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The role of damage associated molecular patterns during posttraumatic coagulopathy

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For Mom and Dad, to whom I owe everything.

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Abbreviations

AD	Aqua distillated
ADP	Adenosine diphosphate
AIS	Abbreviated injury scale
ANOVA	Analysis of variance
APC	Activated protein C
AT	Antithrombin
ATC	Acute traumatic coagulopathy
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BGA	Blood gas analysis
BL	Baseline
BSA	Bovine serum albumin
CD	Cluster of differentiation
cf	cell free
CFT	Clot formation time
CI	Confidence interval
CPD	Citrate phosphate dextrose buffer
CT	Clotting time
CTRL	Control
DAMP	Danger/Damage associated molecular pattern
DIC	Disseminated intravascular coagulation
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (beta-aminoethyl ether)-N,N,N´,N´-tetra acetic acid
ELISA	Enzyme linked immunosorbent Assay
F	Factor of coagulation
FC	Flow cytometry
FACS	Fluorescent absorbance cell sorting
FPX	Fondaparinux

GP	Glycoprotein
GTP	Guanosine triphosphate
H_2SO_4	sulfuric acid
H	Histone
HBSS	Hank´s buffered Saline Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIST	Histones
HMGB1	High mobility group box 1
HS	Hemorrhagic shock
INR	International normalized ratio
ISS	Injury severity score
KCl	Potassium-chloride
KH_2PO_4	potassium dihydrogen phosphate
LI	Lysis index
LT	Lethal triad
MCF	Maximum clot firmness
MCP	2-methacryloyloxethyl phosphocholine
$MgCl_2$	Magnesium dichloride
MODS	Multiple organ dysfunction syndrome
MOF	Multiple organ failure
Na_2HPO_4	sodium hydrogen phosphate dehydrate
NaH_2PO_4	Monosodium phosphate
NaCl	Sodium-chloride
$NaHCO_3$	Sodium bicarbonate
NET	Neutrophil extracellular trap
PAR-1	Protease activated receptor 1
PBS	Phosphatidyl buffered saline
PFP	Platelet free plasma
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PRR	Pattern recognition receptor
PTT	Partial thromboplastin time
RAGE	Receptor of advanced glycation end products

RT	Room temperature
ROTEM	Rotational thrombelastometry
s	soluble
SD	Standard deviation
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TIC	Trauma induced coagulopathy
TLR	Toll like receptor
USA	United states of America
vWF	van Willebrand Factor

SI-Units utilized in this study

°C	Degree(s) of Celsius
g	Gram(s)
L	Liter(s)
M	Molar
m	meter
min	Minutes
s	Seconds

1. Introduction

1.1. Definition of Polytrauma and its relevance in the contemporary world

Given the fact that, even in modern times, millions of deaths each year are caused by traumatic injury, severe trauma is one of the world's most relevant burdens on global health and is expected to be even more so in the future [68],[44],[98]. In Germany, polytrauma is commonly described as multiple injuries occurring in different regions of the body, one of which or the combination of all injuries is life-threatening. Road injuries, self-harm, falls and interpersonal violence were found as the most common causes of trauma-induced deaths [44]. In order to standardize research and patient management concerning this condition, current studies often employ the Injury Severity Score (ISS), when characterizing the extent of major injuries [15]. Pursuing the same goal, a new unitary definition for the pathology, called the New Berlin Definition of polytrauma, was proposed using objective scores like the Abbreviated Injury Scale (AIS) alongside altered clinical parameters [76], [80]. Commonly, three peaks in the mortality rates after an incident are described in the so called trimodal death distribution of trauma victims: immediate (at the site of injury), early (4-24h) and late (>72h) mortality [97]. While traumatic hemorrhage and injuries to the central nervous system were identified as the most common causes of immediate and early death, 60% of the patients with lethal outcome after more than three days post severe injury died of secondary causes such as multiple organ failure (MOF) [85], [97]. Even though this model has been questioned recently, immediate mortality and delayed mortality after polytrauma can still be differentiated [81],[20]. Regarding the delayed deaths of secondary causes, the onset of detrimental pathophysiological processes can often be found at the time of injury. In some cases, massive blood loss at the site of trauma can lead to hypovolemia and thus insufficient oxygen supply on a cellular level. This life-threatening state is defined as hemorrhagic shock (HS) [16]. Following severe injury and thus massive cell death, a complex-pathophysiological response is seen in individuals after trauma [50]. In the case of serious injury or HS, the inflammatory response can become dysregulated in an overshooting manner and may therefore be dangerous for the patient. When activated in an excessive manner, this condition is called systemic inflammatory

response syndrome (SIRS) [50]. Both SIRS and HS are associated with multiple organ dysfunction syndrome (MODS) or even MOF and can lead to late trauma-related death by secondary causes [16], [50]. As hemorrhage and inflammation are linked closely to coagulation and especially coagulation disorders, pathologies regarding the hemostasis are of great significance for the understanding of both early and late death from polytrauma [41], [100].

