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Mediators of trauma-induced secondary cardiac injury

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To my family

Let us choose for ourselves our path in life,
and let us try to strew that path with flowers
Émilie Du Châtelet (1706-1794)

Publication Index

Results from my doctoral thesis have partially been published in the following publications:

(**I**) Weber B, **Lackner I**, Baur M, Fois G, Gebhard F, Marzi I, Schrezenmeier H, Relja B, Kalbitz M. Effects of Circulating HMGB-1 and Histones on Cardiomyocytes-Hemadsorption of These DAMPs as Therapeutic Strategy after Multiple Trauma. *J Clin Med.* 2020 May 11;9(5):1421. doi: 10.3390/jcm9051421. (IF 3.303)

(**II**) Weber B*, **Lackner I***, Knecht D, Braun CK, Gebhard F, Huber-Lang M, Hildebrand F, Horst K, Pape HC, Ignatius A, Schrezenmeier H, Haffner-Luntzer M and Kalbitz M. Systemic and Cardiac Alterations after Long Bone Fracture. *Shock*. 2020 Dec;54(6):761-773. doi: 10.1097/SHK.0000000000001536. (IF 3.048)

(**III**) **Lackner I**, Weber B, Baur M, Fois G, Gebhard F, Pfeifer R, Cinelli P, Halvachizadeh S, Lipiski M, Cesarovic N, Schrezenmeier H, Huber-Lang M, Pape HC, Kalbitz M and TREAT Research Group. Complement activation and organ damage after trauma- differential immune response based on surgical treatment strategy. *Front Immunol*. 2020 Jan 31;11:64. doi: 10.3389/fimmu.2020.00064. eCollection 2020. (IF 6.429)

(**IV**) **Lackner I**, Weber B, Baur M, Haffner-Luntzer M, Eiseler T, Fois G, Gebhard F, Relja B, Marzi I, Pfeifer R, Halvachizadeh S, Lipiski M, Cesarovic N, Pape HC, Kalbitz M and TREAT Research Group. Midkine Is Elevated After Multiple Trauma and Acts Directly on Human Cardiomyocytes by Altering Their Functionality and Metabolism. *Front Immunol*. 2019 Aug 21;10:1920. doi: 10.3389/fimmu.2019.01920. eCollection 2019. (IF 6.429)

(**V**) **Lackner I***, Weber B*, Knecht D, Horst K, Relja B, Gebhard F, Pape HC, Huber-Lang M, Hildebrand F, Kalbitz M; TREAT research group. Cardiac Glucose and Fatty Acid Transport After Experimental Mono- and Polytrauma. *Shock*. 2020 May;53(5):620-629. doi: 10.1097/SHK.0000000000001400. (IF 3.048)

Copies of these publications are attached in the appendix.
Other publications with my contribution, which are not associated with this thesis:

Lackner I, Weber B, Miclau T, Holzwarth N, Baur M, Gebhard F, Teuben M, Halvachizadeh S, Cinelli P, Pfeifer R, Lipiski M, Cesarovic N, Haffner-Luntzer M, Pape HC, Kalbitz M, TREAT Research Group. Reaming of femoral fractures with different reaming irrigator aspirator systems shows distinct effects on cardiac function after experimental polytrauma. *J Orthop Res*. 2020 Aug 22. doi: 10.1002/jor.24830. (IF 3.14)

Baur M, Weber B, **Lackner I**, Gebhard F, Pfeifer R, Cinelli P, Halvachizadeh S, Teuben M, Lipiski M, Cesarovic N, Pape HC, Kalbitz M. Structural alterations and inflammation in the heart after multiple trauma followed by reamed versus non-reamed femoral nailing. *PLoS One*. 2020 Jun 25;15(6):e0235220. doi: 10.1371/journal.pone.0235220. eCollection 2020. (IF 2.776)

Haffner-Luntzer M, Weber B, Lam C, Fischer V, **Lackner I**, Ignatius A, Kalbitz M, Marcucio RS, Miclau T. A novel mouse model to study fracture healing of the proximal femur. *J Orthop Res*. 2020 Mar 31. doi: 10.1002/jor.24677. Online ahead of print. (IF 3.14)

Lackner I*, Weber B*, Chakraborty S, Braumüller S, Huber-Lang M, Gebhard F, Kalbitz M. Toll-Like Receptor-Mediated Cardiac Injury during Experimental Sepsis. *Mediators Inflamm*. 2020 Jan 10;2020:6051983. doi: 10.1155/2020/6051983. eCollection 2020. (IF 3.545)

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Weber B, Mendler MR, **Lackner I**, Pressmar J, Haffner-Luntzer M, Höfler S, Braun CK, Hummler H, Schwarz S, Kalbitz M. Tissue damage in the heart after cardiac arrest induced by asphyxia and hemorrhage in newborn pigs. *Pediatr Res*. 2019 Jul 23. Doi: 10.1038/s41390-019-0505-6. (IF 3.123)

Weber B, Mendler MR, **Lackner I**, von Zelewski A, Höfler S, Baur M, Braun CK, Hummler H, Schwarz S, Pressmar J, Kalbitz M. Lung injury after asphyxia and hemorrhagic shock in newborn piglets: Analysis of structural and inflammatory changes. *PloS One*. 2019 Jul 5;14(7):e0219211. Doi: 10.1371/journal.pone.0219211. eCollection 2019. (IF 2.776)

Foertsch S, **Lackner I**, Weber B, Füchsl AM, Langgartner D, Wirkert E, Peters S, Fois G, Pressmar J, Fegert JM, Frick M, Gündel H, Kalbitz M, Reber SO. Sensory contact to the stressor prevents recovery from structural and functional heart damage following psychosocial trauma. *Brain Behav Immun*. 2019 May 11. Pii: S0889-1591(19)30081-9. Doi: 10.1016/j.bbi.2019.05.013. (IF 6.306)

Haffner-Luntzer M, **Lackner I**, Liedert A, Fischer V, Ignatius A. Effects of low-magnitude high-frequency vibration on osteoblasts are dependent on estrogen receptor α signaling and cytoskeletal remodeling. *Biochem Biophys Res Commun*. 2018 Sep 18;503(4):2678-2684. Doi: 10.1016/j.bbrc.2018.08.023. Epub 2018 Aug 7. (IF 2.559)

Haffner-Luntzer M, Kovtun A, **Lackner I**, Mödinger Y, Hacker S, Liedert A, Tuckermann J, Ignatius A. Estrogen receptor α- (ERα), but not ERβ-signaling, is crucially involved in mechanostimulation of bone fracture healing by whole-body vibration. *Bone*. 2018 May;110:11-20. Doi: 10.1016/j.bone.2018.01.017. Epub 2018 Feb 3. (IF 4.455)

References referring to publications with my contribution are highlighted throughout my dissertation.

Table of Contents

List of Abbreviations

ACE	Adverse cardiac events
AIS	Abbreviated Injury Scale
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ATLS	Advanced Trauma Life Support
C3a	Complement factor C3a
C5a	Complement factor C5a
C3aR	C3a receptor
C5aR1/C5aR2	C5a receptor 1/ C5a receptor 2
CCI	Chronic critical illness
CD36/FAT	Fatty acid translocase
CLP	Cecal ligation and puncture
CLRs	C-type lectin receptors
CMs	Cardiomyocytes
CPK	Creatinine phosphokinase
CRP	C-reactive protein
Cx 40/43/45	Connexin 40/43/45
DAMPs	Damage-associated molecular patterns
DALYs	Disability-adjusted life years
DCO	Damage control orthopaedics
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
ETC	Early total care
GLUT1/4	Glucose transporter 1/4
HFABP	Heart fatty acid binding protein
HMGB1	High-mobility group box 1 protein
HS	Hemorrhagic shock
HSPs	Heat shock proteins
ICU	Intensive care unit
IFC	Inflammation cocktail
IL	Interleukin
ISS	Injury severity score

LCFA	Long-chain free fatty acids
LPS	Lipopolysaccharides
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MBL	Mannose-binding lectin
Mdk	Midkine
mtDNA	Mitochondrial DNA
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
NCX	Sodium-calcium exchanger
NETs	Neutrophil extracellular traps
NFκB	Nuclear factor kappa B
NLRs	Nod-like receptors
NLRP3	Nod-like receptor family protein 3
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
PTC	Polytrauma cocktail
RAGE	Receptor for advanced glycation end products
RIG-I	Retinoic-acid-inducible-protein 1
ROS	Reactive oxygen species
RT-qPCR	Reverse transcribed quantitative polymerase chain reaction
SERCA2A	Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a
SIRS	Systemic inflammatory response syndrome
SLC27A1/A6	Solute carrier protein 27 type A1/A6
SOFA	Sequential organ failure assessment
SR	Sarcoplasmic reticulum
ssRNA	Single stranded RNA
TIC	Trauma-induced coagulopathy
TISCI	Trauma-induced secondary cardiac injury
TLRs 2/4/9	Toll-like receptors 2/4/9
TNF	Tumor necrosis factor
WHO	World Health Organization

1. Introduction

1.1 Trauma - a clinical and scientific challenge

To date, trauma is a major clinical and scientific challenge. Injuries to tissues and organs are caused by traffic-, workplace-, domestic- and sport accidents but also by civil-, military- and terroristic violence. Consequently, trauma can affect anyone, anywhere and anytime over a lifespan [1]. According to the World Health Organization (WHO), trauma accounts for 10% of deaths and 16% of disabilities worldwide [2]. Even more importantly, trauma is the leading cause of death in patients between 5 and 44 years of age in industrial nations and trauma deaths are continuing to rise [3, 4]. In the developing world, the majority of serious traumatic injuries are due to blunt trauma from motor vehicle crashes and pedestrian injuries, followed by falls from height [5]. The most frequently injured organ by a major blunt trauma vector is the head, followed by the chest, the abdomen and by the extremities [6-8]. Besides severe blunt trauma, penetrating injuries are frequently recorded in countries in which firearms are easily accessible, such as in the USA or in South Africa [9-11]. These trauma victims mostly suffer from severe gunshot or stabbing injuries [12]. Furthermore, severely injured patients often sustain hemorrhagic shock (HS) at hospital admission [8]. In the United States, traumatic injuries belong to one of the most common causes of death as well as to a significant socioeconomic burden [13, 14]. This liability is due to the fact that mainly young people suffer from trauma and severe injuries, resulting in a large number of years lost due to premature death, or in a large number of years living with disabilities. In high-income countries, road traffic injuries, falls, interpersonal violence and self-inflicted injuries are the main injury-related causes of disability-adjusted life years (DALYs). DALYs characterize the burden of injuries by combining the number of years of life lost from premature death with the loss of health from disability among persons with nonfatal injuries [3, 15].

Many of the severely injured patients who survive trauma develop persistent low-grade organ dysfunction and require prolonged intensive care, which is also described as chronic critical illness (CCI) [16, 17]. CCI is a disastrous condition for the patients and their families, accompanied by major financial burdens [18].

Multiple trauma is defined as a combination of severe and simultaneous injuries, affecting more than one body region or organ system, whereby at least one injury or

the combination of several injuries is life-threatening [19, 20]. The injury severity is characterized by the injury severity score (ISS). The calculation of the ISS is based on the Abbreviated Injury Scale (AIS). For determination of the AIS, injuries of any body region are ranked from 1 to 6, 1 being minor injury and 6 maximal or non-survivable injury. The ISS is the sum of squares of the highest AIS scores, comprising the three most severely injured body regions [21, 22]. Therefore, multiple trauma is defined by ISS $\geq$ 16 [23, 24]. The early clinical provision of multiply injured patients is often based on the Advanced Trauma Life Support (ATLS) principles. The death due to severe injuries is described in a trimodal distribution, including three peaks [25-27]. The first peak occurs within seconds to minutes after trauma. During this early period, death generally results from apnea due to severe brain damage, high spinal cord injury or due to rupture of the heart, the aorta or other large blood vessels. Very few of these patients can be saved. The second peak develops within minutes to several hours following severe injury. During this peak, death is mostly due to subdural and epidural hematomas, hematopneumothorax, ruptured spleen, liver lacerations, pelvic fractures and due to injuries resulting in a significant blood loss and hemorrhagic shock. The third peak appears several days to weeks after initial injury. This peak is often due to multiple organ dysfunction and sepsis [25-27]. Over the last years, significant improvements in the trauma management were committed, wherefore a change of the death distribution course after severe trauma is under debate [28].

During the last decade, trauma research significantly improved the preclinical as well as the early clinical care of severely injured patients, resulting in a reduced mortality as well as in an improved clinical outcome. Trauma research contributed to an enhanced early management of severely injured patients including airway management, cervical spine immobilization, immediate hemorrhage control, fluid resuscitation, control of trauma-induced coagulopathy (TIC) and damage control surgery [29-32]. However, the development of post-traumatic adverse events, such as a hyper-acute immune response are often associated with the development of Multiple Organ Dysfunction Syndrome (MODS) and death. In addition, infections can frequently occur in the course of this hyper-acute immune response [33]. These adverse events following severe trauma are still a clinical but also a scientific challenge. Various pathways and cells of the innate and acquired immune system were recently described to be involved in this initially hyper-inflammatory process [1, 34, 35]. However, a detailed understanding about the cellular and molecular events is still lacking.

Therefore, further research especially on the inflammatory process following trauma is required, which might improve the early management of the severely injured patients.

1.2 Innate immune response to trauma

Locally at the site of injury and systemically, an acute danger response is induced after trauma, including the rapid activation of the innate immune system [1, 33]. The purpose of this rapid activation is on the one hand to restrict further organ damage as well as to promote tissue healing and regeneration [35]. Furthermore, this immediate activation of the innate immune system provides an instant defense of invading microorganisms and pathogens into damaged tissues and open wounds [35]. But often this enhanced activation of the innate immune system contributes to the development of severe complications, resulting finally in death of the injured patient [36]. Severe injuries are often associated with a so-called Systemic Inflammatory Response Syndrome (SIRS), which can develop early after trauma. SIRS describes a whole-body systemic inflammatory response, which is associated with a large number of clinical conditions. Besides infections, which are one major cause of SIRS, non-infectious pathologic conditions such as pancreatitis, ischemia, tissue injury, hemorrhagic shock and multiple trauma can contribute to the development of SIRS. SIRS can include different clinical manifestations such as a body temperature greater than 38°C or lower than 36°C, enhanced heart rate ($>$ 90 bpm), tachypnea and a rise in white blood cell count [37]. After trauma, SIRS arises from the systemic release of endogenous factors, called damage-associated molecular patterns (DAMPs) [38, 39]. DAMPs are released from damaged and necrotic cells but also from activated cells of the innate immune system, such as neutrophils [39, 40]. DAMPs include different molecules such as heat shock proteins (HSP), monosodium urate, high-mobility group box protein 1 (HMGB1), extracellular ATP and nucleic acids such as mitochondrial DNA (mtDNA). Additionally, fragments of extracellular matrix (ECM) compounds such as hyaluronic acid, collagen, elastin, fibronectin and laminin stimulate inflammation [41]. Hence, DAMPs are able to activate further immune cells like neutrophils and monocytes via specific receptors, triggering an intensified inflammatory response after trauma [33, 42, 43]. Furthermore, DAMPs are also potent activators of the complement system [44, 45]. As a result, activated immune cells and the activation of the complement system lead to the systemic release of more inflammatory mediators, such as inflammatory cytokines and chemokines, generating an inflammatory boost [46]. This intense systemic

inflammation often results in multiple organ failure (MOF), which is one cause of death of severely injured patients especially in the third peak following initial injury [25, 27, 47]. Moreover, nosocomial infections and sepsis often occur days to weeks after severe trauma [48, 49]. Likewise, sepsis and septic shock induce MOF, leading finally to death of the patients [50, 51].

1.3 Mediators and signaling pathways of the innate immune system

The innate immune system is a universal and ancient form of host defense against infections. It includes special cells and signaling pathways, which detect damaged tissue and promote repair of injured cells and tissues. Cells of the innate immune system are able to sense specific alarm signals, triggering an increased inflammatory response. The innate immune response is activated immediately after recognition of specific exogenous and endogenous danger molecules, also called alarmins, including pathogen-associated molecular patterns (PAMPs) and DAMPs. These specific molecules are recognized by leucocytes via specific receptors, termed pattern-recognition receptors (PRRs). PRRs are mainly expressed by monocytes, macrophages, neutrophils and dendritic cells but also by lymphocytes, fibroblasts and epithelial cells [52]. Stimulation of these cells by PRRs provokes a systemic inflammatory response by releasing further inflammatory cytokines and chemokines. Moreover, through autocrine and paracrine effects local neutrophils and macrophages are activated. By endocrine effects, the production of acute phase proteins in the liver is induced, contributing to an enhanced systemic inflammatory response [52]. In addition, cytokines activate endothelial cells and increase their vascular permeability, facilitating the entrance of more immune cells into tissue on site of infection [1, 52, 53]. Likewise, chemokines recruit immune cells to side of infection and injury [54].

The PRR-family imply different receptors, such as toll-like receptors (TLRs), nod-like receptors (NLRs), receptor for advanced glycation end products (RAGE), purigenic receptors and complement receptors [1, 55]. The TLR-signaling pathways play a crucial role for immune response after severe trauma [56]. In humans, ten different TLRs are described, recognizing miscellaneous specific molecular patterns from pathogens such as lipoproteins, dsRNA, lipopolysaccharides (LPS), flagellin, ssRNA and oligonucleotides. After specific pathogen recognition, TLRs induce a diverse spectrum of cellular reactions, including the generation of inflammatory cytokines and chemokines, which is primarily mediated via the nuclear factor kappa B (NFκB)

pathway [57]. The immune dysfunction process following trauma is mainly mediated via the pattern-recognition receptors TLR2, TLR4, and TLR9 [55].

Damage-associated molecular patterns (DAMPs)

DAMPs include endogenous nuclear, mitochondrial or cytosolic molecules, which have under normal conditions different physiological functions inside the cell. DAMPs are defined as substances, which are released immediately after trauma and which are also responsible for immune cell activation. Accordingly, the systemic amount of DAMPs can reflect the severity of trauma. Moreover, plasma levels of DAMPs in severely injured patients correlated with extent of the inflammatory response as well as with the injury severity [58, 59]. Furthermore, DAMPs are specified as substances which induce a pro-inflammatory response on cultured cells with a clearly elucidated mechanism of activation [60]. Hereinafter, some specific selected DAMPs are described:

After severe trauma, nuclear and mitochondrial nucleic acids are released from damaged cells into the circulation. Systemic mitochondrial DNA (mtDNA) correlated with the occurrence of acute lung injury (ALI) as well as with the severity of SIRS and with MODS [61-64]. Furthermore, monocytes produced and released interleukin (IL)-6 and IL-8 in presence of nuclear nucleic acids [65, 66].

Another damage molecule is the high-mobility group box 1 (HMGB1) protein. HMGB1 is a nuclear chaperone protein, which regulates gene transcription under physiological conditions by bending the DNA and facilitating the gene transcription. After severe trauma, HMGB1 is released from stressed, damaged or dead cells into the circulation [67]. Systemic HMGB1 concentrations increased within 6 h after trauma, persisting for 24 h post-injury [68]. Post-traumatic systemic HMGB1 release correlated with the ISS of the patients and also with the development of SIRS, MODS, with an enhanced immune response and with an increased mortality rate [59, 69]. Furthermore, high systemic HMGB1 levels were associated with pulmonary dysfunction during long-term mechanical ventilation [70].

Extracellular histones represent a further class of DAMPs. Histones are intra-nuclear, highly conserved cationic proteins, fulfilling under physiologic conditions different intracellular functions such as enhancing the structure and stability of chromatin and the epigenetic regulation of DNA [71]. Extracellular histones are octamers, which are composed of different subgroups of histones, defined as core histones (H2A, H2B, H3

and H4) and as linker histones (H1 and H5) [72]. After trauma, histones are systemically elevated and were further associated with trauma-induced lung injury [73]. During the inflammatory response, neutrophils of the innate immune system release neutrophil extracellular traps (NETs), containing DNA scaffolds, which are coated with histones and toxic granule proteins. The released NETs protect the host of invading microbes by killing bacteria, nominating the NETs as powerful and very potent weapon of neutrophils [74]. The process of NET formation is also called NETosis. NETosis is either mediated via a lytic pathway, resulting in death of neutrophils or via a vital pathway. During the vital pathway of NETosis, neutrophils release nuclear DNA in the extracellular space by nuclear budding and vesicular trafficking, maintaining the cellular capability of chemotaxis and phagocytosis [75, 76]. The lytic or suicidal pathway includes a stepwise progression of chromatin decondensation, nuclear swelling and spilling of nucleoplasm into the cytoplasm, resulting in plasma membrane rupture and in cell death of the neutrophils [75]. This suicidal pathway is also thought to represent a beneficial immune response to capture pathogens by releasing web-like structures of DNA, coated with histones and elastase [75]. Consequently, enhanced systemic concentrations of extracellular histones are present after severe trauma. Extracellular histones are associated with MOF during sepsis and also after trauma [73, 77]. Furthermore, systemic extracellular histones are nominated as major mediators of MODS in critically ill patients [78], in patients with acute liver injuries [79] as well as in patients with kidney injuries [80]. Extracellular histones acted via the TLRs2/4/9 in a murine model of fatal liver injury [81] and of sterile inflammatory liver injury [82]. They also induced nod-like receptor protein 3 (NLRP3) inflammasome activation in Kupffer cells during sterile inflammatory liver injury [83]. Moreover, extracellular histones have also direct toxic effects on cells by interacting with their phospholipid membrane, resulting in an enhanced cellular permeability and severe cellular damage [84-86].

Further important DAMPs are Heat-Shock proteins (HSPs) and the S100 proteins, which were released after severe trauma, triggering and increased immune response [87, 88].

Pathogen-associated molecular patterns (PAMPs)

PAMPs are exogenous microbial molecules, which are released from penetrating pathogens such as bacteria, viruses and fungi. PAMPs include different molecules, for

instance endotoxins, flagellins, double-stranded RNA and peptidoglycans. However, they share a number of special recognizable biochemical features [89]. These specific recognition patters are detected by cells of the innate immune system, alerting the organism to the invading pathogens [90]. PAMPs are detected by TLRs, NLRs, C-type lectin receptors (CLRs) and retinoic-acid-inducible protein 1 (RIG-I)-like receptors [55]. As a result, the pathogens and the pathogen-infected cells are destroyed and eliminated from the body by activated cells of the immune system [90].

Inflammatory cytokines and chemokines

Inflammatory cytokines are key mediators during the inflammatory response after trauma, by binding to specific receptors on their target cells, triggering a second messenger cascade, resulting in alterations and activation of the target cells. Inflammatory cytokines are mainly released by activated immune cells such as macrophages but also from epithelial cells and act via autocrine, paracrine and endocrine effects on neighboring cells and organs. Cytokines activate endothelial cells of blood vessels, increase the permeability of the vascular walls, attract immune cells of the adaptive immune system to the site of infection, activate leucocytes as well as lymphocytes and induce the production of the acute phase proteins C-reactive protein (CRP) and mannose-binding lectin (MBL) in the liver. Chemokines are small proteins, which are dominantly released from activated phagocytes and dendritic cells and function as chemoattractant for leukocytes. Chemokines mobilize neutrophils, monocytes as well as different effector cells from blood and attract them to the source of infection. Moreover, chemokines promote the extravasation of leucocytes into infected tissue [57].

The inflammatory cytokines and chemokines tumor necrosis factor (TNF), IL-1, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18 and midkine (Mdk) play an important role as inflammatory mediators after trauma [91, 92]. One important pro-inflammatory cytokine is TNF, which is mainly produced by macrophages and T-cells. Another crucial pro-inflammatory cytokine is IL-1β, which is primarily released by macrophages and endothelial cells after trauma. Both, IL-1β and TNF are systemically enhanced in severely injured patients [42]. Besides pro-inflammatory effects, IL-1β and TNF show a variety of effects such as activation of blood coagulation, induction of gene expression and the increase of the permeability of the blood-brain-barrier [93, 94]. More importantly, TNF is associated with septic shock and death of septic patients

[57]. Yet another important inflammatory cytokine, which is systemically released after trauma is IL-6. Enhanced systemic IL-6 levels are associated with the development of post-traumatic complications such as MODS and thus with an increased mortality rate [95, 96]. Moreover, increased IL-6 and TNF concentrations in plasma of patients suffering from burn injuries correlate with the response to infections [97].
Another inflammatory cytokine is the heparin-binding growth- and differentiation factor Mdk, which is associated with different inflammatory diseases such as myocarditis but also with various autoimmune and autoinflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and Chron's disease [98-100]. In the context of trauma, Mdk is also enhanced systemically after tissue injury and after different traumatic injuries such as bone fracture, traumatic spinal cord injury and burn injury [92, 101-103]. Furthermore, Mdk is associated with SIRS and septic shock [104] and is likewise considered as a new biomarker for different cardiovascular diseases such as chronic heart failure and coronary artery disease [105, 106]. In conclusion, the enhanced release of pro-inflammatory cytokines and chemokines after trauma can result in an increased systemic, life-threatening inflammatory response in the severely injured patient.

Reactive oxygen species

Reactive oxygen species (ROS) are a group of highly reactive chemicals containing predominantly oxygen, interacting with various intracellular macromolecules and are thus associated with a variety of human disorders such as chronic inflammation and age-related diseases. However, ROS are also essential for numerous biological functions like cell survival, proliferation, differentiation and for the immune response [107]. ROS are mainly produced by phagocytes via the NADPH oxidase NOX2 in a so-called 'respiratory burst' [108]. Many candidates could induce an oxidative burst, including the TLR-signaling, which can enhance the mitochondrial ROS production [109]. Besides their direct pathogenic effects, ROS also induce the NLRP3 inflammasome activation, promote the neutral killer (NK) cell-mediated cytolysis, trigger the differentiation of dendritic cells and induce T- and B-cell activation [110-113]. Consequently, ROS can stimulate the activation of the innate and the acquired immune system, resulting in an intensified inflammatory response. The activation of the NLRP3 inflammasome was associated with cardiomyocyte (CM) dysfunction during sepsis [114].

The complement system

The complement system is part of the innate immune system, consisting of various plasma proteins, which trigger an inflammatory response after activation, defeating pathogens by opsonization. The complement system can be activated by microorganisms but also immediately after trauma. In the case of severe trauma, excessive activation of the complement cascade lead to complement depletion and to an early 'complementopathy' [44]. The complement system is considered as a main driver of whole body inflammation and of SIRS [115]. Activation of the complement system occurs rapidly after recognition of invading microorganisms via direct recognition of PAMPs. The complement activation is characterized by structural rearrangements, proteolytic cleavages and by the assembly of proteolytic and lytic complexes [116]. Complement activation is mediated via different activation pathways, including the classical pathway, the alternative pathway and the lectin pathway. The classical pathway is antibody-dependent and is activated when antibodies bind to specific components on the surface of pathogens. The alternative pathway is independent of specific antibody-binding and is activated by direct binding of specific danger signals such as bacteria, yeast- and virus infected cells but also by binding to specific danger-signaling peptides. The lectin pathway is activated when mannose-binding lectin (MBL) binds mannose-containing surface proteins on pathogenic surfaces [117]. The activation of the complement system induces the formation of the C3-convertase, which covalently binds to pathogenic surfaces and causes the cleavage of C3 into C3a and C3b. C3b appears as opsonin by specifically labelling pathogens for elimination. Following, the C5-convertase is generated, cleaving C5 into C5a and C5b [57]. In the terminal stage of complement activation, the lytic membrane attack complex (MAC) is formed in membrane of pathogens, disturbing their protective cell wall by inducing lysis of the invading pathogens. Moreover, sub-lytic MAC complex can induce signaling, for example it activates the NLRP3 inflammasome and provokes the release of pro-inflammatory cytokines such as IL-1β [116, 118]. As described above, during the activation of the complement system, the complement activation products C3a and C5a are locally and systemically generated. The anaphylatoxin C3a trigger an inflammatory response in the body and act also directly as antimicrobial agent [119]. C5a can also induce inflammation. Immediately after multiple trauma, the circulating complement activation products C3a and C5a increased significantly in blood of severely injured patients [120]. Enhanced systemic C3a/C3 ratios were

associated with the development of ARDS and of MODS in severely injured patients [120-122]. Moreover, systemic C5a correlated with the ISS of the patients and with development of MODS [123]. The complement activation products act via their G-protein coupled receptors C3aR and C5aR1. Signaling through C3aR and C5aR1 induces chemotaxis, oxidative burst, histamine release, and leukotriene and interleukin synthesis in different immune cells [116, 124].

1.4 Immune dysfunction following trauma

As described above, a massive systemic inflammatory response is provoked after severe trauma, including the activation of various immune cells and signal cascades [33]. Normally, the pro-inflammatory as well as the anti-inflammatory effects are balanced, eliminating pathogens from the body and inducing tissue repair and regeneration [1, 125, 126]. However, if the severity of the injury is substantial and is further accompanied by additional hemorrhagic shock or extensive surgical procedures, the innate immune response can become disbalanced with subsequent severe consequences [127]. Imbalanced immune responses after trauma may result in a vicious cycle of tissue injury and harmful immunological processes, leading to infections, sepsis and to the development of MODS [1]. Consequently, the establishment of the immune balance seems crucial for the overall outcome of severely injured patients. Therefore, different trauma-care concepts have been developed to ensure the adequate provision of injuries as well as to restrict additional tissue damage, which is caused by extensive surgery [128, 129]. In general, the adequate stabilization technique of fractures after multiple trauma is crucial for the early management and can improve the overall outcome of critically injured patients [130]. The ‘early total care (ETC)’ concept, including intramedullary nailing is considered as the gold standard for stabilization of isolated femoral shaft fractures [131]. Reaming of the femoral shaft was described as highly beneficial with regard to improving mechanical properties as well as by providing an osteogenic stimulus, resulting in a lower rate of non-union fractures as well as in a shorter fracture consolidation time [132, 133]. However, in case of multiple injuries and hemorrhagic shock reaming of the medullary shaft is considered as a ‘second hit’, associated with severe post-traumatic complications such as fat embolism, ARDS development and MODS [91, 131, 134-136]. Another concept for the treatment of fractures is the ‘damage control orthopaedics (DCO)’ strategy, including the stabilization of fractures by external

fixation. Compared to ETC, fracture stabilization according to DCO principles is less invasive, it provides an adequate stabilization of fractures and it implies a decreased operative time. Additionally, fracture treatment according to DCO is associated with a diminished systemic inflammatory response [137] as well as with a reduced risk for organ failure [138, 139]. However, there is also the danger for infections and the need for a second operative surgery delays the patient's mobilization [140, 141]. Therefore, the choice of the treatment strategy is essential for the inflammatory process following trauma and might imply the overall outcome of the critically injured patients.

One therapeutic option for the alleviation of systemic inflammation is the filtration of blood by hemadsorption [142-144]. Hemadsorption columns are composed of small polymer beads, filtering various DAMPs, pro-inflammatory cytokines, PAMPs, anaphylatoxins and toxic metabolites from human whole blood [145]. Currently, hemadsorption is routinely used for patients in intensive care unit (ICU) with sepsis or SIRS but not routinely after trauma [146-148].

1.5 Cardiac damage after trauma

1.5.1 Blunt cardiac injuries

Cardiac injury following trauma is a common cause of death, which is often underestimated [149]. Blunt cardiac injuries are an independent predictor for a poor overall outcome after multiple trauma [149]. The incidence of blunt chest trauma in multiply injured patients (ISS $\geq$ 16) amounts to 45% (TraumaRegister® Deutsche Gesellschaft für Unfallchirurgie (DGU)/ Annual Report 2018). Pathologically, blunt cardiac damage includes injuries to anatomical cardiac structures such as the myocardium, comprising cardiac concussion, contusion, valvular disruption and pericardial effusion [150]. Additionally, injuries to coronary arteries and to the septum were reported after trauma. Blunt cardiac injuries could be either induced by direct mechanical impact on bony structures such as the sternum or the vertebrae or by shear stress forces [150, 151]. Blunt cardiac injuries result in cellular damage, necrosis and in extravasation of blood cells into myocardial tissue. As a result of cellular damage, the myocardial cells release creatinine phosphokinase (CPK) and cardiac troponins (I and T) into the circulation [151-153]. Severe blunt cardiac injuries as well as the systemic elevation of cardiac troponins correlate with increased frequency of dysrhythmias, ventricular fibrillation, with sudden cardiac arrest, and enhanced mortality of severely injured patients [149, 152, 154].

1.5.2 Cardiac structural alterations

In translational trauma models there is evidence for cardiac damage after blunt chest trauma in rats [155], after multiple trauma in mice [156] and after multiple trauma in pigs [157]. After experimental multiple trauma, the pigs exhibited cardiac structural alterations, which were associated with post-traumatic cardiac dysfunction [157]. Alterations of the cardiac structure protein α-actinin were observed after multiple trauma in pigs. α-actinin is a Z-line protein, which is co-localized with L-Type Ca^{2+} channels, stabilizing the muscle contractile apparatus in CMs. These Z-disc proteins have been shown to act as responder to stretch or mechanical tension [158]. After experimental multiple trauma, the protein expression of α-actinin was reduced in left ventricles of pigs *in vivo*, which was linked to a reduced cardiac ejection fraction [157]. Furthermore, the cardiac structure protein desmin was also altered after multiple trauma in pigs [157]. Desmin is a major component of the cardiac intermediate filaments and is also located in the Z-lines, forming a physical link between the nucleus, contractile proteins, mitochondria and sarcoplasmic reticulum [159]. After multiple trauma, the protein expression of desmin was upregulated in left ventricles of pigs *in vivo* [157]. Desminopathies, such as aggregates of desmin alter the heart biomechanics as well as cardiac calcium dynamics [160]. Alterations in desmin expression were associated with impaired cardiac function, heart failure and with diastolic dysfunction [157, 161, 162]. Interestingly, alterations in left ventricular α-actinin and desmin expression were also demonstrated in mice during chronic psychosocial stress [163].

Cardiac gap junction proteins such as connexin 43 (Cx43) are located in the intercalated discs of the CMs and serve as intercellular communicating proteins [164]. Furthermore, Cx43 has a key function in maintenance of normal cell electrophysiology by controlling the sodium current amplitude. Cx43 mediates the current which is necessary for generation of action potentials by allowing the transfer of current between cells. In a conditional knock-in murine model, where upon tamoxifen injection the wild-type Cx43 was cardio-specifically replaced by a truncated form of the protein, the loss of Cx43 function resulted in damage of the sodium current amplitude [165]. After multiple trauma in pigs and after blunt chest trauma in rats, alterations in Cx43 location in left ventricles were observed. The Cx43 was translocated from the intercalated discs into the cytosol of the CMs, though without changes in the total Cx43 protein amount [155, 157]. The translocation of Cx43 was associated with a loss of

Cx43 function. Mice with a cardio-specific knockout of Cx43 developed sudden cardiac death from spontaneous ventricular arrhythmias [166]. Furthermore, in rat CMs impairment of Cx43-mediated intercellular communication was associated with slowing down of Ca^{2+} transient propagation velocity and with re-entrant arrhythmias *in vitro* [167]. Additionally, Cx43 plays an important role during inflammation [168, 169] and is also involved in cellular damage by transferring ROS in a model of sepsis-induced intestinal injury [170].

1.5.3 Secondary cardiac damage after trauma

Besides direct cardiac damage, recently published clinical reports have demonstrated that trauma also causes cardiac dysfunction and myocardial failure several days or months after trauma, even in the absence of direct myocardial or thoracic injuries [171, 172]. This phenomenon in absence of direct myocardial damage is also defined as trauma-induced secondary cardiac injury (TISCI) and is associated with an increased mortality rate and long term morbidity [173]. TISCI is characterized by the systemic release of the specific cardiac damage markers troponin I and the heart-fatty acid binding protein (HFABP). Both markers correlate with an increased risk for the occurrence of adverse cardiac events (ACEs), including angina, arrhythmia, myocardial infarction, cardiogenic shock, cardiac death and death of the severely injured patients [173-175]. The incidence of ACEs in severely injured patients were shown to be independent of direct mechanical chest trauma but are related to the degree of overall injury [174]. The mechanisms leading to TISCI are still elusive but in previous studies it was demonstrated that the admission levels of endogenous catecholamines might play an important role. The enhanced levels of catecholamines were mostly due to increased blood loss and hypoperfusion of the injured patients [176]. A possible mechanism for the development of TISCI might be apoptosis of the CMs, caused by an early activation of myocardial caspase-12 [177].

Furthermore, the enhanced systemic release of different inflammatory cytokines and the following myocardial leucocyte infiltration are associated with the development of TISCI [178]. Secondary cardiac injuries also occur after direct myocardial damage and are mainly related to increased inflammation. In humans, several inflammatory mediators such as IL-1β, IL-6 and TNF are systemically increased after multiple trauma, hemorrhage and after burn injury [95, 179, 180]. All of these inflammatory mediators have been previously shown to be cardio-depressive *in vitro* by impairing

myocardial cell contractility of rat CMs [181]. Of note, the occurrence of ACEs and TISCI correlated with systemic IL-6 levels of the severely injured patients [173, 176]. Moreover, IL-1β and IL-6 were elevated locally in cardiac tissue in pigs after multiple trauma and were associated with impaired cardiac function *in vivo* [157]. Furthermore, systemic troponin I and HFABP levels were linked to cardiac damage and cardiac dysfunction in pigs after multiple trauma [157]. In addition, in critically injured patients the systemic levels of IL-1β, IL-6, IL-8, IL-10 and TNF correlated with cardiac injury [182], ACEs [173] and with the development of MODS [183]. Moreover, these inflammatory cytokines were further associated with a higher risk for in-hospital death after trauma [173, 182, 183]. Consequently, secondary cardiac dysfunction might occur after direct but also after indirect myocardial damage and might be further provoked by increased systemic and local levels of cardio-depressive inflammatory mediators. However, the underlying mechanisms are still poorly understood.

DAMPs such as HMGB1, extracellular histones and nucleic acids might play a role in the development of secondary cardiac injury and post-traumatic cardiac dysfunction. These mediators correlated with the ISS of the severely injured patients and were further associated with an increased mortality rate [58, 63, 73]. HMGB1 was released into blood circulation within 30 min after trauma and correlated with the severity of tissue injury, tissue hypoperfusion, early post-traumatic coagulopathy, hyperfibrinolysis, systemic inflammation, activation of the complement system as well as with an increased mortality rate of the severely injured patients [59]. Furthermore, systemic HMGB1 levels were linked to the development of nosocomial infections, sepsis and MODS after severe trauma [183, 184]. Inhibition of HMGB1 attenuated cardiac injury in ischemia-reperfusion (I/R) injury in mice [185]. Furthermore, in hemorrhagic shock and resuscitation (HS/R) injury in rats, inhibition of the HMGB1-TLR4 axis was cardio-protective [186]. In the clinical setting, circulating extracellular histones have been linked to trauma-induced lung injury and to increased mortality in severely injured patients [73]. In a murine model of sepsis, the presence of extracellular histones in plasma was associated with an accumulation in the heart *in vivo* [187] and also with an accumulation on the plasma membrane and in the cytosol of CMs *in vitro* [188]. Incubation of rat CMs with extracellular histones increased the amount of cytosolic ROS, increased intracellular calcium concentrations $[Ca^{2+}]_i$, impaired the mitochondrial membrane potential and disturbed calcium signaling of the cells. These disruptive effects of the extracellular histones could be mediated via TLR2- and TLR4-

signaling [188]. However, the exact mechanisms of CM injury induced by extracellular histones and HMGB1 remain still unclear.

Besides DAMPs and inflammatory cytokines, the complement activation products C3a and C5a are elevated systemically after severe trauma in humans [44]. Activation of the complement system and the systemic release of the anaphylatoxins C3a and C5a might also play a role in the development of post-traumatic secondary cardiac injury. C5a was associated with dramatic contractile dysfunction in CMs *in vitro* and also with impaired cardiac function *in vivo* during experimental sepsis, which was mediated via the receptors C5aR1 and C5aR2 [189, 190]. CMs, which were exposed to C5a released the cardio-depressive inflammatory mediators IL-1β and TNF *in vitro* [191]. Furthermore, in presence of C5a the amount of cytosolic ROS increased in rat CMs *in vitro*, indicating a disturbed redox-balance of the cells. Cardiac dysfunction of the CMs in presence of C5a could be mediated via the activation of mitogen-activated protein kinase (MAPK) and via Akt phosphorylation [192].

Moreover, impaired cardiac function during experimental sepsis is characterized by alterations in the expression of the calcium handling proteins sarcoplasmic/endoplasmic reticulum ATPase2 (SERCA2a), sodium-calcium exchanger (NCX) and of the Na^+/K^+-ATPase [189]. Ca^{2+} is a central player of excitation-contraction coupling of the CMs. A strict regulation of Ca^{2+}-handling is crucial for cardiac function and includes Ca^{2+} cycling between the cytosol and the extracellular space as well as between the cytosol and the intracellular space. Ca^{2+} is stored in the sarcoplasmic reticulum (SR) [193]. Once the cell membrane is depolarized, Ca^{2+} enters the cytosol, raising local Ca^{2+} concentrations, inducing a Ca^{2+} release from the SR. Following, the global cytosolic Ca^{2+} concentrations increase, leading to a contraction of the cells via conformational changes in the troponin-tropomyosin complex, allowing the myofilaments acting and myosin to slide past one another [194, 195]. For relaxation, Ca^{2+} must be removed from the cytosol. Ca^{2+} is transported back into the SR by SERCA2a. SERCA2a mediates the calcium uptake in the SR of CMs, which is important for myocardial contraction and relaxation, maintaining normal cardiac function [196]. NCX and Na^+/K^+-ATPase transport Ca^{2+} back into the extracellular space [193]. The NCX as well as the Na^+/K^+-ATPase play also important roles in maintenance of cardiac function and myocardial contractility [197]. Alterations in SERCA2a, NCX and Na^+/K^+-ATPase are associated with different cardiac pathologies, including heart failure and post-ischemic cardiac injury [197] and

might also contribute to the development of post-traumatic cardiac dysfunction and secondary cardiac injury.

Additionally, the glycolytic- and fatty acid metabolism in CMs might also play an important role in the development of secondary cardiac injury and post-traumatic cardiac dysfunction. Metabolic reprogramming is important for host defense and inflammation. In inflammatory conditions, a metabolic switch from oxidative phosphorylation to aerobic glycolysis is induced by inflammatory cytokines and TLRs in dendritic cells, macrophages and T-lymphocytes [198-200]. Similarly, CMs are also able to undergo metabolic reprogramming and change to aerobic glycolysis, which is especially seen in patients with chronic heart failure as well as with ischemic cardiomyopathy [201]. Enhanced glucose uptake is mediated via the glucose transporter (GLUT) 1 and GLUT4 [202]. This adaptive response of the CMs is also called myocardial hibernation and serves for maintenance of cell viability and CM contractility in state of energy deficiency in the septic heart [203]. Nevertheless, enhanced glycolysis of the CMs was also associated with cardiac dysfunction during sepsis and after endotoxemic shock [204, 205].

As mentioned, inflammation is associated with a massive production of ROS and with enhanced oxidative stress of the cells. Elevated oxidative stress in CMs was recently demonstrated during sepsis, impairing their mitochondrial electron transfer system, resulting finally in mitochondrial dysfunction and aggravated contractile function of the heart muscle [206]. In permeabilized heart fibers from septic mice, the oxidative phosphorylation (OXPHOS) capacity of the mitochondrial electron transfer system was reduced due to defects in complex-I and complex-II of the mitochondrial respiratory chain. The defects were further linked to cellular and mitochondrial damage, caused by enhanced inflammation [207]. Thus, oxidative stress accompanied by mitochondrial dysfunction might also contribute to the development of secondary cardiac injury after trauma.

Summarized, secondary cardiac injury and the development of post-traumatic cardiac dysfunction after direct and indirect myocardial damage can be considered as multifactorial disorder. Miscellaneous local and systemic DAMPs, inflammatory mediators and complement activation products might impair cardiac function after trauma by disturbing redox-balance, calcium signaling and intercellular communication between the CMs. These detrimental effects might be multifactorial, synergistic and also intensified in the condition of multiple trauma. However, the specific molecular

effects of the individual DAMPs and inflammatory mediators as well as of their combinations in the development of secondary cardiac injury and post-traumatic cardiac dysfunction are rarely investigated and remain mostly unidentified. Therefore, these effects have to be addressed more closely in order to gain a better understanding about the cardio-depressive impacts, resulting in post-traumatic cardiac dysfunction. An enhanced mechanistic understanding might help future diagnostics and therapeutic strategies, which might be transferable to human clinical situation.

1.6 Aim of the thesis

Although post-traumatic cardiac damage has been demonstrated after severe multiple trauma, detailed knowledge about the underlying molecular mechanisms leading to this pathologic condition is still lacking. Moreover, post-traumatic secondary cardiac injury after multiple trauma has been recently depicted as a multifactorial effect, including the interaction of various systemic DAMPs, inflammatory cytokines and complement activation products. However, the specific impacts of the individual mediators on human CMs *in vitro* are poorly described. Apart from this, a precise distinction between direct and indirect post-traumatic cardiac damage *in vivo* does not exist.

