Cx43 are more vulnerable to myocardial infarction [97], findings that hint to a more complex role of the connexins in cardiac damage. Further, various other connexins are expressed to a varying extent in the ventricles, atria and the conductive system of the heart [30]. Spatial alterations after trauma have so far only been described for Cx43 and associated CGJ proteins [73], but not for other connexins. Future studies will inevitably have to map the connexin expression and distribution across the heart tissue and its role in the development of pots-traumatic cardiac dysfunction and arrhythmias.

4.1.3.2 Quantitative analysis of Cx43 in heart tissue

Densitometric analysis of the IHC preparations demonstrated a slight but non-significant enhancement of the Cx43 signal, confirmed by WB analysis. Comparable investigations showed significantly increased levels of Cx43 in pig hearts after seventy-two hours [73] and moderately enhanced levels of Cx43 after twenty-four hours with normalisation after five days in rats [72]. In contrast, Cx43 protein levels have been reported to decrease during prolonged hypoxia [163], inflammation [45] and sepsis [24]. This contradiction in results

may stem from underlying differences in models or species. Furthermore, infectious inflammation, e.g. during sepsis may result in a different response than sterile inflammation, e.g. after trauma. As far as the presented results are concerned, considering the absence of statistical significance, the findings may be coincidental. Either way, more data is needed to establish a more comprehensive understanding of the time course of Cx43 expression and its related mechanisms.

Basically, quantitative changes of the Cx43 protein expression may either be an unspecific manifestation of structural cell damage or reflect a specific response to extern stimuli with protective properties. Modulation of Cx43 expression, as shown for atorvastatin appears to alleviate cell damage from ischemia/ reperfusion injury [14] and down-regulation of Cx43 in-vitro accelerates apoptosis of murine neonatal CM [160]. The slight increase of protein amounts of Cx43 in heart tissue homogenates may be an early response to acute stress after systemic inflammation.

4.2. Limitations of the study

4.2.1 Limitations of the methods

Some limitations to the methods applied must be considered. Exsanguination of the mice after experimental PT and HS was performed by piercing the apex of the heart. Although all considered sections of the hearts were located near to the atrioventricular valves and all heart sections have been screened for histological damage from exsanguination, this mode of exsanguination could have influenced certain measurements. Yet, as there were no detectable signs of damage in either groups but still significant differences concerning the evaluated damage markers, alleged influences can be considered negligible.

Immunohistochemistry, though valuable in determining spatial expression patterns and in distinguishing from epitope expression on cardiomyocytes and stromal cells has limitations in stating actual expression levels. As routine sections are about 4 µm in thickness, cells are typically cut on different levels, i.e. epitopes from the surface as well as the cytosol are exposed. Therefore, high levels of antibody-binding do not necessarily reflect high levels of functional active, i.e. surface-bound epitopes. This holds especially true for receptor

expression as shown for C5aR1 and C3aR, but also for HMGB1, which might be ligated to its respective surface receptor or be residual in the extracellular matrix or the cytosol.

Another point of criticism may be the lack of time curves in measurements, rendering the data from the PT and HS trauma model a mere snapshot of a range of parameters that are very likely to behave dynamically upon the organism's reaction in time. This gap in data is in large part due to the very limited blood volume of mice and also to the declared intention to limit animal numbers in scientific research. Although this study for the first time demonstrated alterations in the heart at such an early time point as four hours, the course of the physiological and pathophysiological reactions needs to be assessed in further studies.

4.2.2 Limitations of the study design

Due to small sample numbers in this study, the resulting power for statistical testing is low. Thus, non-significant results from statistical analysis may lead to type-II errors in data interpretation. Larger studies, both in-vitro and in-vivo are needed to confirm changes that were hinted to in this study. Another considerable deficiency of the presented work is, that the assessment of functional data on the heart and in-vitro CM such as echocardiography and life-cell imaging were beyond the scope of this study. Thus, it is not possible to conclude any functional impairment of the heart resulting from the molecular and structural changes presented. To fully understand the extent of cardiomyopathy after trauma, future studies need to address functional performance of the heart.

4.3. Conclusions

With a relevant model of PT and HS in mice, this study demonstrated substantial alterations to the molecular structure of the heart at an early time point after traumatic impact. These changes seem to occur prior to morphological changes and loss of cellular integrity as measured by a pathohistological damage score. Besides a significant increase of both specific and non-specific damage markers, indicating (sub-) cellular damage, changes in expression of complement receptors and inhibitors on the cell surface and spatial alterations of the CGJ protein Cx43 demonstrate, that the cardiac cells react to trauma, shock and systemic inflammation in manifold ways. The implication of these findings on the functionality of the heart and the circulation and further, their relevance for patient care in the emergency room and the intensive care unit remain to be evaluated. Nonetheless, the data of this study provide several conceivable mechanisms to consider for the improvement of outcomes in trauma patients, namely agents interacting with the complement system or DAMPs such as histones. For the latter, it was possible to show significant effects on the integrity of CM in-vitro. Last but not least, awareness of changes in the electrical conduction system may motivate trauma surgeons and anaesthetists to carefully monitor trauma patients in order to detect and treat life-threatening cardiac arrhythmias.

Considering the hypotheses, laid out under 1.5, the following conclusions can be drawn:

1) PT and HS do cause cellular damage as can be deducted from the significant increase in plasma levels of the cardiac specific damage marker cTnI. Moreover, tissue levels of HMGB1 were significantly increased after trauma and shock, which may at least in part be a manifestation of perceivable CM damage. However, the assumption, that this damage is perceivable via histomorphological evaluation could not be verified in this study.
2) Data from this study does not conclusively support the assumption, that the complement system is systemically activated, four hours after PT and HS as measured by plasma levels of C3a and C5a.
3) The traumatic impact and the following inflammatory response in the organism alter the detectable immunohistochemical signal of the complement receptors

C5aR1 and C3aR. These alterations in measured signal density can be assumed to reflect changes in respective protein expression.

4) MAC formation in cardiac tissue, as measured by signal density of IHC preparations was increased early after PT and HS. Since the detected differences were not statistically significant, the hypothesis cannot conclusively be supported.
5) PT and HS in a rodent model lead to delocalisation of Cx43, which was obviously detectable in IHC preparations. Quantitative evaluation of total protein levels of Cx43 in cardiac tissue via IHC analysis and WB did not show statistically significant differences.
6) Exposure of transdifferentiated human cardiomyocytes to bovine histones in-vitro did increase supernatant levels of cTnI and HMGB1, indicating cellular damage or an inflammatory reaction or both. C3a and C5a failed to mediate cTnI or HMGB1 release from human cardiomyocytes.