1.2. Coagulation cascade and hemostasis

In order to understand the pathology of a dysregulated coagulation system, a general understanding of the physiological process is required. Hemostasis is the ability to contain leakage of blood after vessel injury. It is generally divided into two phases: primary hemostasis, characterized by vasoconstriction, platelet adhesion, activation and aggregation as well as primary platelet-based clot formation and secondary hemostasis, characterized by the humoral coagulation system, creating a well-structured fibrin clot. Platelets, as important parts of the primary hemostasis, are characterized as anucleated cells. Following vascular damage and thus exposure of subendothelial matrix, van Willebrand factor (vWF) and collagen are exposed to the vessel lumen. By binding to glycoprotein (GP) Ib/IX/V complex (vWF) or GPIa/IIa (collagen) of the platelet surface, platelets are tied to the site of vascular injury and thereby activated. Upon activation, they release granules and flip phosphatidylserine on their surface, leading to a procoagulant phenotype [70], [82], [96]. These granules contain, among other molecules, agonists of platelets themselves, further activating them and drawing in other platelets to aggregate with them and forming a first hemostatic plug [93]. Simultaneously, the coagulation cascade is activated by tissue factor (TF) released by the subendothelial matrix.

The coagulation pathway is well known as a protease cascade that, as primarily described, works in a waterfall manner [28]. The coagulation cascade proteins, known as coagulation or clotting factors, are in large parts serine proteases that mainly become activated upon cleavage of other coagulation factors. The extrinsic pathway, designated as the strongest initiation of coagulation, is activated by TF also known as Factor (F) III. Upon damage to the endothelium, TF is released by the subendothelial matrix and binds to FVII, leading to an activation of FVII to F VIIa which build the extrinsic tenase complex (FIII / FVIIa) in the presence of calcium, also known as FIV. This complex activates prothrombin, also known as FII, to thrombin. Thrombin itself has the ability to amplify coagulation by activation of Factor XI, VIII and V, indirectly leading to the assembly of the intrinsic tenase complex, thus

activating a self-amplifying thrombin generation. The main driver of isolated intrinsic pathway activation is the contact pathway, where FXII can be autoactivated to FXIIa upon contact with polyanionic surfaces [26]. By a positive feedback loop, comprising kallikrein, FXIIa is produced in a relevant concentration and thus further activates FXI to FXIa, and in consequence FIX to FIXa. As mentioned above, FIXa complexes with FVIIIa, which can be obtained by FXa or FIIa activation, on platelet-provided phospholipid surfaces, building the so-called intrinsic tenase, resulting in the production of FXa and thus thrombin. Next to its auto-amplifying possibilities thrombin can also activate platelets by protease activated receptor-1 (PAR1) and -4 [55]. When activated, platelets release FV and change their surface composition, providing a surface for the so-called prothrombinase complex (FXa/FVa). This complex of FVa and FXa represents the main source of thrombin during coagulation. Thrombin can also cleave both soluble and platelet-bound fibrinogen (FI) to fibrin (FIa). As fibrin is immobilized by binding to GP IIb/IIIa on the platelet surface, it undergoes polymerization and builds the backbone of a clot. F XIIa is crucial for the integrity of a stable clot, which can be activated by the complex of FIIa, FIa and FXIII and functions by organizing and stabilizing fibrin structures (Figure 1).