Therefore, the overarching hypothesis of the doctoral thesis was:
Pro-inflammatory mediators significantly contribute to the development of trauma-induced secondary cardiac damage

To verify this overarching hypothesis, the following questions were addressed:
(**1**) Which effects exhibit DAMPs and inflammatory mediators on CMs *in vitro*?
(**2**) Is there a clear differentiation between direct and indirect post-traumatic cardiac damage *in vivo*?
(**3**) Can the post-traumatic cardiac damage be reduced by hemadsorption?

To address the first question (**1**), the effects of DAMPs (extracellular histones and HMGB1), inflammatory cytokines (Mdk and TNF) and complement activation products (C3a and C5a) on human CMs were determined *in vitro*. To answer the second question (**2**), systemic levels of inflammatory mediators and local cardiac alterations, including structural, inflammatory and metabolic changes were examined *in vivo* after experimental multiple trauma and were compared to changes after isolated long bone fracture. Finally, for the third question (**3**) the filtration capacity of a hemadsorption column for extracellular histones and Mdk was analyzed *ex vivo*.

2. Methods

A detailed description of the performed experiments are published in the following publications: [**208-212**].

Briefly, for the *in vivo* experiments male pigs (*Sus scrofa domestica*) were used. For the experiments in the publications [**208**, **209**, **212**], pigs underwent experimental multiple trauma, including blunt chest trauma, liver laceration, femur fracture and hemorrhagic shock (ISS $\geq$ 27). The femur fracture was treated either by reamed or non-reamed intramedullary nailing [**208**, **209**, **212**] according to ETC or by an external fixator according to DCO principles [**212**]. The observation period was 6 [**208**, **209**] or 72 h [**212**]. For the experiments in publication [**211**], pigs received an isolated femur fracture, followed by fracture provision according to ETC or DCO principles. The observation period was 72 h. Hemodynamic parameters and cardiac function were assessed by transthoracic [**211**, **212**] or transesophageal echocardiography [**208**, **209**]. Blood glucose concentration was determined by blood gas analysis (BGA) [**212**]. For the *in vivo* experiments published in [**211**], male mice (*mus musculus*) received and osteotomy of the right femur, followed by a stabilization with an external fixator according to DCO principles. Systemic levels of cardiac damage markers, DAMPs and inflammatory mediators were assessed by enzyme-linked immunosorbent assays (ELISAs) and local cardiac alterations were investigated by RT-qPCR, immunohistochemical analysis or by western blot analysis.

For the *in vitro* experiments, human cardiomyocytes (iPS) or murine HL-1 cells were used. Cells were exposed for 6 h either to recombinant Mdk [**209**], recombinant human HMGB1 and mixed extracellular histones from calf thymus [**210**], recombinant human C3a and C5a [**208**], recombinant human TNF [**211**], to a defined inflammation cocktail (IFC) [**211**] or to a defined polytrauma cocktail (PTC) [**212**]. The treated cells were compared to control cells, which were only exposed to phosphate buffered saline (PBS). Cell viability as well as caspase-3/7 activity was determined by luminescence assays. Calcium signaling was analyzed by fluorescence microscopy and mitochondrial respiration was investigated by Seahorse XFe96 flux analyzer. Finally, mRNA expression levels were determined by RT-qPCR and the release of specific molecules into supernatant fluids were analyzed by ELISA.

For the *ex vivo* experiments published in [**209**, **210**] human blood plasma from multiple injured patients was used. Blood was drawn from multiple injured patients (ISS ≥ 16) in the emergency room at hospital admission. The blood plasma levels of Mdk [**209**] or of extracellular histones [**210**] were determined before and after filtration by a hemadsorption column. Furthermore, the filtration capacity for Mdk and extracellular histones of the respective hemadsorption column was analyzed by an *ex vivo* dose series.
For the *ex vivo* experiments published in [**211**] human blood samples from patients with metaphyseal/diaphyseal fractures of long bones (femur, tibia, humerus, radius, ulna) were used. Systemic levels of cardiac damage markers, DAMPs and inflammatory mediators were determined by enzyme-linked immunosorbent assays (ELISAs).

For the statistical analysis, the GraphPad Prism 7.0 software was used. In case of three or more treatment groups, the data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's or Turkey's multiple comparison test. Comparisons between two treatment groups were executed by an unpaired student t-test. $p \leq 0.05$ was considered as statistically significant. All values were expressed as mean ± SEM.

3. Results and Discussion

3.1 Which effects exhibit DAMPs and inflammatory mediators on CMs *in vitro*? [**208-212**]

Inflammation cocktail (IFC) and polytrauma cocktail (PTC)

After trauma, many damage molecules and inflammatory mediators are released from injured tissue and activated immune cells, triggering a systemic inflammatory response in order to restrict tissue damage and to promote regeneration. The inflammatory cytokines IL-1β, IL-6, IL-8 and TNF and the complement activation products C3a and C5a were previously demonstrated to be systemically elevated after severe multiple trauma [44, 157, 213] but also after isolated long bone fracture [214] and were further described as cardio-depressive by impairing CM function *in vivo* as well as *in vitro* [157, 189]. Also, extracellular histones and HMGB1 were shown to be released into blood after trauma, aggravating CM function *in vivo* and *in vitro* [188, 215]. However, the effects of these mediators in combination on human CMs *in vitro* are unknown.

In the present study, human CMs were treated with a defined inflammation cocktail (IFC), including the inflammatory mediators IL-1β, IL-6, IL-8, TNF, C3a and C5a [**211**]. In presence of IFC, the amount of cytosolic ROS significantly increased in the cells, suggesting a disturbed redox-balance [**211**]. Additionally, the mitochondrial respiration of the cells was impaired after exposure to the IFC, which was manifested by a reduced mitochondrial basal respiration, maximal mitochondrial respiration as well as by a decreased mitochondrial spare respiratory capacity [**211**]. The IL-1β and NLRP3 mRNA expression was altered in the CMs when treated with the IFC, indicating a NFκB-mediated inflammatory intracellular response, which was further associated with cardiac dysfunction [**211**, 216]. Furthermore, the human CMs were treated with a defined polytrauma cocktail (PTC) consisting of IL-1β, IL-6, IL-8, TNF, C3a, C5a and of extracellular histones, mimicking the inflammatory condition of severe multiple trauma *in vitro* [**212**]. In presence of PTC the expression of HFABP and CD36 mRNA was significantly reduced, whereas expression of GLUT1 mRNA was significantly enhanced [**212**]. Increased expression of GLUT1 was likely linked to the condition of myocardial hibernation in the CMs, including the downregulation of oxygen consumption and the shift to aerobic glycolysis for cardiac ATP production [203]. This adaptive response of the CMs maintain their viability in state of energy deficiency.

Summarized, a combination of various DAMPs and inflammatory mediators, which are elevated after trauma have harmful effects on human CMs *in vitro* by disturbing their redox-balance, impairing their mitochondrial respiration, increasing intracellular inflammation and by affecting their metabolic state. However, these effects might be multifactorial and mediated through synergistic pathways of the different mediators. In order to reveal the specific effects of the distinct DAMPs and inflammatory mediators, human CMs were treated with selected pro-inflammatory agents and their impacts were analyzed *in vitro*.

Midkine (Mdk)

The heparin-binding growth- and differentiation factor Mdk is associated with different traumatic conditions as well as with diverse cardiac pathologies [105, 217]. However, the role of Mdk after severe multiple trauma and its effects on human CMs *in vitro* is unknown.

In the present study, Mdk was found in the blood plasma of multiple injured patients at hospital admission as well as in pigs 6 h after experimental multiple trauma, suggesting some role of Mdk in the post-traumatic inflammatory process [**209**]. Furthermore, Mdk acted directly on human CMs *in vitro*. Recombinant Mdk was bound on the surface of the cells after 30 min and was finally taken up by the CMs after 60 min incubation. Thereby, Mdk was predominantly located around the nucleus of the cells, which may result in gene regulatory effects, but without impairing the CMs viability [**209**]. Moreover, the frequency of calcium signals decreased in the CMs *in vitro* in presence of Mdk, assuming induction of bradycardic effects. These detrimental effects might be provoked by the observed alterations in mRNA expression of SERCA2a and NCX [**209**]. Likewise, the mitochondrial basal respiration as well as the mitochondrial spare respiratory capacity of the human CMs was reduced after Mdk exposure, confirming its cardio-depressive effects [**209**]. Also, the Caspase-3/7 activity was significantly enhanced in the CMs when exposed to Mdk [**209**]. The cardio-depressive effects of Mdk *in vitro* might be further mediated by the purigenic receptor subtype 7 (P2X7) and also via TLR2 and TLR4 [**209**]. Other studies showed that upregulation of the TLRs resulted in enhanced inflammation, increased cardiac injury and finally in cardiac contractile dysfunction [218-220]. Additionally, other authors stated that the upregulation of the TLRs induced a sensitization of cells to other DAMPs such as extracellular histones and HMGB1, aggravating cardiac damage [73, 221].

Tumor necrosis factor (TNF)

The inflammatory cytokine TNF is systemically enhanced in severely injured patients [42] and was recently described as cardio-depressive, impairing CM function *in vivo* as well as *in vitro* [181, 222]. However, the distinct effects of TNF on human CMs *in vitro* were not depictured yet.

In the present study, TNF was detected systemically after isolated long bone fracture in mice as well as in humans, suggesting some role of TNF in the development of trauma-induced secondary cardiac injury [**211**]. The *in vitro* exposure of human CMs to TNF impaired their mitochondrial function, which was manifested by a reduced mitochondrial basal respiration, maximal mitochondrial respiration as well as by a decreased mitochondrial spare respiratory capacity [**211**]. Additionally, the amount of cytosolic ROS increased [**211**], indicating increased intracellular oxidative stress. Furthermore, the cells released troponin I in supernatant fluids when exposed to TNF, evidencing myocardial damage [**211**]. As in presence of the IFC, the IL-1β and NLRP3 mRNA expression was altered in the cells after TNF exposure, suggesting NFκB-mediated intracellular inflammation, which might be primarily induced by TNF [**211**]. Moreover, the CMs released extracellular histones in supernatant fluids when treated with TNF, which might further aggravate myocardial injury [**211**].

The complement system

The complement system is immediately activated after severe multiple trauma leading to depletion of complement factors and to the development of ‘complementopathy’, which is accompanied by a massive systemic generation of the anaphylatoxins C3a and C5a [44]. Activation of the complement system after trauma was further associated with the development of ARDS and MOF [223, 224]. C3a and C5a were recently shown to be cardio-depressive by impairing cardiac function of mice during sepsis *in vivo* but also by disturbing contractility and relaxation of rat CMs *in vitro* [189, 225]. However, the distinct effects of C3a and C5a on human CMs *in vitro* were not described so far.

As described previously in humans, the pigs in the present study showed an early activation of the complement system within the first 6 h after experimental multiple trauma, which was linked to a compromised cardiac function [**208**]. Furthermore, exposure with recombinant C3a and C5a resulted in direct effects on human CMs *in vitro*. Incubation of CMs with C5a increased the cellular expression of the C5aR1, followed by an arrangement of the C5aR1 in tubulo-vesicular structures as well as

around the nucleus [**208**]. Additionally, the Caspase-3/7 activity was significantly enhanced in human CMs when exposed to recombinant C3a and C5a [**208**]. In presence of recombinant C3a and C5a, the calcium handling and calcium signaling of the human CMs was disrupted, developing bradycardia by enhanced build-up of intracellular Ca^{2+} [**208**]. These destructive effects might be caused by alterations in the expression of SERCA2a and NCX mRNA [**208**]. Moreover, the mitochondrial respiration of the human CMs was impaired in presence of C3a and C5a, accompanied by a reduced HFABP mRNA expression. Finally, there was some evidence for myocardial hibernation of the human CMs in presence of C3a and C5a, maintaining their cell viability in a state of energy deficiency [**208**].

HMGB1 and extracellular histones

In severely injured patients, the plasma levels of DAMPs correlated with the extent of the inflammatory response [60, 226] and reflected the severity of trauma. Moreover, circulating histones correlated with the Sequential Organ Failure Assessment (SOFA) score, endothelial damage and with activation of the coagulation system [78]. Extracellular histones have been linked to trauma-induced ALI and to cardiomyopathy during sepsis [73, 188, 227]. Moreover, enhanced release of extracellular histones was shown after blunt chest trauma in rats and after multiple trauma in pigs and were associated with impaired cardiac function and cardiac damage [155, 157, 228]. HMGB1 is systemically released after multiple trauma and is considered as a reliable prognostic marker for critically injured patients. Circulating levels of HMGB1 rose in patients with traumatic hemorrhagic shock, sepsis and with MODS [229-231]. HMGB1 is associated with different cardiac pathologic conditions such as cardiomyocyte dysfunction, cardiac hypertrophy and myocardial ischemia [215, 232], nominating both, HMGB1 as well as extracellular histones as powerful cardio-depressive mediators. However, a detailed understanding about the cardio-depressive mechanisms induced by circulating extracellular histones and HMGB1 in human CMs *in vitro* and their corresponding role in the development of post-traumatic cardiac dysfunction is still lacking.

In the present study, exposure of human CMs to extracellular histones reduced the viability of the cells, which may crucially cause cardio-depressive effects [**210**]. Furthermore, extracellular histones as well as HMGB1 impaired calcium signaling and calcium handling of the human CMs *in vitro* by altering the build-up of calcium signals

in the cells [**210**]. Likewise, the frequency of calcium signals decreased in human CMs in presence of extracellular histones and HMGB1, translating into bradycardic effects [**210**]. Alterations in calcium signaling were described previously by other groups and were associated with impaired CM function [189]. Moreover, in the present study the *in vitro* exposure of murine HL-1 cells to different concentrations of HMGB1 and extracellular histones reduced their cell viability as well as their metabolic activity, confirming the harmful impacts of HMGB1 and extracellular histones [**210**]. Additionally, extracellular histones as well as HMGB1 impaired the mitochondrial respiration of the human CMs, which was evident by a reduced mitochondrial basal respiration as well as by a decreased spare respiratory capacity [**210**]. In other studies, the systemic occurrence of extracellular histones and HMGB1 after multiple trauma was also linked to impaired systolic and diastolic function in pigs [157, 228]. For the first time, we could show in our study that extracellular histones themselves are able to induce NET formation of neutrophils [**211**]. In the trauma setting, this could amplify the immune response and thereby contribute to the development of post-traumatic cardiac injury [**211**].

In conclusion, particular DAMPs like HMGB1 and extracellular histones and inflammatory mediators such as Mdk, TNF, C3a and C5a exhibit detrimental effects on human and murine CMs *in vitro*, affecting their viability, their cellular caspase-3/7 activity, their cytosolic ROS production, their calcium signaling as well as their mitochondrial respiration, resulting finally in impaired CM function and myocardial damage. Consequently, these harmful effects of DAMPs and of the individual inflammatory mediators might be aggravated after severe trauma and subsequent complications. Therefore, it is tempting to speculate that addressing the systemic mediators after severe trauma by therapeutic approaches might alleviate trauma-induced secondary cardiac injury and cardiac dysfunction.

3.2 Is there a clear differentiation between direct and indirect post-traumatic cardiac damage *in vivo*? [**208**, **209**, **211**, **212**]

Blunt cardiac injuries are an independent predictor for a poor overall outcome after multiple trauma and post-traumatic myocardial dysfunction is primarily linked to direct mechanical damage of the heart [149]. Additionally, the inflammatory response following severe trauma was also shown to be crucially involved in the development of post-traumatic secondary cardiac damage [157]. It was also demonstrated that secondary cardiac injuries can also occur even in the absence of direct myocardial damage [173, 178]. However, evidence for secondary cardiac injury after isolated long bone fracture is lacking. Also, a clear differentiation between direct and indirect post-traumatic cardiac damage *in vivo* has not been defined yet. In our studies, we analyzed the systemic but also the local cardiac alterations after experimental multiple trauma and compared them to the cardiac alterations after isolated long bone fracture.

Systemic and local inflammation

Trauma is accompanied by an immediate activation of the innate immune response, including a massive systemic release of DAMPs and inflammatory mediators. The complement system is part of the innate immune system and was shown to be directly activated after severe multiple trauma in humans [44]. However, a detailed knowledge about complement activation after multiple trauma with regard to different surgical treatment strategies of fracture fixation is still lacking. In the present study, the complement system was instantly activated after multiple trauma in pigs [**208**] but also after isolated long bone fracture in pigs, mice and humans [**211**]. After multiple trauma, the early activation of the complement system was associated with an impaired cardiac function of the pigs [**208**]. The complement activation seemed predominantly mediated via the alternative pathway rather than the classical pathway [**208**]. Noteworthy, the complement activation correlated with invasiveness of the fracture treatment, and pigs with reamed nailing of the femoral shaft showed an intensified systemic as well as local cardiac complement activation [**208**]. Other authors proposed that medullary reaming prior nailing was associated with increased inflammation leading to SIRS, ARDS, MODS, MOF and finally to death of severely injured patients [131, 134, 135, 233]. The activation of the complement system might also play a crucial role in the development of post-traumatic secondary cardiac injury in the absence of direct myocardial damage. In the present study, the systemic C3a and C5a levels raised significantly in mice as

well as in humans after long bone fracture [**211**]. Furthermore, the expression of C5aR1 decreased in left ventricles of pigs 72 h after femur fracture [**211**]. Other authors speculated that the decreased C5aR1 expression after multiple trauma might be due to internalization of the receptor, caused by enhanced activation of the complement system, which was further linked to impaired cardiac function [157].

As described above, activation of the innate immune system following trauma is characterized by a systemic release of inflammatory mediators. The heparin-binding growth- and differentiation factor Mdk was recently described to be systemically elevated after long bone fracture and was further associated with some impaired fracture healing [92, 234, 235]. To the best of our knowledge, we showed for the first time that Mdk might also play an important role in the context of severe multiple trauma. In the present study, Mdk was found in blood plasma of multiply injured patients at hospital admission as well as in blood plasma of pigs 6 h after experimental multiple trauma, nominating Mdk as an inflammatory biomarker for multiple trauma [**209**]. The systemic Mdk levels correlated with the invasiveness of the fracture treatment and seemed predominant in pigs with reamed femoral nailing, which might contribute to a 'second hit' response after trauma [**209**]. Of note, the systemic Mdk levels were linked to an aggravated cardiac function after multiple trauma *in vivo* [**208**]. As described previously, Mdk also exhibited detrimental effects on human CMs *in vitro*, impairing CM function [**209**]. Therefore, the systemic release of Mdk might contribute to the development of post-traumatic cardiac damage after both, long bone fracture and multiple trauma [**209**]. Furthermore, we showed in our study that the inflammatory cytokines TNF and IL-6 were likewise systemically released after isolated long bone fracture in mice and in humans [**211**]. Increased systemic levels of IL-6 and TNF were demonstrated previously by others after severe multiple trauma and were further associated with CM injury [181, 213]. Moreover, we showed that extracellular histones were released into blood after isolated femur fracture in pigs, mice, and humans, persisting in humans up to 14 days after fracture [**211**]. In other studies, extracellular histones were also systemically elevated after severe multiple trauma [157, 228] and were further related to impaired cardiac function and myocardial damage [157, **210**, 228].

Summarized, an enhanced systemic- and local cardiac inflammation was found after severe multiple trauma but also after isolated long bone fracture. This augmented inflammatory response might be triggered by various mediators such as DAMPs,

complement activation products and inflammatory cytokines. All of them were recently shown to be cardio-depressive *in vitro* and might further add to the development of post-traumatic cardiac dysfunction and secondary cardiac injury *in vivo*. Regarding the inflammatory response to trauma and the local alterations in the heart and in CMs, no clear differences appeared between severe multiple trauma and isolated long bone fracture, suggesting that even remote isolated trauma can result in alterations in the heart, comparable with the situation after direct cardiac injury.

Local structural alterations

Alterations in cardiac structure proteins were described after multiple trauma in pigs and mice as well as after blunt chest trauma in rats. As a result, these structural changes were suggested to be an effect of blunt cardiac damage and were further associated with the development of post-traumatic cardiac dysfunction and myocardial injury [155-157]. Nevertheless, structural cardiac alterations were also demonstrated during chronic psychosocial stress in mice, indicating a mechanically independent mechanism [163]. However, evidence for cardiac structural alterations after isolated long bone fracture as well as their role in the pathomechanism of trauma-induced secondary cardiac injury are still lacking.

In the present study, the pigs showed cardiac structural alterations in left ventricles 72 h after isolated long bone fracture, which might contribute to the development of secondary cardiac injury [**211**]. There, the expression of the cardiac structure protein α-actinin increased significantly in left ventricles [**211**], in contrast to the left ventricular α-actinin expression in pigs 72 h after multiple trauma [157]. Changes in α-actinin expression after multiple trauma were associated with impaired cardiac function [157]. Furthermore, the left ventricular expression of desmin significantly increased 72 h after isolated long bone fracture in pigs [**211**], as described previously after experimental multiple trauma [157]. Other authors reported that cardiac desminopathies can lead to cardiomyopathy, conduction defects and to arrhythmias [236, 237]. Moreover, we showed in our study a re-localization of the gap junction protein Cx43 in left ventricles of pigs 72 h after femur fracture [**211**]. The Cx43 was translocated from the intercalated discs into the cytosol of the CMs, though without changes in whole protein amount [**211**]. Endocytosis of Cx43 was described previously after experimental multiple trauma and was further associated with a disruptive spread of electrical activation of the CMs as well as with severe arrhythmias and cardiac dysfunction [157, 166].

Despite initial assumptions, cardiac structural alterations were also present after isolated long bone fracture even in the absence of direct myocardial damage. Thus, no distinct effects between direct and indirect cardiac damage were manifested with regard to local cardiac structural alterations. Therefore, these alterations might be due to enhanced inflammation rather than to blunt cardiac damage. Consequently, these local cardiac structural alterations might also contribute to the development of trauma-induced secondary cardiac injury in the presence of indirect cardiac damage.

Cardiac glucose- and fatty acid transport

It was recently demonstrated that metabolic reprogramming plays a critical role in host defense and inflammation. CMs are also able to undergo metabolic reprogramming and shift from oxidative phosphorylation of long-chain free fatty acids (LCFA) to aerobic glycolysis [201]. The post-traumatic release of inflammatory cytokines exaggerates catabolism, triggering hyperglycemia, leading to an increased mortality of multiply injured patients [238-240]. However, little is known about the impact of multiple trauma and isolated femur fracture on cardiac glucose- and fatty acid transport. Also, the role of cardiac glucose- and fatty acid transport in the development of post-traumatic cardiac dysfunction is unknown.

In the present study, multiple trauma induced an early hypermetabolic stress response in pigs, which was reflected by increased blood glucose concentrations immediately after trauma, developing early hyperglycemia [**212**]. After initial hyperglycemia, the systemic glucose levels dropped in pigs, developing hypoglycemia after 72 h. Furthermore, the cardiac protein expression of the GLUT1 increased after 72 h, indicating excessive glucose consumption after multiple trauma [**212**]. Early hyperglycemia in pigs was previously linked to impaired cardiac function *in vivo* [157]. After long bone fracture, the pigs also showed a late hypoglycemia after 72 h but without an initial hyperglycemia as after experimental multiple trauma [**212**]. Interestingly, the cardiac glycogen concentration was significantly reduced after isolated long bone fracture but not after experimental multiple trauma [157, **212**]. In other studies, increased amounts of cardiac glycogen were associated with protection against trauma-induced cardiac damage [241]. In contrast, in the present study a reduction in hepatic glycogen concentrations indicated enhanced glycolysis after experimental multiple trauma but also after isolated long bone fracture [**212**]. Furthermore, the mRNA expression of the fatty-acid translocase (FAT/CD36)

decreased after multiple trauma, whereas the expression of the HFABP, the solute carrier 27 receptor (SLC27) type A1 and of the SLC27A6 significantly increased in the left ventricles of multiply injured pigs, suggesting an enhanced demand for LCFA due to the fast glucose consumption early after trauma [**212**]. After isolated long bone fracture, the mRNA expression of SLC27A6, FAT/CD36 and HFABP decreased significantly after 72 h, indicating a reduced cardiac consumption of LCFA [**212**]. The upregulation of HFABP, SLC27A1 and SLC27A6 might correlate with the severity of trauma and with cardiac dysfunction *in vivo* [**212**].

Summarized, the post-traumatic environment *in vivo* altered the cardiac substrate utilization by adjusting different transporters, which were involved in glucose- and fatty acid transport. Alterations were observed after experimental multiple trauma but also after isolated long bone fracture, suggesting a crucial and ubiquitous role of cardiac glucose- and fatty acid transport after trauma. These cardiac adaptions might correlate with trauma severity and seemed to be involved in the development of trauma-induced secondary cardiac injury in presence and absence of direct myocardial damage.

Cardiac function

Severe blunt cardiac injuries are characterized by impaired cardiac function, leading eventually to dysrhythmias, ventricular fibrillation and to sudden cardiac arrest of severely injured patients [149, 152, 154]. Furthermore, a depressed cardiac function was described after multiple trauma in pigs, which was manifested by a reduced cardiac shortening and ejection fraction 72 h after trauma [157]. Additionally, the systemic release of cardiac damage markers such as troponin and HFBAP were also demonstrated after multiple trauma in pigs and were further associated with aggravated cardiac function [157]. Interestingly, impaired cardiac function accompanied by a systemic elevation of troponin and HFABP were also described in the context of TISCI, even in the absence of direct myocardial damage [171, 173, 176, 242, 243]. However, the effects of isolated long bone fractures on cardiac function have not been investigated yet. Also, a distinct differentiation between experimental multiple trauma and isolated long bone fracture with regard to post-traumatic cardiac dysfunction has not been described so far.

In the present studies, the pigs revealed hemodynamic alterations 6 h after experimental multiple trauma but also 72 h after isolated long bone fracture, including decreased systolic-, diastolic and mean arterial pressure (MAP) as well as an

increased heart rate (HR) [**208**, **211**]. We further observed enhanced systemic troponin I and HFABP levels in pigs as well as in humans after long bone fracture, indicating myocardial tissue damage [**211**]. The systemic troponin I release in humans after long bone fracture was age-independent [**211**]. Elevated systemic levels of troponin I and HFABP were previously reported after experimental multiple trauma and were associated with impaired cardiac function and increased mortality [157, 228]. The troponin I release after fracture in the present study was not mediated through enhanced apoptosis of CMs but might be induced by the augmented levels of DAMPs and inflammatory mediators [**211**].

Summarized, hemodynamic alterations, impaired cardiac function as well as the systemic elevation of myocardial injury markers were observed after experimental multiple trauma but also after isolated long bone fracture. Consequently, aggravated cardiac function after trauma is not strictly due to blunt cardiac injury. The depressed cardiac function after experimental multiple trauma and after isolated long bone fracture was linked to systemic and local inflammation as well as to cardiac structural alterations. Based on the results of our studies, a clear distinction between direct and indirect post-traumatic cardiac damage *in vivo* was not identifiable and future investigations are necessary for clarification. In absence of direct myocardial damage cardiac alterations appeared, which were comparable to those after blunt cardiac injury. Consequently, it was not possible to precisely differentiate the distinct effects leading to secondary cardiac damage and cardiac dysfunction after multiple trauma. However, the contribution of exemplary individual injuries to the development of secondary cardiac injury was demonstrated.

3.3 Can the post-traumatic cardiac damage be reduced by hemadsorption? [**209**, **210**]

Therapeutic approaches for treatment of post-traumatic cardiac dysfunction are limited. Antibodies have been recently applied, improving cardiac function in mice after cecal ligation and puncture (CLP)- induced sepsis [188, 244] as well as in guinea pigs after burn injury [222]. Another promising therapeutic approach is the filtration of blood by hemadsorption [142-144]. Hemadsorption is routinely used in critically ill patients, receiving intensive care [146-148]. It was recently demonstrated that HMGB1 could be filtered from blood by hemadsorption, reducing HMGB1 levels up to 97% [145]. We showed for the first time that hemadsorption is also applicable for the reduction of circulating extracellular histones [**210**]. High levels of extracellular histones were reduced by hemadsorption up to 92% *ex vivo*. Moreover, blood plasma from multiple injured patients could be also cleared from extracellular histones [**210**]. Hemadsorption was also shown by others to capture inflammatory cytokines from the blood such as IL-1β, IL-6 and IL-10 [245]. In the present study, we demonstrated that Mdk was also reduced in human blood by hemadsorption. The hemadsorption column filtered Mdk dose-dependently up to 95% *ex vivo* [**209**]. Moreover, Mdk was significantly reduced from human blood plasma [**209**]. Therefore, it is likely that hemadsorption represents a promising therapeutic approach for prevention of post-traumatic cardiac damage especially with regard to the multifactorial generation [**209**].

Summarized, hemadsorption might be a very auspicious therapeutic approach especially for the mitigation of post-traumatic cardiac damage. However, this absorption column filters various proteins from human blood in a nonspecifically manner. As a result, essential anti-inflammatory mediators and other proteins which are important for a balanced inflammatory response after trauma might also be removed. Currently, hemadsorption is not routinely applied after trauma and further clinical studies are necessary for the confirmation of its clinical benefit regarding the reduction of post-traumatic cardiac damage.

4. Summary

The release of DAMPs from injured tissue, followed by the rapid activation of the innate immune system and by the generation of various inflammatory mediators play a crucial role after trauma. Through this activation, organ damage is restricted and the repair of injured cells and tissue is promoted. However, an imbalanced immune response, including the excessive liberation of DAMPs and inflammatory mediators can lead to the development of SIRS, MODS, MOF and finally to death of severely injured patients.

[208]: In this publication we showed that the complement system is rapidly activated after multiple trauma in pigs. There, the complement activation was mediated via the alternative pathway rather than the classical pathway. Moreover, the complement activation after experimental multiple trauma correlated with the invasiveness of fracture treatment and seemed the most enhanced in pigs with conventional reamed intramedullary nailing. Alterations in left ventricular protein and mRNA expression of C3aR, C5aR1 and C5aR2 indicated local cardiac complement activation after experimental multiple trauma. The immediate activation of the complement system was further associated with impaired cardiac function, which was evident by hemodynamic alterations as well as by a reduced cardiac ejection fraction. *In vitro* exposure of human CMs to recombinant C3a and C5a impaired CM function by aggravating cellular mitochondrial respiration and calcium signaling of the cells as well as by enhancing cellular caspase-3/7 activity. Therefore, the immediate activation of the complement system after multiple trauma might be crucially involved in the development of post-traumatic cardiac damage.

[209]: We further showed that the heparin-binding growth and differentiation factor Mdk plays a role in the context of multiple trauma. Mdk was found in blood plasma of multiple injured patients as well as in blood plasma of pigs 6 h after multiple trauma. After multiple trauma in pigs, the systemic Mdk levels correlated with the invasiveness of fracture treatment and seemed the most elevated after conventional reamed intramedullary nailing. Moreover, Mdk affected human CMs *in vitro* and the exposure of the cells to recombinant Mdk impaired their calcium signaling as well as their mitochondrial respiration, evidencing cardio-depressive effects of Mdk. These detrimental effects were probably mediated via TLR-signaling. Consequently, the

systemic release of Mdk after multiple trauma might also contribute to the generation of post-traumatic cardiac dysfunction. Hemadsorption might be a therapeutic option for mitigation of post-traumatic cardiac damage, filtering Mdk from blood plasma.

[210]: We could further demonstrate that elevated DAMPs such as extracellular histones and HMGB1 have harmful effects on human as well as on murine CMs *in vitro*. Incubation of the cells with these DAMPs reduced their viability, their metabolic activity, impaired their mitochondrial respiration and disturbed their calcium signaling. However, high levels of extracellular histones were removed by hemadsorption. As a result, systemic extracellular histones and HMGB1 might be involved in the pathomechanism of post-traumatic cardiac damage by aggravating CM function. Hemadsorption might be a therapeutic option to alleviate cardiac damage after trauma.

[211]: Regarding secondary cardiac injury after long bone fracture, we showed cardiac structural alterations in pigs 72 h after isolated femur fracture. Furthermore, there was evidence for myocardial damage after long bone fracture in pigs as well as in humans, which was manifested by enhanced systemic troponin I levels. The pigs also showed hemodynamic alterations 72 h after femur fracture. Myocardial damage as well as cardiac structural alterations after isolated long bone fracture were linked to elevated systemic levels of extracellular histones, C3a, C5a and TNF. In addition, the *in vitro* exposure of human CMs to a defined IFC (IL-1β, IL-6, IL-8, TNF, C3a and C5a) impaired the CM function. The cells showed enhanced cellular ROS production and impaired mitochondrial respiration. Therefore, secondary cardiac injury after isolated long bone fracture might be induced by enhanced systemic inflammation but also by local cardiac alterations, independently of direct myocardial damage.

[212]: Finally, we showed that the cardiac glucose- and fatty acid transport might play a role after multiple trauma but also after isolated femur fracture. The left ventricular expression of various receptors, which are involved in cardiac glucose- (GLUT1, GLUT4) and fatty acid transport (FAT/CD36, HFABP, SLC27A1, SLC27A6), was altered in pigs 72 h after isolated long bone fracture and after multiple trauma. The alterations correlated with the severity of trauma, suggesting cardiac adaptions of glucose- and fatty acid transport in response to trauma. Additionally, the *in vitro* exposure of human CMs to a defined PTC (IL-1β, IL-6, IL-8, TNF, C3a, C5a and

extracellular histones) induced alterations in cellular mRNA expression of cardiac glucose- and fatty acid transporters. These alterations suggested myocardial hibernation of the CMs in order to maintain viability and cellular function. Therefore, the cardiac glucose- and fatty acid transport might be also involved in the development of trauma-induced cardiac damage.

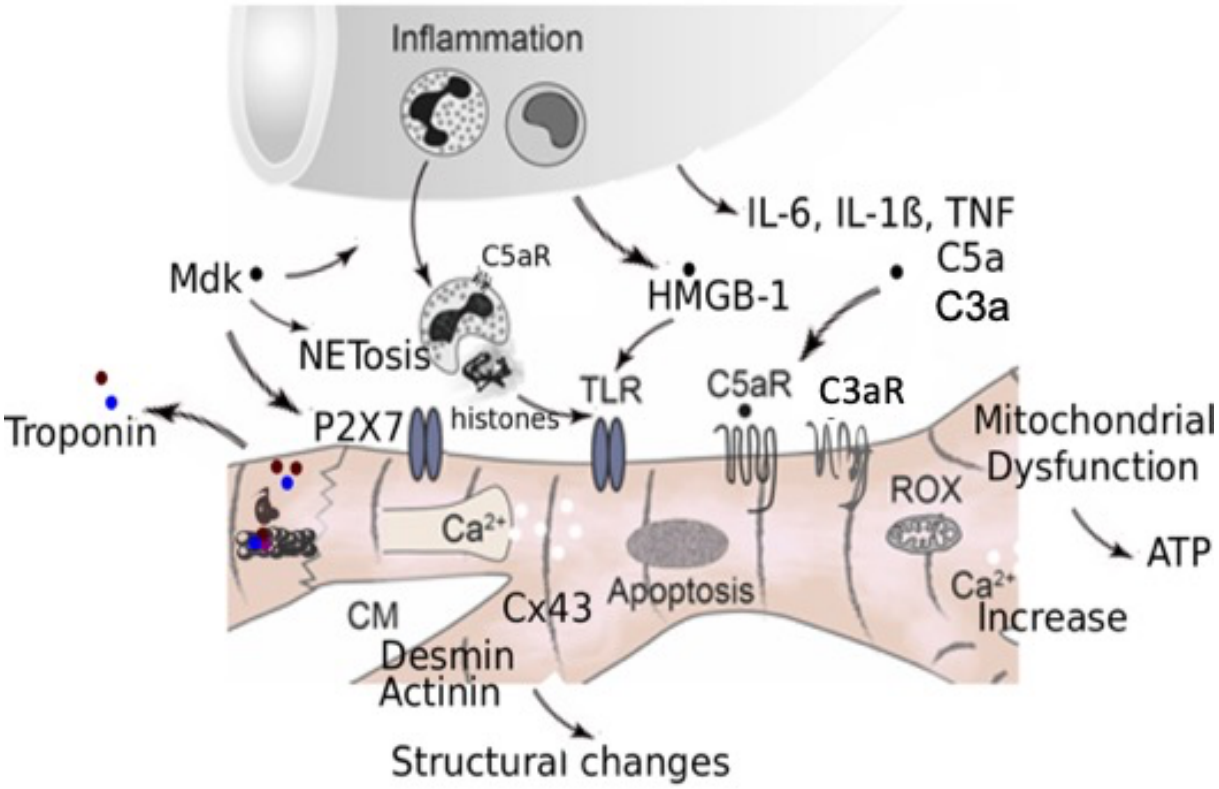

Fig. 1: Mediators and cellular mechanisms of trauma-induced secondary cardiac injury (adapted and modified by Lackner et al. 2019 [**209**], © by the authors, CC BY 4.0, https://creativecommons.org/licenses/by/4.0/)

After trauma, various DAMPs such as HMGB1 and extracellular histones are released from injured tissue and damaged cells. The systemic DAMP elevation is followed by the immediate activation of the innate immune system, including the generation of pro-inflammatory cytokines (IL-1β, IL-6, TNF and Mdk) and complement activation products (C3a and C5a). Furthermore, activated neutrophils of the innate immune system release extracellular histones by NETosis. These inflammatory mediators act

via different receptors (P2X7, TLRs and C5aR1) on human CMs *in vitro*, impairing their cellular function by disturbing the cellular calcium signaling, by aggravating mitochondrial respiration, by enhancing intracellular ROS production and by increasing cellular caspase-3/7 activity. Moreover, these pro-inflammatory mediators might also contribute to the development of trauma-induced secondary cardiac damage *in vivo*. Besides enhanced systemic and local cardiac inflammation, trauma-induced secondary cardiac damage *in vivo* is further associated with an altered left ventricular expression of α-actinin and desmin as well as with a left ventricular redistribution of Cx43. Additionally, trauma-induced secondary cardiac damage is further characterized by the systemic elevation of troponin I, indicating myocardial injury.

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Curriculum vitae

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Statutory Declaration

I hereby declare that I wrote the present dissertation with the title:

"**Mediators of trauma-induced secondary cardiac injury**"

independently and used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works. I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis" [Rules of the University of Ulm for Assuring Good Scientific Practice].

Ulm,

Ina Lackner

6. Appendix

Original publication (I)

Weber B, **Lackner I**, Baur M, Fois G, Gebhard F, Marzi I, Schrezenmeier H, Relja B, Kalbitz M. **Effects of Circulating HMGB-1 and Histones on Cardiomyocytes-Hemadsorption of These DAMPs as Therapeutic Strategy after Multiple Trauma.** *Journal of Clinical Medicine.* 2020 May 11;9(5):1421. doi: 10.3390/jcm9051421.

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IL performed the *in vitro* experiments and prepared the figures for the manuscript

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Article

Effects of Circulating HMGB-1 and Histones on Cardiomyocytes–Hemadsorption of These DAMPs as Therapeutic Strategy after Multiple Trauma

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Abstract: Background and purpose: The aim of the study was to determine the effects of post-traumatically released High Mobility Group Box-1 protein (HMGB1) and extracellular histones on cardiomyocytes (CM). We also evaluated a therapeutic option to capture circulating histones after trauma, using a hemadsorption filter to treat CM dysfunction. Experimental Approach: We evaluated cell viability, calcium handling and mitochondrial respiration of human cardiomyocytes in the presence of HMGB-1 and extracellular histones. In a translational approach, a hemadsorption filter was applied to either directly eliminate extracellular histones or to remove them from blood samples obtained from multiple injured patients. Key results: Incubation of human CM with HMGB-1 or histones is associated with changes in calcium handling, a reduction of cell viability and a substantial reduction of the mitochondrial respiratory capacity. Filtrating plasma from injured patients with a hemadsorption filter reduces histone concentration ex vivo and in vitro, depending on dosage. Conclusion and implications: Danger associated molecular patterns such as HMGB-1 and extracellular histones impair human CM in vitro. A hemadsorption filter could be a therapeutic option to reduce high concentrations of histones.

Keywords: hemadsorption; early myocardial damage (EMD), DAMPs; post-traumatic; calcium handling; mitochondrial dysfunction

1. Introduction

Early myocardial damage (EMD) after trauma is complex and multifactorial. Patients with elevated troponin levels in the emergency rooms have a higher injury severity score (ISS) and require often catecholamines [1,2].

Post-traumatic cardiac injury can be caused by mechanical forces on the heart, particularly in the case of blunt chest trauma [3–5] or by inflammation, which mediates secondary cardiac damage. Severe trauma often leads to a systemic inflammatory response, accompanied by the release of danger

associated molecular patterns (DAMPs), such as the high mobility group box-1 protein (HMGB-1) and extracellular histones. In humans, the release of HMGB-1 after trauma has been associated with injury severity, the activation of the complement system and increased mortality rates [6].

Extracellular histones have been linked to trauma-induced lung injury [7,8] and to cardiomyopathy during sepsis in mice [9]. In traumatized patients, enhanced levels of circulating histones were correlated with the Sequential Organ Failure Assessment score, endothelial damage and the activation of the coagulation system [8]. In experimental blunt chest trauma in rats and in experimental multiple trauma in pigs and mice, we observed a systemic release of extracellular histones [3–5].

HMGB-1 and extracellular histones act via Toll-like receptors (TLRs), which trigger the proinflammatory cytokine signalling, which is known to be cardio-depressive: Histones have been shown to act via TLR 2 and 4 on cardiomyocytes [9]. Furthermore, the role of the TLR9 interaction with histones has been described in liver injury [10]. TLR2 and 4 are activated on neutrophils by HMGB-1 [11]. In mice, the interaction between TLR 4 and HMGB-1 has been described in the development of liver injury [12]. Extracellular histones or HMGB-1 interaction with TLR4 increased the release of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL-)1ß and IL-6, which were shown to be cardio-depressive [13–16]. Additionally, histones were linked to the release of HMGB-1 via cell damage in liver, lung and kidney injuries in mice [13]. HMGB-1 is known to provoke cardiomyocyte dysfunction in cardiac hypertrophy, heart failure [17] and myocardial ischemia [18]. Extracellular histones are associated with increased intracellular reactive oxygen species, intracellular calcium in rodent cardiomyocytes and reduced mitochondrial membrane potential and ATP concentration [9].

We propose that HMGB-1 and extracellular histones can set off the development of post-traumatic cardiac dysfunction We also investigated whether hemadsorption may provide a therapeutic option to reduce the negative effects of extracellular histones. Previously, it was shown that hemadsorption improved the outcomes of patients with endotoxemia [19,20], necrotizing fasciitis and septic shock [21]. While the resorption of HMGB-1 by hemadsorption filter systems has been described before, we assessed the resorption capacity in regard to extracellular histones.

2. Material and Methods

2.1. In Vitro Incubation of Human Cardiomyocytes (CMs) with HMGB-1 and Histones

Human iPSC-derived cardiomyocytes (iPSC-CMs) were purchased from Cellular Dynamics, USA. The iPSC-CMs #11713 from Cellular Dynamics were donated from a healthy female donor. The age group at collection was 35–39 years and iPSC-CMs were obtained by episomal reprogramming of PBMC. The cells were cultured for 10 days at 37 °C and 7% CO_2 in iCell maintenance medium (Cellular Dynamics, Madison, WI, USA). After 10 days cells were incubated with either 100 ng/mL recombinant human HMGB-1 (R&D Systems) or with a mixture of 20 µg/mL extracellular histones (Sigma, St Louis, Missouri, USA) for 6 h at 37 °C and 7% CO_2. To assess caspase-3/7 activity in treated cardiomyocytes the Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) was used. Furthermore, cell viability was measured in the presence of histones or recombinant human HMGB-1 by using the Cell Titer-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Additionally, the appearance of HMGB-1 in the supernatant of cardiomyocytes cultured in the presence of histones were detected by using a HMGB-1-ELISA (lBL international, Hamburg, Germany). For all experiments $n = 6$.

2.2. In Vitro Incubation of HL-1 Cells with HMGB-1

For in vitro experiments, the murine cardiac muscle cell line (HL-1 cells) (Sigma Aldrich, St. Louis, MO, USA) was used. Murine HL-1 cells were cultured in HL-1 expansion medium at 37 °C in an atmosphere of 5% CO_2. Following this, HL-1 cells were incubated with HMGB-1 (R&D Systems) for 6 h. Cell viability was detected by using a Cell Titer-Glo®Luminescent Cell Viability Assay (Promega, Madison, WI, USA) in the presence of different concentrations of HMGB-1 (1 µg/mL, 100 ng/mL,

10 ng/mL). Moreover, we analysed the metabolic activity of the cells by MTT assay (Invitrogen, Waltham, MA, USA) in the presence of HMGB-1 dose-dependently (after 24 h of incubation). For all experiments $n = 6$.