5 Summary

Morbidity and mortality from severe trauma and traumatic bleeding pose a significant burden on global health. Severe trauma, bleeding and consecutive shock result in a systemic inflammatory response syndrome (SIRS), evoked by a plethora of soluble mediators, both released from dying cells and actively secreted from parts of the immune system and may subsequently lead to multi-organ dysfunction. The pathophysiological mechanisms of post-traumatic SIRS resemble in part the systemic inflammation occurring during sepsis. In sepsis, these processes lead to an acute depression of the cardiac function with structural and morphological correlates in the cardiomyocytes (CM). While recent findings propose CM impairment after severe trauma as well, the exact pathological mechanisms and their implications for the clinical management remain unclear. The objective of this study was to characterise the morphological and molecular response of CM during the very early time period after traumatic injury.

To evaluate the impact of multiple trauma and haemorrhagic shock (HS), specimens from a well-characterised rodent model of polytrauma (PT) and HS were analysed. To simulate PT, mice were exposed to blunt chest trauma, traumatic brain injury and a closed femur fracture under anaesthesia. Shortly after infliction of PT, a pressure-controlled HS was induced by blood-drawing to achieve a mean arterial pressure of 30 mmHg. After 60 minutes (min) of shock, mice were reperfused with crystalloids, and pressure was kept steady for another 120 min via volume perfusion and catecholamine administration on demand. Then, mice were sacrificed in deep anaesthesia and hearts were explanted. Untreated animals served as controls.

From plasma, concentrations of cardiac troponin I (cTnI) and of the activated complement components 3 and 5 (C3a and C5a) were measured. To evaluate morphological alterations, a new histopathological damage score was developed and evaluated. High mobility group box nuclear protein 1 (HMGB1) and cleaved caspase 3 (CC3) as markers for cell-injury and apoptosis, as well as C5a receptor 1 (C5aR1) and C3a receptor (C3aR), the tissue-bound membrane attack complex (MAC) and the complement-inhibitor CD59 (cluster of differentiation 59) were analysed by immunohistochemistry (IHC). For spatial and

quantitative evaluation of the gap junction protein (CGJ) connexin 43 (Cx43), IHC analysis and western blotting were carried out. In additional translational experiments, cultured human transduced CM were treated with anaphylatoxins C3a and C5a and histones for 6 hours (h), 12 h and 24 h respectively and cTnI and HMGB1 concentrations in the supernatant were quantified.

The present study did show a significant increase of the cTnI plasma concentration, reflecting CM injury at this early time point after PT and HS. Evaluation of haematoxylin and eosin stained sections and IHC analysis of CC3 could rule out marked cellular necrosis or apoptosis as well as marked muscle ruptures and bleedings. However, PTHS animals presented with significantly increased tissue levels of HMGB1 levels, suggesting cell damage beyond the detectability of morphological alterations. Further, C5aR1 signal was significantly increased on IHC analysis while there was a significant drop in the C3aR signal. A significant decrease of the CD59 signal was accompanied with a slight, although not statistically significant increase of tissue bound MAC. These findings suggest an impact of the complement system on the response of CM to systemic inflammation after PT and HS, although in this study, no statistically significant change in C3a/ C5a plasma concentrations was detectable. IHC analysis of Cx43 showed spatial alterations and possible disruption of the CGJ, while quantification of Cx43 in the tissue did fail to show significant results. On translation into an in-vitro model of human CM, release of HMGB1 and cTnI could be reproduced by treatment with histones representing circulating damage-associated molecular patterns, whereas stimulations with C3a and C5a did not cause an increase of those markers in the supernatant.

The results of this study provide first evidence of an early cardiac cell injury 4 h after PT and HS both on a systemic and a cellular level in the absence of microscopically perceivable CM damage. Further changes of the expression pattern of complement receptors, MAC and CD59 could be observed, leading to the assumption that the complement system is involved in the post-traumatic response of CM. The disintegration of the important CGJ protein Cx43 may lead to arrhythmia and further aggravate possible affections of the cardiac function after trauma and HS. To which extent these structural alterations translate to a clinically significant impairment of the cardiac function needs to be addressed in future investigations.

6 References

1. Abrams S T, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang S S, Brohi K, Kipar A, Yu W, Wang G, Toh C: Circulating histones are mediators of trauma-associated lung injury. Am. J. Respir. Crit. Care Med., 187: 160-169(2013)

2. Alcalá H E, Sharif M Z, Samari G: Social Determinants of Health, Violent Radicalization, and Terrorism: A Public Health Perspective. Health Equity, 1: 87-95(2017)

3. Alhamdi Y, Abrams S T, Cheng Z, Jing S, Su D, Liu Z, Lane S, Welters I, Wang G, Toh C: Circulating Histones Are Major Mediators of Cardiac Injury in Patients With Sepsis. Crit. Care Med., 43: 2094-2103(2015)

4. Ali Ali B, Lefering R, Fortun Moral M, Belzunegui Otano T: Epidemiological comparison between the Navarra Major Trauma Registry and the German Trauma Registry (TR-DGU(R)). Scand. J. Trauma. Resusc. Emerg. Med., 25: 107(2017)

5. Allam R, Darisipudi M N, Tschopp J, Anders H J: Histones trigger sterile inflammation by activating the NLRP3 inflammasome. Eur. J. Immunol., 43: 3336-3342(2013)

6. Allam R, Scherbaum C R, Darisipudi M N, Mulay S R, Hägele H, Lichtnekert J, Hagemann J H, Rupanagudi K V, Ryu M, Schwarzenberger C, Hohenstein B, Hugo C, Uhl B, Reichel C A, Krombach F, Monestier M, Liapis H, Moreth K, Schaefer L, Anders H: Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. J. Am. Soc. Nephrol., 23: 1375-1388(2012)

7. Amara U, Flierl M A, Rittirsch D, Klos A, Chen H, Acker B, Bruckner U B, Nilsson B, Gebhard F, Lambris J D, Huber-Lang M: Molecular intercommunication between the complement and coagulation systems. J. Immunol., 185: 5628-5636(2010)

8. Atefi G, Zetoune F S, Herron T J, Jalife J, Bosmann M, Al-Aref R, Sarma J V, Ward P A: Complement dependency of cardiomyocyte release of mediators during sepsis. FASEB J., 25: 2500-2508(2011)

9. Avlas O, Fallach R, Shainberg A, Porat E, Hochhauser E: Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. Antioxid. Redox Signal., 15: 1895-1909(2011)

10. Baker S P, O'Neill B, Haddon W, Long W B: The injury severity score: a method for describing patients with multiple injuries and evaluating emergency care. J. Trauma, 14: 187-196(1974)

11. Bansal V, Fortlage D, Lee J G, Costantini T, Potenza B, Coimbra R: Hemorrhage is More Prevalent than Brain Injury in Early Trauma Deaths: The Golden Six Hours. Eur. J. Trauma. Emerg. Surg., 35: 26-30(2009)