In order to obtain homeostasis of a fluent blood flow, coagulation has to be strictly regulated. This is mainly achieved by tissue factor pathway inhibitor (TFPI), antithrombin (AT) and activated protein C (APC) among many other factors. As the name suggests, TFPI mostly impairs the TF-pathway. Functioning as a protease inhibitor, TFPI impacts FXa and FVIIa/TF-complex. TFPI is mainly bound to the endothelium but a soluble form has also been described. However, the most prominent circulating serine protease inhibitor is AT. In particular it impairs the coagulation factors IIa and Xa, but also IXa, XIa and XIIa via complex formation [11]. AT effectiveness can be increased 1000-fold while bound to a glycosaminoglycan subgroup called Heparins. This effect is pharmaceutically utilized to treat patients with an elevated risk of thrombosis. One of the latest additions to this subgroup of drugs is a specifically produced, synthetic pentasaccharide inducing AT-dependent selective factor Xa inhibition, called Fondaparinux (FPX) [84].

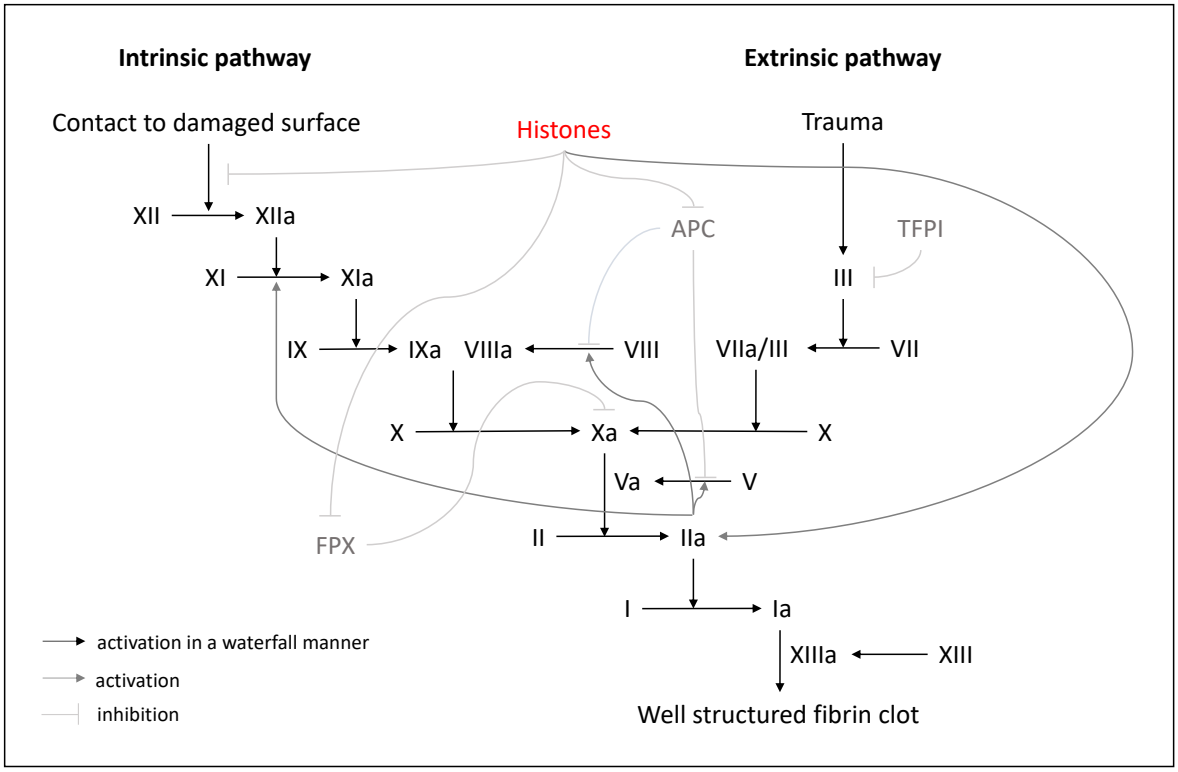

Figure 1 ***Coagulation cascade and impact of physiological anticoagulant agents and histones.*** *Displaying the well-known coagulation cascade with the addition of anticoagulant agents as described before. Also, the pro and anticoagulant effects of histones are shown. This Damage associated molecular pattern (DAMP) appears to interact with the hemostatic system in several way including direct impact on the coagulation cascade, effects on platelets and impairment of anticoagulant agents.*