2.3. Calcium Measurements

For calcium measurements, human CM were incubated with 20 µg/mL histones or with 100 ng/mL HMGB-1 for 60 min before the start of the experiments as well as for the duration of the experiment. To measure changes in intracellular Ca^{2+} concentration, cells were loaded with 5 µM Fura-2 (ThermoScientific, Waltham, MA, USA) for 30 min (in the presence of pharmacological compounds if needed). Fluorescence imaging was performed on a cell observer inverse microscope (Zeiss, Jena, Germany). Cells were illuminated for 90 ms at a rate of 2 Hz at each excitation wavelength (340 and 380 nm). Images were acquired using MetaFluor (Molecular Devices, Ismaning, Germany). Fura-2 ratios were calculated with ImageJ and the data obtained were analysed with the Matlab script PeakCaller [22]. The images were loaded in ImageJ and after background subtraction Fura-2340/380 ratios were calculated. The Fura-2 ratio traces representing changes in cytoplasmic Ca^{2+} concentration were analysed with the Matlab script PeakCaller [22] that allows to obtain the values of rise and decay time and height of calcium transients. For all experiments $n = 6$.

2.4. Mitochondrial Respiration

Mitochondrial respiration was analysed by using the Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA). For this experiment, human cardiomyocytes (iPS) were seeded in special Seahorse XFe96 cell culture plates (Agilent Technologies, Santa Clara, CA, USA) and were cultured for 10 days in iCell maintenance medium (Cellular Dynamics, Madison, WI, USA) at 37 °C and 7% CO2. After the cultivation, cells were treated either with 20 µg/mL extracellular histones or with 100 ng/mL HMGB1 in iCell maintenance medium (Cellular Dynamics, Madison, WI, USA) for 5 h at 37 °C and 7% CO2 and for an additional hour with either 20 µg/mL extracellular histones or with 100 ng/mL HMGB1 in Agilent Seahorse XF DMEM medium pH 7.4 (Agilent Technologies, Santa Clara, CA, USA), supplemented with 1 mM sodium pyruvate (Sigma Aldrich, St. Louis, MO, USA), 2 mM L-Glutamine (ThermoFisher, Waltham, MA, USA) and 50 mM glucose (Sigma Aldrich, St. Louis, MO, USA) at 37 °C and 7% CO2. After exposure of the cells to extracellular histones or HMGB1, the mitochondrial respiration was measured. Therefore, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA) was used. With the Seahorse XF Cell Mito Stress Test mitochondrial function of cells can be assessed and multiple parameters are obtained in one assay, including basal respiration, maximal respiration and spare respiratory capacity. During the entire experimental procedure, the oxygen consumption rate (OCR) in pmol/min is measured continuously by the Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA). The mitochondrial respiration was assessed as follows: during the experimental process, 2 µM oligomycin, 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and 0.5 µM antimycin A and rotenone were added to the cells by programmed injection in defined intervals and after each addition the OCR of the cells were measured. After the experiments, the different parameters for basal respiration, maximal respiration and spare respiratory capacity were calculated by using the Seahorse Wave 2.4 software (Agilent Technologies, Santa Clara, CA, USA). Following this, the obtained parameters of mitochondrial respiration were further normalized on the total amount of mitochondria of the cells. Therefore, the cells were fixed with 4% formalin at 4 °C overnight after the experiment. Then, the cells were stained with 0.2% Janus-Green solution, which specifically stains the mitochondria of cells. Afterwards, cells were washed and resolved with 0.5 M hydrochloric acid. Optical density (OD) was measured at 630 nm, correlating with the amount of cellular mitochondria. Then, the oxygen consumption rate (OCR) values of the mitochondrial parameters were normalized to the OD 630 nm values, respectively. Results were evaluated using Seahorse Wave 2.4 software (Agilent Technologies, Santa Clara, CA, USA). For all experiments $n = 6$.

2.5. Plasma Samples of Polytrauma Patients

Human plasma samples from 20 multiple injured patients with a history of acute blunt or penetrating trauma and an ISS ≥16 were collected after hospital admission to the University Hospital of the Goethe-University Frankfurt. The plasma collection was approved by the institutional ethics committee (312/10), in accordance with the Declaration of Helsinki. All enrolled patients signed the written informed consent form themselves or written informed consent was obtained from the nominated legally authorized representative on the behalf of participants in accordance with ethical standards. Exclusion criteria were younger than 18 or older than 80 years of age, severe burn injury, acute myocardial stroke, cancer or chemotherapy, immunosuppressive drug therapy, HIV, infectious hepatitis, acute cytomegalovirus (CMV) infection and/or thromboembolic events.

Blood samples were withdrawn in ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt, Nürmbrecht, Germany) directly after admission. The samples were kept on ice until centrifugation at 2100× *g* for 15 min. Then the supernatant was collected and stored at −80 °C until assay.

2.6. Hemadsorption in Plasma of Multiple Injured Patients

We developed small hemadsorption-columns for a volume of 150 µL plasma by using a Cytosorb® 300-column (CytoSorbents Inc., Monmouth Junction, NJ, USA). 300 µL of the content of the column was aliquoted in Ultrafiltration Spin-Columns (0.45 cutoff; Merck Milipore, Billerica, MA, USA) and was centrifuged before incubation with plasma of multiple trauma patients. After 6 h of incubation an additional centrifugation followed. The samples were then stored at −80 °C. Furthermore, we investigated a dose-depending hemadsorption-curve of histones (mixture of histones). Therefore, histones of calf thymus were diluted in Aqua dest. with the concentrations 700, 500, 300, 200, 100, 50 and 25 µg/mL. They were incubated for 6 h on a plate shaker and were centrifuged before measuring the histone concentrations.

2.7. Statistical Procedures

All values are expressed as mean ± SEM. Data were analysed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Students *t*-test was used in the case of comparison of two groups. $p \leq 0.05$ is considered statistically significant. GraphPad Prism 7.0 software was used for statistical analysis (GraphPad Software, Incorporated, San Diego, CA, USA).

3. Results

3.1. Decreased Apoptosis in Human Cardiomyocytes and Alterations of Calcium Handling in Presence of HMGB-1 and Histones

In the presence of histones, cell viability of cardiomyocytes was significantly reduced (Figure 1A), whereas the detection of pro-apoptotic caspase was neither changed in the histone nor in the HMGB-1 treated CM (Figure 1B). We also investigated changes in calcium handling of CM in the presence of histones or HMGB1. The mean height calcium signal was reduced in the presence of HMGB-1 (Figure 1C), while the mean rise time of the calcium signal was neither changed in the presence of histones nor after incubation with HMGB-1 (Figure 1D). In Figure 1E, we demonstrate the increase of the mean decay time of the calcium signal in presence of histones. Furthermore, the frequency of the calcium signal in CM was significant reduced in the presence of histones, as well as after incubation with HMGB-1, which was associated with bradycardia of the CMs in vitro (Figure 1F).

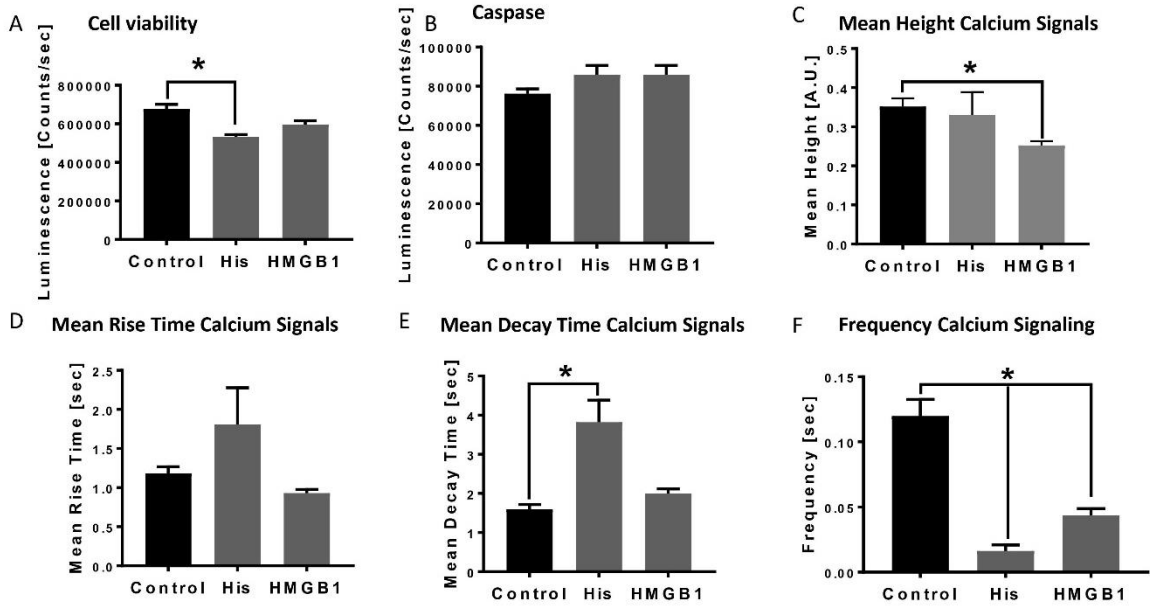

Figure 1. Decrease in cardiomyocytes viability, apoptosis and alterations of calcium handling in the presence of HMGB-1 and histones: Cell viability of human cardiomyocytes (Luminescence in counts/sec) in the presence of 20 µg/mL histones (His) and 100 ng/mL HMGB-1 (37 °C, 6 h) compared to the control (PBS) (**A**). Caspase-3/7 activity (counts/sec) in human CMs in the presence of histones and HMGB-1 (**B**). Mean Height calcium signals (A.U) in the presence of histones and HMGB-1 compared to the control (**C**). Mean rise time (sec) (**D**) and mean decay time (sec) (**E**) of calcium peaks of human CMs in the presence of histones and HMGB-1. Frequency of calcium signals (sec) in human cardiomyocytes treated 20 µg/mL histones and 100 ng/mL HMGB-1 (**F**). Results are presented as mean ± SEM, for all experiments $n = 6$. Indicated results were significant * $p < 0.05$.

3.2. Decrease in HL-1 Cell Viability and Metabolic Activity in Presence of Different HMGB-1 Concentrations

Cell viability of HL-1 cells were significantly reduced in the presence of 1 µg/mL, 100 ng/mL as well as 10 ng/mL HMGB-1 (Figure 2A). By conducting the MTT assay, we detected a reduction of metabolic activity in the HL-cells in the presence of all tested HMGB-1 concentrations (Figure 2B).

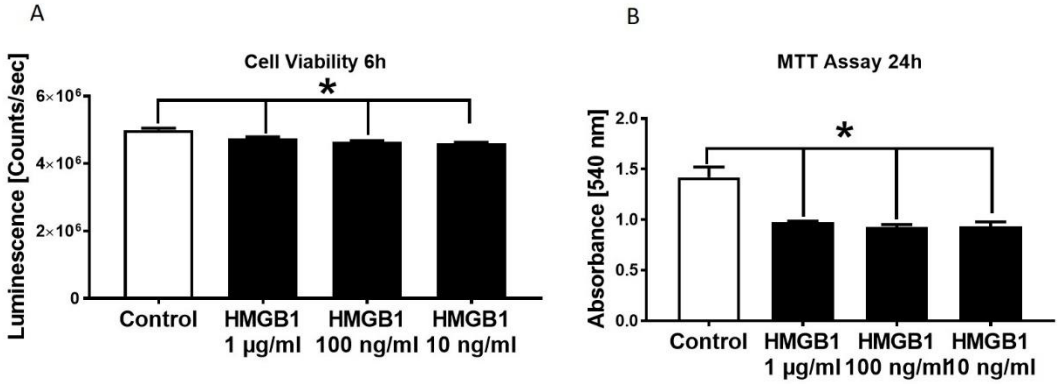

Figure 2. Decrease in HL-1 cell viability and metabolic activity in the presence of different HMGB-1 concentrations. HL-1 cell viability in the presence of different HMGB-1 concentrations after 6 h of incubation compared to control-HL-1 cell viability (**A**). Metabolic activity measured in HL-1 cells in the presence of different HMGB-1 concentrations incubated for 24 h compared to controls (**B**). $n = 6$. Results were significant * $p < 0.05$.

3.3. Metabolic Alterations of Human Cardiomyocytes in Presence of Histones

Metabolic alterations of human CMs in the presence of a mixture of histones or HMGB-1 were investigated. The basal respiration decreased tendentially in the presence of histones or HMGB1 compared to the control group (Figure 3A,D). Moreover, we observed a decrease of the maximal respiration capacity in the presence of both HMGB-1 and histones (Figure 3B,E). In addition, the spare respiratory capacity decreased significantly in the presence of histones or HMGB-1 (Figure 3C,F).

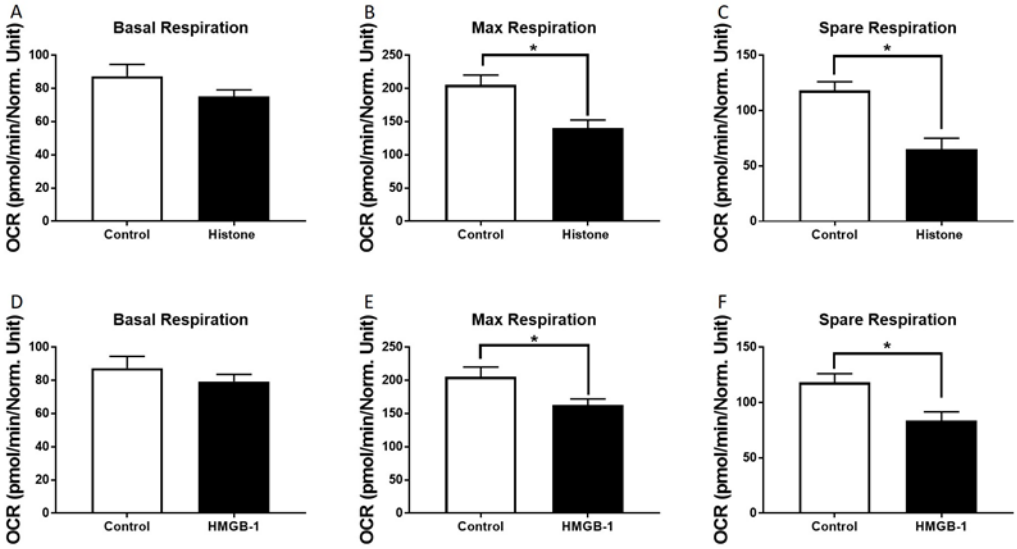

Figure 3. Metabolic alterations of human cardiomyocytes (CM) in the presence of histones: Basal respiration (OCR in pmol/m in/Norm. unit) of human CMs in the presence of 20 µg/mL histones (37 °C, 6 h) (**A**). Maximal respiratory capacity (OCR in pmol/m in/Norm. unit) of human CMs in the presence of 20 µg/mL histones compared to the control (PBS) (**B**). Spare respiration (OCR in pmol/m in/Norm. unit) of human CMs in the presence of 20 µg/mL histones (37 °C, 6 h) (**C**). Basal respiration (**D**), max respiratory capacity (**E**) and spare respiration (**F**) of human CM in the presence of 100 ng/mL HMGB-1. Results are presented as mean SEM, for all experiments $n = 6$. Results are significant * $p < 0.05$, statistical analysis with *t*-test.

3.4. Hemadsorption—A Therapeutic Option to Eliminate Systemic Extracellular Histones

To evaluate the therapeutic potential of hemadsorption we analysed the absorption capability and capacity for extracellular histones by CytoSorb®300. After incubation of different histone concentrations, the levels decreased between 92% and 99% within 6 h (Figure 4A). Moreover, extracellular histone concentration in blood samples of multiple injured patients collected at admission to the hospital significantly dropped after absorption by the hemadsorption filter (Figure 4B).

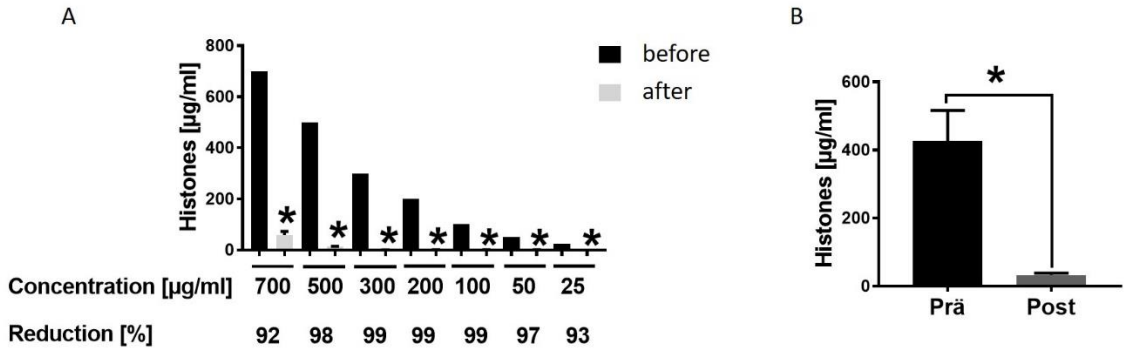

Figure 4. Hemadsorption—a therapeutic option to eliminate systemic extracellular histones. (**A**). Hemadsoption of different concentrations of histones (700, 500, 300, 200, 100, 50, 25 µg/mL) presented as percentage of reduction (%) after 6 hrs incubation time; for all concentrations $n = 6$. (**B**). Histone levels in blood plasma of 22 multiple injured humans (µg/mL) in the Emergency room (pre) and after hemadsorption for 6 h (post). Results were presented as SEM, * $p < 0.05$.

4. Discussion

In this report, we demonstrated the detrimental effects of HMGB-1 and histones on cardiomyocytes. Recent studies revealed an increase of circulatory histones after experimental blunt chest trauma in rats [4], after multiple trauma in pigs [3] and of circulating HMGB-1 in pigs after multiple trauma [23]; observed together with depressed cardiac function [3]. The presented data underpins that both HMGB-1 and extracellular histones impair myocardial functions, leading to consequences such as a deceleration of frequency of CMs and an impaired mitochondrial respiration in vitro.

The role of HMGB1 on cardiac function and the potential detrimental effects are controversial so far. On the one hand, HMGB-1 is systemically elevated in models of myocarditis and ischemic myocardial infarction in mice [24]. In addition, the application of anti-HMGB-1 antibody in hemorrhagic shock in mice was associated with decreased systemic release of cardiac enzymes, reduced local ATP depletion and systemic levels of inflammatory mediators like TNF and IL-1ß [25]. Extracellular HMGB-1 binds directly to TLR 4 as well as to the receptor for advanced glycation end products (RAGE), which was linked to the production and release of inflammatory cytokines [14,15] such as TNF, IL-1ß and IL-6 [16], which were shown to be cardio-depressive [26]. An increase of systemic IL-6 concentration has been linked to cardiac dysfunction, demonstrated as a reduction of stroke volume, cardiac output and the performance of the left ventricle [27,28]. In rat CMs the presence of TNF and IL-1ß led to dysfunction of the calcium balance: it prolongs the calcium transient duration and therefore the action potential, as well as leads to asynchronous calcium release during electrical stimulation. Further, these cytokines increased the vulnerability of the sarcoplasmic reticulum for spontaneous calcium leakage. TNF and IL-1ß depressed calcium transient, the contractility and therefore have been linked to arrhythmogenicity in ventricular rat CM [29]. The presence of IL-1ß could be associated with prolonged action potential duration, the reduction of the transient potassium current of 35%, therefore a reduced repolarization in CM and again the increase of diastolic sarcoplasmic calcium leakage. Together, these changes led to high potential of cardiac arrythmias [30]. Furthermore, RAGE-knock out mice showed lower myocardial inflammation and fibrosis compared to wild-type mice in a model of inflammatory heart disease [31]. Besides, these effects HMGB-1 was shown to influence the post-traumatic development of microvascular thrombosis and endothelial cell activation via inhibition of the anticoagulant protein C pathway mediated by the thrombin-thrombomodulin complex, and further stimulated tissue factor expression on monocytes [32]. High plasma levels of HMGB1 are associated with increased complement activity as indicated by elevated soluble C5b-9 plasma levels that are generated during the late phase of complement activation [6]. The connection between complement activation and cardiac depression is well described after trauma [33] and sepsis [34,35].

On the other hand, Limana, et al. (2005) [36] injected low-dose HMGB-1 in the left ventricles of infarcted mice hearts and observed a partial reconstitution of the defect by a myocardial population after one week compared to the beginning of scar formation in non-HMGB-1 treated animals. The intervention with HMGB-1 resulted in the formation of new myocytes within the infarcted portion of the wall via the proliferation and differentiation of endogenous cardiac c-kit+ progenitor cells [36]. In addition, ejection fraction measured by echocardiography improved in the locally HMGB-1 treated animals after myocardial infarction [36]. The application of HMGB-1 after myocardial infarction also decreased levels of IL-1, IL-6, IL-10 and vascular endothelial growth factor (VEGF) [37]. Transgenic mice with an overexpression of cardiac-specific HMGB-1 were protected against the consequences of myocardial infarction [38], whereas animals with HMGB1 null mutation are nonviable [39].

However, our in vitro data demonstrates a deceleration of the natural frequency of human CMs, as well as changes in the calcium handling in the presence of HMGB-1 and a reduction in mitochondrial respiration. This clearly indicates the negative effects of HMGB-1.

Therefore, therapeutic elimination of high systemic concentrations of HMGB-1 might be an option to reduce cardiac dysfunction after trauma. microRNA-26a inhibited the HMGB-1 expression in an ischemic/reperfusion model, which is correlated with less infiltration of inflammatory cells and a decreased cytokine release [40]. Moreover, an overexpression of microRNA-142-3p targeting HMGB-1 gene in mouse cardiomyocytes presented a significantly lower apoptotic rate as controls after [41]. However, the context of trauma cardiac damage is complex and multifactorial. Therefore, a multipotent therapeutic option could be a hemadsorption filter, which was described to be useful for the elimination of not only HMGB-1 [42] but also other inflammatory molecules such as cytokines, midkine or active complement components [43,44]. The presented report demonstrates the initial usefulness of hemadsorption filters to eliminate extracellular histones. Further studies need to evaluate the therapeutic clinical use after trauma.

Recently circulating histones were discussed as new biomarkers after trauma [45,46]. In previous studies we demonstrated that histones bind to the surface of rat CMs [9]. Incubation of human cardiomyocytes with histones increased the levels of cytosolic reactive oxygen species (ROS) [9]. This observation was dose-dependent [9]. Increased intracellular ROS levels were associated with increased cytosolic calcium concentration in CMs by modulating calcium handling proteins [47] and by blocking the sarcoplasmic endoplasmic reticulum-transporting ATPase (SERCA) [48]. The data revealed that calcium signalling in human CMs was disturbed in the presence of histones and HMGB-1. The frequency of calcium signals decreased either in the presence of HMGB-1 or histones. This was in line with findings obtained from Langendorff perfused mouse hearts in the presence of histones, which showed sinus bradycardia as well as the development of ventricular bigeminy in the ECG [9]. Moreover, this report shows that the mean decay time of the single calcium peaks were altered in human CMs in the presence of histones, which was in accordance with earlier findings demonstrating disturbed calcium handling by enhanced build-up of (Ca^{2+}) [34]. Apart from that, it is also known that histones interact with the phospholipid-membrane of cells, which leads to higher permeability and a calcium influx in cells [8,49–51]. Rat CM incubated with histones featured increased intracellular calcium, which was demonstrated to be dependent on the expression of TLR2 and 4. In the absence of TLR2 or 4 increases of intracellular calcium in cardiomyocytes in the presence of extracellular histones were ameliorated [9].

Increased intracellular calcium concentration have been linked to the cell toxicity of extracellular histones [8]. Alhamdi et al. (2015) [52] cultured cardiomyocytes with the plasma of patients with sepsis, resulting in a significant reduction of cell viability after incubation with serum containing > 75 µg/mL histones compared to incubation with the sera of healthy controls [52]. In the present study, the cell viability of human cardiomyocytes was decreased in the presence of 20 µg/mL extracellular histones. The caspase 3/7 activity did not change compared to the controls.

As presented in Figure 1, the presence of histone led to changes in mitochondrial respiration, especially the spare respiratory capacity, which was impaired. These findings were in accordance with

earlier studies demonstrating reduced mitochondrial membrane potential in isolated rat CMs in the presence of extracellular histones [9].

Therapeutically, anti-histone antibodies have been applied experimentally to reduce high systemic histone concentrations [52,53]. Furthermore, the application of anti-histone antibodies improved cardiac dysfunction in mice with cecal ligation and puncture induced sepsis [9,52]. Hemadsorption is routinely used in critically ill patients [19–21]. Here, we proved for the first time that this filter system reduced high concentrations of extracellular histones. Therefore, the hemadsorption is a promising therapeutic option in severely injured patients because of its multifactorial character. It has been shown to not only reduce extracellular histones and HMGB1 but also complement factor C5a and cytokines [19–21,42]. Hemadsorption may have to mitigate the consequences of these factors that have all been linked to cardiac damage and cardiac dysfunction. In further clinical studies, the advantages of hemadsorption in multiple injured patients has to be evaluated in regard to its ability to ameliorate EMD.

One major limitation of the present study is the missing testing of CMs function in the presence of a patient's plasma before and after hemadsorption. A potential depression of CMs function could be examined by analysis of the mitochondrial respiration (basal, spare and maximal respiration) as well as of the CMs contraction in the presence of non-filtered and filtered polytrauma plasma. Furthermore, the positive effects of hemadsorption could be investigated by intracellular calcium measurements, including the analysis of the mRNA expression of important calcium-handling proteins (NCX or SERCA). Further experiments could also include structural changes in human CMs in the presence of filtered and non-filtered polytrauma plasma, for example the measurement of connexins, intracellular troponin, alpha-actinin or desmin. Although these experiments seem interesting and would fit well in the context of the manuscript, they are beyond its scope.

The focus of the present report lies in the distinct effects of HMGB-1 and extracellular histones on human CMs functionality. In a previous study, we treated human CMs with a defined polytrauma-cocktail, including miscellaneous inflammatory cytokines, complement activation products and DAMPs (IL-1ß, IL-6, IL-8, TNF, C5a, C3a, HMGB1 and extracellular histones), mimicking the inflammatory conditions of multiple trauma in vitro [54]. In this study, we demonstrated that the entirety of these inflammatory molecules acted detrimentally on the CMs, impairing their cellular glucose and fatty acid transport, which might contribute to impaired cardiac function after multiple trauma [55]. However, our main focus lies on the specific effects of the individual components, therefore we treated the cells either with HMGB1 or with extracellular histones in the present study. Moreover, we did not use the plasma before and after hemadsorption, because we cannot exclude that the effects on CMs were mediated via other cytokines or DAMPs. The hemadsorption filter system was shown to reduce unpacifically the concentration of a wide range of inflammatory- and damaging molecules, therefore it is used for treatment of patients with SIRS or sepsis. In order to understand the complex pathomechanism of post-traumatic cardiac dysfunction, the particular influence of the individual DAMPs on CMs should be tested primarily as well as the filtration capacity of the hemadsorption column for these specific molecules. Moreover, in order to further verify the distinct effects of the single components, neutralizing antibodies might be used. However, this was not the goal of the present study and should be investigated in detail in future studies. A correct assignment between damaging mediators, their effects on CMs and the benefit of a mediator's reduction is only possible due to precise testing of the individual mediators.

5. Conclusions

To summarise, this study confirms that nuclear proteins provoke cardiac damage and cardiac dysfunction after trauma. HMGB-1 and extracellular histones are released after multiple trauma. Despite their positive roles in the immune reaction, these mediators are associated with detrimental effects on cardiomyocytes including their viability, calcium handling and mitochondrial function.

The study also shows that hemadsorption is a useful therapeutic option to reduce DAMPs concentrations and to potentially ameliorate EMD after trauma.

Author Contributions: Regarding contributions of the authors B.W., I.L., M.B., B.R., G.F. performed the experiments including cell culture experiments and ELISAs. B.W. and M.K. primarily wrote the paper. B.R., I.M., F.G., H.S. and M.K. contributed to the experimental design and data analysis and coordinated the study and supervised financial support for the studies. All authors made substantial contributions to the conception and design of the study and participated in drafting the article. All authors gave final approval of the version to be published.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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SYSTEMIC AND CARDIAC ALTERATIONS AFTER LONG BONE FRACTURE

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ABSTRACT—The purpose of this study was to reveal possible consequences of long-bone fracture on cardiac tissue and to analyze the role of systemically elevated danger associated molecular patterns, complement anaphylatoxins and cytokines. Blood samples of mice, pigs, and humans after a fracture were analyzed by ELISAs for complement component 5a (C5a), tumor necrosis factor (TNF), and extracellular histones. *In vivo* results were completed by *in vitro* experiments with human cardiomyocytes treated with TNF and extracellular histones. The influence of histones and human plasma after fracture on isolated human polymorphonuclear leukocytes (PMNs) was investigated. An elevation of TNF, C5a, and extracellular histones after long bone fracture was measured. Moreover, the appearance of systemic troponin I levels was observed and structural changes in connexin 43 and desmin were detected. Further, the presence of TNF leads to elevation of reactive oxygen species, troponin I release, and histone appearance in supernatant of human cardiomyocytes. Incubation of human PMNs with histones and plasma of patients after fracture lead to formation of neutrophil extracellular traps. Present results suggest that structural alterations in the heart might be consequences of the complement activation, the release of extracellular histones, and the systemic TNF elevation in the context of a long bone fracture.

KEYWORDS—Complement system, connexin43, damage control orthopedics, early total care, HFABP, polymorphonuclear leukocytes, troponin I

INTRODUCTION

Physical trauma are an increased burden for the society (1) and reflect a major challenge for the future. Despite direct cardiac damage after physical trauma, there is rising evidence for trauma-induced secondary cardiac structural and functional damage (2, 3). After multiple trauma in pigs, a functional and structural damage of the heart has been described in previous studies (4); as well as a linkage between systemic cardiac troponin I levels of multiple injured patients and the corresponding survival (5). Further, an association between hip fracture and the appearance of coronary heart disease was made (6, 7). Therefore, the question arises whether and how single long bone fractures may affect the heart.

In the present study, different animal models and human samples were analyzed to investigate whether a long bone fracture and the inflammatory consequences are linked to cardiac alterations. In particular, the role of tumor necrosis factor (TNF), complement component 5a (C5a), and extracellular histones after fracture were investigated. TNF is well known to be elevated after fracture (8, 9) and to be cardio-depressive (10, 11). TNF induces apoptosis in cardiomyocytes and leads to cardiac dysfunction in different models. (10, 12–14). Activation of the complement system, especially C5a, caused cardiomyocyte dysfunction during sepsis (15). Likewise, after fracture the complement system has been shown to be activated (16, 17). Furthermore, C5a leads to an activation of neutrophil granulocytes, which are able to form neutrophil extracellular traps (NETs) (18, 19), containing DNA and extracellular histones. Extracellular histones are described as a mediator of septic cardiomyopathy. Histones caused disequilibrium in the redox status and intracellular $[Ca^{2+}]i$ levels in cardiomyocytes (CMs) and disturbed functional and electrical responses of hearts perfused with histones and induced defects in mitochondrial function (20). Therefore, the NET formation by neutrophils in patients with bone fracture will be investigated in regard of its role in amplifying systemic release of damage-associated molecular patterns (DAMPs) and consequently causing organ damage.

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The authors report no conflicts of interest.

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MATERIALS AND METHODS

Mice

All animal experiments were performed according to the international regulations for the care and use of laboratory animals and were approved by the responsible government authority (No. 1096 and 1149, Regierungspräsidium Tübingen, Germany). They were performed according to the guidelines of the Federation of European Laboratory Animal Science Association (FELASA). Mice received either sham treatment or femur osteotomy for 6 and 24 h, respectively. For the respective groups, five to seven animals were used. Briefly, an osteotomy (0.4 mm) was performed at the mid-shaft at the right femur of 12 weeks old, male C57BL/6J-Mice, purchased from Charles River (Sulzfeld, Gemany). General anesthesia was conducted with 2% isoflurane, mice received tramadol for analgesia. The femur fracture was stabilized by using an external fixator (RISystem, Davos, Switzerland). Blood plasma from mice was collected at the end of the experiments, 6 and 24 h after fracture or sham procedure, by puncture of the heart under deep anesthesia. 1 mL whole blood was collected, centrifuged at 4,000 ×g for 10 min and plasma was stored at −80°C. Details of the animal experiment and sample collection were already published in Kroner et al. (21) and Kovtun et al. (22).

Pigs

Animals and anesthesia—All the procedures conformed to the Society of Laboratory Animal Science as well as the National Animal Welfare Law and gained approval from the responsible government authority (“Landesamt für Natur, Umwelt, und Verbraucherschutz”: LANUV-NRW, Germany: AZ TV-No.: 84–02.04.2014.A265). They were performed according to the guidelines of the FELASA. Animal experiments adhere to the Animal Research: Reporting of *In Vivo* Experiments guidelines for reporting animal research. In the interest of limiting animal numbers, the samples were obtained from a recently conducted prospective randomized experimental study performed by the TREAT Research Group. The model has been previously described in detail by Horst et al. (23). Briefly, eleven 12 to 16-week-old male pigs with a mean body weight of 30 ± 5 kg (Sus scrofa dormestica, Tierzucht GmbH Heinrich, Heinsberg, Germany) were included in the study. Anesthesia was induced and maintained during the study period of 72 h with propofol (1–2 mg/kg body weight). The pigs were orotracheally intubated and ventilation was conducted in biphasic positive airway pressure mode in a lung protective ventilation (6–8 mL/kg body weight). Vital parameters were monitored by electrocardiographic (ECG) recordings and ECG-synchronized pulse oximetry. Fluids were administered by continuous crystalloid infusion (Sterofundin ISO; 2 mL/kg body weight /h).

Fracture surgery—Pigs underwent a femur fracture (n = 8) or sham procedure (n = 3). The Sham procedure included anesthesia but no femur fracture. The fracture group (n = 8) was randomized in two therapy arms (n = 4); external fixation of the femur fracture corresponding to damage control orthopedics (DCO) or femoral nailing appropriate to early total care (ETC) principles.

Sample collection—Whole venous blood samples, containing all physiological blood components, were collected from *V femoralis* at baseline as well as 1.5, 3.5, 5.5, 24, 48, and 72 h after fracture. At each time point, 6 mL whole venous blood was collected from the pigs. Blood loss was adjusted adequately by continuous crystalloid administration (Sterofundin ISO; 2 mL/kg body weight /h). Samples were kept on ice and serum was obtained by centrifugation (2,000 × g for 15 min at 4°C). After centrifugation, serum was removed and stored at −80°C until analysis. Heart tissue of left ventricles was obtained 72 h after multiple trauma and fixed with 4% formalin followed by embedding in paraffin.

Humans

Human blood sample collection was approved by the Ethical Committee of the University Medical Center Ulm (No. 438/15) as recently published (24) and was in accordance with the Declaration of Helsinki. All the enrolled patients signed the written informed consent form themselves or written informed consent was obtained from the nominated legally authorized representatives on the behalf of participants in accordance with ethical standards. All the methods were performed in accordance with the ethical guidelines of the Ethical Committee of the University Medical Center Ulm (No. 438/15).

In total, 26 patients (age: 32–97 years, mean: 75 years) with metaphyseal/diaphyseal fractures of long bones (femur, tibia, humerus, radius, ulna) treated surgically at the University Medical Center Ulm between January 2016 and January 2018 were included. Exclusion criteria were polytrauma, pregnancy, bone diseases except primary osteoporosis, intake of bisphosphonates or parathyroid hormone, rheumatoid arthritis, open fractures grade 3 or 4 according to Tscherne and Oestern, hepatic or nephritic insufficiency, cancer, intake of steroids, intake of immunosuppressive medication, chemotherapy in the last 3 months, and artificial ventilation after surgery. In further subgroup analyses, patients with femur fracture (AO-31 A1/A2/A3/B2; n = 19) were assigned to two groups at d0: male fracture patients (n = 6, age: 32–97 years, mean: 69 years) and female fracture patients (n = 13, age: 57–87 years, mean: 78 years). Furthermore, 20 healthy volunteers (10 males aged 24–57, mean: 37; 10 females aged 27–87 years, mean: 47 years) donated one blood sample each as controls. The collective of the analyzed serum was published previously by Fischer et al. (24). Peripheral venous blood was obtained from each patient at the day 0 after fracture event. Additionally, blood from seven patients was obtained at day 14 after fracture. Blood samples were centrifuged to get serum, which was stored at −80°C until analysis. Details of sample collection and the patient cohort were previously published by Fischer et al. (24).

ELISAs

Serum and plasma samples were analyzed using ELISA kits according to the manufacturer’s instructions. Histones in mice plasma, pig serum, and human plasma were measured by using a cell death detection ELISA kit (Hoffmann-La Roche, Indianapolis, Ind). A histone mixture (containing H2, H2A, H2B, H3, H4) (Sigma, St.-Louis, Mo) was used to establish a standard curve. Cardiac troponin I concentrations in mouse plasma, pig serum samples, human plasma, and supernatant of human cardiomyocytes were measured by using species-specific ultrasensitive troponin-I ELISA kits (Life Diagnostics, West Chester, Pa) and cardiac troponin I human simple Step ELISA Kit (Abcam, Cambridge, UK). C5a was measured in patient and mouse plasma by using a C5a ELISA (DRG Diagnostics, Marburg, Germany; R&D Systems, McKinley, Minn). IL-6 was measured in plasma/serum of mice/humans with fracture by IL-6 ELISA (R&D Systems, McKinley, Minn). TNF concentrations were measured in mice plasma samples by TNF ELISA (R&D Systems, McKinley, Minn). Human TNF was detected in serum of fracture patients measured by TNF ELISA (Abcam, Cambridge, UK).

Immunohistochemistry

For immunohistochemistry porcine, formalin-fixed left ventricles were used. For complement component 5a receptor (C5aR1) staining polyclonal Rabbit anti CD88/C5aR1 (Acris Antibodies, Herford, Germany) was used as primary antibody. For Connexin (Cx43) staining rabbit anti-pig Cx43 (Cell Signaling Technology, Danvers, Mass) was used. Nitrotyrosine staining was performed using antinitrotyrosine (Merckmillipore, Darmstadt, Germany). Dako REAL Detection System (Dako, Glostrup, Denmark) was used as a secondary system. Signal density was measured in nine randomly chosen, distinct fields of vision from each slide using an Axio ImagerM.1 microscope and the Zeiss AxioVision software 4.9 (Zeiss, Jena, Germany). Results are presented as mean density of each group (arbitrary units).

Confocal imaging

For the confocal imaging, the left ventricles of pigs 72 h after fracture (DCO and ETC) (n = 8) or after sham treatment (n = 3) were analyzed. Therefore, formalin fixed and paraffin embedded heart tissue from left ventricles were used from the pigs.

For α-actinin-2 staining rabbit anti-α-actinin (clone N1N3) (GeneTex, Ivine, Calif) was used as primary and goat antirabbit (AF-488) as secondary antibody (Jackson Immuno research Laboratories, West Grove, Pa). For desmin staining mouse antidesmin (GeneTex, Irvine, Calif) was deployed as primary and goat antimouse (AF-647) as secondary antibody (Jackson Immuno Research Laboratories, West Grove, Pa). Following staining sections were mounted with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, Calif). Confocal imaging was performed using Leica SP8 (Leica microsystems, Wetzlar, Germany). Evaluation of fluorescence-intensity was conducted by the Software Image J x1 (25). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed by using CF 488 TUNEL Apoptosis Detection Kit (Biotium, Fremont, Calif). A counterstain with Höchst 33342 (Sigma, Darmstadt, Germany) was done. Results of TUNEL positive nuclei were presented in percentage of whole nuclei number.

Determination of RNA expression of C5aR1 in pig left ventricle homogenates by real-time quantitative PCR analysis

Total RNA was isolated from pig heart homogenates using TRIZOL (Life Technologies, Carlsbad, Calif) according to manufacturer’s instructions. cDNA was then obtained by using SuperScript IV VILO Master Mix with ezDNAse enzyme (Thermo Fisher, Waltham, Mass) and was then amplified by using PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, Calif). Amplification was performed by using Quant Studio 3 (Applied Biosystems, Waltham, Mass). mRNA expression of the respective genes was normalized to

GAPDH expression. Calculation of the relative quantitative mRNA expression was done with the cycle threshold method$^{\Delta\Delta Ct}$ algorithm. *C5aR1*-Primer: Forward: 5'-AGAATATGGACCCCATGGTTG-3', Reverse: 5'-GTTCTGG-AAGAGGCATCCAC-3'

In vitro incubation of cardiomyocytes (CMs) with inflammation-cocktail (IF-C) and TNF

Human cardiomyocytes (iPS) (Cellular Dynamics, Madison, Wis) were cultured for 10 days at 37°C and 7% CO_2 in iCell medium (Cellular Dynamics, Madison, Wis). After 10 days an incubation with either inflammation-cocktail (IF-C), including 500 ng/mL C3a, 10 ng/mL C5a, 250 pg/mL IL-1β, 500 pg/mL IL-6, 150 pg/mL IL-8, and 10 ng/mL TNF, or with only 250 pg/mL TNF (at 37°C and 7% CO_2) for 6 h followed. After 6 h, supernatant and cell lysates were frozen at −20°C. Supernatant was used to measure extracellular histones detected by cell death detection ELISA kit (Hoffmann-La Roche, Indianapolis, Ind) and to investigate troponin I concentration by using human troponin I simple step ELISA kit (Abcam, Cambridge, UK). To assess caspase-3 in treated cardiomyocytes, Caspase-Glow 3/7 Assay (Promega, Madison, Wis) was used. Cytosolic reactive oxygen species (ROS) were measured by immunofluorescence by using CellROX Deep Red Reagent (Life Technologies, Carlsbad, Calif). Cell lysates were used to isolate mRNA for qPCR measurements. cDNA was then obtained by using SuperScript IV VILO Master Mix with ezDNAse enzyme (Thermo Fisher, Waltham, Mass) and was then amplified by using PowerUp SYBR Green Master Mix (Life Technologies, Carlsbad, Calif). Amplification was performed by using Quant Studio 3 (Applied Biosystems, Waltham, Mass). mRNA expression of the respective genes was normalized to GAPDH expression. Calculation of the relative quantitative mRNA expression was done with the cycle threshold method ΔΔCT algorithm. The following primers were utilized: for mRNA expression of *IL-1β* forward 5'-GCAAGGGCTTCAGGCAGGCCGCG-3' and reverse 5'-GGTCATT-CTCCTGGAAGGTCTGTGGGC-3'; for *NLRP3* mRNA expression forward 5'-CTTCTCTGATGAGGCCCAAG-3' and reverse 5'-GCAGCAAACTG-GAAAGGAAG-3'; mRNA of *C5aR1* forwards 5'-GGAGACCAGAACAT-GAACTCCTT-3' and reverse 5'-ATCCACAGGGGTGTTGAGGT -3'; for C3aR mRNA expression forwards 5'-AGACAGGACTCGTGGAGACA-3' and reverse 5'-AGACGCCATTGCTAAACTTCAAA-3'.

Mitochondrial respiration with seahorse XFe96 analyzer

Human cardiomyocytes (iPS) (Cellular Dynamics, Madison, Wis) were seeded in special Seahorse XFe96 cell culture plates (Agilent Technologies, Santa Clara, Calif) and were cultured for 10 days in iCell maintenance medium (Cellular Dynamics, Madison, Wis) at 37°C and 7% CO_2. After the cultivation, cells were treated either with 250 pg/mL TNF or with the above mentioned IF-C for 6 h at 37°C and 7% CO^2. After exposure of cells to TNF or IF-C, mitochondrial respiration was measured. Mitochondrial respiration was measured by using the Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, Calif) and the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, Calif). Immediately after the experiment, cells were fixed with 4% formalin at 4°C overnight. Then cells were stained with 0.2% Janus-Green solution, washed and resolved with 0.5 M hydrochloric acid. OD was measured at 630 nm. The oxygen consumption rate (OCR) values were normalized to the OD 630 nm values, respectively. Results were evaluated using Seahorse Wave 2.4 software (Agilent Technologies, Santa Clara, Calif).

In vitro incubation with histones

Human cardiomyocytes (iPS) (Cellular Dynamics, Madison, Wis) were cultured for 10 days at 37°C and 7% CO_2 in iCell medium (Cellular Dynamics, Madison, Wis). After 10 days an incubation with 100 μg/mL histones (Sigma, St Louis, Mo) for 6, 12, and 24 h followed. Control cells were incubated with PBS in iCell medium. Troponin I was measured by human cardiac troponin simple step ELISA kit (Abcam, Cambridge, UK) in supernatant of treated cardiomyocytes.

Isolation and treatment of human polymorphonuclear leucocytes (PMNs)

Human blood sample collection was approved by the Ethical Committee of the University Medical Center Ulm (local Approval no 94/14). Human blood plasma was used to isolate human PMNs from healthy control patients.