12. Baroldi G, Mittleman R E, Parolini M, Silver M D, Fineschi V: Myocardial contraction bands. Definition, quantification and significance in forensic pathology. Int. J. Legal Med., 115: 142-151(2001)

13. Bertinchant J P, Polge A, Mohty D, Nguyen-Ngoc-Lam R, Estorc J, Cohendy R, Joubert P, Poupard P, Fabbro-Peray P, Monpeyroux F, Poirey S, Ledermann B, Raczka F, Brunet J, Nigond J, de la Coussaye, J E: Evaluation of incidence, clinical significance, and prognostic value of circulating cardiac troponin I and T elevation in hemodynamically stable patients with suspected myocardial contusion after blunt chest trauma. J. Trauma, 48: 924-931(2000)

14. Bian B, Yu X, Wang Q, Teng T, Nie J: Atorvastatin protects myocardium against ischemia-reperfusion arrhythmia by increasing Connexin 43 expression: A rat model. Eur. J. Pharmacol., 768: 13-20(2015)

15. Bolliger D, Görlinger K, Tanaka K A: Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. Anesthesiology, 113: 1205-1219(2010)

16. Bosmann M, Grailer J J, Ruemmler R, Russkamp N F, Zetoune F S, Sarma J V, Standiford T J, Ward P A: Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. FASEB J., 27: 5010-5021(2013)

17. Boyd J H, Mathur S, Wang Y, Bateman R M, Walley K R: Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. Cardiovasc. Res., 72: 384-393(2006)

18. Braun C K, Kalbitz M, Halbgebauer R, Eisele P, Messerer D A C, Weckbach S, Schultze A, Braumuller S, Gebhard F, Huber-Lang M S: Early structural changes of the heart after experimental polytrauma and hemorrhagic shock. PLoS One, 12: e0187327(2017)

19. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss D S, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. Science, 303: 1532-1535(2004)

20. Burk A, Martin M, Flierl M A, Rittirsch D, Helm M, Lampl L, Bruckner U, Stahl G L, Blom A M, Perl M, Gebhard F, Huber-Lang M: Early complementopathy after multiple injuries in humans. Shock, 37: 348-354(2012)

21. Butcher N E, D'Este C, Balogh Z J: The quest for a universal definition of polytrauma: a trauma registry-based validation study. J. Trauma. Acute Care. Surg., 77: 620-623(2014)

22. Butcher N, Balogh Z: Update on the definition of polytrauma. Eur. J. Trauma Emerg. Surg., 40: 107-111(2014)

23. Campobasso C P, Dell'Erba A S, Addante A, Zotti F, Marzullo A, Colonna M F: Sudden cardiac death and myocardial ischemia indicators: a comparative study of four immunohistochemical markers. Am. J. Forensic Med. Pathol., 29: 154-161(2008)

24. Celes M R, Torres-Duenas D, Alves-Filho J C, Duarte D B, Cunha F Q, Rossi M A: Reduction of gap and adherens junction proteins and intercalated disc structural

remodeling in the hearts of mice submitted to severe cecal ligation and puncture sepsis. Crit. Care Med., 35: 2176-2185(2007)

25. Celes M R N, Prado C M, Rossi M A: Sepsis: Going to the Heart of the Matter. Pathobiology, 80: 70(2012)

26. Chen R, Kang R, Fan X, Tang D: Release and activity of histone in diseases. Cell. Death Dis., 5: e1370(2014)

27. Cohen M J, Brohi K, Calfee C S, Rahn P, Chesebro B B, Christiaans S C, Carles M, Howard M, Pittet J: Early release of high mobility group box nuclear protein 1 after severe trauma in humans: role of injury severity and tissue hypoperfusion. Crit. Care, 13: R174(2009)

28. Copes W S, Champion H R, Sacco W J, Lawnick M M, Keast S L, Bain L W: The Injury Severity Score revisited. J. Trauma, 28: 69-77(1988)

29. Danon A, Zeevi-Levin N, Pinkovich D Y, Michaeli T, Berkovich A, Flugelman M, Eldar Y C, Rosen M R, Binah O: Hypoxia causes connexin 43 internalization in neonatal rat ventricular myocytes. Gen. Physiol. Biophys., 29: 222-233(2010)

30. Davis L M, Kanter H L, Beyer E C, Saffitz J E: Distinct gap junction protein phenotypes in cardiac tissues with disparate conduction properties. J. Am. Coll. Cardiol., 24: 1124-1132(1994)

31. Demir F, Guzel A, Kat C, Karadeniz C, Akdemir U, Okuyucu A, Gacar A, Ozdemir S, Guvenc T: A combination of methylprednisolone and quercetin is effective for the treatment of cardiac contusion following blunt chest trauma in rats. Braz. J. Med. Biol. Res., 47: 766-772(2014)

32. Denk S, Weckbach S, Eisele P, Braun C K, Wiegner R, Ohmann J J, Wrba L, Hoenes F M, Kellermann P, Radermacher P, Wachter U, Hafner S, McCook O, Schultze A, Palmer A, Braumuller S, Gebhard F, Huber-Lang M: Role of Hemorrhagic Shock in Experimental Polytrauma. Shock, 49: 154-163(2018)

33. Diener K R, Al-Dasooqi N, Lousberg E L, Hayball J D: The multifunctional alarmin HMGB1 with roles in the pathophysiology of sepsis and cancer. Immunol. Cell Biol., 91: 443(2013)

34. Ding H, Yang J, Chen P, Yang J, Bo S, Ding J, Yu Q: The HMGB1-TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis. Gene, 527: 389-393(2013)

35. Drouin S M, Kildsgaard J, Haviland J, Zabner J, Jia H P, McCray P B, Tack B F, Wetsel R A: Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. J. Immunol., 166: 2025-2032(2001)

36. Dunbar H: The Medicine and Surgery of Homer. Br. Med. J., 1: 48-51(1880)

37. Edouard A R, Felten M, Hebert J, Cosson C, Martin L, Benhamou D: Incidence and significance of cardiac troponin I release in severe trauma patients. Anesthesiology, 101: 1262-1268(2004)

38. Ehrnthaller C, Amara U, Weckbach S, Kalbitz M, Huber-Lang M, Bahrami S: Alteration of complement hemolytic activity in different trauma and sepsis models. J. Inflamm. Res., 5: 59-66(2012)

39. Ehrnthaller C, Ignatius A, Gebhard F, Huber-Lang M: New insights of an old defense system: structure, function, and clinical relevance of the complement system. Mol. Med., 17: 317-329(2011)

40. Ekaney M L, Otto G P, Sossdorf M, Sponholz C, Boehringer M, Loesche W, Rittirsch D, Wilharm A, Kurzai O, Bauer M, Claus R A: Impact of plasma histones in human sepsis and their contribution to cellular injury and inflammation. Crit. Care, 18: 543(2014)