1.3. Platelet function in and beyond hemostasis

Next to the humoral coagulation system, cellular participation in hemostasis plays a key role. As mentioned above, platelets can multiply their own aggregation, activation and thus coagulation activation. This is mostly due to the release of granules and surface changes following activation by platelet agonists like thrombin or adenosine diphosphate (ADP) [82], [109]. In general, these anucleated cells contain multiple kinds of granules that in turn store different agents. Three major groups are classified as alpha-, delta (dense)-, and lambda (lysosome)- granules [6]. While vWF, fibrinogen, P-selectin and GP IIb/IIIa, among other cytokines and chemokines, are stored in alpha granules, delta granules contain platelet-derived amines and bioactive ions alongside ADP, adenosine triphosphate (ATP) and guanosine triphosphate (GTP) [6]. However, recent findings suggest that there are subgroups of alpha granules that can be released selectively [89]. Moreover, the quantity and quality of exocytotic activity is dependent on the concentration and type of agonist [31]. The exposure of p-selectin on their surface, which is stored in alpha granules by resting platelets and is expressed rapidly upon activation, is the most common characteristic of

“classical” platelet activation by activating agents like ADP or thrombin [109]. It is also described that agonists of platelet Toll-like receptors (TLRs) can induce alternative platelet-activating effects without alpha granule secretion [21]. TLRs are part of the pattern recognition receptor (PRR) family and induce intracellular signaling upon the binding of specific agonists. Of the ten TLR types identified in humans, TLR 1, 2, 4, 6 and 9 were found on human platelets [23], [94]. However, the clinical relevance of these platelet bound PRRs is still being discussed. While it had been shown that agonists specific for TLR2 and 4 were not able to induce or modulate platelet activation, recent findings indicate TLR-induced thrombocytopenia and hint towards an important role of platelet-derived TLR signaling for an inflammatory response including e.g. tumor necrosis factor (TNF) production [22], [53], [54],[8],[43].

In recent years the crosstalk between platelets and innate or even adaptive immunity was put into focus in many research efforts [6], [92]. For example, upon activation platelets were found to be able to express CD 40 ligand (CD40L=CD154), a well-known player in adaptive immunity [48]. Also, CD40L provided by activated platelets can even be released in a soluble form, known as sCD40L [47]. Both soluble and membrane-adherent CD40L modulate the immune response and inflammatory cytokine production, but also contribute to thrombosis [32], [64]. Furthermore, platelets not only modulate the immune response but also immunological events can also activate platelets and coagulation in a process known as immunothrombosis [34].

In summation, platelets and coagulation and their crosstalk with the immune continuum have been found to play a key role in both hemorrhage and MOF [34].

1.4. Trauma-induced coagulopathy and the impact of the lethal triad

As the impact of hemostatic pathology on the outcome of trauma victims became more obvious, two different ways for early trauma-associated bleeding disorders were described. Massive bleeding as an immediate consequence of polytrauma is mostly caused by vascular injury itself but is also worsened by impairment of coagulation known as trauma-induced coagulopathy (TIC) [13], [17]. Here, it will be differentiated between acute traumatic coagulopathy (ATC) and coagulopathy associated with resuscitation [59]. The largest contributer to this effect is the so-called lethal triad (LT) in trauma. The main actors

of this condition are hypothermia, acidosis and hemodilution which are often seen in trauma victims [25]. Following HS or insufficient oxygenation of tissue, the so-called metabolic switch of cells can produce lactic acid and thereby lead to an acidotic environment. While trauma victims often suffer from hypothermia due to hemorrhage and insufficient heat retention, hemodilution is generally due to iatrogenic infusion of crystalloid liquids following substantial blood loss. Due of the increased mortality rate when synchronically present, the condition coined the name “lethal triad” in trauma [63]. It has been shown that each condition per se impairs coagulation but together they seem to amplify their effects [18], [29], [63], [105]. For example, a direct impairment of acidosis in regard to FVII has been shown, as well as a direct impact on the exocytotic capacity of platelets [35], [69]. Overall, this drastic attenuation of the hemostatic process can lead to and aggravate an enormous blood loss clinically manifested as HS [16].