For PMN isolation 30 mL human whole blood was collected with S-Monovette, coated with 3.2% citrate (Sarstedt, Nümbrecht, Germany). Whole blood was mixed with 0.9% NaCl solution (Fresenius Kabi, Bad Homburg, Germany). Afterward, whole blood was layered at Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and was centrifuged for 30 min with 340 × g at room temperature. Cells from whole blood, containing also leukocytes, were separated by density gradient during centrifugation. After centrifugation, the supernatant was discarded and the pellet, containing the leukocytes, was collected. The pellet was incubated for 30 min with Dextran (from Leuconostoc spp, Sigma, St. Louis, Mo). After incubation, the pellet was discarded and the supernatant was filled up with 0.9% NaCl (Fresenius Kabi, Bad Homburg, Germany) and was once again centrifuged for 5 min with 340 × g. After this centrifugation, the supernatant was discarded, the pellet was lysed by adding double-distilled water and was refilled afterward with 2.7% NaCl solution (Sigma Aldrich, St. Louis, Mo). Afterward, another centrifugation step followed for 5 min with 340 × g. Then, the supernatant was discarded and the pellet, containing the neutrophils, was resuspended in Hanks' balanced salt solution (HBSS, Sigma Aldrich, St. Louis, Mo) Isolated neutrophils were cultured with DMEM (Gibco, Paisley, Scotland, UK), supplemented with 10% fetal bovine serum, 1% L-Glutamin, and 1% penicillin/streptomycin for 1.5 h. Isolated neutrophils were incubated with a mixture of histones (Sigma, St.-Louis, Mo). For Figure 6B histone concentrations of 0.05, 0.5, 1, 10, and 20 μg/mL and LPS concentrations of 5, 10, 20, and 50 μg/mL were used and incubated for 2 h.

Isolated PMNs were also incubated with serum from patients with a fracture or from healthy controls for 2 h. The used serum samples from fracture patients as well as from healthy controls were randomly selected from the present serum collective of the study. Additional propidium iodide (Sigma, St. Louis, Mo) and Hoechst was added to the wells for staining extracellular DNA/NETs. After incubation PMNS were washed twice with PBS, were fixed with 4% Formalin, and were mounted with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, Calif). The whole experiment was repeated three times.

Statistical procedures

All values were expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett, Tukey, or Holm-Sidak multiple comparison test. $P \leq 0.05$ was considered statistically significant. GraphPad Prism 7.0 software was used for statistical analysis (GraphPad Software Inc, San Diego, Calif).

RESULTS

Systemic inflammation and release of extracellular histones after fracture in mice, pigs, and humans

To determine systemic inflammation after long bone fracture, the systemic release of the inflammatory and cardio-depressive mediators C5a, C3a, TNF and of extracellular histones were measured in mouse, pig, and human blood samples (Fig. 1). Mice subjected to femur fracture demonstrated increased systemic complement activation by enhanced release of C5a (Fig. 1A) and of C3a (Fig. 1B) compared with sham-treated animals 6 h after fracture. Systemic IL-6 concentrations in blood samples of mice 6 h after fracture were elevated significantly, whereas systemic TNF was enhanced slightly but not significantly (Fig. 1, C and D). Extracellular histone concentration in plasma was elevated 24 h after fracture in mice (Fig. 1E). In pigs subjected to femur fracture, extracellular histones in serum samples were significantly increased 1.5 and 3 h after trauma compared with baseline (Fig. 1F). In humans, extracellular histones (Fig. 1G) were increased systemically compared with healthy controls 0 and 14 days after meta/diaphyseal fracture of long bones. Further, after fracture C5a (Fig. 1I) and TNF (Fig. 1H) were slightly elevated systemically at day 0 and 14 days after fracture compared with healthy controls.

Hemodynamic alterations in pigs after fracture and systemic Troponin I concentrations in pigs and humans after fracture

Since mice, pigs, and humans showed increased levels of inflammatory mediators after long bone fracture, which were all previously shown to be cardio-depressive, cardiac injury was analyzed in the respective species (Table 1, Fig. 2). To

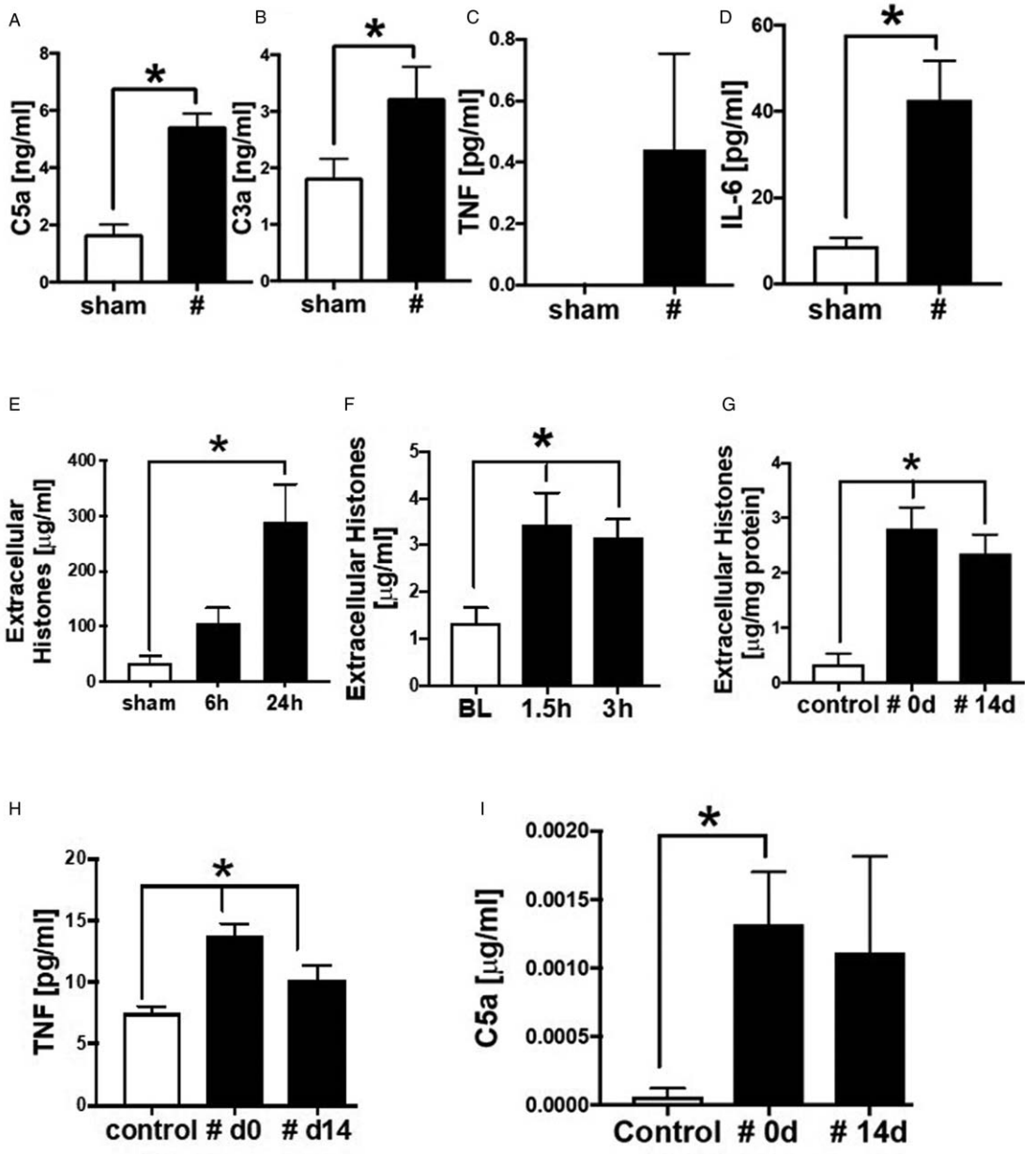

Fig. 1. **Systemic inflammation and release of extracellular histones after fracture in mouse, pig and human.** A, C5a concentration in plasma of mice with a fracture (#), compared with sham treated animals measured 6 h after fracture, n = 5–7. B, C3a concentration in µg/mL in plasma of mice with a fracture (#), compared with sham-treated animals measured 6 h after fracture, n = 5–7. (C) TNF levels in µg/mL and (D) Il-6 levels in pg/mL in plasma of mice with fracture, compared with sham-treated animals measured 6 h after fracture, n = 5–7. E, Extracellular histone appearance in plasma from mice (C57BL/6) 6 and 24 h after fracture or after sham treatment. F, Time course for extracellular histone appearance in serum from pigs after fracture (black bars, n = 48) compared with sham (white bars, n = 3). G, Time course for extracellular histone appearance in plasma from patients with fracture (#), at day of fracture (0 d, n = 25) and 14 days after fracture (14 d, n = 6) (both black bars). H, TNF concentration in pg/mL in plasma from patients with fracture (#), at day of fracture (0 d, n = 26) and 14 days after fracture (14 d) (both black bars, n = 7), compared with healthy controls (white bar) n = 20. I, C5a concentration in plasma from patients with fracture (#), at day of fracture (0 d, n = 26) and 14 days after fracture (14 d, n = 7) (both black bars), compared with healthy controls (white bar), n = 20. * differences to sham/baseline (BL)/ control procedure were significant, $P < 0.05$.

estimate cardiac injury after fracture in pigs and humans, troponin I as a specific marker for cardiac cell damage was assessed at different time points after trauma (Fig. 2). Cardiac troponin I was elevated systemically in pooled serum from pigs of both treatment groups (DCO and ETC) 1.5 and 3 h after trauma compared with baseline (Fig. 2A). The troponin I release could not be explained by apoptosis of cardiac cells: no differences in apoptotic positive cardiomyocytes were measured between sham and fracture animals (Fig. 2B). Furthermore, cardiac troponin I in human plasma was detected after fracture. Here, an elevation at the day of fracture (day 0) was measured in plasma of patient with fracture compared with healthy controls (Fig. 2C). The systemic troponin I release from humans after fracture was independent of age and there was no statistic correlation between systemic troponin I release and age ($R^2 = 0.003$) (Fig. 2D).

Pigs showed increased systemic inflammation as well as the presence of the myocardial injury marker troponin I; therefore,

TABLE 1. **Mean values (± SD) of hemodynamic parameters of pigs after long bone fracture receiving fracture provision either according to damage control orthopedics (DCO) or to early total care principles (ETC)**

	n	BL	3.5 h	5.5 h	24 h	48 h	72 h	*P* value
HR	12	94.6	78.1	83.8	67.7	67.8	76.8	0.001
MAP	12	73.1	73.8	77.6	68.5	81.2	85.5	<0.001
RR	12	99.1	105.3	105.7	104.6	121.1	116.9	<0.001

P values were determined for changes over time per group. n = 12 animals per group. Heart rate (HR) in beats/min, mean arterial pressure (MAP) in mm Hg and systolic RR-interval in ms.

the hemodynamic parameters of the animals were analyzed after long bone fracture. To determine the hemodynamic alterations in pigs after long bone fracture, the heart rate (HR), the mean arterial pressure (MAP), as well as the systolic RR-interval were measured by ECG recordings (Table 1). The data from the pigs of both treatment groups (DCO and ETC) were combined for the analysis (n = 12). The HR decreased significantly during the observation period. Moreover, the MAP as well as the systolic RR interval increased significantly over the observation time.

Structural alterations in the porcine heart after long bone fracture: α-actinin, desmin, and translocation of Cx43

Since pigs showed hemodynamic alterations, the left ventricular expression of the cardiac structure proteins α-actinin and desmin were analyzed (Fig. 3). Both structure proteins are associated with impaired cardiac function after trauma. The expression of α-actinin in CMs was increased 72 h after fracture, followed by DCO treatment compared with sham procedure (Fig. 3A), which was determined by fluorescence intensity measurement. Comparable results showed the measurement of fluorescence intensity of desmin staining of the left ventricles. The expression of desmin was increased in the ETC compared with sham treated animals (Fig. 3B).

Left ventricular alterations of the gap junction protein Cx43 are associated with severe arrhythmia. Therefore, alterations of the left ventricular expression of Cx43 after long bone fracture were analyzed in pigs. To determine whether gap junction proteins in the heart were altered after fracture immunohistochemistry staining of Cx43 gap junction protein in left ventricular tissue sections was performed. In sham-treated animals Cx43 was located in intercalated discs whereas Cx43 was translocated and scattered into the cytosol (Fig. D) after fracture followed by DCO or ETC. No differences in total Cx43 protein expression in left ventricles after femur fracture were observed in pigs (Fig. 3C).

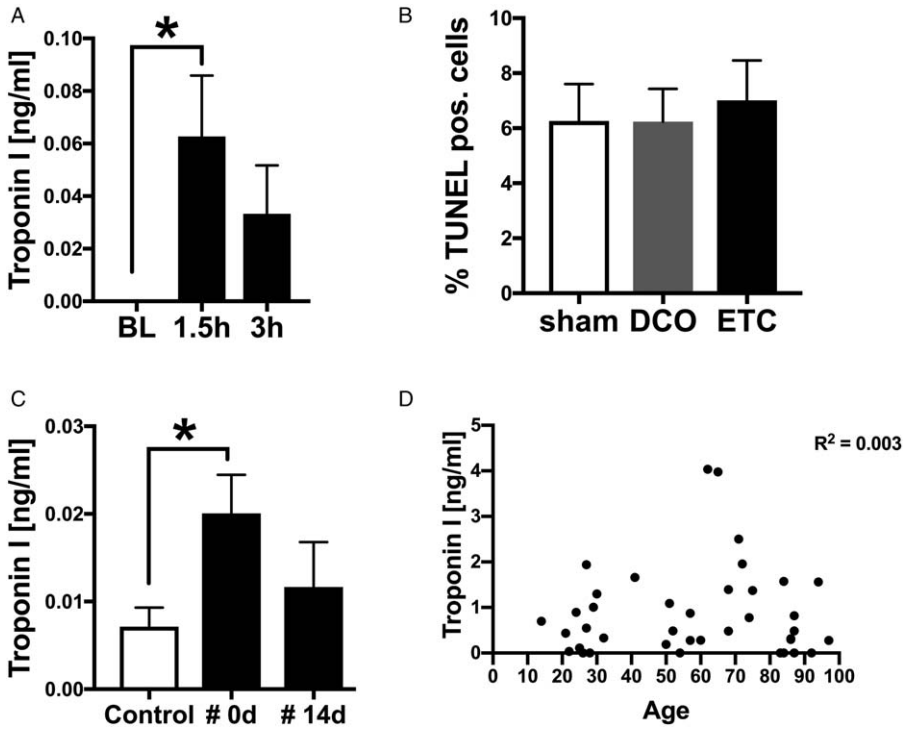

FIG. 2. **Troponin I concentration in pig and human after fracture.** A, Time course of cardiac specific troponin I in ng/mL in serum of pigs after fracture (n = 8) compared with sham procedure (n = 3). B, Apoptotic cells in percentage detected by TUNEL staining of left ventricle in sham (n = 3) and after fracture and damage control treatment (DCO, n = 4) or early total care (ETC, n = 4) treatment. C, Concentrations of cardiac troponin I in ng/mL in plasma of patients with fracture (#) at day of fracture (0 d, n = 26) and at day 14 after fracture (14 d, n = 7) compared with healthy controls (white bars, n = 20). Correlation analysis if systemic troponin I values (D). Systemic troponin I values (ng/mL) were correlated with the age of the patients. $R^2 = 0.003$. * differences to control procedure were significant, $P < 0.05$.

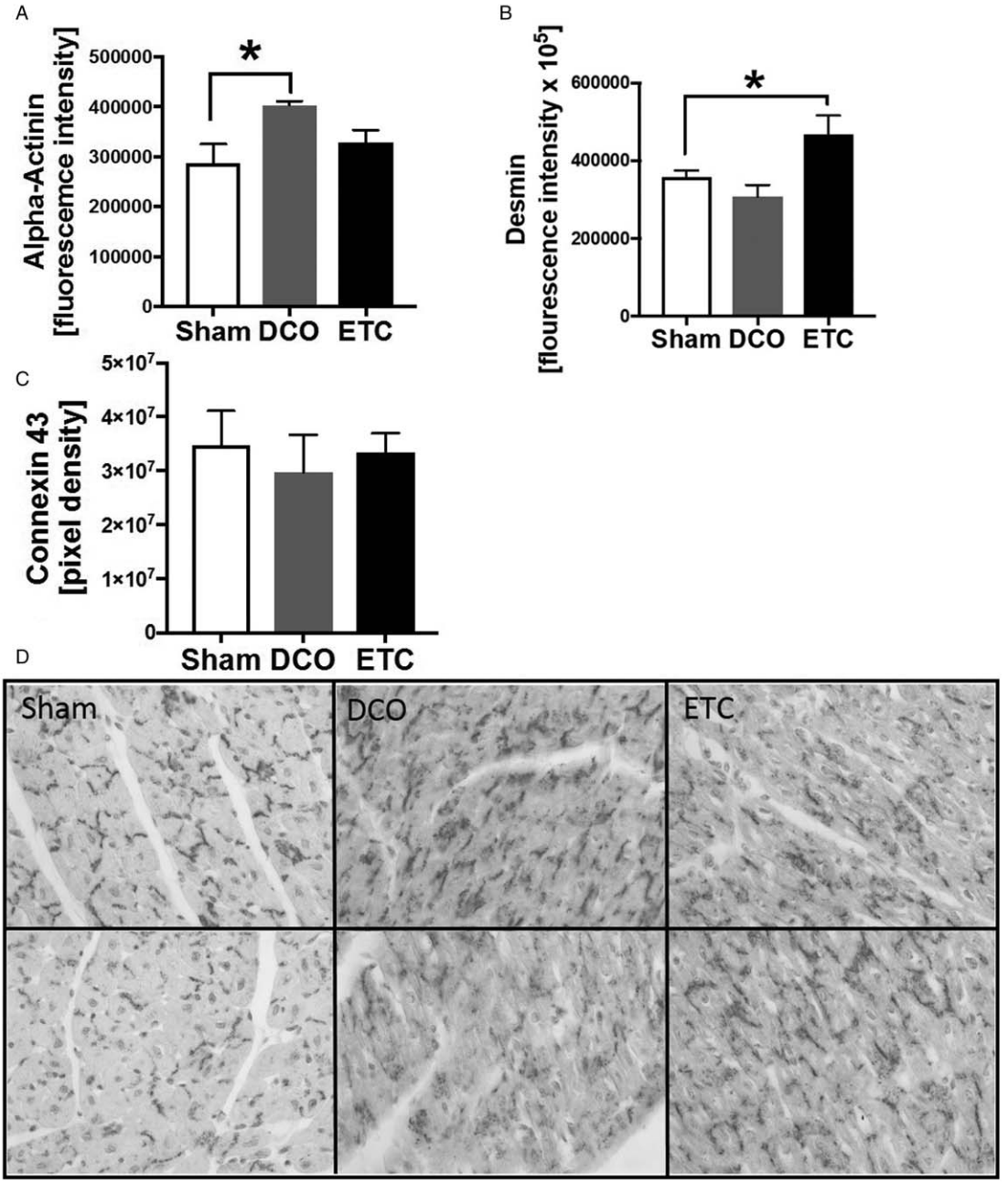

FIG. 3. **Structural alterations in the porcine heart after fracture: α-actinin, desmin, and translocation of Cx43.** A, Fluorescence intensity of α2-actinin staining and confocal imaging in animals treated with fracture and Damage Control Orthopedics (DCO, n = 4), or fracture and Early Total Care (ETC, n = 4) compared with sham (n = 3). B, Fluorescence intensity of α2-desmin staining and confocal imaging in animals treated with fracture and Damage Control Orthopedics (DCO, n = 4), or fracture and Early Total Care (ETC, n = 4) compared with sham (n = 3). C, Results of density measurement of Connexin 43 (Cx43) staining in left ventricular tissue after fracture and DCO (n = 4) or ETC (n = 4) treatment and in sham-treated animals (white bar, n = 3). D, Representative distribution of Cx43 staining of the left ventricle of sham-treated animals and pigs after fracture and damage control (DCO, middle) treatment and early total care (ETC, right), * differences to sham were significant, $P < 0.05$.

Local inflammation in porcine heart after long bone fracture

After trauma, the activation of the complement system is associated with impaired cardiac function (Fig. 4). Since mice and humans showed increased systemic levels of the complement activation products C3a and C5a after long bone fracture, the left ventricular expression of the C5a receptor 1 (C5aR1) was determined in pigs after isolated long bone fracture. There was a reduction of the complement receptor C5aR1 72 h after fracture in both treatment groups (DCO and ETC) compared with the sham group (Fig. 4A), whereas mRNA expression of *C5aR1* was not significantly influenced by fracture in pigs, after 72 h (Fig. 4B).

In vitro influence of inflammatory mediators and TNF on human cardiomyocytes

Mice, pigs, and humans showed increased systemic levels of inflammatory mediators after isolated long bone fracture;

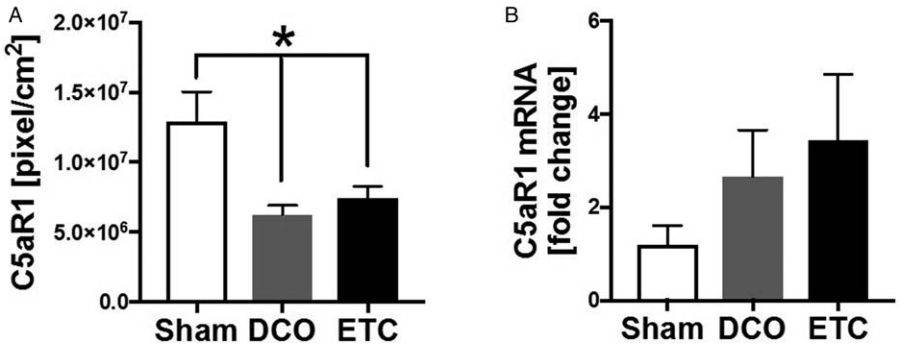

FIG. 4. **Local inflammation.** A, Changes in density of C5aR1 staining in left ventricular tissue of pigs 72 h after fracture, followed by DCO (n = 4) or ETC (n = 4) treatment as well as in sham-treated pigs (n = 3). B, mRNA expression of C5aR in left ventricles of pigs 72 h after fracture and DCO (n = 4) or ETC treatment (n = 4) or sham procedure (n = 3) * differences to sham were significant, $P < 0.05$.

therefore, the effects of the TNF as well as of a defined inflammation-cocktail were analyzed on human cardiomyocytes *in vitro* (Figs. 5 and 6). In the presence of inflammation-cocktail and in the presence of TNF, the mitochondrial respiration was impaired compared with control cells (Fig. 5A). The mitochondrial basal respiration (Fig. 5B), the mitochondrial spare respiratory capacity (Fig. 5C), as well as the mitochondrial maximal respiration (Fig. 5D) were significantly decreased in the cells when exposed to inflammation-cocktail and to TNF. Furthermore, the amount of cytosolic ROS significantly enhanced in the presence of inflammation-cocktail (Fig. 5E), compared with the control cells. The mRNA expression of *IL-1β* significantly decreased in the presence of inflammation-cocktail (Fig. 5F), whereas mRNA expression of

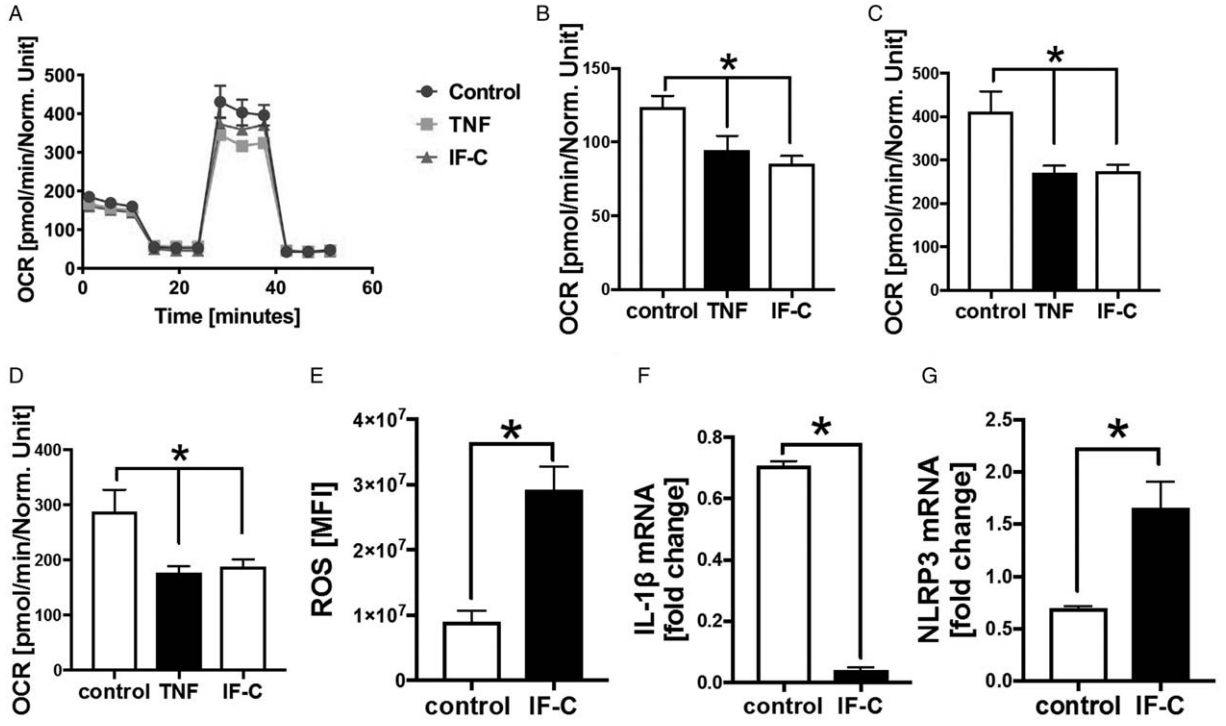

FIG. 5. ***In vitro*** **influence of inflammation-cocktail (IF-C) or TNF (250 pg/mL) on human cardiomyocytes.** A, Mitochondrial respiration of human cardiomyocytes during MitoStress Assay, measured with Seahorse XFe96 Analyzer. Oxygen consumption rate (OCR) in pmol/min/Norm. Unit. Cells were treated for 6 h either with inflammation-cocktail (IF-C) or with 250 pg/mL TNF. Control group was only treated with cell culture medium. n = 6. B, Mitochondrial basal respiration of human cardiomyocytes. Oxygen consumption rate (OCR) in pmol/min/Norm. Unit. Cells were treated with inflammation cocktail (IF-C) or with 250 pg/mL TNF for 6 h. Control group was only treated with cell culture medium. Mitochondrial respiration was measured with MitroStress Assay using the Seahorse XFe96 Analyzer. n = 6. C, Mitochondrial spare respiratory capacity of human cardiomyocytes. OCR) in pmol/min/Norm. Unit. Cells were treated with inflammation cocktail (IF-C) or with 250 pg/mL TNF for 6 h. Control group was only treated with cell culture medium. Mitochondrial respiration was measured with MitroStress Assay using the Seahorse XFe96 Analyzer. n = 6. D, Mitochondrial maximal respiration of human cardiomyocytes. Oxygen consumption rate (OCR) in pmol/min/Norm. Unit. Cells were treated with inflammation cocktail (IF-C) or with 250 pg/mL TNF for 6 h. Control group was only treated with cell culture medium. Mitochondrial respiration was measured with MitroStress Assay using the Seahorse XFe96 Analyzer. n = 6. E, Reactive oxygen species (ROS) in human cardiomyocytes treated with inflammation-cocktail (IF-C) for 6 h and controls (control) assessed by Cell ROX Red Reagent, n = 6. mRNA expression of IL-1β (F) and NLRP3 inflammasome (G) of human cardiomyocytes treated with inflammation-cocktail (IF-C). Control cells were only treated with cell culture medium. mRNA expression in fold change. * differences to control were significant, $P < 0.05$.

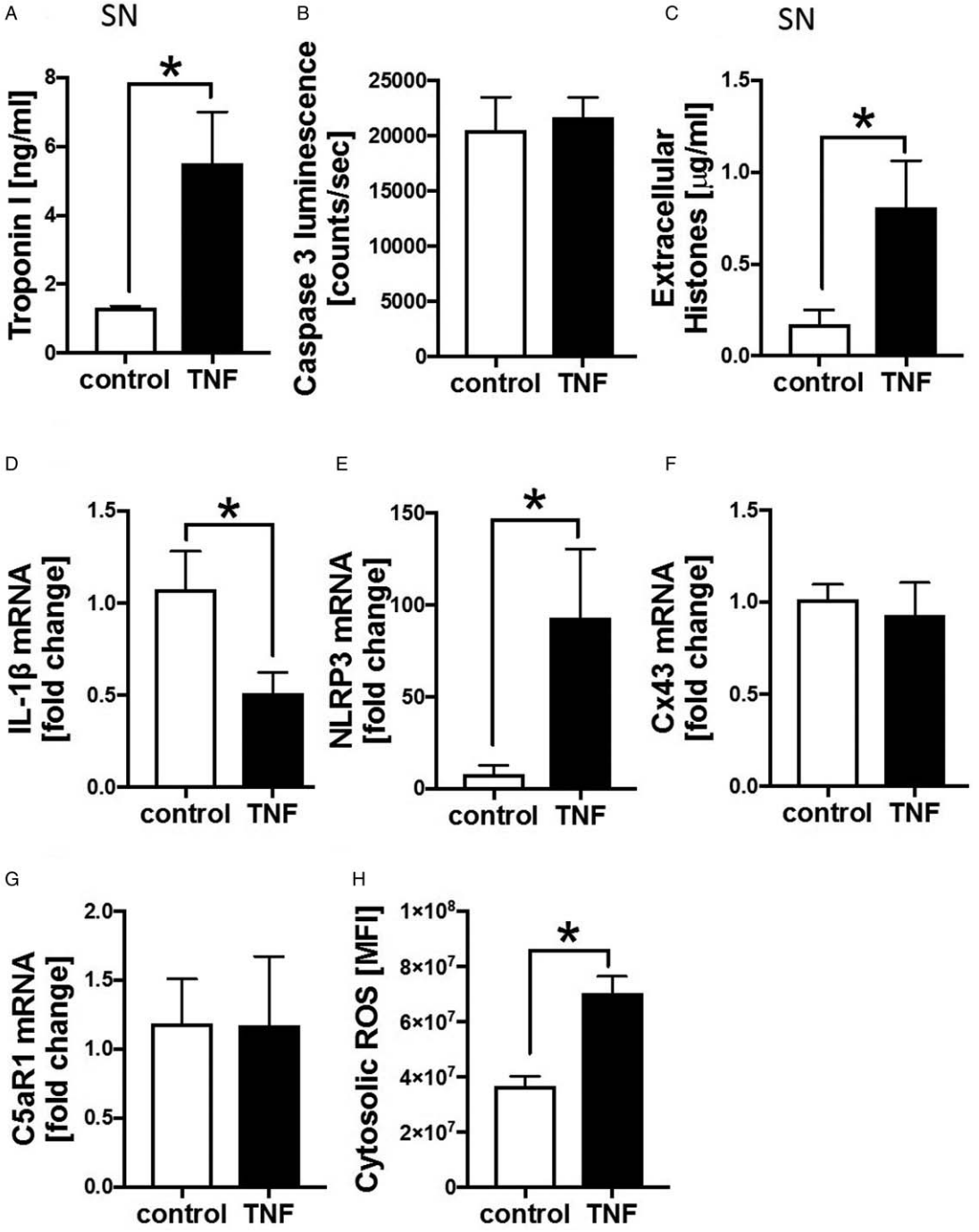

FIG. 6. ***In vitro* influence of TNF (250 pg/mL) presence to human cardiomyocytes.** A, Troponin I concentration in ng/mL in supernatant (SN) of human cardiomyocytes treated with 250 pg/mL TNF for 6 h compared with control cardiomyocytes measured by ELISA, n = 6. B, Caspase 3 luminescence in counts/seconds measurement of human cardiomyocytes after incubation in the absence (control) and in the presence of 250 pg/mL TNF for 6 h (TNF), n = 6. C, Appearance of extracellular histones in µg/mL in supernatant of human cardiomyocytes incubated with 250 pg/mL TNF for 6 h compared with control cardiomyocytes, n = 6 measured by using ELISA. mRNA expression of IL-1β (D), NLRP3 (E), Cx43 (F), and C5aR1 (G) of cardiomyocytes incubated in the absence (control) and in the presence of TNF for 6 h, n = 6. H, Reactive oxygen species (ROS) in human cardiomyocytes treated with TNF (TNF) for 6 h and controls (control) assessed by Cell ROX Red Reagent, n = 6, *differences to control were significant, $P < 0.05$.

NLRP3 inflammasome significantly enhanced in the cells (Fig. 5G), compared with the control cells.

Human CMs were incubated with recombinant human TNF for 6 h. Here, troponin I concentrations in supernatants were significantly increased in the presence of TNF (Fig. 6A). Activity of caspase 3 indicating CM apoptosis was not elevated in the presence of TNF (Fig. 6B). In contrast, extracellular histone concentration in supernatant was significantly elevated in the presence of TNF (Fig. 6C). Further, TNF was associated with reduced mRNA expression of *IL-1β* in CMs compared with controls after 6 h (Fig. 6D), whereas mRNA expression of *NLRP3* in the presence of TNF was increased (Fig. 6E). Neither the expression of *Cx43* (Fig. 6F) nor *C5aR1* (Fig. 6G) was affected by the presence of TNF of human CMs. Though,

cytosolic ROS was elevated in CMs in the presence of TNF compared with CM in the absence of TNF (Fig. 6H).

Effects of extracellular histones

Pigs and humans showed enhanced systemic levels of extracellular histones; consequently, we analyzed the ability of human PMNs to make NET formation in the presence of extracellular histones, amplifying the inflammatory response after isolated long bone fracture (Fig. 7). The incubation of human PMNs with histones led to NET-formation in a dose-dependent manner, measured by immunofluorescence of extracellular DNA (Fig. 7A), whereas the positive control was accomplished by LPS-incubation. Additionally, incubation of PMNs with plasma of patients with fracture resulted in a significant increase in extracellular DNA compared with PMNs treated with plasma from healthy controls (Fig. 7B). Incubation of human CMs with histone mixture for 6, 12, and 24 h was associated with increased troponin I concentrations in supernatant compared with controls (Fig. 7C).

DISCUSSION

In this study, we showed a systemic elevation of different inflammatory biomarkers and DAMPs, such as C5a, TNF, and extracellular histones after long bone fracture in the species human, pig, and mouse. Moreover, we observed hemodynamic and structural cardiac alterations in pigs, which might indicate for a secondary cardiac damage after isolated femur fracture.

Structural alterations of cardiac tissue after fracture were indicated by an increased α-actinin expression in pig hearts after femur fracture, with DCO treatment. We previously showed alterations in cardiac α-actinin expression in pigs after experimental polytrauma, which was associated with impaired cardiac function. α-actinin is linked with L-type Ca^{2+}-channels and reacts as responder to mechanical stretch by hemodynamic adaption (26). Interestingly, Z-disc-associated proteins like α-actinin are not only altered due to direct mechanical trauma on the heart as shown after multiple trauma in pigs, but also to fracture. Alterations in α-actinin expression might be to systemic inflammation after fracture. Furthermore, the expression of desmin increased in the present study in porcine heart tissue, which was previously shown after experimental polytrauma in pigs (4). Cardiac desminopathies lead to cardiomyopathy, conduction defects and to arrhythmias (27, 28). Desminopathies were associated with impaired mechanical properties of CMs and alterations of the calcium amplitude (29). Increased desmin concentrations were observed in guinea pigs with heart failure (30) and in mice with diastolic dysfunction (31).

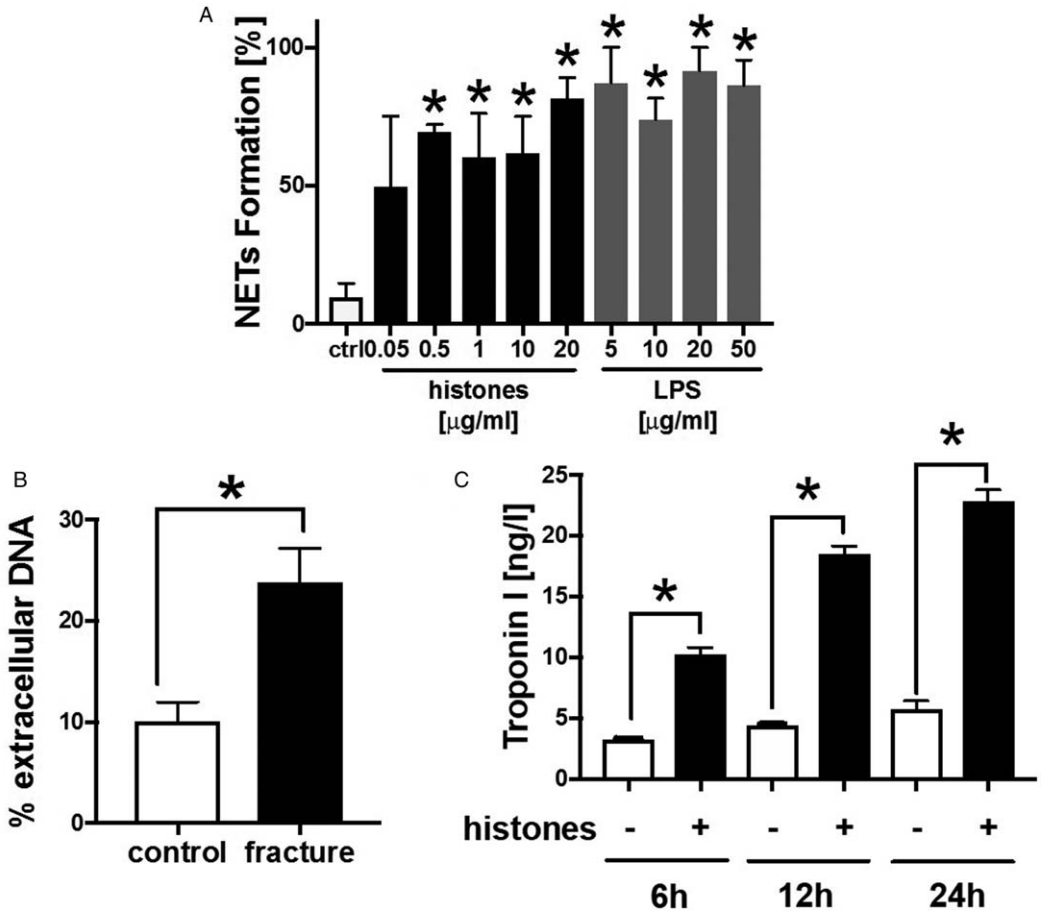

FIG. 7. **Effects of extracellular histones.** A, NET formation in human PMNs in percentage after treatment with different concentrations of histones (mix of histones) and LPS for 6 h compared with control-PMNs, n = 6. B, Extracellular DNA in percentage measured in PMNs treated with plasma of healthy controls (n = 4) and of patients with fracture (n = 6) for 2 h. C, Troponin I concentration in supernatant of human cardiomyocytes treated with histones (6, 12, 24 h, +) compared with untreated cardiomyocytes (−), n = 6. *differences to controls were significant $P < 0.05$.

Additionally, the distribution and function of the ryanodine receptor is affected by modification of desmin (32). Alterations in cardiac desmin expression might be also to systemic inflammation. TNF was previously described to induce the cleavage of desmin by caspase-6, leading to a loss of localization of desmin at the intercalated discs of CMs (33). Moreover, remodeling of gap junction proteins and relocalization of Cx43 were described in a mouse model of desmin-related cardiomyopathy (34). In the present study, pigs showed likewise a translocation of Cx43 from the intercalated discs into the cytosol of the CMs after isolated long bone fracture. Endocytosis of Cx43 was associated with changes in spread of electrical activation and was further associated with arrhythmias and cardiac dysfunction (35, 36). To our knowledge this is the first time that alterations in cardiac z-disc and gap junction proteins were described after an isolated long bone fracture. Summarized, in the present study, pigs showed alterations in the left ventricular expression of cardiac structure proteins as well as in the localization of the gap junction protein Cx43 after isolated long bone fracture. These structural cardiac alterations after long bone fracture might predispose to cardiac dysfunction and to cardiac damage.

Interestingly, there was evidence for cardiac injury after fracture due to increased levels of circulating troponin I in humans and pigs. Currently, troponin I is considered an important diagnostic marker for cardiac damage in the clinical use, as well as after trauma (37). Troponin I has been reported to be elevated after heart contusion in 15% to 45% of the cases and was described as a reliable tool for the detection of post-traumatic heart complications, especially in combination with echocardiography (38, 39). Enhanced systemic troponin values were associated with increased catecholamine requirement as well as with an increased mortality rate of the patients after trauma (5). In previous studies we observed systemic troponin I elevations after experimental polytrauma in mice and pigs (4, 40) and after experimental blunt chest trauma in rats (41). There, enhanced troponin values were associated with impaired cardiac function and cardiac damage. However, here we describe a systemic, but mechanically-independent elevation of troponin I, which might indicate for cardiac injury after long bone fracture in humans and in pigs. The systemic troponin I elevation in this study was not dependent on the age of the patients. The findings of this study are in contrast to those of a previous study in which no cardiac troponin I but skeletal troponin I was systemically detectable after soft tissue- and orthopedic injuries in humans (42). Nevertheless, we do not think that the release of skeletal troponin I distorted the present data due to highly specificity and sensitivity of the used immunoassays. The release of cardiac troponin I in pigs was not due to cardiomyocyte apoptosis, but might be induced by inflammatory cytokines. Notably, the systemic cardiac troponin I by itself is not a sufficient diagnostic tool for myocardial injury and patients with systemic elevated troponin I values must have at least one of five further diagnostic criteria for the definition of myocardial injury (43, 44). However, no echocardiographic parameters were assessed from the patients in the current study but this issue has to be addressed in future studies to show myocardial injury after long bone fracture. Moreover, the systemic release of cardiac troponin I was described in various diseases and also in the context of emotional and psychosocial stress (44–46). Certainly, we could not exclude stress-induced systemic release of cardiac troponin I in humans, but we think that the release of cardiac troponin I in this study might be due to the enhanced inflammation after long bone fracture. This inflammatory condition of long bone fracture might predispose to cardiac injury.

Based on the findings of cardiac structural alterations and of enhanced systemic cardiac troponin I levels in pigs after long bone fracture, we analyzed hemodynamic parameters. The HR of the animals decreased significantly during the observation period, whereas the MAP as well as the systolic RR-interval significantly increased over the observation time. These hemodynamic findings might indicate for cardiac functional alterations after fracture and might be further linked to cardiac structural changes and to cardiac injury. However, this assumption of cardiac dysfunction after long bone fracture has to be examined in more detail in future studies by echocardiographic analysis. Summarized, the occurrence of systemic troponin I as well as the alterations in hemodynamic parameters might indicate for cardiac injury after isolated long bone fracture.

In this context the activation of the complement system may play an important role. Especially, the complement activation product C5a was elevated after long bone fracture in mice and humans in the present study. C5a and its receptors (C5aR1/2) were associated with cardiac dysfunction during experimental sepsis in mice (47). During ischemia/reperfusion, the complement system plays a critical role in endothelial transmigration, developing cardiac diseases. In the absence of the C5aR, the expression of matrix metalloprotease and junction adhesion molecule -A was reduced, leading to reduced infarct size and to leucocyte recruitment (48). Considering other cardiac pathologies, C5a was described as powerfully cardio-depressive via C5aR1 interactions on the membrane of cardiomyocytes. This C5a-C5aR1 interaction reduced cardiomyocyte contractility and the ability of relaxation, leading to reduced cardiac output, to amongst multi-organ failure, to cardiac shock and to sudden cardiac death (20, 49, 50). Moreover, C5a triggers a massive increase of cellular ROS and $[Ca^{2+}]i$ in isolated rat CMs (15). Disturbed calcium homeostasis leads to impaired electrophysiological function of CMs and consecutively aggravated contractility (15). Increased ROS further reduced left ventricular function (51). Furthermore, C5a induces the release of cytokines such as IL-6 and TNF in CMs *in vitro* (52). In addition, C5a is able to induce release of extracellular histones, which is ameliorated in the absence of C5aR1 (20, 53). *In vitro* activation of neutrophils by C5a leads to enhanced histone concentrations, caused by neutrophil extracellular trap (NET) formation (18). Summarized, the increased systemic C5a levels might contribute to the present cardiac alterations after isolated long bone fracture.