41. Faist E, Baue A E, Dittmer H, Heberer G: Multiple organ failure in polytrauma patients. J. Trauma, 23: 775-787(1983)

42. Fattahi F, Kalbitz M, Malan E A, Abe E, Jajou L, Huber-Lang M S, Bosmann M, Russell M W, Zetoune F S, Ward P A: Complement-induced activation of MAPKs and Akt during sepsis: role in cardiac dysfunction. FASEB J., 31: 4129-4139(2017)

43. Fattahi F, Ward P A: Complement and sepsis-induced heart dysfunction. Mol. Immunol., 84: 57-64(2017)

44. Fauvel H, Marchetti P, Chopin C, Formstecher P, Nevière R: Differential effects of caspase inhibitors on endotoxin-induced myocardial dysfunction and heart apoptosis. Am. J. Physiol. Heart Circ. Physiol., 280: H1614(2001)

45. Fernandez-Cobo M, Gingalewski C, Drujan D, De Maio A: Downregulation of connexin 43 gene expression in rat heart during inflammation. The role of tumour necrosis factor. Cytokine, 11: 216-224(1999)

46. Frohlich M, Lefering R, Probst C, Paffrath T, Schneider M M, Maegele M, Sakka S G, Bouillon B, Wafaisade A, Committee on Emergency Medicine, Intensive Care and Trauma Management of the German Trauma Society Sektion NIS: Epidemiology and risk factors of multiple-organ failure after multiple trauma: an analysis of 31,154 patients from the TraumaRegister DGU. J. Trauma. Acute Care. Surg., 76: 8(2014)

47. Ganter M T, Brohi K, Cohen M J, Shaffer L A, Walsh M C, Stahl G L, Pittet J F: Role of the alternative pathway in the early complement activation following major trauma. Shock, 28: 29-34(2007)

48. Ganz J C: Head injuries in the 18th century: the management of the damaged brain. Neurosurgery, 73: 75; discussion 176(2013)

49. Gawande N, Tumram N, Dongre A: Cardiac Changes in Hospitalized Patients of Trauma. Shock, 42: 211-217(2014)

50. Gewurz H, Zhang X H, Lint T F: Structure and function of the pentraxins. Curr. Opin. Immunol., 7: 54-64(1995)

51. Ghannaee Arani M, Fakharian E, Ardjmand A, Mohammadian H, Mohammadzadeh M, Sarbandi F: Ibn Sina's (Avicenna) Contributions in the Treatment of Traumatic Injuries. Trauma. Mon., 17: 301-304(2012)

52. Giannoudis P V, Harwood P J, Court-Brown C, Pape H C: Severe and multiple trauma in older patients; incidence and mortality. Injury, 40: 362-367(2009)

53. Goddard C M, Allard M F, Hogg J C, Walley K R: Myocardial morphometric changes related to decreased contractility after endotoxin. Am. J. Physiol., 270: 1446(1996)

54. Hack C E, De Groot E R, Felt-Bersma R J, Nuijens J H, Strack Van Schijndel, R J, Eerenberg-Belmer A J, Thijs L G, Aarden L A: Increased plasma levels of interleukin-6 in sepsis. Blood, 74: 1704-1710(1989)

55. Hack C E, Nuijens J H, Felt-Bersma R J, Schreuder W O, Eerenberg-Belmer A J, Paardekooper J, Bronsveld W, Thijs L G: Elevated plasma levels of the anaphylatoxins C3a and C4a are associated with a fatal outcome in sepsis. Am. J. Med., 86: 20-26(1989)

56. Hadjizacharia P, O'Keeffe T, Brown C V R, Inaba K, Salim A, Chan L S, Demetriades D, Rhee P: Incidence, risk factors, and outcomes for atrial arrhythmias in trauma patients. Am. Surg., 77: 634-639(2011)

57. Halbgebauer R, Braun C K, Denk S, Mayer B, Cinelli P, Radermacher P, Wanner G A, Simmen H P, Gebhard F, Rittirsch D, Huber-Lang M: Hemorrhagic shock drives glycocalyx, barrier and organ dysfunction early after polytrauma. J. Crit. Care, 44: 229-237(2017)

58. Hashmi S, Al-Salam S: Acute myocardial infarction and myocardial ischemia-reperfusion injury: a comparison. Int. J. Clin. Exp. Pathol., 8: 8786-8796(2015)

59. Hecke F, Schmidt U, Kola A, Bautsch W, Klos A, Köhl J: Circulating complement proteins in multiple trauma patients--correlation with injury severity, development of sepsis, and outcome. Crit. Care Med., 25: 2015-2024(1997)

60. Hoesel L M, Niederbichler A D, Schaefer J, Ipaktchi K R, Gao H, Rittirsch D, Pianko M J, Vogt P M, Sarma J V, Su G L, Arbabi S, Westfall M V, Wang S C, Hemmila M R, Ward P A: C5a-Blockade Improves Burn-Induced Cardiac Dysfunction. J. Immunol., 178: 7902-7910(2007)

61. Hoesel L M, Niederbichler A D, Ward P A: Complement-related molecular events in sepsis leading to heart failure. Mol. Immunol., 44: 95-102(2007)

62. Horst K, Hildebrand F, Pfeifer R, Hübenthal S, Almahmoud K, Sassen M, Steinfeldt T, Wulf H, Ruchholtz S, Pape H C, Eschbach D: Impact of haemorrhagic shock intensity on the dynamic of alarmins release in porcine poly-trauma animal model. Eur. J. Trauma. Emerg. Surg., 42: 67-75(2016)

63. Horstick G, Heimann A, Götze O, Hafner G, Berg O, Böhmer P, Becker P, Darius H, Rupprecht H J, Loos M, Bhakdi S, Meyer J, Kempski O: Intracoronary application of C1 esterase inhibitor improves cardiac function and reduces myocardial necrosis in an experimental model of ischemia and reperfusion. Circulation, 95: 701-708(1997)

64. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, Liao X, Billiar T, Xu J, Esmon C T, Tsung A: Endogenous histones function as alarmins in sterile inflammatory liver injury through Toll-like receptor 9 in mice. Hepatology, 54: 999-1008(2011)

65. Huber S, Biberthaler P, Delhey P, Trentzsch H, Winter H, van Griensven M, Lefering R, Huber-Wagner S: Predictors of poor outcomes after significant chest trauma in multiply injured patients: a retrospective analysis from the German Trauma Registry (Trauma Register DGU®). Scand. J. Trauma. Resusc. Emerg. Med., 22: 52(2014)

66. Huber-Lang M, Lambris J D, Ward P A: Innate immune responses to trauma. Nat. Immunol., 19: 327-341(2018)

67. Huebener P, Pradere J P, Hernandez C, Gwak G Y, Caviglia J M, Mu X, Loike J D, Jenkins R E, Antoine D J, Schwabe R F: The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. J. Clin. Invest., 125: 539-550(2015)