1.5. Disseminated intravascular coagulopathy and the impact of damage-associated molecular patterns

Disseminated intravascular coagulation (DIC) is a well-documented serious condition, that, despite abundant research, diagnostic criteria and modern treatment, is still associated with a markedly high mortality [42], [104]. DIC can lead to micro- and macrovascular thrombosis by an overshooting activation of the coagulation system ultimately leading to MOF [39], [65]. This condition is mostly associated with SIRS and clinical conditions like sepsis and trauma [38], [99], [101]. There are two main types of DIC: One characterized by hyperfibrinolysis resulting in hemorrhage and one characterized by an overshooting thrombin generation leading to micro- and macrovascular thrombosis, eventually resulting in organ dysfunction [40]. The consumption of coagulation factors by mislead and misplaced microvascular thrombosis can also result in hemorrhage. Nowadays, many pathomechanisms such as a tissue factor overexpression by monocytes, platelet activation, impairment of anti-coagulation pathways, fibrinolysis and immunothrombosis are known [45], [62], [70], [112]. However, in recent years the focus of research shifted to pathogen- and damage-associated molecular patterns (DAMPs) as causes of DIC [52],[34]. HS, just like tissue trauma itself, can end up in massive cell death and in turn to a considerable release of DAMPs into the circulation [24], [50], [58], [86]. DAMPs are molecules, that are set free upon cell stress or tissue injury and function by strong activation of the immune response without the presence of pathogens [83]. Thereby, the discussion mainly revolves around

histones and high mobility group box 1 (HMGB1) [46], [71]. While physiologically interacting with deoxyribonucleic acid (DNA) by linking it with intranuclear proteins, HMGB1 can become a mediator of thrombosis and DIC when its plasma concentration rises [14], [46], [103]. HMGB1 has been found to induce prolonged PTT times in mice when admitted together with thrombin, but not on its own [51]. Even though the interaction with TLRs is assumed, recent research shows HMGB1 binding to receptors for advanced glycation end products (RAGE) on platelets and not TLRs [77], [108],[3].

Similar to HMGB1, all histone proteins H1, H2A, H2B, H3, H4 and H5 are mostly found intranuclearly as molecules complexed with DNA, keeping up the integrity of chromosomes [19]. Histone release in trauma and inflammation is mainly caused by two mechanisms: neutrophil extracellular trap (NET) generation by activated neutrophil granulocytes and cell death per se [12], [19], [110]. Once released, they contribute to multiple severe conditions like DIC or organ dysfunction [2], [57], [71]. DAMPs can further drive systemic inflammation and thereby MOF, potentially leading to death even multiple days after the trauma incident [61], [85], [111]. When NETs were found to induce platelet aggregation, DNA and histones, the core constituent of NETs, were particularly in question to mediate platelet and thereby coagulation activation [73], [36], [107]. Negatively charged cell-free (cf) DNA was found to activate the intrinsic coagulation cascade whereas histones showed to dose-dependently induce thrombin generation independently of a blockage of FXII and thereby the intrinsic coagulation pathway [56], [75], [91]. After histones were found to activate and aggregate platelets similar to common platelet activators like ADP or collagen, a direct interaction of histones and platelets became evident [37]. These findings were further validated as histones were found to induce thrombin generation in platelet rich plasma (PRP) but not in platelet free plasma (PFP) [74]. Histones are said to be able to interact directly with platelets in a TLR 2 and 4 dependent manner, thus leading to platelet activation and thrombin generation [91]. However, they are also known to have effects through binding to TLR 9 [47], [103]. Yet, when complexed to octamers, like in the cell's nucleus, histones fail to induce thrombin generation [74]. In addition to direct coagulation activation, histone-induced impairment of thrombomodulin leads to less protein C activation (Figure 1) [7]. Altogether, the activation of platelets and thereby the initiation of coagulation, attenuation of the antithrombotic agents and their direct cytotoxicity can all contribute to DIC development [95].

1.6. Objectives of the investigation

As controversies about the mechanisms of action of single DAMPs exist, it is the aim of this work to shed light on the impact of both HMGB1 and histones on platelets and coagulation. The goal of this thesis is to depict the effect of specific DAMPs (histones, HMGB1) combined with LT on platelets and coagulation in a Fondaparinux (FPX)-based PRP model. First, it was necessary to establish and characterize a PRP-model, where platelets could still function without significant changes in coagulating function compared to common assessments of the hemostatic system, although the blood was only treated with FPX beforehand. Then, this model was used to investigate the effects of different stimuli on platelets and the coagulation system in the absence of red or white blood cells.

Based on the results of these investigations, further testing was required to improve the comprehension of the underlying mechanisms. Therefore, both platelet function and plasmatic coagulation were more specifically examined after addition of DAMPs.