Furthermore, in the present study, extracellular histones were elevated systemically after long bone fracture in mice, pigs, and humans, which was also described previously after experimental multiple trauma (4), blunt chest trauma (41), during sepsis (20, 54) and in humans after trauma (55). Histones were released from damaged cells after trauma (56, 57), mediated by neutrophils and their NETs (58). Increased levels of circulating histones were associated with acute lung injury and with

septic complications after burn injury (59). Perfusion of mouse hearts with extracellular histones leads to reduction of left ventricular pressure and to impaired contractility (20). Besides, incubation of human CMs with histones induced an increase in cytosolic ROS in a dose-dependent manner (20). The present data showed similarly an elevation of ROS in human CMs in the presence of TNF. ROS induce a cytosolic calcium increase by modulating and blocking calcium handling proteins. Further, histones are able to interact with the phospholipid-membrane of cells, which lead to higher permeability and consequently to enhanced cellular calcium influx (55, 60–62). Interestingly, in this study the *in vitro* exposure of human CMs to TNF induced extracellular histone release into supernatant fluids, which might additionally enhance the cardio-depressive effects. This additional release of extracellular histones from CMs might further recruit other immune cells such as neutrophils, intensifying the systemic immune response. Moreover, the CMs themselves are affected by the release of extracellular histones as described above, leading to an enhanced cardio-depressive effect, which might predispose to cardiac dysfunction. Summarized, increased systemic levels of extracellular histones after long bone fracture may affect the heart in the present study, by inducing the systemic release of troponin I.

In the present report we demonstrated to the best of our knowledge for the first time that histones themselves are able to activate human neutrophils by inducing NET-formation. Neutrophils are present in fracture hematoma after bone injury (17). Consequently, this may result in an amplification of local as well as of systemic histone concentrations, which might contribute to compromised organ function after long bone fracture. Moreover, histone application in mice induces the systemic release of further cytokines such as TNF, IL-1ß, IL-6, and IL-10 (63), which is mediated via histone-TLRs interactions (64–68).

Besides C5a and extracellular histones, the release of TNF after long bone fracture is well described and correlated with the results in the present study (8, 9). The systemic release of TNF in the present study might also contribute to the cardiac alterations after long bone fracture. Blockade of TNF in experimental burn injury has been shown to improve cardiac function (69). Also, TNF disturbed the maximum extent of cardiac myocyte shortening in cultured rat CMs, which was reversible after the removal of TNF (14). However, the systemic conditions after single long bone fracture might quite differ from them after severe polytrauma (with blunt chest trauma) or during sepsis. After polytrauma, the development of cardiac dysfunction is obvious due to the extent of systemic inflammation by the release of inflammatory cytokines and damages molecules, induced by severe tissue trauma. During sepsis the inflammation has a much larger extent compared with fracture, since additional involvement of exogenous factors induces cardiac dysfunction and multiple organ failure. Moreover, patients who died from sepsis often exhibit suppressed immune response, which makes translation to systemic condition of single long bone fracture difficult (70). Nevertheless, we think that the molecular mechanisms which affect the heart after single long bone fracture might be the same as after polytrauma or during sepsis. The absence of C5aR1/2 improved cardiac function in mice (15) during sepsis. Moreover, usage of an antibody with neutralizing activity to histone H2A and H4 ameliorated cardiac function in septic mice (20). Furthermore, inhibition of TNF production in endotoxemic rats preserved myocardial contractile function (71). Consequently, these cardio-depressive mediators are involved in the development of cardiac dysfunction after trauma and during sepsis and might also predispose to cardiac damage after long bone fracture. For sure, many other cytokines are systemically released after fracture such as IL-1β, IL-6, IL-8, TGFβ, and G-CSF, which further may contribute to cardiac alterations after fracture and to the development of cardiac dysfunction (72, 73).

In the present study, we treated human CMs with an inflammation cocktail, including the inflammatory cytokines IL-1β, IL-6, IL-8, and TNF, as well as the anaphylatoxins C3a and C5a, which all have been shown to be elevated systemically after fracture. In the present study, the presence of the inflammation cocktail impaired mitochondrial respiration of the human cardiomyocytes and increased the amount of cellular ROS, which might be mediated via NLRP3 inflammasome activation and IL-1β release. Since the observed effects of inflammation-cocktail on human cardiomyocytes were multifactorial and could be mediated through synergistic interactions, we selected the inflammatory TNF for the further experiments to specify the cardio-depressive effects of the single fracture-specific inflammatory cytokines. In the present study, presence of TNF increased the release of troponin I, extracellular histones as well as the amount of cytosolic ROS in human CMs *in vitro*. The caspase-3 activity as well as the number of apoptotic cells in left ventricles of pigs were not altered in the present study, indicating an apoptosis-independent release of troponin I after long bone fracture. This is in contrast to previous studies, which showed a correlation between myocardial TNF concentrations, cardiac dysfunction, and CM apoptosis during sepsis as well as in chronic heart failure (12, 13). Furthermore, incubation of CMs with plasma of mice receiving mechanical trauma increased apoptosis in cells, which was ameliorated in the presence of an anti-TNF-antibody (74). Also, elevation of caspases 3, 8, 9, and the pro-apoptotic factor BCL-2 associated X protein were described in isolated rat CMs in the presence of IL-1ß and TNF (10). Therefore, the release of the cardio-depressive inflammatory cytokine TNF after isolated long bone fracture might contribute to the present cardiac structural alterations.

One major limitation of this study is the age discrepancy between the pigs and the humans. Whilst the pigs are juvenile, the patients in this study are adult. These age differences may bias the data and should be always considered when investigating and interpreting cardiac function, cardiac morphology but also systemic inflammation after fracture. Consequently, further studies have to be performed with younger patients to determine cardiac alterations after fracture. Despite the age differences of the used species we were able to show indications for cardiac alterations and cardiac injury in both species. Moreover, besides the difficulties in interpreting the data, the authors want to underline that one advantage of the present study is the comparison between the different species and models with regard to cardiac alterations after single long bone fracture.

Summarized, in the present study we demonstrated systemic as well as local cardiac alterations after isolated long bone fracture. These alterations were linked to the systemic release of inflammatory mediators and DAMPs. Finally, the observed systemic as well as local cardiac alterations after isolated long bone fracture might predispose for secondary cardiac injury. However, this has to be addressed in more detail in future studies.

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Complement Activation and Organ Damage After Trauma—Differential Immune Response Based on Surgical Treatment Strategy

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Background: The complement system is part of the innate immunity, is activated immediately after trauma and is associated with adult respiratory distress syndrome, acute lung injury, multiple organ failure, and with death of multiply injured patients. The aim of the study was to investigate the complement activation in multiply injured pigs as well as its effects on the heart *in vivo* and *in vitro*. Moreover, the impact of reamed vs. non-reamed intramedullary nailing was examined with regard to the complement activation after multiple trauma in pigs.

Materials and Methods: Male pigs received multiple trauma, followed by femoral nailing with/without prior conventional reaming. Systemic complement hemolytic activity (CH-50 and AH-50) as well as the local cardiac expression of C3a receptor, C5a receptors1/2, and the deposition of the fragments C3b/iC3b/C3c was determined *in vivo* after trauma. Human cardiomyocytes were exposed to C3a or C5a and analyzed regarding calcium signaling and mitochondrial respiration.

Results: Systemic complement activation increased within 6 h after trauma and was mediated via the classical and the alternative pathway. Furthermore, complement activation correlated with invasiveness of fracture treatment. The expression of receptors for complement activation were altered locally *in vivo* in left ventricles. C3a and C5a acted detrimentally on human cardiomyocytes by affecting their functionality and their mitochondrial respiration *in vitro*.

Conclusion: After multiple trauma, an early activation of the complement system is triggered, affecting the heart *in vivo* as well as *in vitro*, leading to complement-induced

cardiac dysfunction. The intensity of complement activation after multiple trauma might correlate with the invasiveness of fracture treatment. Reaming of the femoral canal might contribute to an enhanced "second hit" response after trauma. Consequently, the choice of fracture treatment might imply the clinical outcome of the critically injured patients and might be therefore crucial for their survival.

Keywords: multiple trauma, cardiac dysfunction, femoral nailing, conventional reaming, inflammation

INTRODUCTION

The complement system is part of the innate immunity and is activated immediately after trauma in response to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) (1). During complement activation, structural rearrangements, proteolytic cleavage, and the assembly of proteolytic and lytic complexes occur in a complex signal cascade, leading finally to the destruction and elimination of pathogens (2). In multiply injured patients, the complement system is rapidly activated after polytrauma, leading to the so-called trauma-induced "complementopathy" shortly after injury. This early complementhopathy is accompanied with a massive systemic release of the anaphylatoxins C3a and C5a in serum of multiply injured patients, triggering the innate immune response after severe trauma (3). C3a as well as C5a plasma concentrations correlate with the severity of trauma and are predictors for the development of the acute respiratory distress syndrome (ARDS) and of multiple organ failure (MOF), which both often occur after multiple trauma in humans (3–9). Moreover, the development of acute lung injury (ALI) is described in critically injured patients as well as in animals after experimental trauma and is also linked to the activation of the complement system (10–13). In critically injured patients, the early activation of the complement system is mostly mediated via the alternative pathway, correlating with injury severity and with worse clinical outcomes (14). Moreover, complement activation via the classical pathway was also described after trauma and was further associated with an increased mortality rate of the patients (15).

In patients with femur shaft fracture internal fixation by intramedullary nailing is the gold standard (16). Reaming of the femoral canal allows the insertion of a nail with bigger diameter and is further associated with the release of endogenous osteogenic factors in the reaming debris (17). Additionally, the mechanical stability is improved by bigger diameter of the inserted nail (18). Reamed intramedullary nailing is associated with shorter time to union and with lower rates of delayed-union, non-union, and reoperation (19). Nevertheless, femoral reaming has been reported to prime the immuno-inflammatory response, leading to the "second hit" phenomenon in multiply injured patients (17, 20–22). Furthermore, femoral reaming has been linked to post-traumatic complications such as ARDS and multiple organ dysfunction syndrome (MODS) (20–22). The concept of damage control orthopedics (DCO) in patients with multiple trauma and especially in presence of blunt chest trauma is based on these findings (23). With regard to cardiopulmonary consequences during femoral reaming, increased intramedullary pressure has been described. Increased intramedullary pressure was associated with intravasation of bone marrow contents, leading to bone marrow embolization in the lungs, which was further associated with altered cardiopulmonary function (24).

Besides diverse complement-induced organ damage, the activation of the complement system is strongly involved in cardiac pathology. The anaphylatoxin C5a was shown to be strongly cardio-depressive by acting via the C5aR1/2 on the membrane of cardiomyocytes (CMs), inducing defective CM contractility, reduced cardiac output, leading finally to cardiac dysfunction (25–27). Absence of either C5aR1 or C5aR2 attenuates cardiac dysfunction and improves survival of mice during sepsis (28). Moreover, C5a triggers a massive amount of cytosolic reactive oxygen species (ROS) and intracellular calcium [Ca^{2+}i] in isolated rat CMs (28). Enhanced ROS was associated with cardiac remodeling, reduced left ventricular (LV)-function as well as with contractile dysfunction (29, 30). The increased [Ca^{2+}i] affected the homeostasis and the electrophysiological functions of the CMs (28). Moreover, C5a induces defects in CMs contractility and relaxation by altering and disturbing their action potentials, nominating C5a as very powerful cardio-suppressive factor (25, 28). C3a was also shown to be cardio-depressive, leading to cardiac dysfunction, arrhythmia and contractile failure (31). Nevertheless, C3a and C5a seem also crucial during systemic inflammation by enhancing pro-inflammatory and inflammatory danger signaling pathways as well as exhibiting direct antimicrobial effects (32, 33).

In this study, we aim to investigate the early activation of the complement system in multiply injured pigs within the first 6 h after trauma with a focus on the heart. Thereby, systemic complement activation should be examined more detailed with respect to different surgical treatment strategies (reamed vs. non-reamed intramedullary nailing). Moreover, the expression of the receptors for complement activation (C3aR, C5aR1/2) as well as the deposition of C3 cleavage products C3b/iC3b/C3c should be analyzed locally in left ventricular cardiac tissue *in vivo*. Further, we aim to thoroughly investigate the effects of C3a and C5a on human CMs *in vitro*. The goal of the study was to get a closer understanding of the underlying molecular mechanisms leading to complement-induced cardiac dysfunction in the context of multiple trauma.

MATERIALS AND METHODS

Animals

This study presents results obtained from a project using porcine multiple trauma model, conducted by the TREAT research group.

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 138/2017, and were in accordance with Swiss Animal Protection Law and Ordinance. Housing and experimental procedures also conformed to the European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of vertebrate animals used for scientific purposes (Council of Europe no. 123, Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 2011).

25 male pigs weighting 50 ± 5 kg (*Sus scrofa domestica*) were included in the study (mean height: 123.6 cm). General instrumentation, anesthesia, and trauma induction were described previously by Horst et al. (34). Animals were held in controlled environment with 21 ± 3°C room temperature (50% humidity), with a light/dark cycle of 12 h. Water was available for animals *ad libitum*.

Multiple Trauma in Pigs

In the present study, 15 pigs were investigated. Pigs underwent either multiple trauma ($n = 10$) or sham-procedure ($n = 5$). Multiple trauma includes a combination of a penetrating thorax trauma, laparotomy, liver laceration, femur fracture, and hemorrhagic shock (ISS ≥ 27). Control animals underwent sham-procedure ($n = 5$). Femur fracture was induced by a bolt gun (Blitz-Kernen, turbocut JOBB GmbH, Germany), positioned on the mid third of the left femur. For introduction of blunt chest trauma, a pair of panels (steel 0.8 cm, lead 1.0 cm thickness) was placed on the right dorsal lower chest. A shock wave was induced by a bolt shot (Blitz-Kerner, turbocut JOBB GmbH, Germany), which was applied onto the panel using cattle-killing cartridges as previously described (35, 36). Midline-laparotomy was performed by exploring the right upper liver lobe. Penetrating hepatic injury was induced by cross-like incision halfway through the liver tissue. After a short period of uncontrolled bleeding (30 s), liver package was performed. Directly after hepatic packing, pressure-controlled and volume-limited hemorrhagic shock was induced by withdrawing of blood until a mean arterial pressure (MAP) of 30 ± 5 mm Hg was reached. Maximal withdrawal amounts to 45% of total blood volume. The reached MAP was maintained for 60 min. At the end of the shock period, animals were resuscitated according to established trauma guidelines (ATLS®, AWMF-S3 guideline on Treatment of Patients with Severe and Multiple Injuries®) by adjusting FiO_2 and an initial substitution of the withdrawn blood volume with Ringerfundin. Fluid maintenance was performed by infusion of additional fluids (Ringerfundin, 2 ml/kg body weight/h). Further, pigs were rewarmed until normothermia (38.7–39.8°C) was reached. Sham procedure ($n = 5$) included instrumentation and anesthesia but without trauma or hemorrhage. The multiple trauma group ($n = 10$) was randomized in two therapy arms: pigs received either femoral nailing without reaming ($n = 5$) or standard reaming ($n = 5$). In both groups, a shortened conventional tibia nail was introduced.

Follow-Up and Euthanasia

Hemodynamic parameters were continuously monitored for 6 h. Pigs were euthanized under deep, general anesthesia with intravenous Na-Pentobarbital.

Sample Collection

Serum and plasma samples were collected at baseline, 4 and 6 h after multiple trauma and kept on ice. After centrifugation (1,500 g for 12 min at 4°C), serum and EDTA-plasma were removed and stored at −80°C until analysis. Heart tissue samples were obtained 6 h after resuscitation. Tissue of the superficial and the luminal left ventricle was fixed with 4% formalin, followed by embedding in paraffin. Furthermore, tissue was quick-frozen in liquid nitrogen, followed by storage at −80°C until analysis.

Transesophageal Echocardiography in Pigs

Imaging was performed according to the recommendations using a standard cardiac ultrasound machine (Cx50 xMATRIX, Phillips Healthcare, Germany with the X7-2t probe and the S5-1 ultrasound probe for additional transthoracic measurements). Serial imaging was performed before, 4 and 6 h after trauma by an experienced investigator for echocardiography in pigs. The ejection fraction (EF) was calculated as $EF\ (\%) = (EDV–ESV)\ 1/EDV \times 100$ (EDV = end-diastolic volume; ESV = end-systolic volume). Further, blood pressure curves were measured continuously over 6 h. Thereby, following parameters were determined: heart rate (HR) in beats per minute (bpm), systolic, diastolic blood pressure, and mean arterial pressure (MAP) in mmHg at trauma as well as 1, 2, 3, 4, and 6 h after trauma.

Complement Hemolytic Activity

Classical Pathway (CH-50)

Sensitized sheep erythrocytes (Complement Technology Inc., Tyler, TX, USA) were washed once with tris buffered saline (TBS), centrifuged (3 min, 4°C, 500 g) and erythrocytes were re-suspended in GVB^{++} buffer (with Ca^{2+} and Mg^{2+}, pH 7.3) (Complement Technology Inc., Tyler, TX, USA). GVB^{++} buffer contains 0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025% NaN_3, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$, which allows the process of complement activation via the classical pathway. In order to determine 50 and 100% lysis, the erythrocytes were diluted with ddH_2O. The optical density was measured at 415 nm and OD values for 50 and 100% lysis were determined.

Serial dilution of porcine serum from 1:20 through 1:640 was prepared in GVB^{++} buffer (with Ca^{2+} and Mg^{2+}, pH 7.3). Then, sensitized sheep erythrocytes were added to the diluted serum samples and incubated for 30 min at 37°C. Afterwards, ice cold GVBE buffer (with EDTA, pH 7.3) (Complement Technology Inc., Tyler, TX, USA) was added to the samples and samples were centrifuged (3 min, 4°C, 500 g). GVBE buffer contains 0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025% NaN_3, and 10 mM EDTA, which inhibits the complement activation cascade. Afterwards, the supernatant was transferred and optical density from supernatant was measured at 415 nm.

Alternative Pathway (AH-50)

Sensitized rabbit erythrocytes (Complement Technology Inc., Tyler, TX, USA) were washed once with tris buffered saline (TBS), centrifuged (3 min, 4°C, 500 g) and erythrocytes were re-suspended in GVB^0 buffer (without Ca^{2+} and Mg^{2+}, pH 7.3) (Complement Technology Inc., Tyler, TX, USA). GVB^0 buffer contains 0.1% gelatin, 5 mM Veronal, 145 mM NaCl, and 0.025% NaN_3. GVB^0 is a basic buffer which can be used to make other traditional buffers for complement assays. In order to determine 50 and 100% lysis, the erythrocytes were diluted with ddH_2O. The optical density was measured at 415 nm and OD values for 50 and 100% lysis were determined.

Serial dilution of porcine serum from 1:2 through 1:32 was prepared in GVB^0 buffer with 5 mM MgEGTA. The addition of MgEGTA allows the process of complement activation via the alternative pathway. Then, sensitized rabbit erythrocytes were added to the diluted serum samples and incubated for 30 min at 37°C. Afterwards, ice cold GVBE buffer (with EDTA, pH 7.3) (Complement Technology Inc., Tyler, TX, USA) was added to the samples and samples were centrifuged (3 min, 4°C, 500 g). GVBE buffer contains 0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025% NaN_3, and 10 mM EDTA, which inhibits the complement activation cascade. Afterwards, supernatant was transferred and optical density from supernatant was measured at 415 nm.

Immunohistochemical Staining (IHC)

Paraffin sections of left ventricles were dewaxed and rehydrated in a descending series of ethanol. Antigen unmasking was performed by boiling sections at 100°C in 10 mM citrate buffer (pH 6). Unspecific binding sites were blocked with 10% goat serum. Specific antigen binding was performed by incubating sections with the respective primary antibody for C3a receptor (C3aR) (Abcam, Cambridge, UK), C5a receptor 1 (C5aR1) (Acris, Rockville, MD, USA), and cleaved C3 fragments C3b/iC3b/C3c (HycultBiotech, Wayne, PA, USA) for 1 h at RT. Specific antibody binding was detected by using DakoREAL™ Alkaline Phosphatase/RED Detection System (Agilent Technologies, Santa Clara, CA, USA). Cell nuclei were counterstained with Hematoxylin. Sections were investigated by bright field microscopy using Axio Imager M.2 microscope and the Zeiss ZEN 2.3 software (Zeiss, Jena, Germany). Results are presented as mean pixel density. The levels of C3aR, C3b/iC3b/C3c, and C5aR1 in porcine samples were determined by IHC due to the restricted availability of tools.

RNA Isolation From Heart Tissue

RNA from left ventricles was isolated using TRIzol RNA isolation reagent (ThermoFisher, Waltham, MA, USA).

RNA Isolation From Cell Lysates

RNA isolation from cell lysates was performed by using ISOLATE II RNA Mini Kit (Meridian Bioscience, Cincinnati, OH, USA). Remaining DNA was digested by DNaseI (Meridian Bioscience, Cincinnati, OH, USA).

Reverse Transcribed Quantitative Polymerase Chain Reaction RT-qPCR

The respective RNA samples were reverse transcribed in cDNA using SuperScript™ IV VILO™ MasterMix (Invitrogen, Carlsbad, CA, USA). For quantitative PCR the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA, USA) was used. All procedures were performed according to the manufacturer's instructions. For qPCR the QuantStudio3 system (Applied Biosystems, Waltham, MA, USA) was utilized. Quantitative mRNA expression of *porcine C5a receptor-2 (C5aR2)* (forward: 5′-AAGAGATGCTCTCCTGGACCT-3′, reverse: 5′-AAACTGTGTCAGTCCGGCTC-3′) and human *heart fatty acid binding protein (HFABP/FABP3)* (forward: 5′-GCATCACTATGGTGGACGCT-3′, reverse: 5′-AACCCA CACCGAGTGACTTC-3′), *connexin43* (forward: 5′-GGC TTTTAGCGTGAGGAAAGT-3′, reverse: 5′-AAGGCAAGT TCAGGCACTCA-3′), *sarcoplasmic/endoplasmic reticulum ATPase (SERCA2a)* (forward: 5′-CTCCTTGCCCGTGATT CTCA-3′, reverse: 5′-CCAGTATTGCAGGTTCCAGGT-3′), *sodium-calcium exchanger (NCX)* (forward: 5′-GCCTGGTGG AGATGAGTGAG-3′, reverse: 5′-ACAGGTTGGCCAAACA GGTA-3′) was examined and calculated by the cycle threshold method ΔΔCt. Respective genes were normalized using housekeeping gene *glutaraldehyde-phosphate dehydrogenase (GAPDH)* for pig (forward: 5′-GAGTGAACGGATTTGGCC-3′, reverse: 5′-AAGGGGTCATTGATGGCGAC-3′) and for human *GAPDH* (forward: 5′-TCTCTGCTCCTCCTGTTCGAC-3′, reverse: 5′-CCAATACGACCAAATCCGTTGA-3′). Results are presented as mean fold change. The mRNA levels of C5aR2 in porcine samples were determined by qPCR due to the restricted availability of tools.

In vitro Experiments

Human cardiomyocytes (iPS) (Cellular Dynamics, Madison, WI, USA) were cultured for 10 days in maintenance medium (Cellular Dynamics, Madison, WI, USA) at 37°C and in an atmosphere of 7% CO_2. For experiments, human CMs were incubated with either 10 ng/ml C5a or with 500 ng/ml C3a (both from Merck, Darmstadt, Germany) for 6 h at 37°C in an atmosphere of 7% CO_2.

Viability Assay and Caspase-3/7 Assay

Cell viability of human cardiomyocytes was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and Caspase-3/7 activity was examined by using Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA). All procedures were performed according to manufacturer's instructions.

Calcium Measurements

For calcium measurements human cardiomyocytes (iPS) were seeded on 8-well chambers (IBIDI, Munich, Germany). Before the measurements, cells were incubated with either 10 ng/ml C5a or 500 ng/ml C3a 60 min before the start of the experiments, as well as for the duration of the experiment. For measurement of changes in intracellular Ca^{2+} concentration, cells were loaded with 5 μM Fura-2 (ThermoScientific, Waltham, MA,

USA) for 30 min (in presence of pharmacological compounds if needed). After incubation, cells were washed twice with bath solution (in mM: 140 NaCl; 5.4 KCl; $MgCl_2$; 1.8 $CaCl_2$; 5.5 Glucose; 5 Hepes; pH = 7.4). Fluorescence imaging was performed on a Cell Observer inverse microscope with a Zeiss Fluor 40x NA 1.3 oil objective (Zeiss, Jena, Germany). Cells were illuminated for 90 ms at a rate of 2 Hz at each excitation wavelength (340 and 380 nm). Images were acquired using MetaFluor (Molecular Devices, Ismaning, Germany). Fura-2 ratios were calculated with ImageJ and the data obtained were analyzed with the Matlab script PeakCaller (37).

Mitochondrial Respiration

Mitochondrial respiration was analyzed by using the Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA). For analysis of mitochondrial respiration, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA) was used. After the experiment, cells were fixed with 4% formalin at 4°C for overnight. Then, cells were stained with 0.2% Janus-Green solution, washed and resolved with 0.5 M hydrochloric acid. OD was measured at 630 nm. The oxygen consumption rate (OCR) values were normalized to the OD 630 nm values, respectively. Results were evaluated using Seahorse Wave 2.4 software (Agilent Technologies, Santa Clara, CA, USA).

Immunofluorescent Staining of Human Cardiomyocytes

Human cardiomyocytes were incubated for 3 h with 10 ng/ml C5a. Afterwards, cells were washed and fixed with 4% formalin for 15 min at RT. Cells were permeabilized with 0.03% Triton-X for 10 min at RT. Unspecific binding sites were blocked with 10% goat serum. Specific antigen binding was performed by incubating cells with specific primary antibody for C5aR1 (Proteintech, Rosemont, IL, USA) for overnight at 4°C. As second antibody the AlexaFluor-488 IgG (Jackson Immunoresearch, Cambridgeshire, UK) was used. Staining of cytoskeletal actin filaments was performed by using rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA, USA) and cell nuclei were stained with Hoechst. Cells were analyzed by fluorescence microscopy using Axio Imager M.2 microscope and the Zeiss ZEN 2.3 software (Zeiss, Jena, Germany).

Statistical Analysis

All values were expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Data of the *in vitro* experiments were also analyzed by unpaired students *t*-test. $p \leq 0.05$ was considered statistically significant. GraphPad Prism 7.0 software was used for statistical analysis (GraphPad Software, Incorporated, San Diego, CA, USA).

RESULTS

Hemodynamic and Functional Cardiac Parameters of Multiply Injured Pigs

The blood pressure of multiply injured pigs was measured at trauma as well as 1, 2, 3, 4, and 6 h after trauma. The systolic blood pressure significantly decreased at trauma as well as 1 h after trauma in the group, which received conventional reaming of the fracture compared to the sham group. In group with femoral nailing the systolic blood pressure significantly decreased 1 h after trauma compared to the sham group (**Figure 1A**). The diastolic blood pressure significantly decreased 1, 2, and 4 h after trauma in the group with conventional treatment of the fracture compared to the sham group. In group with femoral nailing of the fracture, the diastolic blood pressure significantly decreased 1 h after trauma compared to the sham group (**Figure 1B**). The mean arterial pressure (MAP) significantly decreased 1 and 4 h after trauma in group with conventional reaming compared to the sham group. In the group with femoral nailing, the MAP significantly decreased 1 h after trauma compared to the sham group (**Figure 1C**). The heart rate significantly increased 1, 2, 3, 4, and 6 h after trauma in the group with conventional reaming compared to the control group. In the group with femoral nailing, the heart rate significantly increased 6 h after trauma compared to the sham group (**Figure 1D**). The cardiac ejection fraction significantly decreased 6 h after trauma in the group, which received conventional reaming of the fracture compared to the sham group (**Figure 1E**).

Complement Hemolytic Activity in Multiply Injured Pigs

CH-50-Classical Pathway

In order to determine early complement activation after multiple trauma, we analyzed the complement hemolytic activity in serum of multiply injured pigs compared to the sham treated animals. Considering the classical pathway of complement activation, the CH-50 lysis curves of the two trauma groups were shifted to the left after 4 and 6 h compared to the control group, needing a higher dilution for lysis of the sensitized erythrocytes due to already consumed complement factors (**Figures 2A,C**). This was also demonstrated in the respective calculated CH-50 values of the CH-50 lysis curves. The CH-50 decreased within 6 h in the polytrauma group, which received internal fixation by femoral nailing (**Figures 2B,D**). In the polytrauma group with conventional reaming of the fracture, the CH-50 also decreased within 6 h after trauma and dropped significantly 6 h after trauma compared to the sham treated group (**Figures 2B,D**). Lower CH-50 values indicate for increased complement activation.

AH-50-Alternative Pathway

Regarding the alternative pathway, the lysis curves of the two trauma groups were shifted to the left after 4 h and 6 h compared to the control group, indicating for already consumed complement factors after trauma (**Figures 3A,C**). This was also reflected in the respective calculated AH-50 values of the AH-50 curves. The AH-50 decreased significantly in both trauma groups

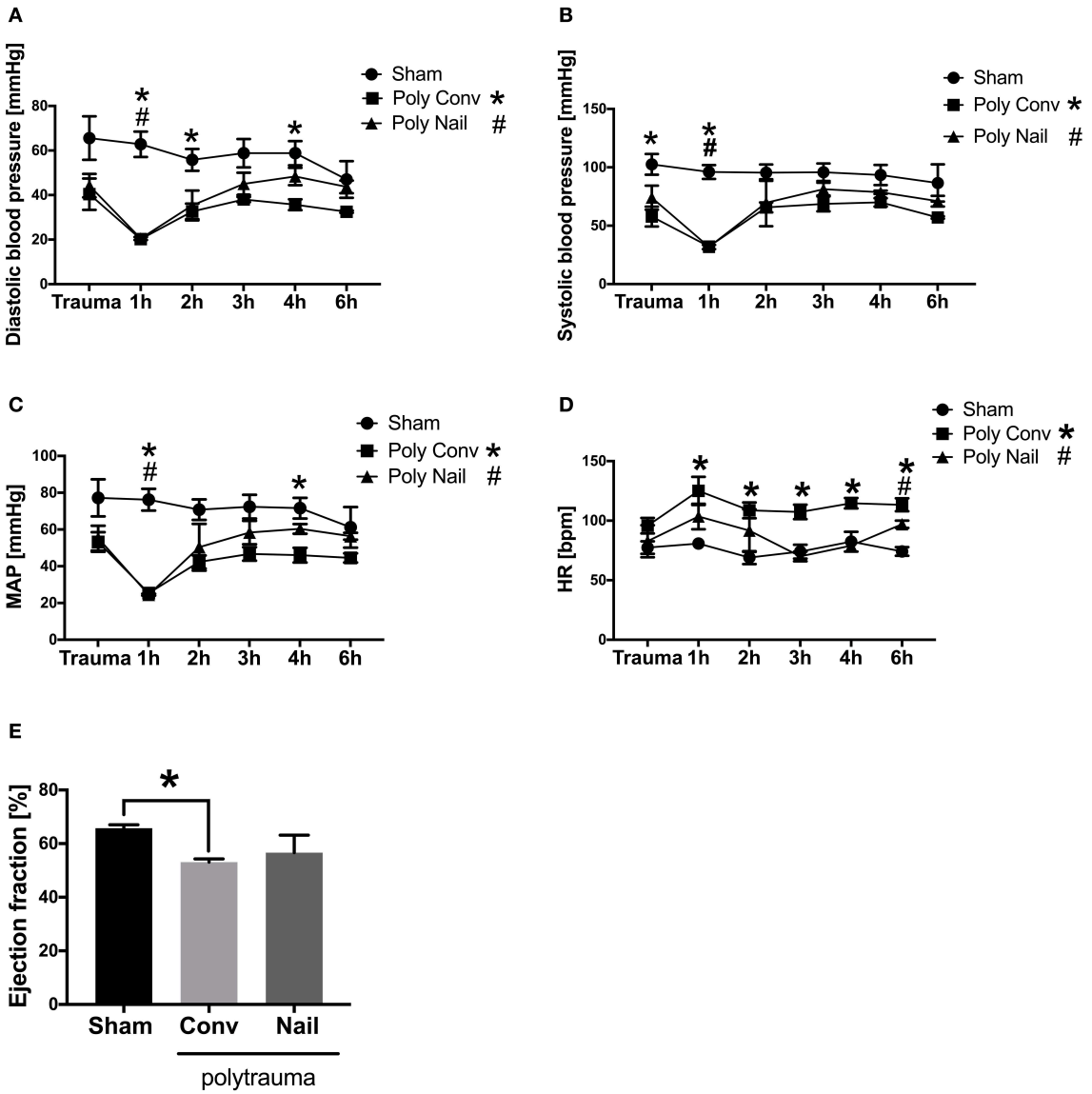

FIGURE 1 | Hemodynamic and functional cardiac parameters of multiply injured pigs. Pigs received multiple injury, followed by femoral nailing of the fracture (poly nail, triangle) or conventional reaming of the fracture (poly conv, square). Control animals underwent sham-procedure (sham, circle). Blood pressure was measured continuously over 6 h and systolic blood pressure (mmHg) **(A)**, diastolic blood pressure (mmHg) **(B)**, mean arterial pressure (MAP) (mmHg) **(C)**, heart rate (HR) (bpm) **(D)** were measured directly at trauma as well as 1, 2, 3, 4, and 6 h after trauma. Cardiac ejection fraction (%) **(E)** was determined by transesophageal echocardiography 6 h after trauma. Results are presented as mean ± SEM. $n = 5$. *$p \leq 0.05$.

after 4 and 6 h, compared to the control group (**Figures 3B,D**). Lower AH-50 values indicate reduced residual activity of the complement system due to already consumed complement factors after trauma.

Local Cardiac Levels of C3aR, C5aR1/2 and Local Deposition of C3 Cleavage Products C3b/iC3b/C3c in Multiply Injured Pigs After 6 h

Since the multiply injured pigs showed complement activation in serum within 6 h after trauma, we next investigated the local levels of the complement receptors C3aR, C5aR1/2, and the local deposition of the C3 cleavage products C3b/iC3b/C3c in heart tissue. In left ventricles, no significant differences in the expression of the C3aR were observed (**Figure 4A**). However, the C3aR expression enhanced slightly but not significantly in left ventricular lumen of both polytrauma groups compared to the sham-treated group (**Figure 4A**). On the ventricular surface, the C3aR expression increased slightly but not significantly in the group with conventional reaming and decreased in the group with femoral nailing, compared to the control group (**Figure 4A**). The deposition of C3 cleavage products C3b/iC3b/C3c significantly increased in the ventricular

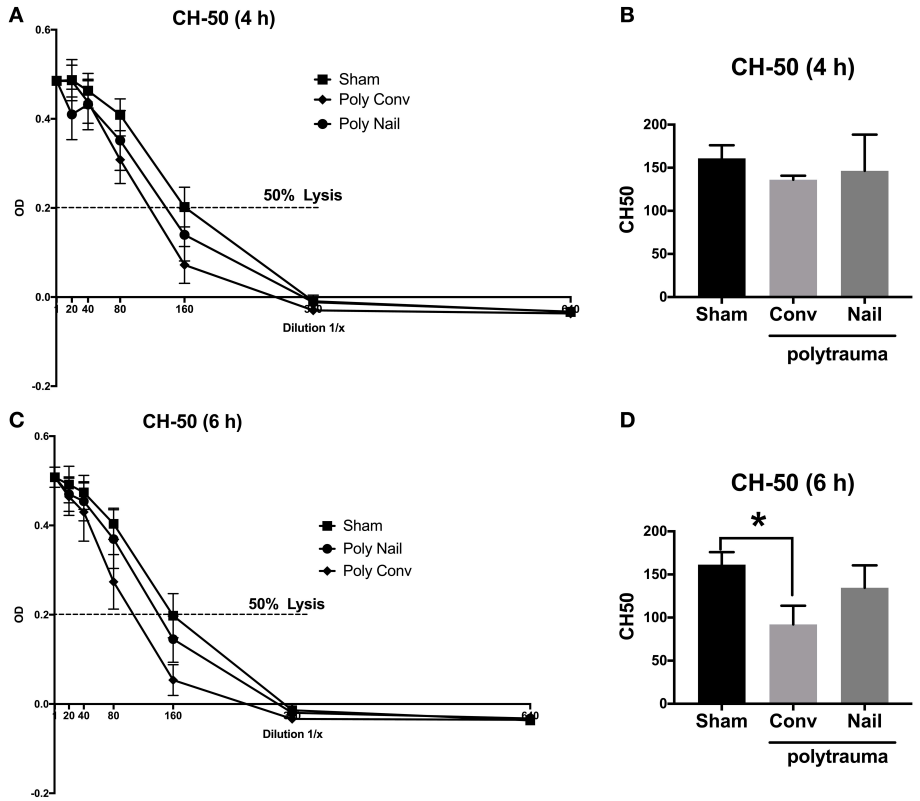

FIGURE 2 | Complement hemolytic activity in serum of multiply injured pigs via the classical pathway (CH-50). Pigs received multiple injury, followed by femoral nailing of the fracture (poly nail, circle) or conventional reaming of the fracture (poly conv, rhombus). Control animals underwent sham-procedure (sham, square). CH-50 was measured at 4 h **(A,B)** and 6 h **(C,D)** after trauma. For CH-50 lysis curves, a serial dilution of the serum from 4 h after trauma **(A)** and 6 h after **(C)** was performed (x-axis, dilution in 1/x) and the respective OD values of the serum were determined at 415 nm (y-axis, OD). Dotted line represents OD value of 50% lysis of sensitized sheep erythrocytes. CH-50 values (1/x) of 4 h after trauma **(B)** and 6 h after trauma **(D)**. Results are presented as mean ± SEM. $n = 5$. $^*p \leq 0.05$.

lumen of the trauma group with conventional reaming, compared to the group with femoral nailing (**Figure 4B**). Moreover, the deposition of C3 cleavage products C3b/iC3b/C3c increased significantly on the ventricular surface of the trauma group with conventional reaming, compared to the control group (**Figure 4B**). In the group with femoral nailing the C3 cleavage products C3b/iC3b/C3c deposition decreased significantly on the ventricular surface compared to the trauma group with conventional reaming (**Figure 4B**). In the left ventricular lumen, expression of the C5aR1 slightly decreased in both polytrauma groups, compared to the control group (**Figure 4C**). No differences in C5aR1 expression were observed on the ventricular surface (**Figure 4C**). The levels of C3aR, C3b/iC3b/C3c, and C5aR1 in porcine samples were determined by IHC due to the restricted availability of tools. The *C5aR2* mRNA expression decreased significantly in the ventricular lumen of the polytrauma group with femoral nailing compared to the group with conventional reaming (**Figure 4D**). Moreover, the *C5aR2* mRNA expression decreased significantly on the ventricular surface of the polytrauma group with femoral nailing compared to the control group. Also, the *C5aR2* mRNA expression decreased in general in the polytrauma group with conventional reaming on the ventricular surface (**Figure 4D**). The levels of C5aR2 in porcine samples were determined by qPCR due to the restricted availability of tools.

Effects of C3a and C5a on Human CMs *in vitro*

Since the complement system is systemically activated after polytrauma in pigs and acts locally on the heart via its receptors, we next investigated the effects of C3a and C5a on human CMs *in vitro*, trying to investigate the effects of the single complement factors on the cells. First, human CMs were cultured in presence of C5a. After an exposure of 3 h, the C5aR1 of the human CMs was translocated from the surface of the CMs into the cytosol of the cells. In parallel, the intracellular fraction of the C5aR1 increased in presence of C5a in the human CMs (**Figure 5A**). Furthermore, the Caspase 3/7 activity significantly enhanced in CMs in presence of C5a and C3a, compared to the control group (**Figure 5B**).

Calcium Signaling and Metabolic Alterations of Human CMs in Presence of C3a or C5a

Next, we investigated the calcium signaling of the human CMs in presence of C3a or C5a. Calcium is the key signaling element

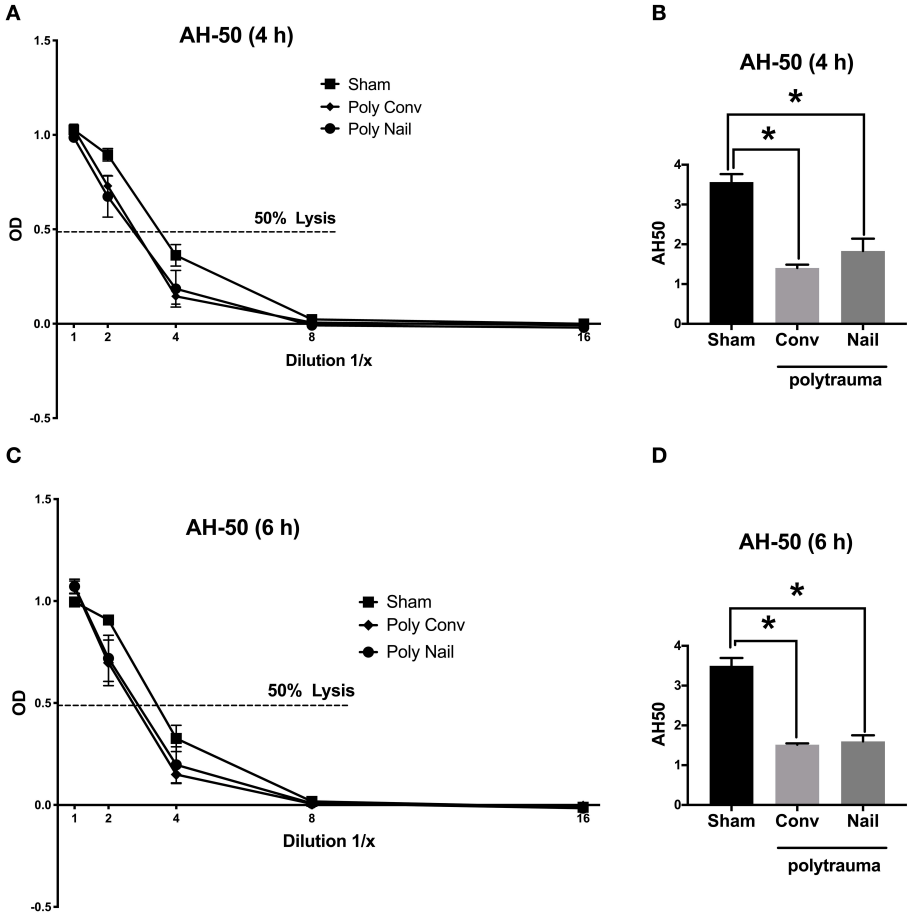

FIGURE 3 | Complement hemolytic activity in serum of multiple injured pigs via the alternative pathway (AH-50). Pigs received multiple injury, followed by femoral nailing of the fracture (poly nail, circle) or conventional reaming of the fracture (poly conv, rhombus). Control animals underwent sham-procedure (sham, square). AH-50 was measured at 4 h **(A,B)** and 6 h **(C,D)** after trauma. For AH-50 lysis curves, a serial dilution of the serum from 4 h after trauma **(A)** and 6 h after **(C)** was performed (x-axis, dilution in 1/x) and the respective OD values of the serum were determined at 415 nm (y-axis, OD). Dotted line represents OD value of 50% lysis of sensitized sheep erythrocytes. AH-50 values (1/x) of 4 h after trauma **(B)** and 6 h after trauma **(D)**. Results are presented as mean ± SEM. $n = 5$. $^*p \leq 0.05$.

in CMs and is crucial for cardiac function. In CMs, calcium transport includes Ca^{2+} cycling between the cytosol and the extracellular space as well as Ca^{2+} cycling between the cytosol and the intracellular calcium stores, the sarcoplasmic reticulum (SR). During depolarization of the cell membrane, the L-type Ca^{2+} channels in the plasma membrane are activated, allowing Ca^{2+} entering the cytosol, raising local Ca^{2+} concentrations. The local increase of Ca^{2+} triggers activation of further Ca^{2+} channels in the SR membrane. Due to the additional Ca^{2+} release from the SR, the local as well as the global systolic Ca^{2+} levels increase. The increased Ca^{2+} concentrations induce contraction of the CMs via conformational changes in the troponin-tropomyosin complex, allowing the myofilaments actin and myosin to slide past one another (38, 39). For relaxation, the Ca^{2+} has to be removed from the cytosol and is transported back into the SR by the sarcoplasmic-/endoplasmic reticulum ATPase (SERCA) and into the plasma membrane via the sodium-calcium exchanger (NCX). The Ca^{2+} cycling process is induced by extra- or intracellular stimuli, affecting the activity state of Ca^{2+}-handling proteins (39). In the present study, the intracellular calcium was labeled with a fluorescent calcium indicator and calcium signaling of the CMs was analyzed by fluorescence microscopy. The frequency of the calcium signals decreased significantly in the CMs after exposed to C3a or C5a (**Figure 5C**). Moreover, the mean decay time of the single calcium peaks increased significantly in presence of C5a (**Figure 5D**). Furthermore, the basal mitochondrial respiration decreased significantly in presence of C3a and C5a, compared to the control group (**Figure 5E**) and the mitochondrial spare respiratory capacity decreased significantly (**Figure 5F**).