68. Hundeshagen G, Herndon D N, Clayton R P, Wurzer P, McQuitty A, Jennings K, Branski L, Collins V N, Marques N R, Finnerty C C, Suman O E, Kinsky M P: Long-term effect of critical illness after severe paediatric burn injury on cardiac function in adolescent survivors: an observational study. Lancet Child Adolesc. Health, 1: 293-301(2017)

69. Hunter J D, Doddi M: Sepsis and the heart. Br. J. Anaesth., 104: 3-11(2010)

70. Ilczuk T, Wasiutynski A, Wilczek E, Gornicka B: The study of the protein complement in myocardial infarction. Immunol. Lett., 162: 262-268(2014)

71. Johnson N B, Hayes L D, Brown K, Hoo E C, Ethier K A: CDC National Health Report: leading causes of morbidity and mortality and associated behavioral risk and protective factors--United States, 2005-2013. MMWR supplements, 63: 3-27(2014)

72. Kalbitz M, Amann E M, Bosch B, Palmer A, Schultze A, Pressmar J, Weber B, Wepler M, Gebhard F, Schrezenmeier H, Brenner R, Huber-Lang M: Experimental blunt chest trauma-induced myocardial inflammation and alteration of gap-junction protein connexin 43. PLoS One, 12: e0187270(2017)

73. Kalbitz M, Schwarz S, Weber B, Bosch B, Pressmar J, Hoenes F M, Braun C K, Horst K, Simon T P, Pfeifer R, Stormann P, Hummler H, Gebhard F, Pape H C, Huber-Lang M, Hildebrand F, TREAT Research Group: Cardiac Depression in Pigs after Multiple Trauma - Characterization of Posttraumatic Structural and Functional Alterations. Sci. Rep., 7: 1(2017)

74. Kalbitz M, Fattahi F, Grailer J J, Jajou L, Malan E A, Zetoune F S, Huber-Lang M, Russell M W, Ward P A: Complement-induced activation of the cardiac NLRP3 inflammasome in sepsis. FASEB J., 30: 3997-4006(2016)

75. Kalbitz M, Fattahi F, Herron T J, Grailer J J, Jajou L, Lu H, Huber-Lang M, Zetoune F S, Sarma J V, Day S M, Russell M W, Jalife J, Ward P A: Complement Destabilizes Cardiomyocyte Function In Vivo after Polymicrobial Sepsis and In Vitro. J. Immunol., 197: 2353-2361(2016)

76. Kalbitz M, Grailer J J, Fattahi F, Jajou L, Herron T J, Campbell K F, Zetoune F S, Bosmann M, Sarma J V, Huber-Lang M, Gebhard F, Loaiza R, Valdivia H H, Jalife J, Russell M W, Ward P A: Role of extracellular histones in the cardiomyopathy of sepsis. FASEB J., 29: 2185-2193(2015)

77. Kalbitz M, Pressmar J, Stecher J, Weber B, Weiss M, Schwarz S, Miltner E, Gebhard F, Huber-Lang M: The Role of Troponin in Blunt Cardiac Injury After Multiple Trauma in Humans. World J. Surg., (2016)

78. Kanse S M, Gallenmueller A, Zeerleder S, Stephan F, Rannou O, Denk S, Etscheid M, Lochnit G, Krueger M, Huber-Lang M: Factor VII-activating protease is activated in multiple trauma patients and generates anaphylatoxin C5a. J. Immunol., 188: 2858-2865(2012)

79. Karch S B, Billingham M E: Myocardial contraction bands revisited. Hum. Pathol., 17: 9-13(1986)

80. Kildsgaard J, Hollmann T J, Matthews K W, Bian K, Murad F, Wetsel R A: Cutting edge: targeted disruption of the C3a receptor gene demonstrates a novel protective anti-inflammatory role for C3a in endotoxin-shock. J. Immunol., 165: 5406-5409(2000)

81. Knoester H, Sol J J, Ramsodit P, Kuipers I M, Clur S B, Bos A P: Cardiac function in pediatric septic shock survivors. Arch. Pediatr. Adolesc. Med., 162: 1164-1168(2008)

82. Korff S, Katus H A, Giannitsis E: Differential diagnosis of elevated troponins. Heart, 92: 987-993(2006)

83. Kumar A, Brar R, Wang P, Dee L, Skorupa G, Khadour F, Schulz R, Parrillo J E: Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. Am. J. Physiol., 276: 265(1999)

84. Kumar A, Thota V, Dee L, Olson J, Uretz E, Parrillo J E: Tumor necrosis factor alpha and interleukin 1beta are responsible for in vitro myocardial cell depression induced by human septic shock serum. J. Exp. Med., 183: 949-958(1996)

85. Kumar A, Kumar A, Paladugu B, Mensing J, Parrillo J E: Transforming growth factor-beta1 blocks in vitro cardiac myocyte depression induced by tumor necrosis factor-alpha, interleukin-1beta, and human septic shock serum. Crit. Care Med., 35: 358-364(2007)

86. Kutcher M E, Xu J, Vilardi R F, Ho C, Esmon C T, Cohen M J: Extracellular histone release in response to traumatic injury: implications for a compensatory role of activated protein C. J. Trauma Acute. Care Surg., 73: 1389-1394(2012)

87. Kytö V, Saraste A, Saukko P, Henn V é, Pulkki K, Vuorinen T, Voipio-Pulkki L: Apoptotic cardiomyocyte death in fatal myocarditis. Am. J. Cardiol., 94: 746-750(2004)

88. Landesberg G, Jaffe A S, Gilon D, Levin P D, Goodman S, Abu-Baih A, Beeri R, Weissman C, Sprung C L, Landesberg A: Troponin elevation in severe sepsis and septic shock: the role of left ventricular diastolic dysfunction and right ventricular dilatation*. Crit. Care Med., 42: 790-800(2014)

89. Lappegard K T, Garred P, Jonasson L, Espevik T, Aukrust P, Yndestad A, Mollnes T E, Hovland A: A vital role for complement in heart disease. Mol. Immunol., 61: 126-134(2014)

90. Levy R M, Mollen K P, Prince J M, Kaczorowski D J, Vallabhaneni R, Liu S, Tracey K J, Lotze M T, Hackam D J, Fink M P, Vodovotz Y, Billiar T R: Systemic inflammation and remote organ injury following trauma require HMGB1. Am. J. Physiol. Regul. Integr. Comp. Physiol., 293: 1538(2007)

91. Li X, Luo R, Jiang R, Meng X, Wu X, Zhang S, Hua W: The role of the Hsp90/Akt pathway in myocardial calpain-induced caspase-3 activation and apoptosis during sepsis. BMC Cardiovasc. Disord., 13: 8(2013)

92. Liu H, Xiao X, Sun C, Sun D, Li Y, Yang M: Systemic inflammation and multiple organ injury in traumatic hemorrhagic shock. Front. Biosci. (Landmark Ed), 20: 927-933(2015)