The following hypotheses have been proposed and addressed:

1. Platelet rich plasma can be gained from FPX-treated whole blood without undue activation of platelets or coagulation.
2. DAMPs (HMGB1 and histones) induce measurable activation of platelets and the coagulation cascade, leading to the consumption of both.
3. The LT itself impairs coagulation in PRP, while enhancing the effects of DAMPs on platelets and coagulation.
4. Histones directly impact platelet physiology, showing both a classical and an atypical platelet response.
5. Histones have a concentration-dependent effect on both platelets and the coagulation cascade.

2. Material and Methods

2.1. Devices and materials

All devices were maintained, cleaned and tested for functionality on a regular basis.

Table 1: Devices and materials

0.5 ml tubes	Sarstedt (Catalog-No.: 72.704.400; Nümbrecht, Germany)
1.5 ml tubes	Sarstedt (Catalog-No.: 72.706.400; Nümbrecht, Germany)
2 ml Eppendorf tubes	Eppendorf AG (Catalog-No.: 0030 120.094; Hamburg, Germany)
15 ml Falcon	Corning Science Mexico S.A. de C.V. (Lot-No.: 19619245; Reynosa, Mexico)
50 ml Falcon	Sarstedt (Catalog-No.: 62.547.254; Nümbrecht, Germany)
BGA-device	ABL 800 Flex (Radiometer Medical ApS, Brønshøj, Denmark).
Centrifuge 1 (big)	Multifuge X3R (Thermo Fisher Scientific; Waltham, USA)
Centrifuge 2 (small)	Fresco 21 (Thermo Fisher Scientific; Waltham, USA)
Collection tube Citrate	Sarstedt (Catalog-No.: 02.1067.001; Nümbrecht, Germany)
Collection tube Neutral	Sarstedt (Catalog-No.: 02.1726.001; Nümbrecht, Germany)
Flow cytometer (FACS)	FACSLyric 2 (Serial-No.: R663029000063; Becton, Dickinson and Company, USA)
Fridge (4°C)	Bosch, Gerlingen, Germany
Fridge (-80°C)	HERZAfreeze (Thermo Fisher Scientific; Waltham, USA)
Heat bath (37°C)	SW 23 (Julabo; Germany)

Incubator (37°C)	Heraeus (Thermo Fisher Scientific; Waltham, USA)
Incubator (34°C)	HERAcell 150i (Thermo Fisher Scientific; Waltham, USA)
Microscope	Bl 200 Bino Plan 630 (VWR, Radnor, Pennsylvania, USA)
Needle	Sarstedt (Catalog-No.: 85.1637.005; Nümbrecht, Germany)
Neubauer-Chambers	Marienfeld Germany (Lauda-Königshofen, Germany)
pH-meter	Sartorius (Göttingen, Germany)
Pipettes/tips	(Brand, Wertheim, Germany/ Sarstedt, Nümbrecht, Germany)
ROTEM	ROTEM delta (Serial-No.: 5022; Tem Innovations GmbH, Munich, Germany)
Tecan absorbance reader	Tecan (Männedorf, Switzerland)
Thrombocount-Reagent	ThromboCount fix (servopax GmbH, Wesel, Germany)
Vortex Genie-2	Scientific industries Inc. (Bohemia, USA)

2.2. Ethical approval and blood collection

The study was approved prior to the beginning of the project by the ethics committee of Ulm University (votum No. 103/20) and complies with the declaration of Helsinki.

Blood was withdrawn from healthy donors with neither a history of a bleeding disorder, nor anticoagulant or antiplatelet drug intake in the last 14 days by cubital venous puncture using a Multifly-Needle 20G tube 80 mm (Catalog-No.: 85.1637.005; Sarstedt, Nümbrecht, Germany). Whole blood was collected into citrate tubes (Catalog-No.: 01.1606.00; Sarstedt, Nümbrecht, Germany), containing 820 µl of 3.2% sodium citrate, or neutral tubes

(Catalog-No.: 02.1726.001; Sarstedt, Nümbrecht, Germany), that were supplemented with Fondaparinux (FPX) (Arixtra, Aspen Pharmacare, Durban, South Africa) at a final concentration of 8 µg/ml, later referred to as FPX tubes. To avoid procoagulant factors released by the endothelium upon venous puncture, the initial 3 ml of blood was drawn in a separated tube and discarded.