Cellular Gene Expression in Human CMs in Presence of C3a or C5a

Since the calcium signaling was altered in human CMs in presence of C3a and C5a, we next investigated the gene expression of the gap junction protein Cx43 as well as of the calcium handling proteins *SERCA2a* and *NCX*. In presence

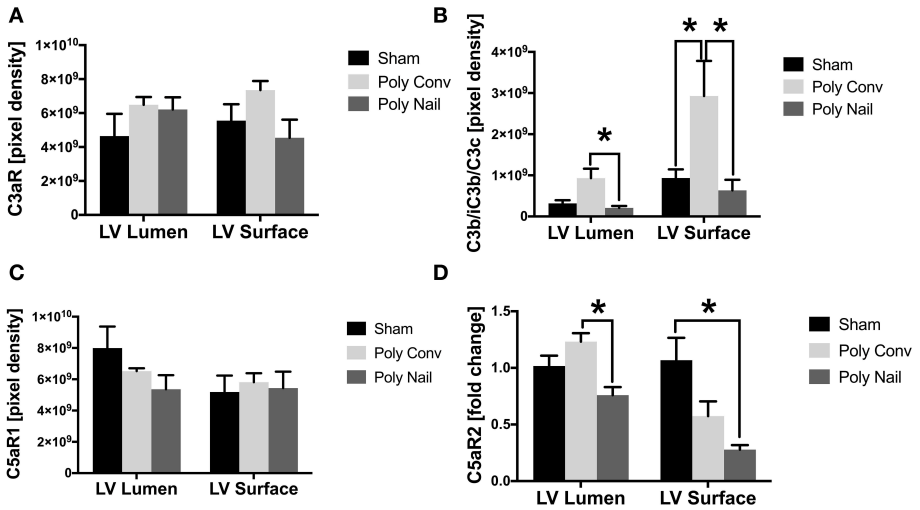

FIGURE 4 | Local cardiac expression of C3a receptor (C3aR), C5a receptors 1 and 2 (C5aR1/C5aR2) and deposition of C3 cleavage products C3b/iC3b/C3c. Pigs received multiple injury, followed by femoral nailing of the fracture (poly nail) or conventional reaming of the fracture (poly conv). Control animals underwent sham-procedure (sham). Tissue of left ventricular lumen and surface were collected 6 h after trauma. Local cardiac expression protein expression of C3aR **(A)**, deposition of C3 cleavage products C3b/iC3b/C3c **(B)** and local expression of C5aR1 **(C)** in control (black), poly conv (light gray), and poly nail (dark gray) pigs, represented as pixel intensity. Local cardiac mRNA expression of *C5aR2* **(D)** in control (black), poly conv (light gray), and poly nail (dark gray) pigs, represented as fold change. Results are presented as mean ± SEM. $n = 5$. $^*p \leq 0.05$.

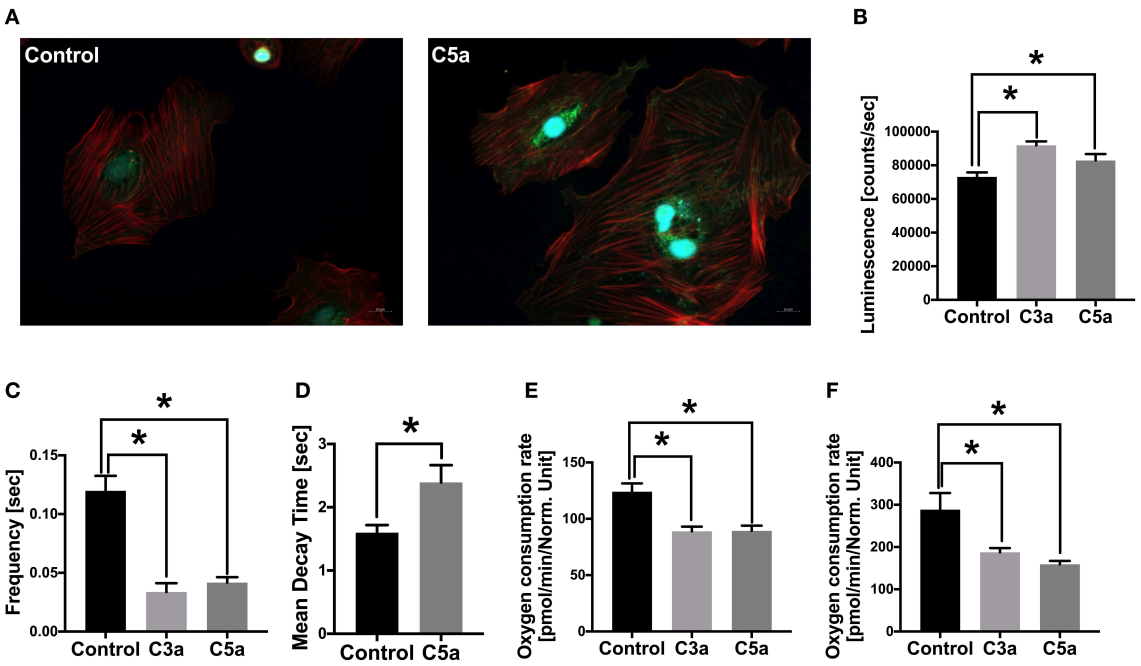

FIGURE 5 | Effects of C3a and C5a on human cardiomyocytes (CMs) *in vitro*. Cellular staining of the C5a receptor 1 (C5aR1) in human CMs in presence of PBS (control) or in presence of C5a **(A)**. Human CMs were treated for 3 h with either PBS or with 10 ng/ml C5a and C5aR1 was stained (green). Cell nuclei were stained with Hoechst (blue) and cytoskeleton was stained with rhodamine-labeled phalloidin (red). Caspase-3/7 activity (counts/s) in human CMs in presence of C3a (light gray) and C5a (dark gray) **(B)**. Frequency of calcium signals (s) of human CMs in presence of C3a and C5a **(C)**. Mean decay time (s) of calcium peaks of human CMs in presence of C5a **(D)**. Basal respiration oxygen consumption rate (OCR in pmol/m in /Norm. unit) of human CMs in presence of C3a and C5a **(E)**. Spare respiratory capacity oxygen consumption rate (OCR in pmol/m in /Norm. unit) of human CMs in presence of C3a and C5a **(F)**. Results are presented as mean ± SEM. $n = 6$. $^*p \leq 0.05$.

of C3a, the *SERCA2a* and *NCX* mRNA expression increased significantly in human CMs, whereas the *Cx43* mRNA expression was not affected (**Figures 6B–D**). In presence of C5a, the mRNA expression of *Cx43* was significantly increased, as well as the mRNA expression of NCX (**Figures 6F,H**). The mRNA expression of *SERCA2a* was not affected in presence of C5a

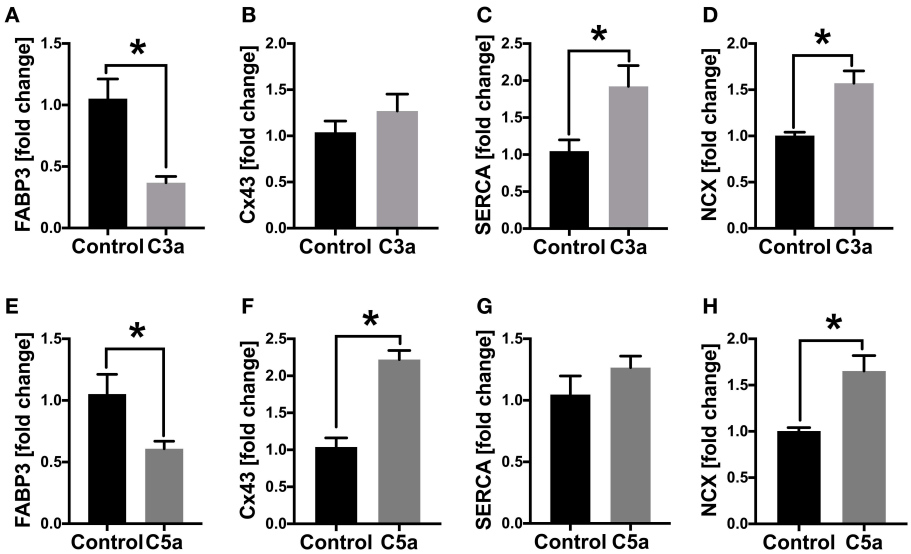

FIGURE 6 | mRNA expression (fold change) of cardiac structure- and gap junction proteins in human cardiomyocytes (CMs) treated with C3a or C5a. Cells were treated for 6 h with either 500 ng/ml C3a or with 10 ng/ml C5a and mRNA expression of *fatty acid binding protein 3 (FABP3)* **(A,E)**, *connexin43* **(B,F)**, *sarco plasmic/endoplasmic reticulum ATPase (SERCA2a)* **(C,G)** and *sodium-calcium exchanger (NCX)* **(D,H)** were determined. Results are presented as mean ± SEM. $n = 6$. $^*p \leq 0.05$.

(**Figure 6G**). In presence of C3a and C5a the mRNA expression of *heart fatty acid binding protein (FABP3/HFABP)* decreased significantly in the human CMs (**Figures 6A,E**).

DISCUSSION

In the present study, we demonstrated an activation of the complement system in multiply injured pigs within the first 6 h after trauma, which is in accordance with previous studies in humans (3, 40). Thereby, the complement activation resulted in impaired cardiac function of the pigs after trauma. The complement activation was mediated via both, classical and alternative pathway. The complement activation after multiple trauma was probably triggered simultaneously but via different activation cascades. In the present study, the early complement activation might be rapidly induced via the alternative pathway rather than the classical pathway, since the AH-50 dropped significantly directly after trauma as well as after 4 and 6 h in both polytrauma groups, which is in accordance to earlier studies (14). This rapid induction of the alternative pathway might be due to the antibody-independent activation of this pathway. The alternative pathway is immediately activated after binding of danger signals from exogenous pathogens but also from damaged tissue, which is the situation after multiple trauma (41, 42). Furthermore, the CH-50 of the polytrauma group with conventional reaming dropped significantly 6 h after trauma, confirming the complement activation also via the classical pathway. In contrast to the alternative pathway, the activation of the classical pathway is antibody-dependent, which might explain the significant drop of the CH-50 only after 6 h (41). Moreover, the complement activation might correlate with the tissue damage and with the release of bone marrow debris into the circulation reflected by the invasiveness of the fracture provision during both, classical- and alternative pathway. Nevertheless, the activation of the complement system might be also mediated via a third pathway, the lectin pathway. The activation of this pathway is induced when mannose-binding lectin (MBL) binds mannose containing surface proteins on pathogenic surfaces (41). The activation of the complement system via the lectin pathway was not examined, wherefore we cannot exclude complement activation via this pathway in the present study. The polytrauma group, which received conventional reaming showed the highest activation of the complement system. Correlations between enhanced inflammation in general and femoral reaming were also demonstrated previously in humans and were accordingly associated with an increased release of tumor necrosis factor (TNF), interleukin (IL)-6 and IL-10 after fracture treatment (20, 43, 44). The intramedullary nailing in multiply injured patients was described as a less invasive method for fracture treatment (45). Nevertheless, femoral nailing and femoral reaming in multiply injured patients was associated with increased inflammation, occurring as "second hit" after trauma (20, 46). During the "second hit" response, invasive surgical interventions may induce systemic inflammation, leading to systemic inflammatory response syndrome (SIRS), ARDS, ALI, MODS, MOF, and finally to death of the trauma patients (20, 47). Summarized, in the present study we demonstrated that the fracture provision with reamed intramedullary nailing induced an enhanced activation of the complement system after multiple trauma, compared to non-reamed femoral nailing. This increased complement activation might contribute to the "second hit" response after multiple trauma. Accordingly, the choice of fracture treatment might imply the clinical outcome of critically

injured patients with respect to the development of SIRS, ARDS, ALI, MODS, and MOF. Consequently, the selection of the treatment strategy might be crucial for survival of the critically injured patients.

The early activation of the complement system may also affect the heart *in vivo*, which was confirmed in the present study by alterations in the left ventricular expression of C3aR, C5aR1/2, and by changes in the deposition of C3 cleavage products C3b/iC3b/C3c, early after trauma. The C3aR expression was enhanced slightly but not significantly in the left ventricles of both polytrauma groups. In humans with non-ischemic heart failure, increased C3aR expression was associated with enhanced cardiac inflammation and progressive heart failure (48). Interestingly, the C3aR expression may correlate with the invasiveness of fracture treatment, indicating augmented inflammation and complement activation during femoral reaming (20). However, in the acute inflammatory phase during ischemia-reperfusion injuries, C3aR activation was shown to reduce the inflammatory response through prevention of leucocyte mobilization (49, 50). In the present study, the deposition of C3 cleavage products C3b/iC3b/C3c were strongly enhanced in the lumen of the left ventricles as well as on the surface of the polytrauma group with conventional reaming, correlating with the invasiveness of the fracture treatment, according to earlier studies (20). Interestingly, in the group with femoral nailing the deposition of C3 cleavage products C3b/iC3b/C3c was significantly decreased on the ventricular lumen and surface, indicating less inflammatory response during trauma (51). An increased amount of deposited C3 cleavage products C3b/iC3b/C3c induces the second inflammatory phase after trauma, resulting in cardiac failure and death (52, 53). In the present study, the expression of C5aR1 was slightly decreased in the lumen of the left ventricle after trauma in both polytrauma groups, which was also described previously after 72 h in an experimental polytrauma model in pigs and after 24 h in an experimental blunt chest trauma model in rats (54, 55). The decreased expression of C5aR1 after trauma was associated with internalization of the receptor, triggered by C5a, which was further associated with CMs dysfunction and compromised cardiac function (25, 56). In contrast, C5aR1 is upregulated during burn injury and after cecal ligature and puncture-induced (CLP) sepsis, mediating complement-induced cardio-depressive effects (25, 28, 57). In the present study, the C5aR1 was slightly upregulated on the surface of the left ventricle of the polytrauma group with femoral reaming, which is in accordance with the above-mentioned studies. Further, C5a elevation during fracture treatment seems to induce a second inflammatory hit after experimental trauma (46). In the present study, the ventricular luminal mRNA expression of the *C5aR2* decreased significantly in the trauma group with femoral nailing, compared to the group with conventional reaming. Interestingly, the mRNA expression of *C5aR2* decreased in both trauma groups on the ventricular surface, compared to sham treated animals. During the inflammatory condition of CLP-sepsis, C5a-C5aR2 interactions were shown to induce excessive amount of cytosolic ROS and $[Ca_i^{2+}]$ in CMs. Further, the absence of the C5aR2 improves heart function in CLP mice (28, 58). Therefore, the here shown posttraumatic downregulation of the *C5aR2* expression in the heart tissue might even be protective for the heart function during complement activation. The differences in the local cardiac expression of C3aR, C5aR1, C5aR2 and in the deposition of C3 cleavage products C3b/iC3b/C3c might be due to inflammatory effects on the cardiac surface. In the present study, we showed that the complement system is activated in pigs early after polytrauma, acting on the heart via its receptors. Also, the local cardiac complement activation correlated with invasiveness of the fracture treatment. Consequently, the selection of the adequate fracture provision might imply the post-traumatic cardiac function. During early complement activation, the anaphylatoxins C3a and C5a are systemically released. Therefore, we investigated the effects of C3a and C5a on human CMs *in vitro*. In the present study we could show that the C5a was actively internalized by the human CMs via the C5aR1. Thereby, the C5aR1 might be endocytosed during this process, which is in accordance with earlier studies (56, 59). After internalization, the C5aR1 was predominantly localized in tubulo-vesicular structures and around the nucleus, which is in accordance with our observations (60). The increased intracellular fraction of C5aR1 in the human CMs confirmed this assumption as well. C5a was shown to disturb cellular calcium homeostasis and electrophysiological functions and induces defects in CMs contractility and relaxation (28–30).

In the present study, the Caspase-3/7 activity significantly increased in human CMs in presence of C3a and C5a *in vitro*, indicating somehow an enhanced cellular apoptosis. Increased cellular apoptosis in presence of C3a was already demonstrated in previous studies. In these studies, caspase-11 expression was induced by the carboxypeptidase B1 (Cpb1)-C3-C3aR pathway by increasing mitogen-activated protein kinase (MAPK) activity and downstream processing of toll-like receptor 4 (TLR4), resulting in cell death (61). Moreover, C3aR is required for upregulation of caspase-4 and 5 in human macrophages, correlating with cell death and worse outcome of septic patients (61). In contrast, C5a was described to protect against apoptosis by inhibiting MAPK-mediated regulation of caspase-3 cascades in neurons (62). Furthermore, C5a inhibits caspase-9 activity in neutrophils via the phosphatidylinositol-3 kinase (PI-3K)/Akt and via the extracellular signal-regulated kinase (EKR) pathway (63, 64), which is not in agreement with the results in our study. However, the enhanced caspase 3/7 activity might be an effect of the CMs to the exposure of C3a and C5a *in vitro*. We could not detect any apoptosis in cardiac tissue *in vivo* (data not shown), which might indicate for complement-independent apoptotic effects. However, in order to investigate apoptotic effects during complement activation *in vivo*, further studies are necessary.

In the present study, the calcium signaling of the human CMs was disturbed in presence of C3a and C5a, which was demonstrated previously in isolated rat CMs in presence of C5a and was further associated with cardiac dysfunction (28). Here, the frequency of calcium signals decreased significantly, resulting in slowed cell beat and bradycardia. Moreover, the mean decay time of the single calcium peaks was altered in human CMs in presence of C5a, indicating a disturbed calcium handling by enhanced build-up of $[Ca^{2+}i]$, as described

previously (28). The disturbed calcium signaling in the human CMs might be induced by increased ROS. Enhanced cytosolic ROS was shown in isolated rat and mouse CMs in presence of C5a or when exposed to CLP-sepsis and was associated with myocardial dysfunction and cardiovascular diseases (65, 66). Contractile dysfunction due to enhanced ROS is mostly induced by modification of different calcium regulatory proteins (29, 30). Alterations in mRNA expression of *SERCA2a* and *NCX* in presence of C3a and C5a in our study confirmed this assumption. Moreover, changes in *SERCA2a* and *NCX* mRNA expression were previously shown during CLP-sepsis and were also associated with impaired cardiac function (28). Alterations in these calcium regulatory proteins were mediated via C5a and its receptors C5aR1/C5aR2 (28). Redox imbalance during C5a as well as during C3a treatment might be induced by the NADPH oxidases Nox1 and Nox2 (67). Finally, we could show that the mitochondrial respiration of human CMs was impaired in presence of C3a and C5a. Thereby, the basal mitochondrial respiration as well as the mitochondrial spare respiratory capacity decreased significantly in the cells when treated either with C3a or with C5a. Detrimental effects of C5a on mitochondrial function were already described in pheocromocytoma-derived PC12 cells. Here, C5a inhibited mitochondrial respiration as well as dehydrogenase and cytochrome c oxidase (COX) activities. Moreover, C5a induced mitochondrial stress and damage in these cells (68). Disruption of mitochondrial membrane potential was also demonstrated previously in isolated rat CMs after addition of extracellular histones (27).

Interestingly, in the present study the mRNA expression of the *heart fatty acid binding protein (HFABP/FABP3)* decreased significantly in the human CMs in presence of C3a and C5a. So far, HFABP was primarily used as systemic biomarker for different cardiovascular diseases such as acute myocardial infarction (AMI) (69). Not to forget, HFABP also plays a dominant role in cardiac fatty acid metabolism by promoting the uptake of Long-chain free fatty acids (LCFA) into the cytoplasm of CMs, by delivering the LCFA to the outer membrane of mitochondria and by accelerating the dissociation of LCFA from albumin (70–72). The downregulation of *HFABP* gene expression might indicate for a reduction of the cardiac utilization of LCFA, switching to a condition of enhanced cellular glycolysis, which was also shown previously after experimental multiple trauma (73). This metabolic switch is also called myocardial hibernation and was demonstrated previously in the septic heart (74). Myocardial hibernation is characterized by enhanced expression of the glucose transporter 4 (GLUT4), preventing cardiac cell damage and LV-dysfunction in the injured hypoxic myocardium by enhancing the glucose uptake (75–77).

In summary, we were able to demonstrate an early activation of the complement system in multiply injured pigs within the first 6 h after trauma, which impair the cardiac function *in vivo*. For the first time we could show differential immune response based on surgical treatment strategy (reamed vs. non-reamed intramedullary nailing), which might contribute to the "second hit" response after trauma. Consequently, the selection of the fracture treatment strategy might imply the overall outcome of the multiply injured patients. The anaphylatoxins C3a and C5a acted detrimentally on human CMs *in vitro* by enhancing cellular apoptosis and by disrupting calcium signaling and mitochondrial respiration. Finally, changes in *HFABP* gene expression suggested that CMs could undergo metabolic switch to myocardial hibernation during complement activation resulting from multiple trauma.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 138/2017.

AUTHOR CONTRIBUTIONS

IL, BW, FG, RP, PC, SH, ML, NC, HS, MH-L, H-CP, and MK designed the research. IL, BW, MB, GF, SH, ML, and NC performed the research. IL wrote the paper. IL and MK analyzed the data. FG, RP, PC, HS, H-CP, and MK contributed new reagents or analytic tools. All authors made substantial contributions to conception and design of the study, participated in drafting the article and gave final approval of the version to be published.

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Midkine Is Elevated After Multiple Trauma and Acts Directly on Human Cardiomyocytes by Altering Their Functionality and Metabolism

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Background and Purpose: Post-traumatic cardiac dysfunction often occurs in multiply injured patients (ISS $\geq$ 16). Next to direct cardiac injury, post-traumatic cardiac dysfunction is mostly induced by the release of inflammatory biomarkers. One of these is the heparin-binding factor Midkine, which is elevated in humans after fracture, burn injury and traumatic spinal cord injury. Midkine is associated with cardiac pathologies but the exact role of Midkine in the development of those diseases is ambiguous. The systemic profile of Midkine after multiple trauma, its effects on cardiomyocytes and the association with post-traumatic cardiac dysfunction, remain unknown.

Experimental Approach: Midkine levels were investigated in blood plasma of multiply injured humans and pigs. Furthermore, human cardiomyocytes (iPS) were cultured in presence/absence of Midkine and analyzed regarding viability, apoptosis, calcium handling, metabolic alterations, and oxidative stress. Finally, the Midkine filtration capacity of the therapeutic blood absorption column CytoSorb ®300 was tested with recombinant Midkine or plasma from multiply injured patients.

Key Results: Midkine levels were significantly increased in blood plasma of multiply injured humans and pigs. Midkine acts on human cardiomyocytes, altering their mitochondrial respiration and calcium handling *in vitro*. CytoSorb®300 filtration reduced Midkine concentration *ex vivo* and *in vitro* depending on the dosage.

Conclusion and Implications: Midkine is elevated in human and porcine plasma after multiple trauma, affecting the functionality and metabolism of human cardiomyocytes *in vitro*. Further examinations are required to determine whether the application of CytoSorb®300 filtration in patients after multiple trauma is a promising therapeutic approach to prevent post-traumatic cardiac disfunction.

Keywords: polytrauma, cardiac dysfunction, fracture treatment, damage associated molecular pattern, toll-like receptor, toll-like receptor signaling, prevention cardiac injury, CytoSorb® 300

INTRODUCTION

According to the World Health Organization (WHO), trauma accounts for 10% of deaths and 16% of disabilities worldwide (1). Multiple trauma in humans (Injury Severity Score, ISS $\geq$ 16) are characterized by a massive release of different inflammatory biomarkers, such as cytokines, and damage associated molecular patterns (DAMPs). This damage affects different organs of the body and can trigger whole-body inflammation after trauma (2, 3). A substantial release of these trauma-dependent molecules is associated with the development of the so-called systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS), which are both associated with an increased mortality (4, 5). Many of the released inflammatory cytokines and DAMPs were recently shown to be cardio-depressive by acting on cardiomyocytes (CMs), altering their calcium handling, redox balance, signaling transduction, and finally resulting in post-traumatic cardiac dysfunction (6, 7). One inflammatory cytokine is the heparin-binding growth- and differentiation factor Midkine (Mdk). Increased Mdk expression is associated with different traumatic conditions such as bone fracture, burn injury, traumatic spinal cord injury, and sepsis (8–11). Increased Mdk in human blood can persist for overall 42 days after fracture (11). Furthermore, Mdk impairs fracture healing by reducing bone formation and increasing neutrophil infiltration during the fracture healing process (12, 13). However, the trauma-dependent elevation of Mdk in multiply injured patients as well as the exact impact of Mdk on the heart after trauma remains unclear. In patients with chronic heart failure, circulating Mdk increases significantly and is regarded as a novel marker, predicting different cardiac events (14, 15). Moreover, Mdk plays a role in ischemic heart injury, myocardial infarction and cardiac hypertrophy (16–18). Nevertheless, the function of Mdk in these different pathologies is still controversial, because in some cases such as ischemic heart injury, chronic heart failure and myocardial infarct, Mdk has positive effects by improving cell survival and cardiac function, inducing angiogenesis and reducing detrimental remodeling (17, 19, 20). In contrast, Mdk reduces cellular survival and induces pathological remodeling as well as fibrosis in patients with cardiac hypertrophy (18). Consequently, the exact effect of Mdk on the heart is ambiguous since Mdk can have beneficial and detrimental effects in cardiac pathology. The function of Mdk as an inflammatory cytokine on the heart during trauma especially requires clarification. After all, Mdk might be a potential therapeutic option in cardiac diseases as well as in the treatment and prevention of post-traumatic cardiac injury (21, 22). Mdk has been shown to play an important role in active myocarditis in patients and in experimental autoimmune myocarditis in mice (23). In these instances, Mdk promotes the recruitment of polymorphonuclear neutrophils (PMNs) and the production of neutrophil extracellular traps (NETs) in cardiac tissues, resulting in impaired systolic function (23). Increased activation and recruitment of neutrophils in cardiac tissue were also observed in humans after trauma and in experimental blunt chest trauma models in rats. In addition, it is linked to increased systemic levels of extracellular histones by NETosis, leading to cardiac dysfunction (24, 25).

In this study, we investigate the Mdk elevation in blood circulation after multiple trauma in pigs and humans. We further aim to thoroughly examine the effects of Mdk on human CMs. With regards to therapeutic options for posttraumatic cardiac dysfunction, the study aims to investigate the usage of CytoSorb® 300 hemadsorption. In clinical settings, CytoSorb® 300 hemadsorption improved the outcome of patients with endotoxemia, necrotizing fasciitis, septic shock, and cardiac surgery (26–29). Furthermore, CytoSorb® hemadsorption resulted in immediate hemodynamic stabilization and increased survival rates in patients with multiple organ failure (30). CytoSorb® 300 consists of highly porous (styrene-co-divinylbenzene) hemadsorbent polymer beads, which can remove substances within 10–60 kDa of molecular weight, such as complement factor 5a, cytokines DAMPs and pathogen associated molecular patterns (PAMPs), from circulating blood (26, 31). Similarly, the high-mobility group box 1 protein (HMGB1) can be removed from blood in a time dependent manner (31). Lastly, the study examines the capacity of CytoSorb® 300 to filtrate Mdk, which may be used as a therapeutic approach for preventing and handling post-traumatic cardiac dysfunction.

MATERIALS AND METHODS

Human Blood Samples

Human plasma from 11 multiply injured patients with a history of acute blunt or penetrating trauma and an ISS $\geq$ 16 was collected after hospital admission in the University Hospital of the Goethe-University Frankfurt with institutional ethics committee approval (312/10), in accordance with the Declaration of Helsinki and following the Strengthening the Reporting of Observational studies in Epidemiology (STROBE)-guidelines (32). All enrolled patients either signed the written informed consent form or written informed consent was obtained from the nominated legally authorized representative of the participants in accordance with ethical standards. Exclusion criteria were the patients being younger than 18 or older than 80 years, presenting severe burn injury, acute myocardial stroke, cancer or chemotherapy, immunosuppressive drug therapy, HIV, infectious Hepatitis, acute CMV infection, and/or thromboembolic events. Control blood samples were collected from healthy volunteers ($n = 6$, 50:50 female male, no comorbidities). Randomization of the groups was not possible during the sample collection. Blood samples were withdrawn in ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt, Nürmbrecht, Germany) directly after admission. The samples were kept on ice until centrifugation at 2,100 g for 15 min. Then, the supernatant was collected and stored at $-80°$C until assay.

Animals

This study presents partial results obtained from a large animal porcine multiple trauma model, conducted by the TREAT research group.

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 138/2017, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conformed to the European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of vertebrate animals used for scientific purposes (Council of Europe no. 123, Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 2011). Twenty-five male pigs weighting 50 ± 5 kg (*Sus scrofa domestica*) were included in the study (mean height, snout-tail length: 123,6 cm). Animals were held in a controlled environment with 21 ± 3°C room temperature (50% humidity), with a light/dark cycle of 12 h. Water was available for animals *ad libitum*. General instrumentation, anesthesia and trauma induction were described previously by Horst et al. (33).

Analgesia and Anesthesia

For premedication, pigs received an intramuscular injection with ketamine (20 mg/kg body weight), azaperone (1–2 mg/kg body weight) and atropine (0.1–0.2 mg/kg body weight). Anesthesia was performed by intravenous application of propofol (2,6-diisopropylphenol) (1–2 mg/kg body weight). Anesthesia was maintained during the study period with propofol (5–10 mg/kg/h). Pain medication was ensured by sufentanyl (1 μg/kg/h) perfusion over the whole observation period.

Multiple Trauma in Pigs

Analgesia and Anesthesia of the animals was maintained during the whole procedure.

Pigs underwent either multiple trauma ($n = 20$) or sham-procedure ($n = 5$). Multiple trauma includes a combination of a penetrating thorax trauma, laparotomy, liver laceration, femur fracture, and hemorrhagic shock (ISS ≥ 27). Control animals underwent sham-procedure ($n = 5$). Femur fracture was induced by a bolt gun (Blitz-Kernen, turbocut JOBB GmbH, Germany), positioned on the mid third of the left femur. The gun was loaded with cattle-killing cartridges (9 x 17; DynamitNobel AG, Troisdorf, Germany). For introduction of blunt chest trauma, a pair of panels (steel 0.8 cm, lead 1.0 cm thickness) was placed on the right dorsal lower chest. A shock wave was induced by a bolt shot (Blitz-Kerner, turbocut JOBB GmbH, Germany), which was applied onto the panel using cattle-killing cartridges as previously described (34, 35). Midline-laparotomy was performed by exploring the right upper liver lobe. Penetrating hepatic injury was induced by cross-like incision halfway through the liver tissue. After a short period of uncontrolled bleeding (30 s), liver package was performed. Directly after the hepatic package, pressure-controlled and volume-limited hemorrhagic shock was induced by withdrawing blood until a mean arterial pressure (MAP) of 30 ± 5 mm Hg was reached. Maximal withdrawal amounts to 45% of total blood volume. The reached MAP was maintained for 60 min. At the end of the shock period, animals were resuscitated according to established trauma guidelines (ATLS®, AWMF-S3 guideline on Treatment of Patients with Severe and Multiple Injuries®) by adjusting FiO_2 and an initial substitution of the withdrawn blood volume with Ringerfundin, fluid maintenance was performed by continuous infusing additional fluids (Ringerfundin, 2 ml/kg body weight/h). Moreover, pigs were rewarmed until normothermia (38.7–39.8°C) was reached. Sham procedure ($n = 5$) included instrumentation and anesthesia but without trauma or hemorrhage. The multiple trauma group ($n = 20$) was randomized in four therapy arms: pigs received either femoral nailing without reaming ($n = 5$), standard reaming ($n = 5$), reamed irrigation and aspiration (RIA I) ($n = 5$) or reamed irrigation and aspiration with reduced diameter and improved control of irrigation and suction (RIA II) ($n = 5$). In all groups a shortened conventional tibia nail was introduced.

Follow-Up and Euthanasia

Hemodynamic parameters were continuously monitored for 6 h. Pigs were euthanized under deep general anesthesia with intravenous Na-Pentobarbital.

This animal model represents a clinically relevant porcine model of severe multiple trauma (pulmonary contusion, extremity injury, liver laceration) with post-traumatic observation period under ICU conditions (33).

Sample Collection

Serum and plasma samples were collected at baseline, 4 and 6 h after multiple trauma and kept on ice. After centrifugation (1,500 g for 12 min at 4°C), serum and EDTA-plasma were removed and stored at −80°C until analysis. Heart tissue samples were obtained 6 h after resuscitation. Tissue of the superficial and the luminal left ventricle was fixed with 4% formalin, followed by embedding in paraffin. Furthermore, tissue was quick-frozen in liquid nitrogen, followed by storage at −80°C until analysis.

Midkine ELISA

For determination of Midkine in human and porcine plasma, as well as for the CytoSorb® 300 experiments, the human Midkine ELISA (R&D Systems, McKinley, MN, USA) was used. All procedures were performed according to manufacturers' instructions. Midkine ELISA was performed by a blinded investigator. Human plasma samples were diluted 1:4 and porcine plasma samples were diluted 1:2.

ips-Cardiomyocyte Cell Culture

Human cardiomyocytes (iPS) (Cellular Dynamics, Madison, WI, USA) were cultured for 10 days in maintenance medium at 37°C and in an atmosphere of 7% CO_2, according to manufacturers' recommendations.

Binding Analysis of FITC-Labeled Midkine

Fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in DMSO. Two mg/ml Midkine (Dianova, Hamburg, Germany), dissolved in 0.1 M $NaHCO_3$ were added to 3 mg/ml fluorescein isothiocyanate (FITC) (Sigma Aldrich, St. Louis, MO, USA) solution and were incubated for 1 h at RT while continuously shaking. Unbound FITC was removed

by using SnakeSkin® dialysis tube (ThermoScientific, Waltham, MA, USA). For dialysis, 1X phosphate buffered saline was used. Human CMs were seeded at a density of 6.3×10^4 cells/cm^2 on ibidi 12-well chamber slides (ibidi, Germany). Afterwards, cells were incubated for 30 and 60 min with 100 ng/ml FITC-labeled Midkine. Cells were washed, fixed with 4% formalin and cell nuclei were counterstained using Hoechst (Sigma Aldrich, St. Louis, MO, USA). Cells were mounted with ProLong® Gold Antifade Mountant (ThermoScientific, Waltham, MA, USA). Cells were analyzed by blinded investigator by using Axio Imager M.2 microscope (Zeiss, Jena, Germany) and the Zeiss ZEN 2.3 software (Zeiss, Jena, Germany). Images were performed with 40x magnification (N.A. 0.75).

Cell Viability Assay

Cell viability was analyzed using Cell Titer-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Cells were seeded with a density of 6.3×10^4 cells/cm^2 on a 96-well plate and treated with different Midkine concentrations (0.05, 0.1, 1 μg/ml) for 3 h, or with 1 μg/ml for different incubation times (0.5, 1, or 3 h). All procedures were performed according to manufacturers' instructions. For all experiments $n = 6$.

Troponin I ELISA

Human CMs were seeded with a density of 6.3×10^4 cells/cm^2 on a 24-well plate and treated for 6 h with 100 ng/ml Midkine at 37°C and 7% CO_2. Supernatant was collected and troponin I in supernatant was determined by using Human Cardiac Troponin I ELISA (Abcam, Cambridge, UK). All procedures were performed according to manufacturers' instructions. For all experiments $n = 6$.

Caspase-3/7 Assay

Human cardiomyocytes were seeded with a density of 6.3×10^4 cells/cm^2 on a 96-well plate and treated with 100 ng/ml Midkine for 6 h at 37°C. Caspase-3/7 activity in human cardiomyocytes was examined by using Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA). All procedures were performed according to manufacturers' instructions. For all experiments $n = 6$.

Live Cell Imaging

Live cell imaging was performed using Leica Microscope SP8 and LAS X software (Leica, Wetzlar, Germany). Cells were seeded with a density of 6.3×10^4 cells/cm^2 on a 96-well plate and were pre-loaded with 5 μM calcium indicator Fluo-3AM (Life Technologies, Carlsbad, CA, USA) and were incubated for 30 min at 37°C and 7% CO_2. After incubation with Fluo-3AM, cells were analyzed immediately. For measurements, cells were placed in special live cell imaging chamber, adjusted at 37°C and 7% CO_2. Cells were incubated with 100 ng/ml Mdk for 30 min and calcium signals were recorded and evaluated by using LAS X software. Cell culture medium was used during measurements. Live cell imaging was performed with 63x magnification (N.A. 1.2, water). Calcium peaks were determined and compared to baseline values. For all experiments $n = 6$.

Calcium Measurements

For calcium measurements, human cardiomyocytes (iPS) were seeded with a density of 6.3×10^4 cells/cm^2 on ibidi 8-well chambers (ibidi, Germany). Before the measurements, cells were incubated with 100 ng/ml Midkine 60 min before the start of the experiments, as well as for the duration of the experiment. For measurement of changes in intracellular Ca^{2+} concentration, cells were loaded with 5 μM Fura-2 (ThermoScientific, Waltham, MA, USA) for 30 min (in presence of pharmacological compounds if needed). After incubation, cells were washed twice with bath solution (in mM: 140 NaCl; 5.4 KCl; $MgCl_2$; 1.8 $CaCl_2$; 5.5 Glucose; 5 Hepes; pH = 7.4). Fluorescence imaging was performed on a Cell Observer inverse microscope (Zeiss, Jena, Germany). Cells were illuminated for 90 min at a rate of 2 Hz at each excitation wavelength (340 and 380 nm). Images were acquired using MetaFluor (Molecular Devices, Ismaning, Germany). Cells were measured in bath solution using 40x magnification (N.A. 1.3) at room temperature. Fura-2 ratios were calculated with ImageJ and the data obtained were analyzed with the Matlab script PeakCaller (36). For all experiments $n = 6$.

RNA Isolation

For qPCR experiments, human CMs were seeded at a density of 6.3×10^4 cells/cm^2 on a 24-well plate and were treated with 100 ng/ml Midkine for 6 h at 37°C and 7% CO_2. Cells were lysed with RLY lysis buffer (Meridian Bioscience, Cincinnati, OH, USA), containing 10 μl/ml β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA). RNA isolation from cell lysates was performed by using ISOLATE II RNA Mini Kit (Meridian Bioscience, Cincinnati, OH, USA). Remaining DNA was digested by DNase I (Meridian Bioscience, Cincinnati, OH, USA) for 15 min at RT as recommended by the manufacturer.

Reverse Transcribed Quantitative Polymerase Chain Reaction (RT-qPCR)

The respective RNA samples were reverse transcribed in cDNA using SuperScript® IV VILO® MasterMix (Life Technologies, Carlsbad, CA, USA). For cDNA transcription, 1–5 ng/ml mRNA were used, and experiment was performed according to manufacturer's instructions. For quantitative PCR, the PowerUp® SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA) was used. All procedures were performed according to the manufacturers' instructions. For qPCR, the QuantStudio3 (Applied Biosystems, Waltham, MA, USA) system was utilized. Five-hundred to seven-hundred ng/ml cDNA were used for quantitative PCR. Quantitative mRNA expression of human *troponin I* (for: 5′-CCTCCAACTACCGCGCGCTTAT-3′, rev: 5′-CTGCAATTTTCTCGAGGCGG-3′), *sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a)* (for: 5′-CTCCTTGCCCGT GATTCTCA-3′, rev: 5′-CCAGTATTGCAGGTTCCAGGT-3′), *ryanodine receptor 1 (RyR1)* (for: 5′-GGGTTCCTGCCC GACATGAG-3′, rev: 5′-GCACAGGTAGCGGTTCACG-3′), Na^+/Ca^{2+} exchanger (NCX) (for: 5′-GCCTGGTGGAGATGAG TGAG-3′, rev: 5′-ACAGGTTGGCCAAACAGGTA-3′), *toll-like receptor 4 (TLR4)* (for: 5′-CCTGCGTGGAGGTGTGAAAT-3′, rev: 5′-CTGGATGGGGTTTCCTGTCAA-3′), *toll-like receptor 9 (TLR9)* (for: 5′-AGACCTGAGGGTGGAAGTGT-3′, rev:

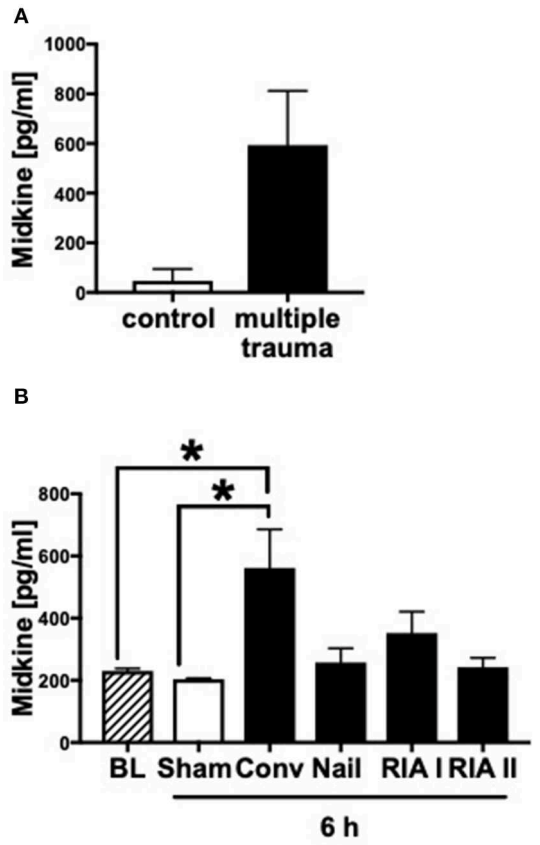

FIGURE 1 | Midkine levels in blood plasma of multiply injured humans and pigs. Midkine levels (pg/ml) in shock room blood plasma from multiply injured patients compared to healthy control group ($n = 10$) **(A)**. Midkine levels (pg/ml) in blood plasma of multiply injured pigs **(B)**. The pigs' femur fracture was either treated with femoral nailing (nailing, $n = 5$), conventional (conv, $n = 5$), or with reamer irrigator aspirator 1 or 2 (RIA I or RIA II, each $n = 5$). Control animals received sham-procedure ($n = 5$). Evaluation of blood plasma at baseline (BL) and 6 h after trauma. Results are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Results are significant $^*p < 0.05$.

5′-CTGGATAGCACCAGTAGCGG-3′) and *purigenic receptor subtype 7 (P2X7)* (for: 5′-CACACCAAGGTGAAGGGGAT-3′, rev: 5′-GGTGTAGTCTGCGGTGTCAA-3′) was examined and calculated by the cycle threshold method ΔΔCt. Respective genes were normalized to expression of the housekeeping gene *glutaraldehyde-phosphate dehydrogenase (GAPDH)* (forward: 5′-TCTCTGCTCCTCCTGTTCGAC-3′, reverse: 5′-CCAA TACGACCAAATCCGTTGA-3′) in order to exclude variations. Quantitative mRNA expression was determined by the double-threshold method (ΔΔCT). Results are presented as mean fold change. For all experiments $n = 6$.

Reactive Oxygen Species (ROS)

For analysis of cellular ROS, human CMs were seeded at a density of 6.3×10^4 cells/cm^2 on ibidi 12-well slides (ibidi, Germany). Human CMs were treated with 100 ng/ml Midkine for 6 h at 37°C and 7% CO_2. After treatment, cells were incubated for another 30 min with 5 μM CellROX® Deep Red Reagent (Life Technologies, Carlsbad, CA, USA) at 37°C and 7% CO_2. Afterwards, cells were fixed with 4% formaldehyde and cell nuclei were stained with Hoechst. Cell were mounted with ProLong® Gold Antifade Mountant. Cells were investigated by blinded investigator by fluorescence microscopy using Axio Imager M.2 microscope and the Zeiss ZEN 2.3 software. Imaging was performed by using 20x magnification (N.A. 0.5). Relative amount of reactive oxygen species was determined by Zeiss ZEN 2.3 software in order to exclude variations. For all experiments $n = 6$.

Mitochondrial Respiration With Seahorse XF Analyzer

Mitochondrial respiration was analyzed by using the Seahorse XFe96 Analyzer (Agilent Technologies, Santa Clara, CA, USA). This extracellular flux analyzer makes it possible to perform highly accurate real-time measurements of cellular metabolism in living cells by simultaneously quantifying the rates of extracellular acidification (ECAR) and oxygen consumption (OCR), and measuring the glycolysis and the mitochondrial respiration of the cells. For the analysis of mitochondrial respiration, the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Santa Clara, CA, USA) was used. The Seahorse XF Cell Mito Stress Test Kit is an optimized solution for assessing mitochondrial function. During the experiment, the ECAR and the OCR were continuously measured, gaining the parameter for the basal (baseline) respiration of the mitochondria. Afterwards, 2 μM oligomycin, 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 μM antimycin A and rotenone were pneumatically injected into the media of the cells. After automatically and gently mixing, the OCR and the ECAR were measured at multiple times after each injection. After the experiment, cells were fixed with 4% formalin at 4°C overnight. Then, cells were stained with 0.3% Janus-Green solution (Sigma Aldrich, St. Louis, MO, USA), washed and resolved with 0.5 M hydrochloric acid. Optical density was measured at 630 nm and OCR values were normalized to OD 630 nm values to exclude variations. Results were evaluated using Seahorse Wave 2.4 software (Agilent Technologies, Santa Clara, CA, USA), gaining the parameter for spare respiratory capacity of the mitochondria. For the analysis of mitochondrial respiration, cells were seeded with a density of 5×10^5 cells/cm^2 on Seahorse XFe96 analyzer cell culture plates (Agilent Technologies, Santa Clara, CA, USA) and incubated for 6 h with 100 ng/ml Midkine and the above- mentioned procedure was performed. For all experiments $n = 6$.