93. Liu Y, Yu M, Shou S, Chai Y: Sepsis-Induced Cardiomyopathy: Mechanisms and Treatments. Front. Immunol., 8: 1021(2017)

94. Lord J M, Midwinter M J, Chen Y, Belli A, Brohi K, Kovacs E J, Koenderman L, Kubes P, Lilford R J: The systemic immune response to trauma: an overview of pathophysiology and treatment. Lancet, 384: 1455-1465(2014)

95. Maass D L, Hybki D P, White J, Horton J W: The time course of cardiac NF-kappaB activation and TNF-alpha secretion by cardiac myocytes after burn injury: contribution to burn-related cardiac contractile dysfunction. Shock, 17: 293-299(2002)

96. Maass D L, White J, Horton J W: Il-1β And Il-6 Act Synergistically With Tnf-α To Alter Cardiac Contractile Function After Burn Trauma. Shock, 18: 360-366(2002)

97. Maass K, Chase S E, Lin X, Delmar M: Cx43 CT domain influences infarct size and susceptibility to ventricular tachyarrhythmias in acute myocardial infarction. Cardiovasc. Res., 84: 361-367(2009)

98. Marras E, Cukon-Buttignoni S, Berton G, Tamari W: Cardiac injury as a rare cause of cardiogenic shock following polytrauma. Eur. Heart J., 35: 3231(2014)

99. Marshall K D, Edwards M A, Krenz M, Davis J W, Baines C P: Proteomic mapping of proteins released during necrosis and apoptosis from cultured neonatal cardiac myocytes. Am. J. Physiol., Cell Physiol., 306: 639(2014)

100. Masiakos P T, Griggs C: The Quiet Room. N. Engl. J. Med., 377: 2411-2412(2017)

101. Mathey D, Schofer J, Schäfer H J, Hamdoch T, Joachim H C, Ritgen A, Hugo F, Bhakdi S: Early accumulation of the terminal complement-complex in the ischaemic myocardium after reperfusion. Eur. Heart J., 15: 418-423(1994)

102. McCain M L, Desplantez T, Geisse N A, Rothen-Rutishauser B, Oberer H, Parker K K, Kleber A G: Cell-to-cell coupling in engineered pairs of rat ventricular cardiomyocytes: relation between Cx43 immunofluorescence and intercellular electrical conductance. Am. J. Physiol. Heart Circ. Physiol., 302: 443(2012)

103. Mehta N J, Khan I A, Gupta V, Jani K, Gowda R M, Smith P R: Cardiac troponin I predicts myocardial dysfunction and adverse outcome in septic shock. Int. J. Cardiol., 95: 13-17(2004)

104. Mokdad A H, Forouzanfar M H, Daoud F, Mokdad A A, El Bcheraoui C, Moradi-Lakeh M, Kyu H H, Barber R M, Wagner J, Cercy K, Kravitz H, Coggeshall M, Chew A, O'Rourke K F, Steiner C, Tuffaha M, Charara R, Al-Ghamdi E A, Adi Y, Afifi R A, Alahmadi H, AlBuhairan F, Allen N, AlMazroa M, Al-Nehmi A A, AlRayess Z, Arora M, Azzopardi P, Barroso C, Basulaiman M, Bhutta Z A, Bonell C, Breinbauer C, Degenhardt L, Denno D, Fang J, Fatusi A, Feigl A B, Kakuma R, Karam N, Kennedy E, Khoja T A, Maalouf F, Obermeyer C M, Mattoo A, McGovern T, Memish Z A, Mensah G A, Patel V, Petroni S, Reavley N, Zertuche D R, Saeedi M, Santelli J, Sawyer S M, Ssewamala F, Taiwo K, Tantawy M, Viner R M, Waldfogel J, Zuniga M P, Naghavi M, Wang H, Vos T, Lopez A D, Al Rabeeah A A, Patton G C, Murray C J: Global burden of diseases, injuries, and risk factors for young people's health during 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet, 387: 2383-2401(2016)

105. Morita S, Furukawa S, Nishi K: Classification of contraction bands using immunohistochemistry. Am. J. Forensic Med. Pathol., 36: 23-26(2015)

106. Mueller M, Herzog C, Larmann J, Schmitz M, Hilfiker-Kleiner D, Gessner J E, Theilmeier G: The receptor for activated complement factor 5 (C5aR) conveys myocardial ischemic damage by mediating neutrophil transmigration. Immunobiology, 218: 1131-1138(2013)

107. Neher M D, Weckbach S, Flierl M A, Huber-Lang M S, Stahel P F: Molecular mechanisms of inflammation and tissue injury after major trauma--is complement the "bad guy"? J. Biomed. Sci., 18: 90(2011)

108. Niederbichler A D, Hoesel L M, Westfall M V, Gao H, Ipaktchi K R, Sun L, Zetoune F S, Su G L, Arbabi S, Sarma J V, Wang S C, Hemmila M R, Ward P A: An essential role for complement C5a in the pathogenesis of septic cardiac dysfunction. J. Exp. Med., 203: 53-61(2006)

109. Niederbichler A D, Westfall M V, Su G L, Donnerberg J, Usman A, Vogt P M, Ipaktchi K R, Arbabi S, Wang S C, Hemmila M R: Cardiomyocyte Function After Burn Injury And Lipopolysaccharide Exposure: Single-cell Contraction Analysis And Cytokine Secretion Profile. Shock, 25: 176-183(2006)

110. Nonaka M: Evolution of the complement system. Subcell. Biochem., 80: 31-43(2014)

111. Noris M, Remuzzi G: Overview of complement activation and regulation. Semin. Nephrol., 33: 479-492(2013)

112. Nußbaum B L, McCook O, Hartmann C, Matallo J, Wepler M, Antonucci E, Kalbitz M, Huber-Lang M, Georgieff M, Calzia E, Radermacher P, Hafner S: Left ventricular function during porcine-resuscitated septic shock with pre-existing atherosclerosis. Intensive Care Med. Exp., 4: 14(2016)

113. Oliveira G H M, Brann C N, Becker K, Thohan V, Koerner M M, Loebe M, Noon G P, Torre-Amione G: Dynamic expression of the membrane attack complex (MAC) of the complement system in failing human myocardium. Am. J. Cardiol., 97: 1626-1629(2006)

114. Osler T, Baker S P, Long W: A modification of the injury severity score that both improves accuracy and simplifies scoring. J. Trauma, 43: 6(1997)

115. Paffrath T, Lefering R, Flohé S: How to define severely injured patients? -- an Injury Severity Score (ISS) based approach alone is not sufficient. Injury, 45 Suppl 3: 64(2014)