2.3. Ex vivo assessment of coagulation and platelet function

2.3.1. Polymer based coating of tubes

In order to enable an inert surface in the reaction tube over time, 0.5 ml and 2 ml tubes were coated with a 3% (weight / volume [w/v]) 2-methacryloyloxethyl phosphocholine (MPC) Lipidure-CM5206 (NOF Corporation, Japan) - ethanol (99.9%; Lot-No.: #STBJ3541; Sigma-Aldrich, St. Lewis, USA) solution. Tubes were filled with 300 µl of coating solution and incubated for 15 minutes under circulatory conditions. Afterwards, tubes were dried at an ambient temperature of 50°C. The process was then repeated to ensure complete coating of the tubes, which was confirmed by Rhodamine staining of sample materials of every batch.

2.3.2. Processing of FPX blood samples for examination of coagulation

After blood collection, FPX tubes were gently inverted, and the contained blood was immediately transferred into MPC-coated tubes and exposed to respective stimulating agents according to experimental protocols (see below). Subsequently, specimens were incubated under constant movement at 37°C (Thermo Fisher Scientific; Waltham, USA) or 34°C (Thermo Fisher Scientific; Waltham, USA) for 30 minutes. Different conditions were simulated as described in the following.

2.3.3. Stimulation with DAMPS

A blend of histones from calf thymus (Ref-No.: 10 223 565 001; Lot-No; Roche Diagnostics GmbH, Mannheim, Germany) was used at concentrations of 50 µg/ml unless otherwise stated. Recombinant HMGB1 (Ref-No.: 1690-HMB; R&D Systems, Minneapolis, USA) was applied at a final concentration of 500 ng/ml.

2.3.4. Lethal triad in whole blood

Lethal triad (LT) was simulated by a 30% Dilution and acidosis at approximately pH=7.15, Ranges of pH-values are given in the respective section and graphs in results. Using 275 µl/ml Jonosteril and 25 µl/ml Jonosteril with a pH adjusted to 1 by titration. To simulate hypothermia, Lethal Triad-samples (LT-samples) were incubated at 34°C. For measurement

of the blood pH, blood gas analysis (BGA) was performed by filling 95 µl heparinized capillary tubes (Clinitubes; Ref-No.: 942-875-D941G-240x5; Radiometer Medical ApS, Brønshøj, Denmark) with samples and analyzing them in an ABL800 Flex (Radiometer Medical ApS, Brønshøj, Denmark).

2.3.5. Platelet rich plasma preparation

Purified platelet rich plasma of FPX- anticoagulated whole blood was generated to analyze the coagulative response, without the impact of other blood cells. Therefore, an FPX tube was gently transferred to a centrifuge immediately after the withdrawal of the blood (Thermo Fisher Scientific; Waltham, USA) and spun at 20°C with at a 150g for 15 minutes. 2 ml of the upper layer were collected into microtubes and carefully homogenized by hand.

2.3.6. Lethal triad simulation in platelet rich plasma

Stimulation of LT in PRP was in accordance with whole blood with the following variations. Dilution of 30 % and acidosis were simulated at pH=7.0, Using 270 µl/ml Jonosteril and 30 µl/ml Jonosteril with a pH adjusted to 1 by titration for a severe LT. In order to assess the impact of a milder LT, pH was set to 7.35, using 30 µl/ml Jonosteril with a pH adjusted to 3 to complement the dilution of 270 µl/ml Jonosteril. Hypothermia was simulated by incubating the specimen at 34°C. BGA for each specimen was performed for pH analysis.

2.3.7. Storing of plasma samples

For later analysis via enzyme-linked immunoblot assay (ELISA), plasma samples were generated. These samples were transferred into 1.5 ml Micro-tubes (Catalog-No.: 72.706.400; Sarstedt, Nümbrecht, Germany) and further anticoagulated with 20 µl/ml Ethylenediaminetetraacetic acid (EDTA) (Lot-No.: BZBV5052; Sigma-Aldrich, St. Lewis, USA). Each specimen was spun twice at 4°C (Fresco 21; Thermo Fisher Scientific, Waltham, USA). In the first step, centrifugation was performed at a force of 800 G for 5 minutes. Following that, approximately 200 µl of supernatant were added in to another 1.5 ml Micro-tube and spun at a force of 16 000 G for two minutes. Specimens were then stored at -80°C before further processing.