CytoSorb® 300 Experiments

For the therapeutic experiments, the CytoSorb® 300 was used (CytosorbensInc., MonmouthJunction, NJ, USA). Therefore, small columns were prepared. An excess of CytoSorb® 300 at the ratio 2:1 (CytoSorb® 300 to plasma samples) was added on the column as recommended by the manufacturers. Human shock room blood plasma samples were added on the columns and were

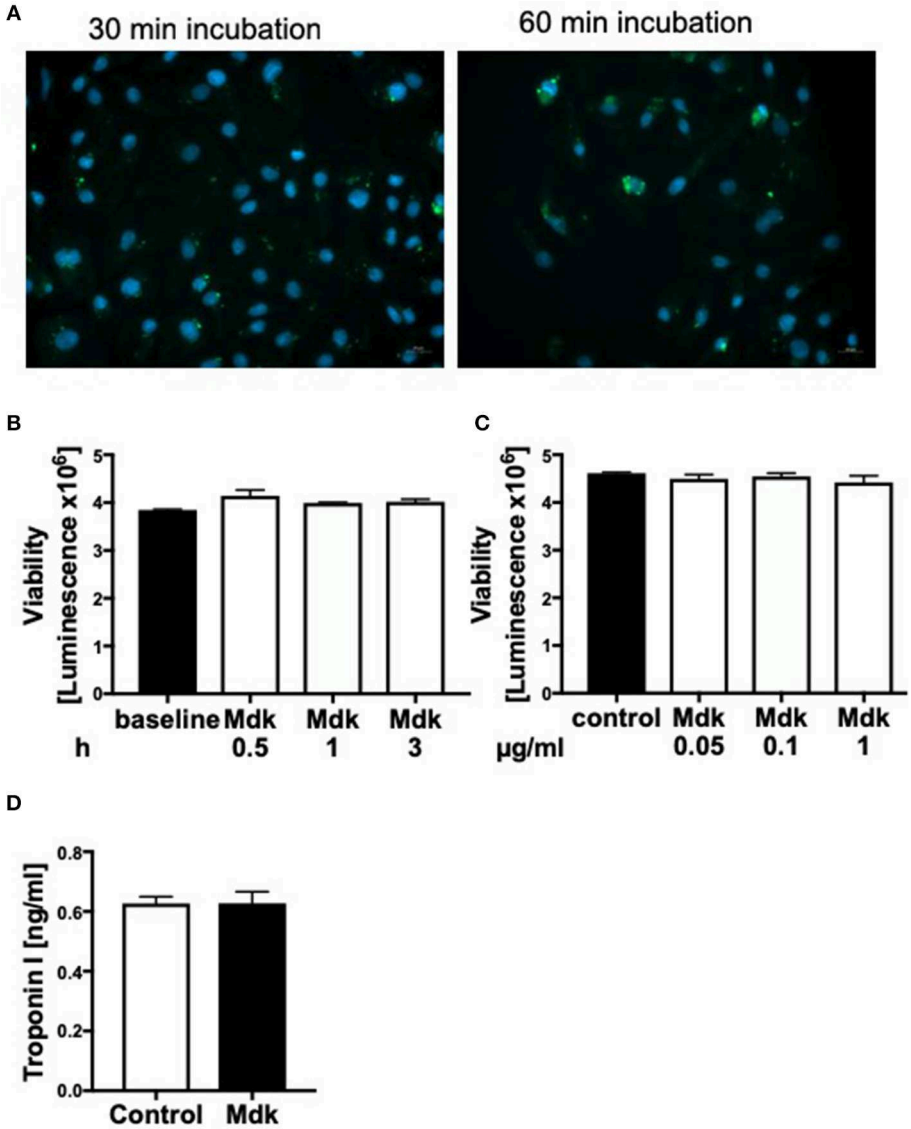

FIGURE 2 | Effects of Midkine on human cardiomyocytes. Immunofluorescence staining of human cardiomyocytes **(A)**. Human cardiomyocytes were treated for 30 and 60 min with 100 ng/ml fluorescein isothiocyanate (FITC)-labeled Midkine (green). Cell nuclei were counterstained with Hoechst (blue). Cell viability of human cardiomyocytes (Luminescence in counts/sec) treated for 0.5, 1, and 3 h with 100 ng/ml Midkine **(B)**. Cell viability of human cardiomyocytes (Luminescence in counts/sec) treated for 3 h with 0.05, 0.1, and 1 µg/ml Midkine **(C)**. Troponin I (ng/ml) in supernatant of human cardiomyocytes, treated for 6 h with 100 ng/ml Midkine **(D)**. Results are presented as mean ± SEM. For all experiments $n = 6$. Data were analyzed by two-tailed, unpaired students *t*-test.

incubated for 6 or 3 h at RT while continuously shaking. For time-doses experiments, different Midkine concentrations (10,000, 5,000, 2,500, 2,000, 1,500, 1,000, 500, 1,000 pg/ml) diluted in PBS with 1% BSA were added on the columns and were also incubated for 6 and 3 h at RT, while continuously shaking. For all experiments $n = 6$.

Statistical Analysis

All values were expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. For the statistical analysis of two groups, unpaired two-tailed students *t*-test was used. $p \leq 0.05$ was considered statistically significant. GraphPad Prism 7.0 software was used for statistical analysis (GraphPad Software, Incorporated, San Diego, CA, USA).

RESULTS

Midkine Plasma Levels in Multiply Injured Humans and Pigs

In humans as well as in pigs, the blood plasma concentrations of Midkine increased after multiple trauma compared to the healthy controls (**Figures 1A,B**). Animals submitted to

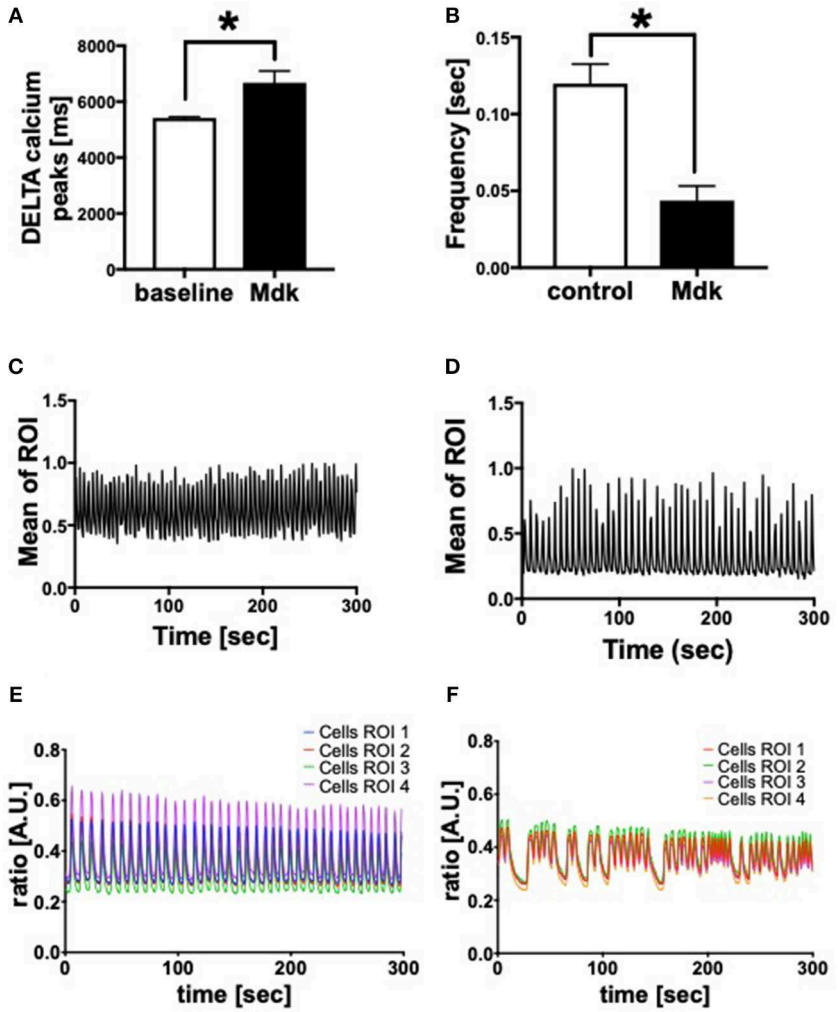

FIGURE 3 | Calcium handling of human cardiomyocytes. Delta calcium peaks (msec) of human cardiomyocytes treated for 30 min with 100 ng/ml Midkine **(A)**. Frequency of calcium signals (sec) of human cardiomyocytes treated for 60 min with 100 ng/ml Midkine **(B)**. Traces of calcium signals of human cardiomyocytes (Mean Calcium peaks of ration of interest (ROI) vs. time in sec) **(C,D)**. Traces of calcium signals of human cardiomyocytes (Ratio of Fura-2 signals in A.U. vs. time in sec) **(E,F)**. Different colors for selected ROI of calcium signals of the cells. For all experiments $n = 6$. Results are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Results are significant $^*p < 0.05$.

reamed femoral nailing showed significantly higher Mdk levels when compared with pigs treated with conventional femoral nailing or with reamer irrigator aspirator treatment (RIA I/II; **Figure 1B**). This indicates that Mdk levels correlate with the invasiveness of the reaming method. In multiply injured pigs, plasma Mdk levels increased significantly after 6 h in the group with conventional reaming of the fracture compared to the control group.

Since plasma Mdk levels increased after multiple trauma, we investigated whether Mdk affects human cardiomyocytes (CMs). After 30 and 60 min the Mdk was actively absorbed into the human CMs and was primarily located around their nucleus *in vitro* (**Figure 2A**).

Cell Viability, Cell Damage, and Calcium Handling of Human Cardiomyocytes

Given that Mdk is actively taken into the cells, we examined whether it then affects the cell viability of the human CMs. The cell viability of the human CMs was neither affected by different Mdk concentrations nor by different incubation times (**Figures 2B,C**). Furthermore, there were no differences in troponin I concentrations in supernatant of the humans CMs treated with Mdk compared to control cells after 6 h (**Figure 2D**). However, the calcium handling of the human CMs was altered after Mdk treatment, which is exemplified by the significant increase in their delta calcium peaks (**Figure 3A**), meaning the cells beat slower in presence of Mdk. Moreover, the frequency of calcium signals

in human CMs decreased significantly in presence of Mdk, developing bradycardic conditions (**Figure 3B**), which is also demonstrated the traces of the calcium signals of the cells (**Figures 3C–F**).

Gene Expression of Human Cardiomyocytes

We showed that Mdk alters the calcium handling in human CMs. Next, we investigated the gene expression of specific cardiac calcium pumps as well as the expression of different receptors, which might be involved in Mdk signaling. In human CMs, the mRNA expression of *SERCA2a*, *NCX*, *TLR4*, *TLR9,* and *P2X7* increased significantly in presence of Mdk compared to control (**Figures 4A,C–F**), indicating for direct effects of Mdk on gene expression of calcium handling proteins. Moreover, the effects of Mdk might be mediated via TLR-P2X7 signaling. The mRNA expression of *RyR1* was unaffected (**Figure 4B**).

Mitochondrial Respiration of Human Cardiomyocytes

In addition, we analyzed the effects of Mdk on the mitochondrial respiration of CMs **Figure 5A**. The basal respiration as well as the spare respiratory capacity of the human CMs decreased significantly after the Mdk treatment (**Figures 5B,C**), indicating detrimental effects of Mdk on mitochondrial respiration.

Intracellular Reactive Oxygen Species (ROS) and Caspase3/7 Activity

As Mdk alters mitochondrial respiration and ATP production of the cells, we next investigated whether Mdk also affects the redox balance of the human CMs. The amount of ROS did not change in human CMs after being treated with Mdk compared to control cells (**Figure 5D**). Although Caspase3/7 activity increased significantly in human CMs in presence of Mdk (**Figure 5E**), indicating for enhanced apoptosis in the cells.

Filtration of Midkine by CytoSorb® 300

Because Mdk is elevated in plasma of multiply injured humans and pigs and acts on human CMs, we examined the potential of a therapeutic approach: the absorption capacity of Mdk from human blood by CytoSorb® 300. After incubation of different Mdk concentrations with CytoSorb® 300, the Mdk levels decreased between 45 and 95% within 6 h (**Figure 6A**). Especially high Mdk concentrations (10,000 pg/ml) were significantly reduced up to 95% after filtration with CytoSorb® 300 after 6 h compared to the 3 h incubation (**Figure 6A**). Moreover, Mdk levels in plasma from multiply injured patients were significantly reduced after incubation with CytoSorb® 300 (**Figure 6B**).

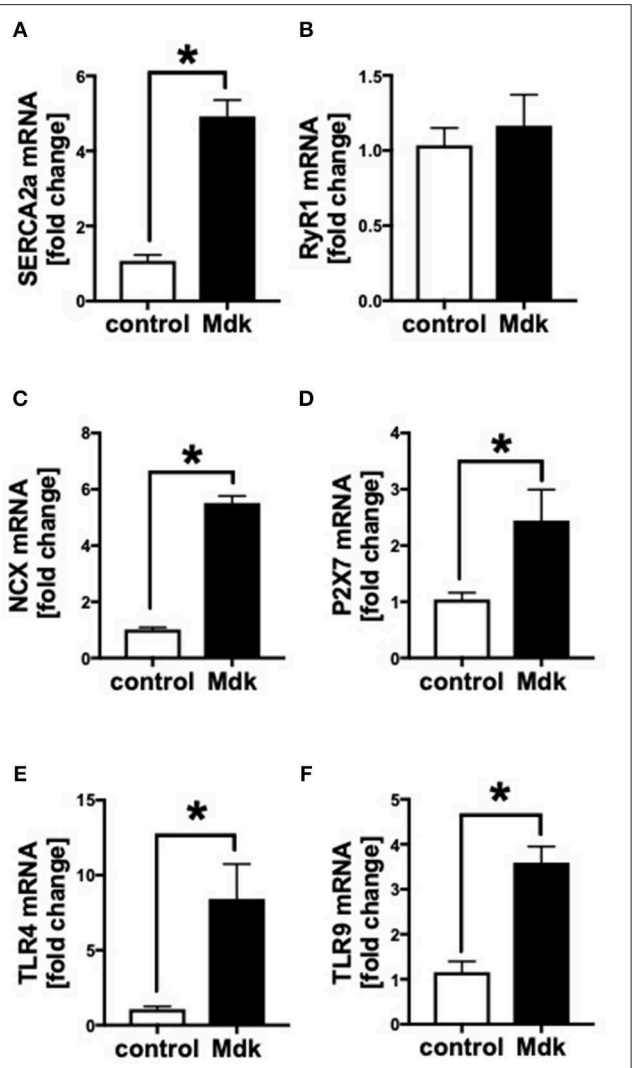

FIGURE 4 | Gene expression of human cardiomyocytes treated for 6 h with 100 ng/ml Midkine. mRNA expression (in fold change) of *sarco/endoplasmatic reticulum* Ca^{2+}*-ATPase* (*SERCA2a*) **(A)**, *ryanodine receptor-1 (RyR1)* **(B)**, *sodium-calcium exchanger (NCX)* **(C)**, *purigenic P2X receptor subtype 7 (P2X7)* **(D)**, *toll-like receptor 4 (TLR4)* **(E)**, *toll-like receptor 9 (TLR9)* **(F)**. Results are presented as mean ± SEM. Data were analyzed by two-tailed, unpaired students *t*-test. For all experiments $n = 6$. Results are significant $^*p < 0.05$.

DISCUSSION

Our study shows for the first time that Mdk is elevated in blood circulation after multiple trauma. This elevation is similar to other traumatic injuries, suggesting that circulating Mdk may act as a novel inflammatory marker for polytrauma (8, 9, 11). Furthermore, we demonstrated that Mdk acts directly on human cardiomyocytes *in vitro* and is actively taken up by these cells, altering their functionality without affecting their viability. We found that Mdk affects the functionality of the human CMs by altering their calcium handling. The delta calcium peaks of the human CMs increased significantly after Mdk treatment, meaning the cells became bradycardic. Moreover, the frequency of the calcium signals in human CMs decreased significantly after Mdk treatment, confirming the bradycardic effect of Mdk. The mRNA expression of the specific cardiac calcium pumps *SERCA2a* and *NCX* also increased significantly after Mdk treatment, suggesting direct effects of Mdk on calcium handling in the cells. The location of Mdk around the cell nucleus

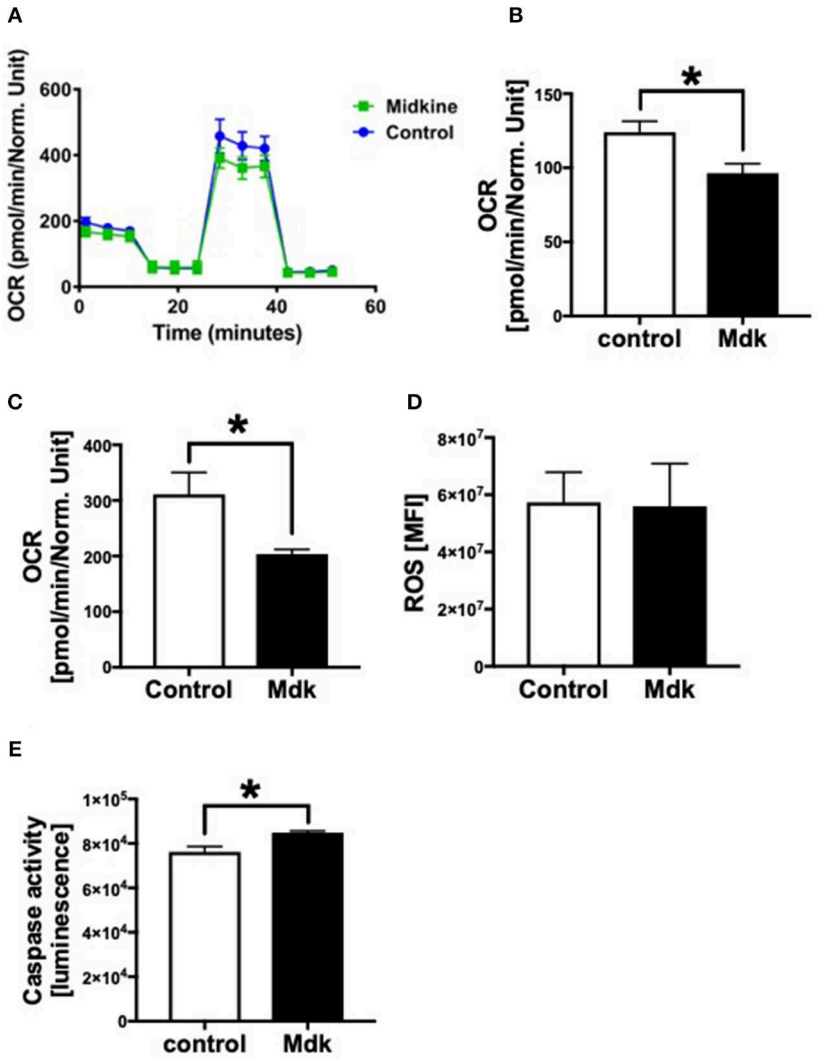

FIGURE 5 | Mitochondrial respiration, cellular reactive oxygen species (ROS) and Caspase 3/7 activity of human cardiomyocytes treated for 6 h with 100 ng/ml Midkine. Oxygen consumption rate (OCR) of human cardiomyocytes (Control, Midkine) during Seahorse MitoStress Assay (OCR in pmol/min/E630 vs. time in min) **(A)**. Basal respiration of human cardiomyocytes (OCR in pmol/min/E630) **(B)**. Spare respiratory capacity of human cardiomyocytes (OCR in pmol/min/E630) **(C)**. Amount of reactive oxygen species (mean fluorescence intensity, MFI) **(D)**. Caspase 3/7 activity (Luminescence in counts/sec) **(E)**. Results are presented as mean ± SEM. For all experiments $n = 6$. Data were analyzed by two-tailed, unpaired students *t*-test. Results are significant $^*p < 0.05$.

of the human CMs confirmed the regulatory effects on cellular gene expression of the calcium handling proteins. Alterations in calcium signals as well as in mRNA expression of *SERCA2a* and *NCX* were also described previously in presence of other trauma-related inflammatory biomarkers and DAMPs as well as in different trauma models and during sepsis, nominating Mdk as a powerful cardio-depressive mediator after trauma and during sepsis (25, 37–42). However, cardiac overexpression of SERCA2a in rodents improved cardiac contractility and relaxation, which might also indicate potential protective effects of Mdk in the heart, which would require to be investigated in future studies (43, 44). We also novelly showed that the basal respiration as well as the spare respiratory capacity of the mitochondria of the human CMs decreased significantly, indicating detrimental effects of Mdk on cellular mitochondrial respiration and energy production. Nevertheless, the amount of cytosolic reactive oxygen species (ROS) was not altered in the human CMs in presence of Mdk. Mitochondrial dysfunction was also depicted previously for other trauma-related biomarkers (7, 45, 46). The detrimental and cardio-depressive effects of Mdk on the human CMs might be mediated *via* the toll-like receptor (TLR) 4, TLR9, and the pyrogenic receptor subtype 7 (P2X7) since the mRNA expression of these receptors was significantly upregulated. All of these receptors have been demonstrated to be involved in the DAMP-associated cardiac signaling pathways in different trauma models (47, 48). The activation of the TLRs results in increased cardiac inflammation, mediated via the nuclear factor κ B (NFκB) (47). This TLR-mediated cardiac

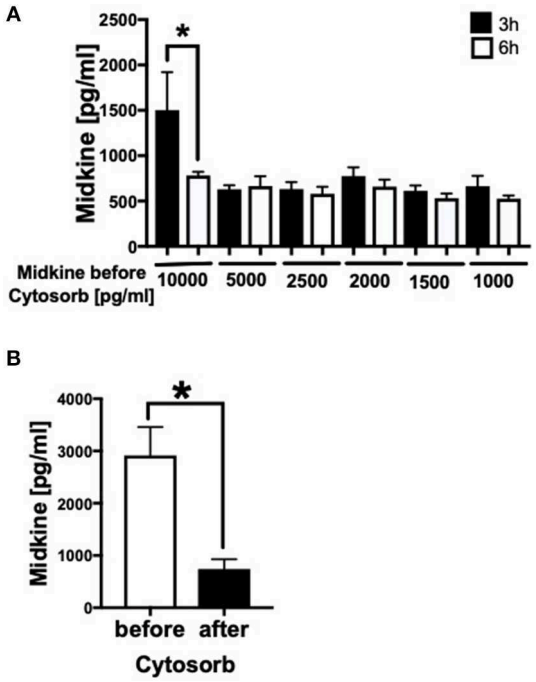

FIGURE 6 | Filtration of Midkine by CytoSorb® 300. Filtration of different Midkine concentrations (10,000, 5,000, 2,500, 2,000, 1,500, 1,000 pg/ml) ($n = 6$) with CytoSorb® 300 after 3 h (black bars) and 6 h (white bars) **(A)**. Midkine concentration before and after filtration with CytoSorb® 300 on the x-axis in pg/ml. Midkine levels (pg/ml) in human shock room blood plasma of multiply injured patients before and after filtration with CytoSorb® 300 ($n = 11$) **(B)**. Results are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. Results are significant $^*p < 0.05$.

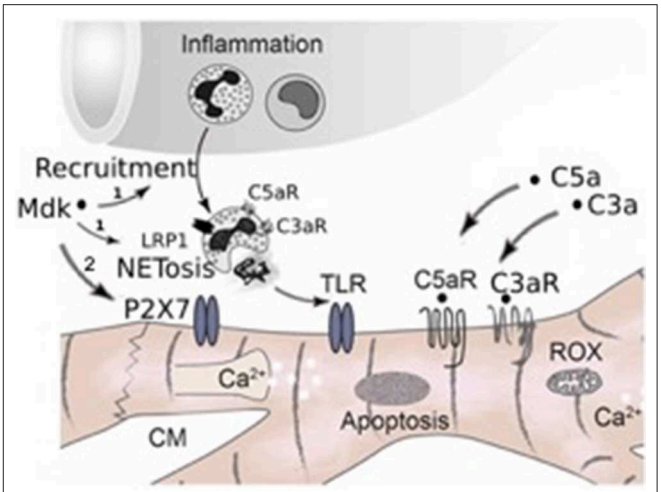

FIGURE 7 | Schematic representation of systemic inflammation after trauma and during sepsis. After trauma Midkine is released systemically. Midkine recruits polymorphonuclear neutrophils (PMNs) and induces the release of extracellular traps (NETosis) via the receptor-related protein 1 (LRP1) on the neutrophils, which was demonstrated by Weckbach et al. (23), (1). The neutrophil extracellular traps (NETs) include extracellular histones, which act via toll-like receptors (TLRs) on the surface of cardiomyocytes, inducing cardiac dysfunction and cardiac damage. Furthermore, Midkine is able to act detrimental on human cardiomyocytes by direct interactions via TLR4, TLR9, and pyrogenic receptor subtype 7 (P2X7), inducing enhanced apoptosis, disturbing calcium signaling and impairing mitochondrial respiration (2, Hypothesis of this manuscript). Additionally, after trauma and during sepsis the complement factors C5a and C3a are released systemically. Both act directly via their receptors (C5aR, C3aR) on cardiomyocytes, leading to cardiac dysfunction. Moreover, the complement factors also induce NETosis of neutrophils via their receptors.

inflammation leads to cardiac injury and finally results in cardiac contractile dysfunction (47–49). Since the mRNA expression of the TLRs was upregulated, the protein expression of these receptors might be increased after Mdk treatment. This might lead to sensitization of the CMs for other systemic circulating DAMPs, such as HMGB1 and extracellular histones, which were elevated after polytrauma, leading to cardiomyocyte dysfunction (7, 24, 50). The P2X7 receptor was also shown to be involved in cardiac contractile dysfunction (51). Interestingly, the Caspase 3/7 activity increased in human CMs after treatment with Mdk, which was demonstrated previously in cardiac tissue *in vivo* in an experimental polytrauma model in pigs (41). So far, Mdk was described as an anti-apoptotic factor by decreasing caspase activity in other cells, such as neurons and HepG2 cells, which is in accordance to unaffected cell viability in the present study (52, 53). The effects of Mdk on cellular apoptosis of human CMs has not been described, so far. In contrast to other cells, human CMs seem to follow different cellular processes and various signal cascades might be involved in Caspase-3/7 activation and activity when these cells were treated with Mdk. Moreover, this phenomenon could also be time-dependent as we solely investigated the Caspase-3/7 activity after 6 h of Mdk exposure. This observation should be the subject of future studies in order to understand the specific effects of Mdk on apoptosis of human CMs.

Therapeutic approaches treating post-traumatic cardiac dysfunction are still limited. In this study, we clearly showed that Mdk is elevated in plasma after multiple trauma and is predominantly detrimental on human CMs, causing the development of post-traumatic cardiac dysfunction. As a consequence, we investigated the efficiency of CytoSorb® 300 in filtering Mdk from human blood plasma. CytoSorb® 300 is an absorption column, composed of porous polymer beads, which is normally used in the intensive care unit (ICU) for septic patients or for patients with SIRS. The filtration potential of CytoSorb® 300 for various trauma-associated cytokines and DAMPs was already demonstrated by others (31, 54). Here, we showed for the first time that CytoSorb® 300 is able to absorb Mdk dose-dependently, filtering high Mdk concentrations (10,000 pg/ml) up to 95%. Moreover, CytoSorb® 300 filtered Mdk from human plasma obtained on admission to the emergency room, making it a very promising therapeutic approach for treatment and prevention of post-traumatic cardiac dysfunction. One huge benefit of using CytoSorb® 300 instead of single antibodies for therapy is that CytoSorb® 300 is able to filter a high amount of many miscellaneous damage- and inflammation

molecules after trauma and not only a single molecule, which is the case of antibody treatment. Furthermore, filtration of Mdk by CytoSorb® 300 might limit other negative effects of Mdk on polytrauma patients, since it was shown that Mdk acts as an inhibitor of fracture healing and that high Mdk serum levels were associated with poor outcome in septic patients. Finally, we found that systemic Mdk is higher after conventional reaming, compared to nailing without reaming and to RIA I/II. Consequently, treatment of the fracture with RIA I/II might be better for fracture outcome as well as for fracture healing after trauma (55). In addition, conventional reaming of the fracture might have other negative effects after trauma (e.g., pulmonary embolism).

One limitation of the study might be the small sample size ($n = 6$) to investigate different treatment approaches for the femur fracture. Consequently, more experiments are needed to find the best and the least invasive treatment approach. The same applies for a possible correlation between fracture treatment approaches and systemic Mdk levels. Because investigated groups were heterogenous, a bigger number of samples might be helpful to extrapolate the results to a clinical population. Another limitation might be that we only used small columns with Cytosorb® 300 polymer beads in our study, trying to mimic the clinical application in ICU. However, as our study was only an experimental approach, clinical studies should be performed, including more patients and larger application approaches of Cytosorb® 300. This may help to- confirm the therapeutic potential of Cytosorb® 300 for the prevention of post-traumatic cardiac dysfunction by filtering Mdk from human blood *in vivo*. Furthermore, it is not possible to mimic *in vitro* the real *in vivo* inflammatory conditions, which occur after trauma. The presence of many different inflammatory mediators and DAMPs and the activation of different signal cascades in the cells lead to post-traumatic cardiac dysfunction. Consequently, it is not possible to specify these detrimental effects on one single mediator like Mdk.

Taken together, in our study we observed for the first time that Mdk is elevated systemically after multiple trauma in humans and pigs, acting cardio-depressive on human CMs by impairing their calcium handling and mitochondrial respiration capacity *in vitro*. PlX27/TLR might be involved in mediating these detrimental effects of MdK (**Figure 7**). In the clinical setting, the hemadsorption filter Cytosorb® 300 might be a powerful tool to remove cardio-depressive mediators from patients' circulation and therefore help to improve cardiac function.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

Human plasma from 11 multiply injured patients with a history of acute blunt or penetrating trauma and an ISS ≥ 16 was collected after hospital admission in the University Hospital of the Goethe-University Frankfurt with institutional ethics committee approval (312/10), in accordance with the Declaration of Helsinki and following the Strengthening the Reporting of Observational studies in Epidemiology (STROBE)-guidelines (32). All enrolled patients signed the written informed consent form themselves or written informed consent was obtained from the nominated legally authorized representative on the behalf of participants in accordance with ethical standards.

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 138/2017, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conformed to the European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of vertebrate animals used for scientific purposes (Council of Europe no. 123, Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 2011).

AUTHOR CONTRIBUTIONS

IL, BW, MB, TE, GF, SH, ML, and NC performed the experiments including animal studies, cell culture experiments, microscopic studies, and ELISAs. IL primarily wrote the paper. MH-L, FG, BR, IM, RP, H-CP, and MK contributed to experimental design and data analysis and coordinated the study and supervised financial support for the studies. All authors made substantial contributions to conception and design of the study, participated in drafting the article, and gave final approval of the version to be published.

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TREAT RESEARCH GROUP

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Cardiac glucose and fatty acid transport after experimental mono- and polytrauma

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Running Title: Trauma induces alterations in cardiac metabolism

Abstract

Objective: The aim of this study was to define the influence of trauma on cardiac glucose and fatty acid transport. The effects were investigated *in vivo* in a porcine mono- and polytrauma model and *in vitro* in human cardiomyocytes, which were treated simultaneously with different inflammatory substances, mimicking post-traumatic inflammatory conditions.

Methods and Results: In the porcine fracture- and polytrauma model, blood glucose concentrations were measured by blood gas analysis during an observation period of 72 h. The expression of cardiac glucose and fatty acid transporters in the left ventricle was determined by RT-qPCR and immunofluorescence. Cardiac and hepatic glycogen storage was examined. Furthermore, human cardiomyocytes were exposed to a defined trauma-cocktail and the expression levels of glucose- and fatty acid transporters were determined. Early after polytrauma, hyperglycaemia was observed. After 48 h and 72 h, pigs with fracture- and polytrauma developed hypoglycaemia. The propofol demand significantly increased post trauma. The hepatic glycogen concentration was reduced 72 h after trauma. Cardiac glucose and fatty acid transporters changed in both trauma models *in vivo* as well as *in vitro* in human cardiomyocytes in presence of proinflammatory mediators.

Conclusions: Monotrauma as well as polytrauma changed the cardiac energy transport by altering the expression of glucose and fatty acid transporters. *In vitro* data suggest that human cardiomyocytes shift to a state alike myocardial hibernation preferring glucose as primary energy source in order to maintain cardiac function.

Keywords: polytrauma, cardiac fatty acid metabolism, cardiac glucose metabolism, myocardial hibernation

Introduction

After polytrauma, patients often exhibit impaired cardiac function and cardiomyopathy, which is associated with an increased mortality rate [22]. In critically ill patients nutrition is challenging because the stress-induced catabolism after injury predisposes to malnutrition. In this context catabolic hormones such as glucagon, catecholamines and cortisol are elevated, which is associated with mobilization of endogenous energy substrates (glucose, amino acids and free-fatty acids). Of note, the delivery of these substrates to the vital organs brain and heart is prioritized [20]. The posttraumatic release of proinflammatory cytokines exaggerates catabolism [44] [17], and this stress response triggers hyperglycaemia, which is associated with increased mortality [33]. During intensive care patients are further challenged by prolonged fasting periods or feeding interruption [34]. In surgical patients malnutrition is associated with delayed healing, higher rates of complications, longer hospital stay and poor outcomes [1]. Combining different single traumata changes the levels of metabolites including glycolysis, tri-carboxylic acid cycle, pentose phosphate and fatty acid pathways [46]. Furthermore, trauma-induced hepatic stress has been associated with stress metabolism, including insulin resistance, and resulting in increased hepatic glucose production but decreased glucose uptake of the skeletal muscle [2]. In regard to the heart, we have previously demonstrated that polytrauma exhibit increased cardiac glycogen concentrations, but decreased glucose transporter 4 (GLUT4) expression in pigs [23]. For proper cardiac function a very tight coupling between ATP production and myocardial contraction is essential, supposing a constant provision of metabolites necessary for ATP production [29]. The heart is capable of utilizing all classes of energy substrates. Thereby, the use of long-chain free fatty acids (LCFA) and carbohydrates is favored [19]. In well oxygenated hearts the primary energy source for cardiomyocytes is the utilization of LCFA [38]. Another important energy source is glycogen, supporting cardiac metabolism

during acute cardiac workload. However, the glycogen turnover rate is usually very low under normal conditions [13]. Nevertheless, due to its capability to change between the different metabolisms, the human heart is able to providing a constant ATP production, and thus a proper function [12, 54]. During such cardiac metabolic reprogramming the oxidation of LCFA is mostly reduced, whereas the glucose consumption increased [4]. This shift from fatty acid oxidation to glucose utilization is especially seen in patients with chronic heart failure as well as with ischemic cardiomyopathy [48]. Given that the intramuscular storage of LCFA is limited, the heart tissue is strongly dependent on systemic supply of LCFA from adipose tissue [30]. LCFA are provided either unbound as free fatty acids or linked to albumin [36]. Its uptake by heart tissue is a complex process, involving multiple steps and many barriers [5]. The rate-limiting factor is the cellular uptake and the passage across the plasma membrane [30] since the LCFA enters cardiomyocytes either inactively by diffusion or actively *via* different transport proteins [9, 56]. Of those, we focused on cardiac fatty acid transporters, including the solute carrier 27 gene family (SLC27), the heart fatty acid binding protein (HFABP) and the fatty acid translocase (FAT/CD36). The CD36 facilitates the uptake by catalyzing the accumulation and integration of protonated LCFA into the outer phospholipid bilayer [49]. HFABP promotes the uptake of LCFA into the cytoplasm of cardiomyocytes and is responsible for the delivery of LCFA from the sarcolemma through the cytoplasm to the outer membrane of mitochondria [16, 30]. Both, CD36 and HFABP accelerate LCFA-dissociation from albumin [11]. SLC27 solute carrier proteins are integral transmembrane proteins, promoting the transport of LCFA actively across the plasma membrane. The SLC27A1 and SLC27A6 are expressed ubiquitous, whereas the SLC27A6 is almost exclusively expressed in cardiac tissue [3, 47]. In cardiomyocytes the glucose transporters GLUT1 and GLUT4 are mainly involved in glucose transport [31].

Trauma is associated with whole-body inflammation, upregulating cytokines, complement factors and damage-associated molecular patterns (DAMPs) like extracellular histones [18, 21, 39, 45]. Although it has been shown that cytokines and endogenous danger molecules may have negative effects on the heart, leading to cardiac suppression and cardiac dysfunction [25, 43], the exact influence and underlying mechanisms are still obscure [15]. Furthermore, little is known about the impact of trauma on cardiac glucose and fatty acid transport. Trauma is able to induce an imbalance between glucose and LCFA utilization in cardiomyocytes, leading finally to impaired cardiac function. Therefore, investigations regarding cardiac glucose and fatty acid transport during trauma are of huge importance [8]. The aim of this report was to define the effects of single- and polytrauma on the cardiac glucose and fatty acid transport *in vivo* as well as *in vitro*.

Materials and Methods

In vivo experiments

Animals

The animal experiments were conducted in the framework of the TREAT consortium. This study presents results from a large animal porcine polytrauma model and all pigs from this animal experiments were enclosed in the present investigations. The model has been previously described in detail by Horst et al. [17]. Animal experiments adhere to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for reporting animal research [28].

All procedures conformed to the Society of Laboratory Animal Science (GV-SOLAS) as well as the National Animal Welfare Law and were approved by the responsible governmental authorities ("Landesamt für Natur, Umwelt und Verbraucherschutz":

LANUV-NRW, Germany: AZ TV-No.: 84-02.04.2014.A265). This study was performed according to the guidelines of the Federation of European Laboratory Animal Science Association (FELASA). For the investigations, n=30 12-16 weeks old male pigs with a mean of 30 ± 5 kg body weight (*Sus scrofa dormestica*, Tierzucht GmbH Heinrich, Heinsberg, Germany) were included in the study.

Anaesthesia

For premedication, pigs received an intramuscular injection with azaperone (3-4 mg/kg body weight. Anaesthesia was performed by intravenous application of propofol (2,6-diisopropylphenol) and sufentanil. Anaesthesia was maintained during the study period with propofol (1-2 mg/kg body weight). Pain medication was ensured by sufentanil (0.05 mg/ml) perfusion over the whole observation period. Vital parameters were monitored on a regular basis by electrocardiographic (ECG), pulse oximetry and blood gas analysis. Data on vital signs (heart frequency, mean arterial pressure, pO_2 and FiO_2) were checked according to the time points of whole blood sampling (1.5, 3.5, 5.5, 24, 48 and 72 h after trauma). Maintaining of body temperature was performed by using a forced-air warming system. Pigs were bedded every 6 h in order to prevent positional damage and organ compromise. Lung function was maintained by lung-protective ventilation and changing position. Liver function was monitored by aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) measurements. Kidney function was controlled by sampling of urine and measuring systemic creatinine values.

Fluids were administered by continuous crystalloid infusion (Sterofundin ISO®; 2 ml/kg body weight /h). The pigs were then orotracheally intubated and ventilation was

conducted in biphasic positive airway pressure (BiPAP) mode [17]. Detailed information about animal care after trauma were described by Horst et al. [17].

Nutrition

Pigs received 16.6 ml/kg/24 h Amminopäd® 10% (Baxter, Unterschleißheim, Germany), except on the day of trauma.

Monotrauma Pig (Fracture)

Pigs underwent either monotrauma with femur fracture (n=12) or sham procedure (n=6). The sham procedure included solely anaesthesia without trauma. The monotrauma fracture group (n=12) was randomized into two therapeutic groups (n=6). One group received an external fixation of the femur fracture corresponding to damage control orthopaedics (DCO), the other group were treated with femoral nailing in accordance to early total care principles (ETC).

Polytrauma Pig

The applied polytrauma porcine model was previously published [17]. In brief, pigs with polytrauma (n=12) underwent a combination of blunt chest trauma, penetrating liver injury and femur fracture, followed by pressure-controlled hemorrhage (mean arterial pressure of 40 $\pm$ 5 mm Hg, maximal withdrawal of 45% of calculated total blood volume) for 90 min. The polytrauma group (n=12) was randomized in two therapeutic arms (n=6); external fixation of the femur fracture corresponding to damage control orthopaedics (DCO) or femoral nailing reflecting early total care (ETC).

Sample collection

Full blood samples were collected prior to trauma, directly as well as 1.5, 3.5, 5.5, 24, 48 and 72 h after trauma. After centrifugation (2000 g, 15 min, 4°C), serum was removed and stored at -80°C until analysis. Heart tissue from left ventricle as well as liver tissue were collected 72 h after trauma and were either fixed with 4% formalin followed by embedding in paraffin or quick-frozen in liquid nitrogen followed by storage at -80°C.

In vitro experiments

Human stem cell-derived cardiomyocytes (iPS) from Cellular Dynamics (Madison, WI, USA) were cultured in iCell Maintenance Medium for 10 days at 37°C in an atmosphere of 7% CO_2. Cells were then treated with polytrauma cocktail (PTC, Table 1), supplemented with 20 µg/ml whole histone fraction from calf thymus (Sigma Aldrich, St. Louis, MO, USA) for 6 h at 37°C in an atmosphere of 7% CO_2. The control group was cultured in iCell Maintenance Medium. The concentrations of the respective cytokines included in the PTC were adjusted to concentrations measured in the blood of polytrauma patients. Concentrations of extracellular histones were adjusted to serum levels in the pigs (13).

RNA Isolation

RNA from left ventricles was isolated using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). Remaining DNA was digested using DNaseI Amplification Grade Kit (Invitrogen, Carlsbad, CA, USA). For RNA isolation from cell lysates, ISOLATE II RNA Mini Kit (Meridian Bioscience, Cincinnati, OH, USA) was used and remaining DNA was digested by DNase I (Meridian Bioscience, Cincinnati, OH, USA).

Quantitative reverse transcribed polymerase chain reaction (RT-qPCR)

The respective RNA samples were reverse transcribed in cDNA using SuperScript™ IV VILO™ MasterMix (Invitrogen, Carlsbad, CA, USA). For quantitative PCR the PowerUp™ SYBR™ Green Master Mix was used (Applied Biosystems, Waltham, MA, USA). All procedures were performed according to the manufactures instructions. For qPCR the QuantStudio3 system (Applied Biosystems, Waltham, MA, USA) was utilized. Following genes were examined from pig and from human, respectively: GLUT1, GLUT4, SLC27A1, SLC27A6, FABP3 and CD36 (Table 3, 4). mRNA expression of the respective genes was normalized to GAPDH expression. Quantitative mRNA expression was calculated by the cycle threshold method $\Delta\Delta$Ct. Results are presented as mean fold change.

Immunofluorescence staining (IF) and PAS staining

Paraffin sections of the respective left ventricles were deparaffinized and rehydrated. Antigen unmasking was performed in 10 mmol/L citrate buffer (pH 6) at 100°C and unspecific binding sites were blocked with 5% goat serum. Specific antigen binding was performed by incubation with the respective first antibody (Table 2). Specific antibody binding was detected with AlexaFluor®488-labeled second antibody (Jackson Immunoresearch, West Grove, PA, USA). Nuclei were counterstained with Hoechst and sections were mounted. Expression of the respective receptors was analyzed by fluorescence microscopy using an Axio ImagerM.1 microscope and the Zeiss AxioVision software 4.9 (Zeiss, Jena, Germany) with 40x magnification (N.A. 0.75). Fluorescence intensities were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and results are presented as mean pixel density of each group.

PAS staining was performed using PAS-staining-Kit (Merckmillipore, Darmstadt, Germany). Signal density was measured using an Axio ImagerM.1 microscope and the

Zeiss AxioVision software 4.9 (Zeiss, Jena, Germany) with 40x magnification (N.A. 0.75). Results are presented as mean density of each group (arbitrary units).

Statistical Analysis

A GraphPad Prism 7.0 software was used for statistical analysis (GraphPad Software, Incorporated, San Diego, Ca, USA). All values are expressed as means ± SEM. Data were analyzed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test. $p<0.05$ was considered statistically significant.

Results

Blood glucose and propofol concentrations during polytrauma, fracture and sham procedure

In order to determine the blood glucose concentrations during trauma, blood gas parameters were measured before, directly as well as 1.5, 3.5, 5.5, 24, 48 and 72 h after trauma. Pigs subjected to polytrauma exhibited hyperglycaemia immediately after trauma in both treatment groups (fig. 1 A). In polytrauma- and in fracture groups, blood glucose levels significantly decreased between 48 h and 72 h in the respective treatment groups compared to their baseline levels, developing hypoglycaemia (fig. 1 A). Blood glucose levels from pigs subjected to fracture increased during the first 5 h, but decreased 24 h thereafter (fig. 1 A). Sham treated animals likewise developed hypoglycaemia after 48 h. Propofol demand increased significantly in all groups after 48 h and 72 h compared to baseline levels (fig. 1 B).