116. Paredes R M, Reyna S, Vernon P, Tadaki D K, Dallelucca J J, Sheppard F: Generation of complement molecular complex C5b-9 (C5b-9) in response to poly-traumatic hemorrhagic shock and evaluation of C5 cleavage inhibitors in non-human primates. Int. Immunopharmacol., 54: 221-225(2018)

117. Pathan N, Franklin J L, Eleftherohorinou H, Wright V J, Hemingway C A, Waddell S J, Griffiths M, Dennis J L, Relman D A, Harding S E, Levin M: Myocardial depressant

effects of interleukin 6 in meningococcal sepsis are regulated by p38 mitogen-activated protein kinase. Crit. Care Med., 39: 1692-1711(2011)

118. Pearn J: Gunpowder, the Prince of Wales's feathers and the origins of modern military surgery. ANZ J. Surg., 82: 240-244(2012)

119. Peng Q, Li K, Smyth L A, Xing G, Wang N, Meader L, Lu B, Sacks S H, Zhou W: C3a and C5a promote renal ischemia-reperfusion injury. J. Am. Soc. Nephrol., 23: 1474-1485(2012)

120. Petrovic D, Zorc-Pleskovic R, Zorc M: Apoptosis and proliferation of cardiomyocytes in heart failure of different etiologies. Cardiovasc. Pathol., 9: 149-152(2000)

121. Pfeifer R, Tarkin I S, Rocos B, Pape H C: Patterns of mortality and causes of death in polytrauma patients--has anything changed? Injury, 40: 907-911(2009)

122. Prioreschi P: Skull trauma in Egyptian and Hippocratic medicine. Gesnerus, 50 (Pt 3-4): 167-178(1993)

123. Rajan G P, Zellweger R: Cardiac troponin I as a predictor of arrhythmia and ventricular dysfunction in trauma patients with myocardial contusion. J. Trauma, 57: 808; discussion 808(2004)

124. Ricklin D, Hajishengallis G, Yang K, Lambris J D: Complement: a key system for immune surveillance and homeostasis. Nat. Immunol., 11: 785-797(2010)

125. Riedemann N C, Neff T A, Guo R, Bernacki K D, Laudes I J, Sarma J V, Lambris J D, Ward P A: Protective effects of IL-6 blockade in sepsis are linked to reduced C5a receptor expression. J. Immunol., 170: 503-507(2003)

126. Rittirsch D, Flierl M A, Nadeau B A, Day D E, Huber-Lang M, Mackay C R, Zetoune F S, Gerard N P, Cianflone K, Köhl J, Gerard C, Sarma J V, Ward P A: Functional roles for C5a receptors in sepsis. Nat. Med., 14: 551-557(2008)

127. Rittirsch D, Redl H, Huber-Lang M: Role of complement in multiorgan failure. Clin. Dev. Immunol., 2012: 962927(2012)

128. Rossaint J, Zarbock A: Pathogenesis of Multiple Organ Failure in Sepsis. Crit. Rev. Immunol., 35: 277-291(2015)

129. Rutledge R, Hoyt D B, Eastman A B, Sise M J, Velky T, Canty T, Wachtel T, Osler T M: Comparison of the Injury Severity Score and ICD-9 diagnosis codes as predictors of outcome in injury: analysis of 44,032 patients. J. Trauma, 42: 489(1997)

130. Salim A, Velmahos G C, Jindal A, Chan L, Vassiliu P, Belzberg H, Asensio J, Demetriades D: Clinically significant blunt cardiac trauma: role of serum troponin levels combined with electrocardiographic findings. J. Trauma, 50: 237-243(2001)

131. Saraste A: Morphologic criteria and detection of apoptosis. Herz, 24: 189-195(1999)

132. Saraste A, Pulkki K: Morphologic and biochemical hallmarks of apoptosis. Cardiovasc. Res., 45: 528-537(2000)

133. Scaffidi P, Misteli T, Bianchi M E: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature, 418: 191-195(2002)

134. Sekiguchi R, Nonaka M: Evolution of the complement system in protostomes revealed by de novo transcriptome analysis of six species of Arthropoda. Dev. Comp. Immunol., 50: 58-67(2015)

135. Shahreyar M, Fahhoum R, Akinseye O, Bhandari S, Dang G, Khouzam R N: Severe sepsis and cardiac arrhythmias. Ann. Transl. Med., 6: 6(2018)

136. Shenoy K S, Jeevannavar S S, Baindoor P, Shetty S: Fatal blunt cardiac injury: are there any subtle indicators? BMJ Case Rep., 2014: (2014)

137. Skinner D L, Laing G L, Rodseth R N, Ryan L, Hardcastle T C, Muckart D J J: Blunt cardiac injury in critically ill trauma patients: A single centre experience. Injury, 46: 66-70(2015)

138. Smith E F, Egan J W, Bugelski P J, Hillegass L M, Hill D E, Griswold D E: Temporal relation between neutrophil accumulation and myocardial reperfusion injury. Am. J. Physiol., 255: 1060(1988)

139. Soriano F G, Nogueira A C, Caldini E G, Lins M H, Teixeira A C, Cappi S B, Lotufo P A, Bernik M M, Zsengeller Z, Chen M, Szabo C: Potential role of poly(adenosine 5'-diphosphate-ribose) polymerase activation in the pathogenesis of myocardial contractile dysfunction associated with human septic shock. Crit. Care Med., 34: 1073-1079(2006)

140. Spruijt N E, Visser T, Leenen L P: A systematic review of randomized controlled trials exploring the effect of immunomodulative interventions on infection, organ failure, and mortality in trauma patients. Crit. Care, 14: R150(2010)

141. Szebeni J, Baranyi L, Savay S, Götze O, Alving C R, Bünger R, Mongan P D: Complement activation during hemorrhagic shock and resuscitation in swine. Shock, 20: 347-355(2003)

142. Tada T, Okada H, Okada N, Tateyama H, Suzuki H, Takahashi Y, Eimoto T: Membrane attack complex of complement and 20 kDa homologous restriction factor (CD59) in myocardial infarction. Virchows Arch., 430: 327-332(1997)

143. Takasu O, Gaut J P, Watanabe E, To K, Fagley R E, Sato B, Jarman S, Efimov I R, Janks D L, Srivastava A, Bhayani S B, Drewry A, Swanson P E, Hotchkiss R S: Mechanisms of cardiac and renal dysfunction in patients dying of sepsis. Am. J. Respir. Crit. Care Med., 187: 509-517(2013)

144. Tao L, Liu H, Gao F, Qu Y, Christopher T A, Lopez B L, Ma X L: Mechanical traumatic injury without circulatory shock causes cardiomyocyte apoptosis: role of reactive nitrogen and reactive oxygen species. Am. J. Physiol. Heart Circ. Physiol., 288: 2811(2005)

145. Thekkudan J, Luckraz H, Ng A, Norell M: Tricuspid valve chordal rupture due to airbag injury and review of pathophysiological mechanisms. Interact. Cardiovasc. Thorac. Surg., 15: 555-557(2012)