Cardiac and hepatic glycogen amount after polytrauma, fracture and sham procedure

Tissue from heart as well as from liver was collected 72 h after polytrauma and after fracture, followed by DCO or ETC treatment or after sham-procedure, respectively. Additionally, liver tissue from non-treated healthy pigs of a historical group was collected and used as control. The hepatic glycogen amounts of sham-treated, fracture and polytrauma pigs decreased compared to healthy control pigs (fig. 2 A, B). Further, the hepatic glycogen amount of fracture pigs treated with ETC and of polytrauma pigs, treated with DCO decreased significantly after 72 h compared to the control group (fig. 2 A, B). Cardiac glycogen storage decreased significantly after 72 h in the fracture group with ETC treatment, compared to the control group (fig. 2 C, D).

Cardiac glucose transport in pigs after fracture

Tissue of left ventricles was collected 72 h after fracture, ensued by DCO or ETC treatment or after sham procedure, respectively. GLUT1 mRNA expression significantly decreased in both, DCO and ETC treated pigs, whereas the GLUT1 protein levels did not differ considerably between the respective treated groups (fig. 3 A, B). GLUT4 mRNA expression significantly increased in pigs treated with ETC (fig. 3 C).

Cardiac fatty acid transport in pigs after fracture

The SLC27A1 mRNA expression did not differ between the respective treated groups, whereas the SLC27A6 mRNA expression decreased significantly in pigs treated with DCO (fig. 4 A, B). The HFABP mRNA expression decreased significantly in both treated groups, whereas on protein level no differences were visible between the respective groups (fig. 4 D, E). The CD36 mRNA expression and the CD36 protein expression decreased significantly in animals treated with DCO (fig. 4 F, G, Supplement fig.1 A).

Cardiac glucose transport in pigs after polytrauma

Tissue homogenates and sections of left ventricles were collected 72 h after polytrauma, followed by DCO or ETC treatment or after sham procedure, respectively. GLUT1 mRNA levels decreased significantly in pigs obtained ETC treatment compared to pigs received sham procedure or DCO treatment (fig. 5 A). On protein level, the extracellular GLUT1 expression did not differ significantly between the groups (fig. 5 B, Supplement fig. 1 B), whereas the intracellular GLUT1 protein expression increased significantly in ventricles from pigs subjected to DCO treatment (fig. 5 C, Supplement fig. 1 C).

Cardiac fatty acid transport in pigs after polytrauma

SLC27A1 mRNA expression increased significantly in pigs with DCO treatment compared to pigs with sham procedure. SLC27A1 mRNA expression decreased significantly in ETC treated pigs compared to pigs with DCO treatment (fig. 6 A). SLC27A6 mRNA expression increased significantly in pigs that received DCO or ETC treatment, whereas the extracellular SLC27A6 protein expression decreased in both treated groups (fig. 6 B, C, Supplement fig. 1 D). HFABP mRNA expression and protein expression increased significantly in pigs obtained DCO treatment (fig. 6 D, E). CD36 mRNA expression decreased significantly in pigs with ETC treatment, whereas the protein expression level of CD36 did not differ significantly between the respective groups (fig. 6 F, G, Supplement fig. 1 E).

Cardiac glucose and fatty acid transport in human cardiomyocytes in presence of polytraumacocktail and extracellular histones

Human cardiomyocytes were incubated for 6 h with PTC and additional extracellular histones. The control group was incubated in cell culture medium. GLUT1 mRNA expression increased significantly in cardiomyocytes treated with PTC and extracellular histones, whereas the GLUT4 mRNA expression did not differ significantly between the treated and the control group (fig. 7 A, B). SLC27A1 and SLC27A6 mRNA expression did not differ considerably between the respective groups (fig. 7 C, D). HFABP and CD36 mRNA expression decreased significantly in cardiomyocytes treated with PTC and extracellular histones (fig. 7 E, F).

Discussion

In this report the effects of severe polytrauma and fracture on cardiac glucose- and fatty acid utilization were described *in vivo* and *in vitro* in presence of an inflammation cocktail. Polytrauma in pigs induced a hypermetabolic stress response, which is reflected by early increased blood glucose concentrations. This trauma-related metabolic stress response is caused by increased concentrations of circulating catecholamines and glucocorticoids, which is also a well-known problem in traumatized patients, and which is further associated with increased mortality rates as well [10, 26]. Of note, adequate pre-medication by analgo-sedation with sufentanil did not reduce high baseline glucose levels in all groups. The depleted glycogen storage in liver by the respective trauma models might also be due to increased glycolysis after trauma. Furthermore, enhanced metabolic rate (20-25%) was observed after trauma and was associated with the severity of trauma [42]. Peripheral insulin resistance during hyperglycaemia probably leads additionally to enhanced hepatic glycolysis and further decreased glucose uptake of the skeletal and cardiac muscle tissue [33]. This hyperglycaemic state is often critical for multiply injured patients, rising their risk for

mortality [26, 33]. Interestingly, cardiac glycogen concentration, which has been associated with protection against trauma-induced cardiac damage [40], decreased in animals after monotrauma, but not after polytrauma as demonstrated before [23]. After initial hyperglycaemia in pigs post fracture and ETC treatment and in pigs post polytrauma, mean systemic glucose concentration dropped below 100 mg/dl. GLUT1 mRNA expression decreased in both trauma models after 72 h, indicating extensive glucose utilization during trauma. GLUT4 expression was not significantly altered between the respective trauma groups [23]. But this was probably due to the trauma-dependent insulin resistance, since insulin predominantly stimulates the GLUT4 expression of the cardiomyocytes [31]. One limitation factor for the present analysis of glucose consumption after trauma might be the small animal numbers, wherefore these results should be interpreted carefully. Therefore, more experiments are necessary to generalize the glucose consumption after trauma in pigs. However, the present data are consistent with the hypothesis of an increased glucose consumption after experimental polytrauma (13).

An increased demand of propofol in all groups during the observation period was observed. Though the propofol concentration was adapted to the depth of anaesthesia of the pigs in the present study. This may result in a metabolic switch from oxidative phosphorylation to glycolysis by targeting mitochondrial complexes I, II and III. Further, increased propofol concentration lead to disrupted β-oxidation, reduced mitochondrial entry of LCFA and consequently to accumulation of free fatty acids in cells [27]. Excess propofol concentrations are known to lead to enhanced levels of acylcarnitines and inhibit transport protein carnitine palmitoyl transferase I (CPT1) and uncouple the mitochondrial respiratory chain in humans [53]. The disturbance of mitochondrial electron transport also causes generation of free oxygen reactive species (ROS), resulting in cardiomyocyte apoptosis [32]. This phenomenon was also observed in humans, called

propofol infusion syndrome (PRIS). PRIS is associated with impaired mitochondrial function and is observed in humans with propofol demands larger than 4 mg/kg per body weight and at exposure times longer than 48 h [50, 53]. In rats, propofol concentration in tissue during intravenous application (20 mg/kg body weight) reached 200 µM and was also correlated with impaired electron flow through the respiratory chain, with coenzyme Q as the main interaction site of propofol [51]. Furthermore, propofol induces contractile dysfunction in the rat myocardium [14]. The upregulation of the GLUT1 in cardiomyocytes during trauma might be protective, preventing propofol-induced apoptosis of cardiomyocytes [37]. GLUT4 expression was probably reduced by the presence of excessive lipids and free fatty acids due to increased propofol infusion [52]. Moreover, excess exposure to saturated long chain fatty acids leads to a state of so-called cardiac lipotoxicity, including impaired cardiac glucose metabolism, impaired insulin and catecholamine signaling, cellular inflammation, excess reactive oxygen species (ROS) production, inhibition of autophagy and activation of apoptosis [6].

Since glucose consumption increased in the present trauma models in pigs with fracture or with polytrauma, cardiac utilization of LCFA was mostly reduced. The CD36 expression was completely downregulated in both trauma models. This was probably caused by a trauma-dependent insulin resistance, since the expression of the CD36 is strongly dependent on insulin stimulation [7]. Decreased CD36 expression could also be due to the profopol-induced shift from β-oxidation to glycolysis [7]. The HFABP expression decreased in pigs 72 h after fracture but in contrast increased in pigs 72 h after polytrauma, receiving DCO treatment. The increased HFABP expression in polytrauma animals indicates some post-production of the protein most likely compensatory to loss of HFABP early after polytrauma [23]. SLC27A1 and SLC27A6 expression also increased in pigs with polytrauma but not in pigs with fracture. The enhanced expression of the SLC27A1, SLC27A6 as well as of HFABP in pigs with

polytrauma might be due to the increased demand for LCFA due to the fast glucose release and consumption directly after trauma. Since the upregulation of SCL27A1, SLC27A6 and HFABP was only observed in pigs with polytrauma, we suggest that the effects were not due to increased circulating propofol concentration but correlate with the severity of trauma.

Summarized, the posttraumatic environment *in vivo*, influenced by inflammation, DAMPs, intensive care nutrition and anaesthesia changed cardiac substrate utilization by adjusting different transporters, which are involved in cardiac fatty acid- and glucose transport. In mono- and polytrauma, cardiomyocytes modified their substrate utilization from LCFA to glucose, indicating for high energy requirement of the heart during trauma. However, after polytrauma, expression of cardiac LCFA transporters increased, indicating for trauma-dependent but propofol-independent cardiac LCFA utilization, due to increased energy requirements and inflammation, correlating with the severity of trauma. Moreover, the observed alterations in the energy metabolism of the multiple injured pigs might cause their disturbed and impaired cardiac function after trauma [23, 24, 41, 55]. In order to get a closer understanding about the molecular mechanisms, leading to a cardiac metabolic switch after trauma *in vivo*, additional observation points before 72 h are required.

In vitro, changes of glucose and LCFA transporters in human cardiomyocytes in presence of an inflammation cocktail confirmed the *in vivo* observed changes. As *in vivo*, the GLUT1 expression increased, whereas the GLUT4 expression was not influenced by trauma, assuming increased glucose transport via the GLUT1 [37].

It is tempting to speculate that the increased expression of the GLUT1 was due to myocardial hibernation, which includes the downregulation of oxygen consumption and the shift to anaerobic glycolysis for cardiac ATP production [35]. Due to this adaptive response, cardiomyocytes are able to maintain their viability in state of energy

deficiency. The responsible enzyme for the induction of the hibernation is the cytochrome oxidase of the electron transport chain in mitochondria [35]. In human cardiomyocytes, the expression of all transporters involved in cardiac fatty acid transport was reduced, confirming the shift to preferred glucose utilization of cardiomyocytes during treatment with DAMPs as well as with inflammatory mediators [35, 37].

In conclusion, the data indicate that human cardiomyocytes skip to the condition of myocardial hibernation, when treated with PTC and extracellular histones, utilizing glucose as primary and preferred energy source in order to maintain cardiac function and contractility of cardiomyocytes.

Authors' contributions: Regarding contributions of the authors I.L., B.W. and D.K performed the experiments including animal studies, cell culture experiments, microscopic studies and ELISAs. I.L. primarily wrote the paper K.H., B.R., F.G., H.C.P., M.H.L, F.H. and M.K. contributed to experimental design and data analysis and coordinated the study and supervised financial support for the studies. All authors made substantial contributions to conception and design of the study and participated in drafting the article. All authors gave final approval of the version to be published.

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Figure Legends

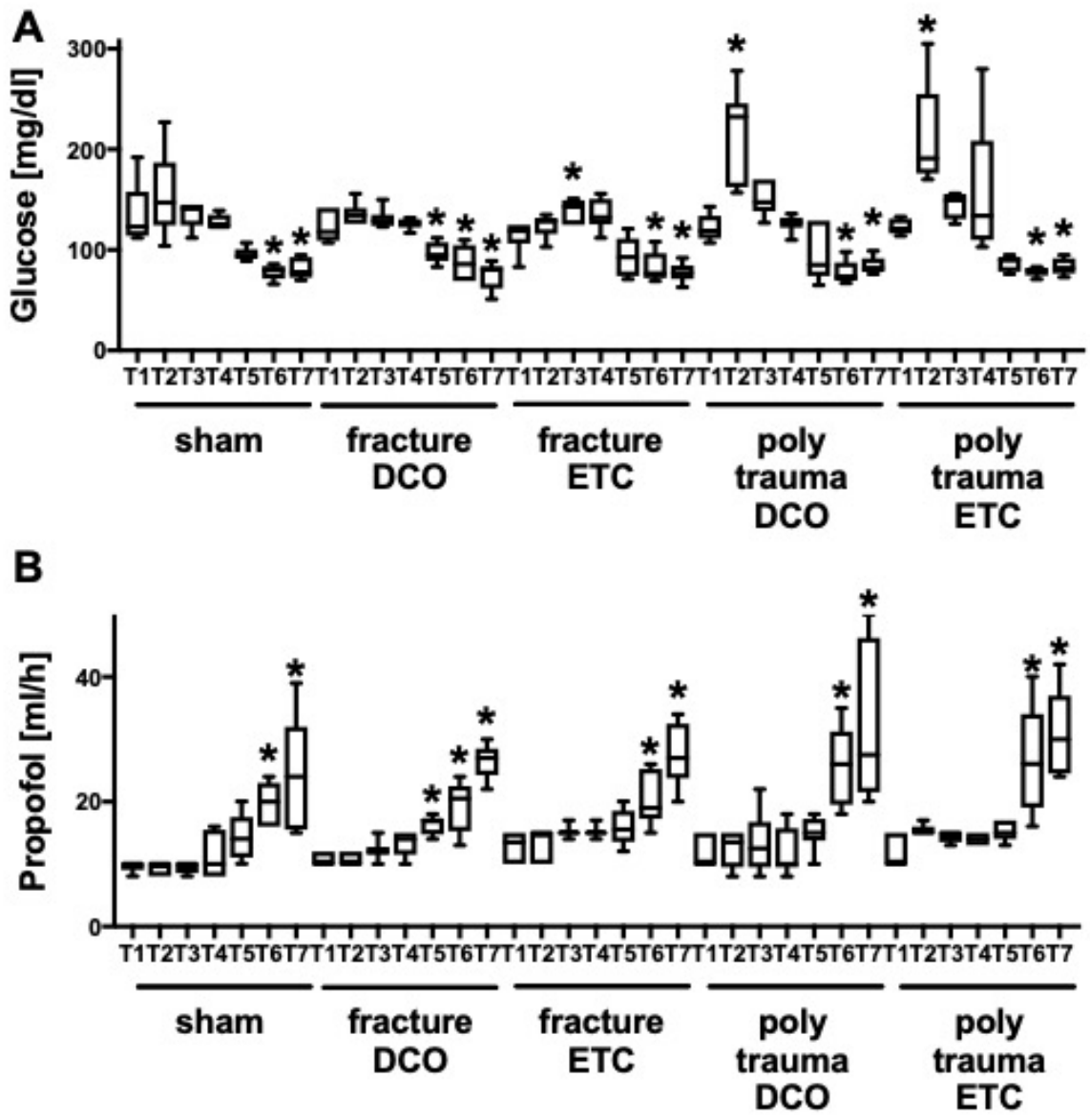


Figure 1: Blood glucose (mg/dl) (A) and propofol (ml/h) (B) values from blood gas analysis (BGA) in pigs with polytrauma, fracture or sham procedure. Pigs received either polytrauma or fracture, followed either by damage control orthopaedics (DCO) treatment or by early total care (ETC) treatment. Control pigs received sham procedure. Time of measurement was directly after trauma (baseline) and 1.5, 3.5, 5.5,

24, 48 and 72 h after trauma. n=6 pigs in each group. *p<0.05 differences to baseline concentrations.

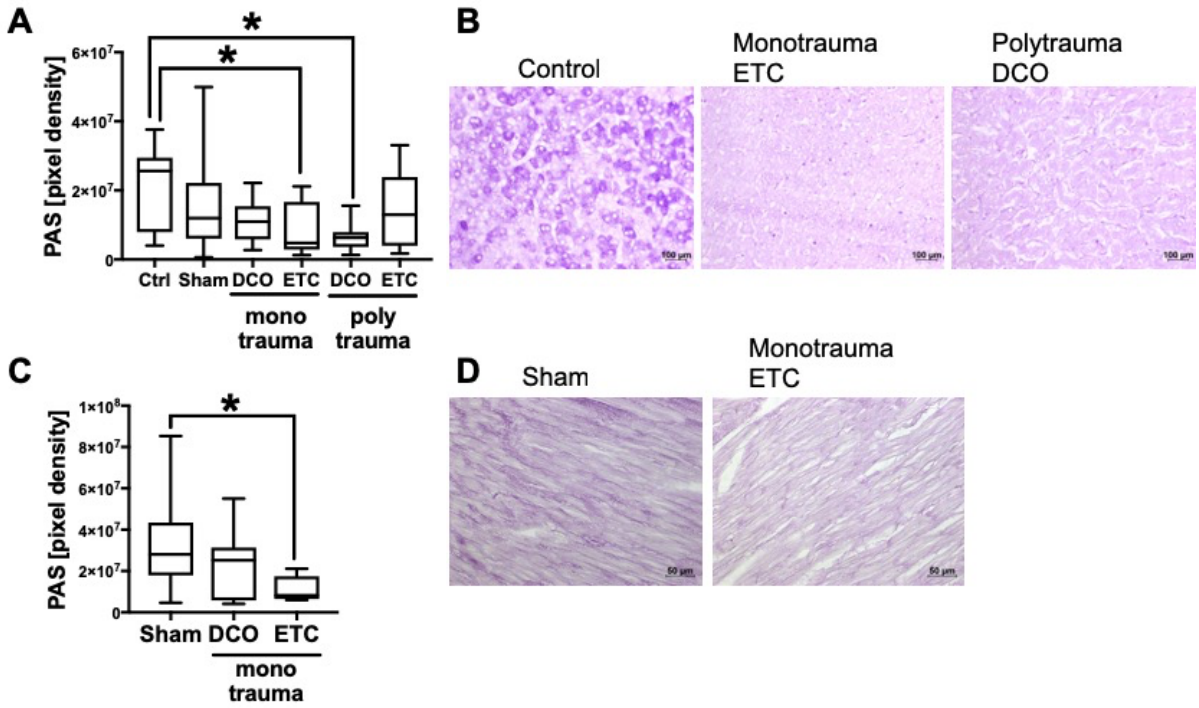


Figure 2: Hepatic (A, B) and cardiac (C, D) glycogen amount (pixel density) of sham-treated, fracture- and polytrauma and control pigs. Pigs either underwent fracture or polytrauma, followed either by damage control orthopaedics (DCO) treatment or by early total care (ETC) treatment. Control animals received sham procedure. Further, liver tissue from non-treated healthy pigs from historical group was collected (control). Liver tissue was collected 72 h after trauma. n=6 pigs in each group. *p<0.05.

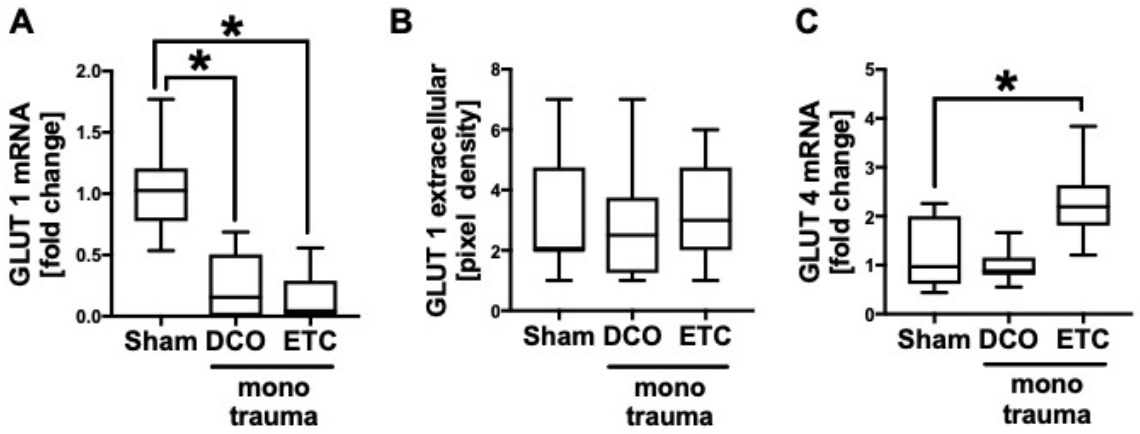


Figure 3: Cardiac glucose transport in pigs with fracture. The fold change of glucose 1 transporter (GLUT1) (A) and glucose 4 transporter (GLUT4) (C) mRNA, as well as the pixel density of the extracellular GLUT1 protein expression (B) is demonstrated. Pigs received fracture, followed either by damage control orthopaedics (DCO) treatment or by early total care orthopaedics (ETC) treatment. Control group received sham-procedure. Left ventricles were collected 72 h after trauma. n=6 pigs in each group. *p<0.05.

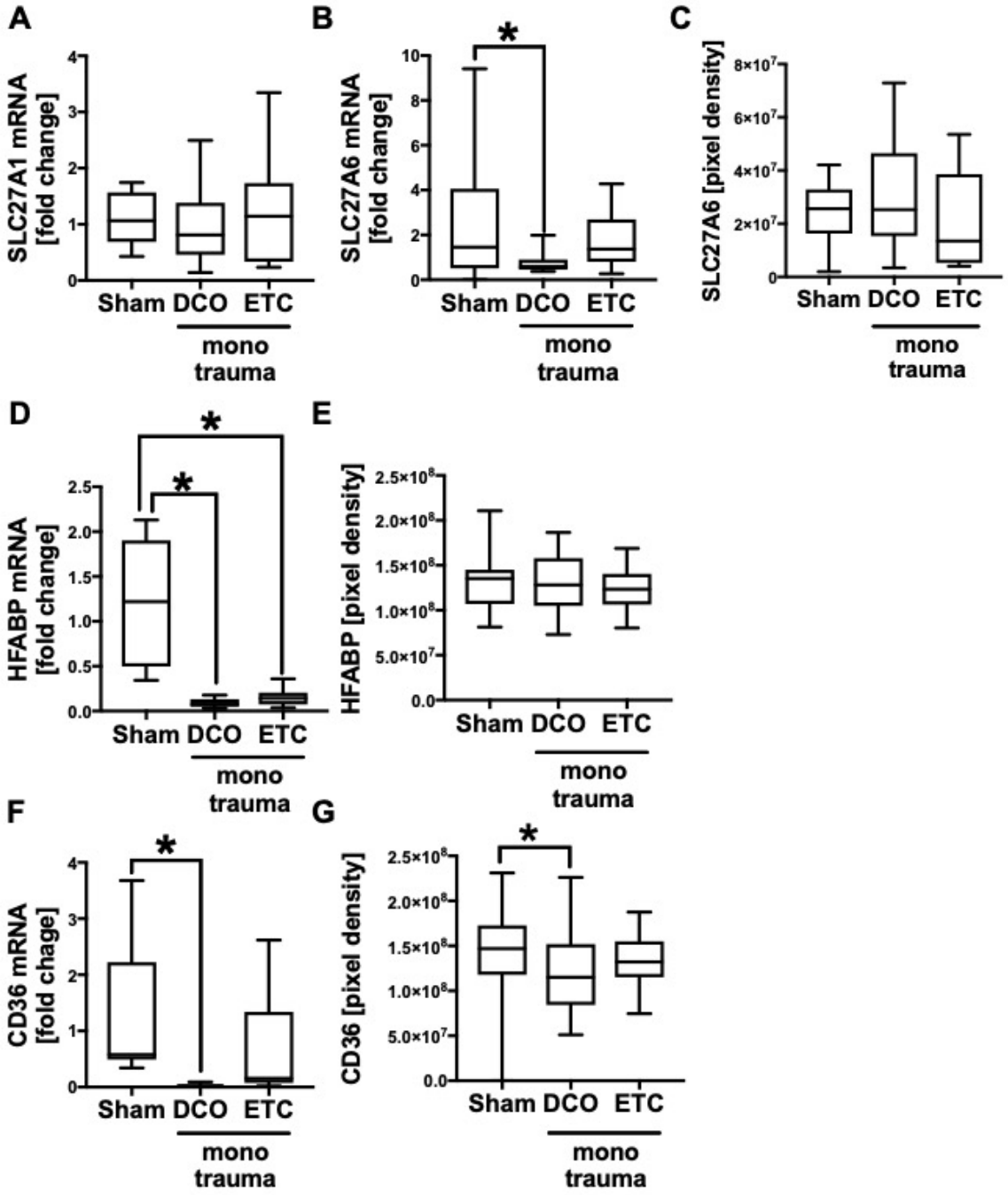


Figure 4: Cardiac fatty acid transport in pigs with fracture. The expressions of fatty acid transporter 1 (SLC27A1) (A), fatty acid transporter 6 (SLC27A6) (B), heart fatty acid binding protein (HFABP) (D) and fatty acid translocase (CD36) (F) mRNA, as well as the pixel density of SLC27A6 (C), HFABP (E) and CD36 (G) protein expression is demonstrated. Pigs received fracture, followed either by damage control orthopaedics (DCO) treatment or by early total care (ETC) treatment. Control groups were sham

treated. Left ventricles were collected 72 h after trauma. n=6 pigs in each group. *p<0.05.

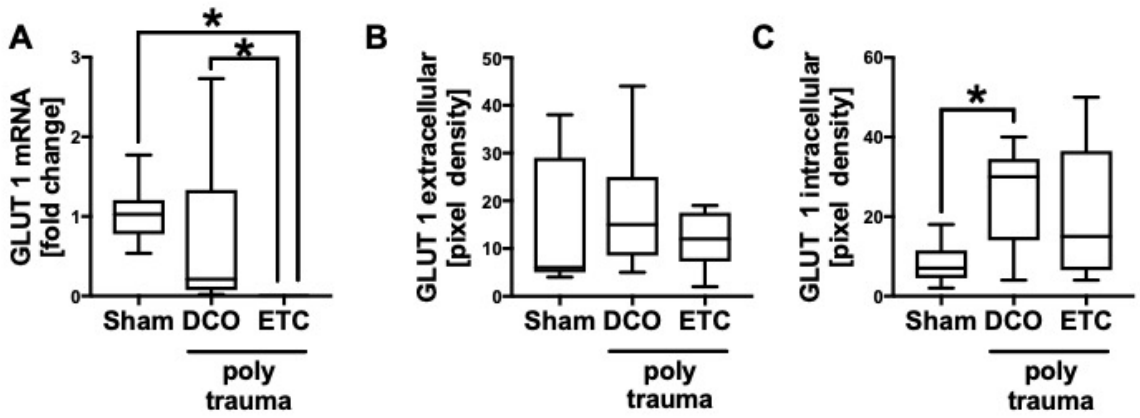


Figure 5: Cardiac glucose transport in pigs with polytrauma. The fold change of Glucose 1 transporter (GLUT1) (A) mRNA, as well as the pixel density of extracellular (B) and intracellular GLUT1 (C) protein expression is demonstrated. Pigs received polytrauma, followed either by damage control orthopaedics (DCO) treatment or by early total care (ETC) treatment. Control group was undergoing sham procedure. Left ventricles were collected 72 h after trauma. n=6 pigs in each group. *p<0.05.

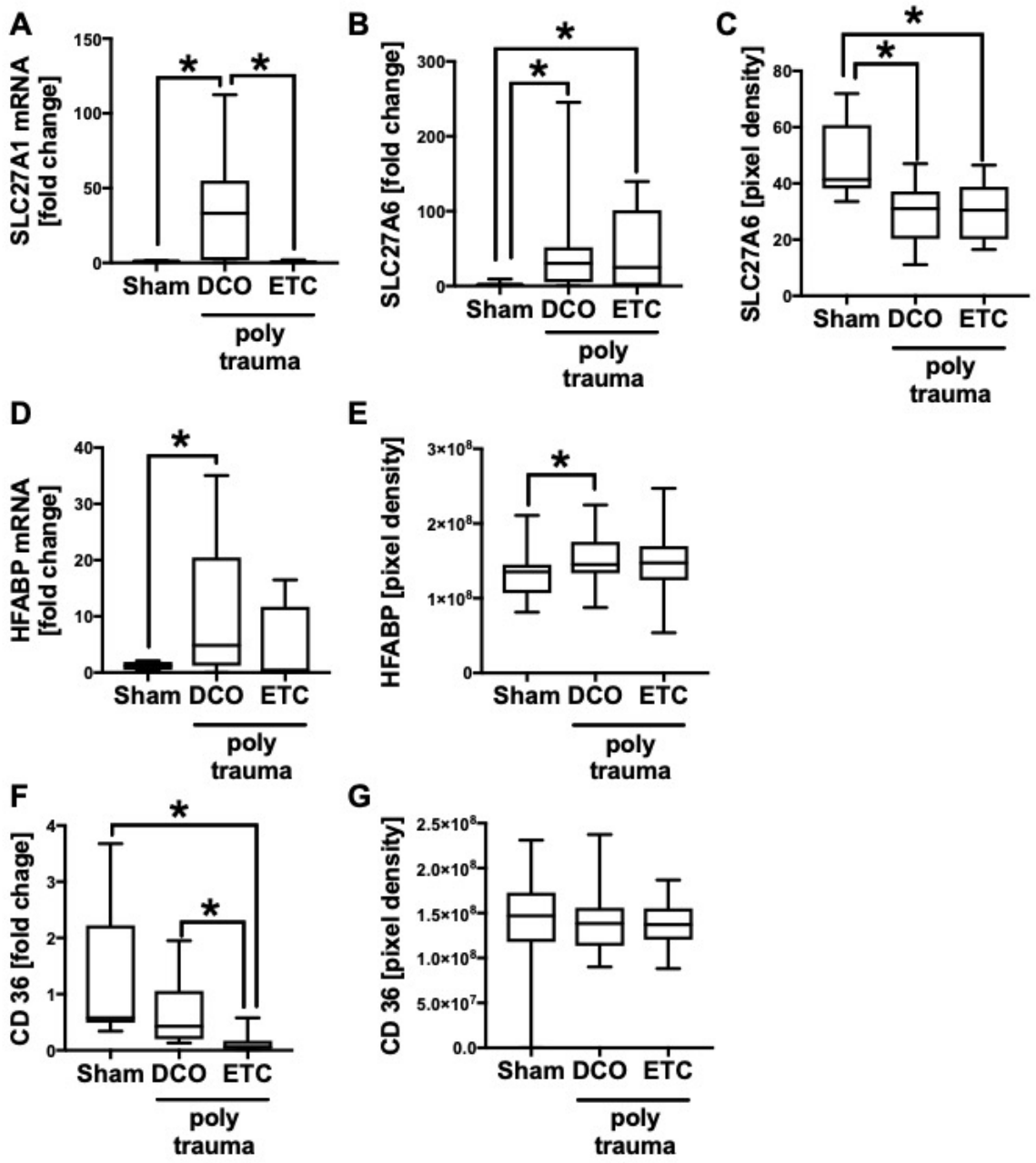


Figure 6: Cardiac fatty acid transport in pigs with polytrauma. The mRNA expression of fatty acid transporter 1 (SLC27A1) (A), fatty acid transporter 6 (SLC27A6) (B), heart fatty acid binding protein (HFABP) (D) and fatty acid translocase (CD36) (G) mRNA as well as the pixel density of extracellular SLC27A6 (C), HFABP (E) and CD36 (G) protein expression is demonstrated. Pigs received polytrauma, followed either by damage control orthopaedics (DCO) treatment or by early total care (ETC) treatment. Control group was undergoing sham procedure. Left ventricles were collected 72 h after trauma. n=6 pigs in each group. *p<0.05.

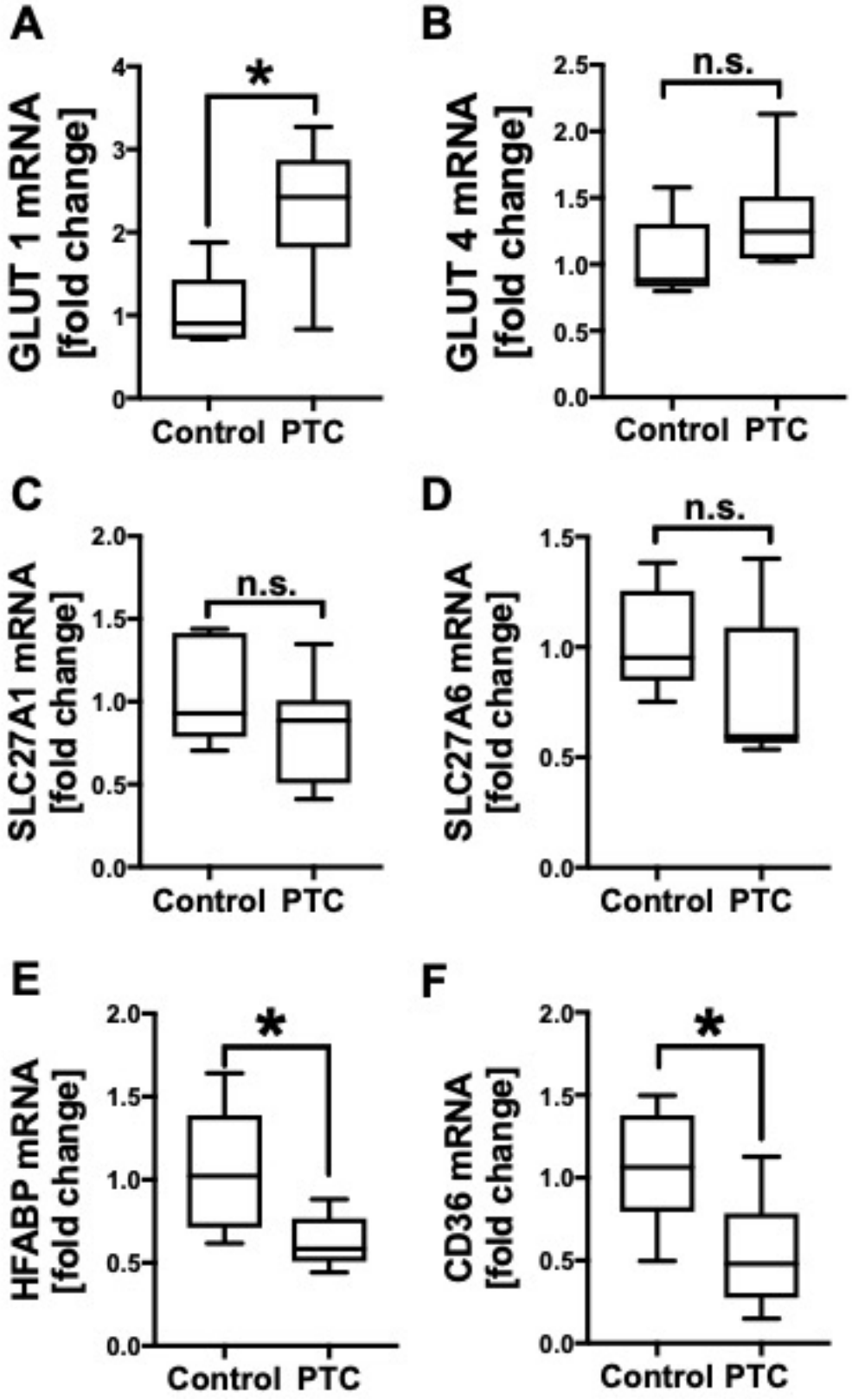
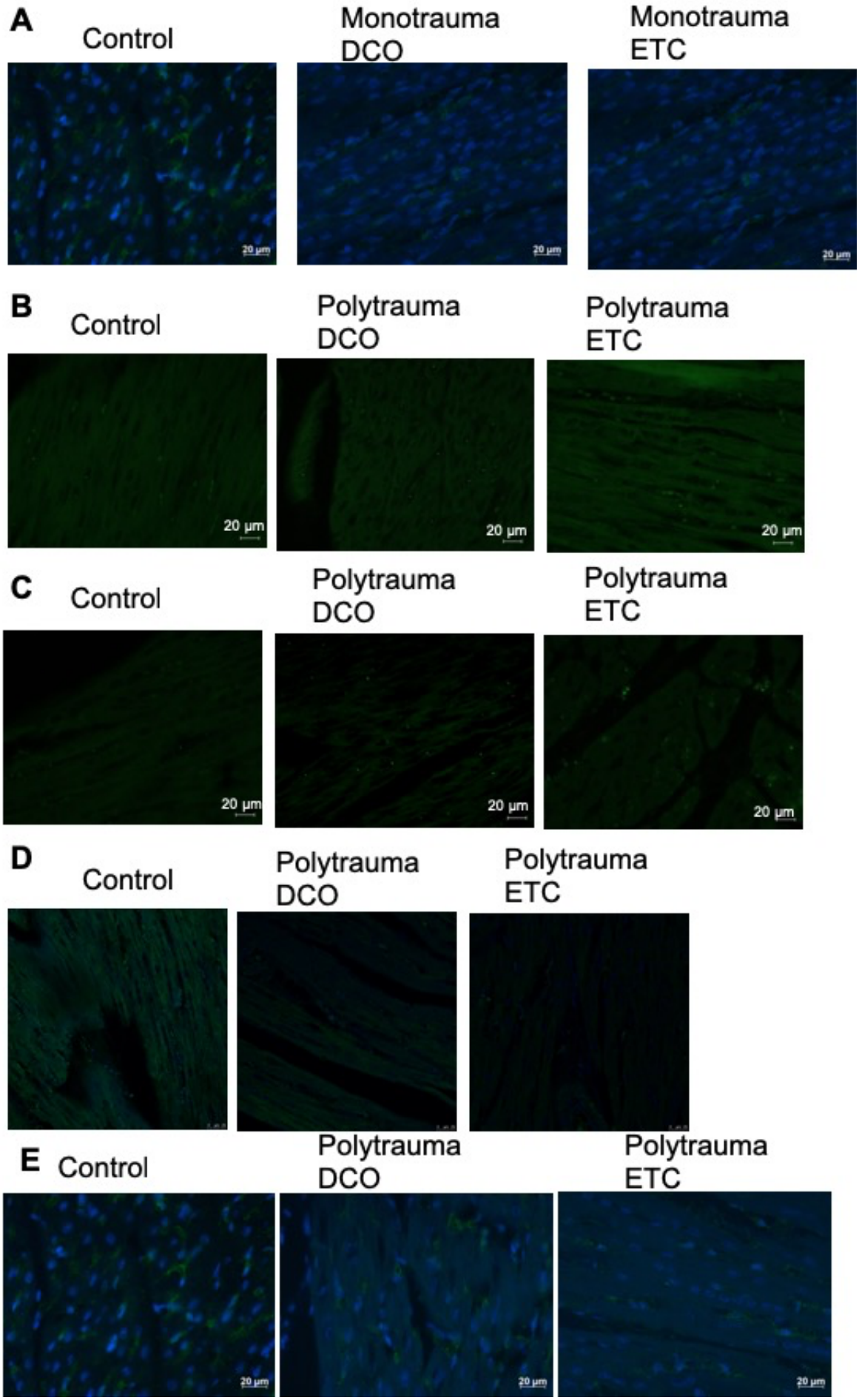


Figure 7: Cardiac glucose- and fatty acid transport in human cardiomyocytes. Human cardiomyocytes were either sham-treated or incubated for 6 h with polytraumacocktail PTC (C3a 500 ng/ml; C5a 10 ng/ml; IL-1β 200 pg/ml; IL-6 500 pg/ml; IL-8 150 pg/ml;

TNFα 10 ng/ml) and 20 μg/ml extracellular whole histone fraction mix. The mRNA expression of glucose transporter 1 (GLUT1) (A), glucose transporter 4 (GLUT4) (B), fatty acid transporter 1 (SLC27A1) (C), fatty acid transporter 6 (SLC27A6) (D), heart fatty acid binding protein (HFABP) (E) and fatty acid translocase (CD36) (F) mRNA is illustrated. n=6 in each group. *$p<0.05$.


A
Control
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Monotrauma ETC
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C
Control
Polytrauma DCO
Polytrauma ETC
D
Control
Polytrauma DCO
Polytrauma ETC
E
Control
Polytrauma DCO
Polytrauma ETC
20 µm

Supplemental Figure 1: Representative images of protein expression in left ventricles of pigs with fracture or experimental polytrauma. Pigs receive fracture provision either by damage control orthopaedics (DCO) principles or by early total care (ETC) principles. Representative images of protein expression of fatty acid translocase (CD36) in pigs with fracture (A). Representative images of protein expression of glucose transporter GLUT1 intracellular (B) and extracellular (C), fatty acid transporter 6 (SLC27A6) (D) and CD36 (E) in left ventricles of pigs with experimental polytrauma.

Supplemental material

Table 1: Ingredients of polytraumacocktail (PTC)

SUBSTANCE	**COMPANY**
C3a (500 ng/ml)	Merck, Kenilworth, NJ, USA
C5a (10 ng/ml)	Merck, Kenilworth, NJ, USA
IL-1β (250 pg/ml)	Peprotech, Rocky Hill, NJ, USA
IL-6 (500 pg/ml)	Biomol, Hamburg, Germany
IL-8 (150 pg/ml)	Biomol, Hamburg, Germany
TNF (10 ng/ml)	Biomol, Hamburg, Germany

Table 2: Antibodies for immunofluorescence

ANTIBODY	**COMPANY**
CD36 (Fatty acid translocase FAT/CD36)	Abcam, Cambridge, UK
HFABP (Heart fatty acid binding protein)	Abcam, Cambridge, UK
GLUT1 (Glucose transporter 1)	Abcam, Cambridge, UK
GLUT4 (Glucose transporter 4)	Abcam, Cambridge, UK

SLC27A1 (Fatty acid transport protein 1)	Biorbyt, San Francisco, CA, USA
SLC27A6 (Fatty acid transport protein 6)	Abcam, Cambridge, UK

Table 3: Human primer for RT-qPCR

GENE	**PRIMER SEQUENCE**
CD36 (Fatty acid translocase FAT/CD36)	5‘-CTTTCCTGCAGCCCAATGGT-3‘ (forward)
	5’-TTGTTGTATGCCACAGCCAG-3‘ (reverse)
FABP3 (Heart fatty acid binding protein)	5‘-GCATCACTATGGTGGACGCT-3‘ (forward)
	5‘-AACCCACACCGAGTGACTTC-3‘ (reverse)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	5‘-TCTCTGCTCCTCCTGTTCGAC-3‘ (forward)
	5‘-CCAATACGACCAAATCCGTTGA-3‘(reverse)
GLUT1 (Glucose transporter 1)	5‘-GAGGACCCAGAGGAAACCCA-3‘ (forward),
	5‘-GAAGGCGGCGAGGAAGAC-3‘ (reverse)

GLUT4 (Glucose transporter 4)	5‘-GGTTCTTTCATCTTCGCCGC-3‘ (forward)
	5‘-TCCCCATCTTCGGAGCCTAT-3‘ (reverse)
SLC27A1 (Fatty acid transport protein 1)	5‘-ATGGCTATGTCAGCGAGAGC-3‘ (forward)
	5‘-GCACGTCACCTGAGAGGTAG-3‘ (reverse)
SLC27A6 (Fatty acid transport protein 6)	5‘-GATGTGTTGAGTTGGGTGCC-3‘ (forward)
	5‘-GCAGTCACTCCAAAACTGGC-3‘ (reverse)

Table 4: Porcine primer for RT-qPCR

GENE	**PRIMER SEQUENCE**
CD36 (Fatty acid translocase FAT/CD36)	5’-TCCACTGTATTCCAACCAGCA-3’ (forward)
	5’-AAGATATCAGTTAGGAGTCCGATGA-3’ (reverse)

FABP3 (Heart fatty acid binding protein)	5‘-GAGACAACGCTTGTTCGGGA-3‘ (forward)
	5‘-TCTCGTAAGTGCGAGTGCAA-3‘ (reverse)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	5‘-GAGTGAACGGATTTGGCC-3‘ (forward)
	5‘-AAGGGGTCATTGATGGCGAC-3‘ (reverse)
GLUT1 (Glucose transporter 1)	5‘-CAATGCTCCCCAGAAGGTGAT-3‘ (forward)
	5‘-CGAGATGCTCTCCCCATAGC-3‘ (reverse)
GLUT4 (Glucose transporter 4)	5‘-ATGTTGCGGATGCTATGGGG-3‘ (forward)
	5‘-CCTCGGGTTTCAGGCACTTT-3‘ (reverse)
SLC27A1 (Fatty acid transport protein 1)	5‘-ACCTATCAGGTGACGTGCTG-3‘ (forward)
	5‘-GAGACATTCTCCCCACGCC-3‘ (reverse)

SLC27A6 (Fatty acid transport protein 6)	5‘-CACCGGAGACCTAATGGTCC-3‘ (forward) 5‘-GTTCCAGTTAGTACACCTGAATGT-3‘ (reverse)