146. Thurman J M, Holers V M: The central role of the alternative complement pathway in human disease. J. Immunol., 176: 1305-1310(2006)

147. Timmermans K, Kox M, Scheffer G J, Pickkers P: Danger in the Intensive Care Unit: Damps in Critically Ill Patients. Shock, 45: 108-116(2016)

148. Tornetta M A, Foley J J, Sarau H M, Ames R S: The mouse anaphylatoxin C3a receptor: molecular cloning, genomic organization, and functional expression. J. Immunol., 158: 5277-5282(1997)

149. Tsai K, Liang H, Yang Z, Lue S, Yang S, Hsu C: Early inactivation of PKCε associates with late mitochondrial translocation of Bad and apoptosis in ventricle of septic rat. J. Surg. Res., 186: 278-286(2014)

150. Tsukamoto T, Chanthaphavong R S, Pape H: Current theories on the pathophysiology of multiple organ failure after trauma. Injury, 41: 21-26(2010)

151. Unnewehr H, Rittirsch D, Sarma J V, Zetoune F, Flierl M A, Perl M, Denk S, Weiss M, Schneider M E, Monk P N, Neff T, Mihlan M, Barth H, Gebhard F, Ward P A, Huber-Lang M: Changes and regulation of the C5a receptor on neutrophils during septic shock in humans. J. Immunol., 190: 4215-4225(2013)

152. Väkevä A, Laurila P, Meri S: Loss of expression of protectin (CD59) is associated with complement membrane attack complex deposition in myocardial infarction. Lab. Invest., 67: 608-616(1992)

153. van Griensven M, Ricklin D, Denk S, Halbgebauer R, Braun C K, Schultze A, Hones F, Koutsogiannaki S, Primikyri A, Reis E, Messerer D, Hafner S, Radermacher P, Biglarnia A R, Resuello R R G, Tuplano J V, Mayer B, Nilsson K, Nilsson B, Lambris J D, Huber-Lang M: Protective Effects of the Complement Inhibitor Compstatin CP40 in Hemorrhagic Shock. Shock, (2018)

154. Vanzant E L, Lopez C M, Ozrazgat-Baslanti T, Ungaro R, Davis R, Cuenca A G, Gentile L F, Nacionales D C, Cuenca A L, Bihorac A, Leeuwenburgh C, Lanz J, Baker H V, McKinley B, Moldawer L L, Moore F A, Efron P A: Persistent Inflammation,

Immunosuppression and Catabolism Syndrome after Severe Blunt Trauma. J. Trauma. Acute Care Surg., 76: 21-30(2014)

155. Wafaisade A, Lefering R, Bouillon B, Sakka S G, Thamm O C, Paffrath T, Neugebauer E, Maegele M, Trauma Registry of the German Society for Trauma Surgery: Epidemiology and risk factors of sepsis after multiple trauma: an analysis of 29,829 patients from the Trauma Registry of the German Society for Trauma Surgery. Crit. Care Med., 39: 621-628(2011)

156. Wang H, Bloom O, Zhang M, Vishnubhakat J M, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue K R, Faist E, Abraham E, Andersson J, Andersson U, Molina P E, Abumrad N N, Sama A, Tracey K J: HMG-1 as a late mediator of endotoxin lethality in mice. Science, 285: 248-251(1999)

157. Ward P A, Gao H: Sepsis, complement and the dysregulated inflammatory response. J. Cell. Mol. Med., 13: 4154-4160(2009)

158. Xu H, Yao Y, Su Z, Yang Y, Kao R, Martin C M, Rui T: Endogenous HMGB1 contributes to ischemia-reperfusion-induced myocardial apoptosis by potentiating the effect of TNF-α/JNK. Am. J. Physiol. Heart Circ. Physiol., 300: 913(2011)

159. Yang R, Harada T, Mollen K P, Prince J M, Levy R M, Englert J A, Gallowitsch-Puerta M, Yang L, Yang H, Tracey K J, Harbrecht B G, Billiar T R, Fink M P: Anti-HMGB1 neutralizing antibody ameliorates gut barrier dysfunction and improves survival after hemorrhagic shock. Mol. Med., 12: 105-114(2006)

160. Yasui K, Kada K, Hojo M, Lee J K, Kamiya K, Toyama J, Opthof T, Kodama I: Cell-to-cell interaction prevents cell death in cultured neonatal rat ventricular myocytes. Cardiovasc. Res., 48: 68-76(2000)

161. Younger J G, Sasaki N, Waite M D, Murray H N, Saleh E F, Ravage Z B, Hirschl R B, Ward P A, Till G O, Ravage Z A: Detrimental effects of complement activation in hemorrhagic shock. J. Appl. Physiol., 90: 441-446(2001)

162. Yu M, Wang H, Ding A, Golenbock D T, Latz E, Czura C J, Fenton M J, Tracey K J, Yang H: HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. Shock, 26: 174-179(2006)

163. Zeevi-Levin N, Barac Y D, Reisner Y, Reiter I, Yaniv G, Meiry G, Abassi Z, Kostin S, Schaper J, Rosen M R, Resnick N, Binah O: Gap junctional remodeling by hypoxia in cultured neonatal rat ventricular myocytes. Cardiovasc. Res., 66: 64-73(2005)

164. Zhang B, Liu Y, Zhang J, Zhang X, Chen W, Yin X, Qi Y: Cortistatin protects myocardium from endoplasmic reticulum stress induced apoptosis during sepsis. Mol. Cell. Endocrinol., 406: 40-48(2015)

165. Zhang C, Mo M, Ding W, Liu W, Yan D, Deng J, Luo X, Liu J: High-mobility group box 1 (HMGB1) impaired cardiac excitation-contraction coupling by enhancing the sarcoplasmic reticulum (SR) Ca(2+) leak through TLR4-ROS signaling in cardiomyocytes. J. Mol. Cell. Cardiol., 74: 260-273(2014)

166. Zhang H, Qin G, Liang G, Li J, Barrington R A, Liu D: C5aR-mediated myocardial ischemia/reperfusion injury. Biochem. Biophys. Res. Commun., 357: 446-452(2007)

167. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser C J: Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature, 464: 104-107(2010)

168. Zhou Y, Li Y, Mu T: HMGB1 Neutralizing Antibody Attenuates Cardiac Injury and Apoptosis Induced by Hemorrhagic Shock/Resuscitation in Rats. Biol. Pharm. Bull., 38: 1150-1160(2015)

169. Zou L, Feng Y, Li Y, Zhang M, Chen C, Cai J, Gong Y, Wang L, Thurman J M, Wu X, Atkinson J P, Chao W: Complement factor B is the downstream effector of TLRs and plays an important role in a mouse model of severe sepsis. J. Immunol., 191: 5625-5635(2013)

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