2.4. Analysis of platelet function and coagulation cascade

2.4.1. BCA protein determination

Due to the dilutional effect of the LT, the total amount protein per specimen was determined in order to attain reference values for enzyme-linked immunosorbent assays (ELISAs). Pierce bicinchoninic acid (BCA) Protein Assay Kit (Ref-No.: 23225, Thermo-

Scientific, Meridian, USA) was used according to the manufacturer's instructions. 10 µl of respective samples and reference solution were transferred in duplicates onto a 96-well plate (Lot-No.: 159908; Thermo Fisher Scientific, Waltham, USA). Therefore, samples were diluted 1:100 in distilled water (AD). After addition of a freshly produced 51:1 mixture of reagent A and reagent B, which was included in the kit, the plate was incubated for 30 minutes at 37 °C. Afterwards absorption was measured at 562 nm wavelength in a Tecan absorbance reader.

2.4.2. Enzyme linked immunosorbent assay

2.4.2.1. ELISA reagents

Table 2: Reagents used for ELISA

Washing buffer	137 mM (8 g) sodium-chloride (NaCl; Prod.-No.: 71380; Sigma-Aldrich, USA) 2 mM (0.2 g) potassium-chloride (KCl; Prod.-No.: 4936; Merck Millipore, USA) 11 mM (1.5 g) of sodium hydrogen phosphate dehydrate (Na_2HPO_4 x $2H_2O$; Prod.-No.: 6580; Merck Millipore, USA) 3 mM (0.4 g) of potassium dihydrogen phosphate (KH_2PO_4; Prod.-No.: 4873; Merck Millipore, USA) 0.4 mM (0.5 g) of tween 20 (Prod.-No.: P-1379; Sigma-Aldrich, USA) 1000 mL Ampuwa (Lot-No.: 13NMP051; Fresinus Kabi, France)
Reagent diluent	0.15 mM (1 g) bovine serum albumin (BSA; Prod.-No.: A-8022-100G; Sigma-Aldrich, USA) 0.4 mM (0.05 g) tween 20 2 mL 0.5 M EDTA (Prod.-No.: 03690-100ML, Sigma-Aldrich, USA) 100 mL of PBS
Stop solution	20 mL 5 normal (N) sulfuric acid (H_2SO_4; Prod.-No. 109912, Merck Millipore, USA) 30 mL of Ampuwa to obtain 50 mL of 2 N H_2SO_4,

2.4.2.2. ELISA procedure

To attain measurements of platelet derived activation molecules, ELISAs were performed for sCD40L and vWF. In a first step, the capture antibody was diluted in Dulbecco`s Phosphate Buffered Saline (PBS) (Lot-No.: 14190-094; Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions at a concentration of 800 ng/ml for CD40L capture antibody and 2 µg/ml for vWF. Afterwards, capture antibody in a total volume of 100 µl was used to coat each well of a 96-well plate (Lot-No.: 163419; Thermo Fisher Scientific; Waltham, USA). The plate was incubated at room temperature (RT) over night. The following day, each well was rinsed three times with 300 µl washing buffer and subsequently incubated with 100 µl reagent diluent for one hour. The wells were rinsed as described previously and plasma-EDTA-samples were diluted 1:5 in reagent diluent, loaded into a total volume of 100 µl and incubated for two hours at RT. The plates then were also rinsed again as described above. 100 µl of detection antibody diluted in reagent diluent to a final concentration of 100 ng/ml for the CD40L detection antibody and 50 ng/ml for the vWF were applied to each well and incubated for an hour. The rinsing step was repeated once more. After addition of 100 µl working dilution of streptavidin conjugated with horse radish peroxidase, plates were incubated for 20 minutes without direct exposure to light. As the final rinsing step was performed, 100 µl substrate solution made of TMB Substrate Reagent Set (Lot-No.: 9141997, Cat-No.: 555214, BD Biosciences, San Diego, California, USA) were applied to each well, and samples were incubated for another 20 min without exposure to direct light. Afterwards, 50 µl of stop solution were added to each well. Plates were gently tapped to ensure mixing. Absorption measurements were performed at 450 nm wavelength and a wavelength correction was set to 540 nm, according to the manufacturer's instructions in a Tecan absorbance reader. All incubation steps were performed using sealed plates at room temperature.

2.4.2.3. ELISA kits

Human vWF-A2-Kit (Lot-No.: P237993; R&D Systems, Minneapolis, USA) and Human CD40Ligand / TNFSF5-Kit (Lot-No.: P151115; R&D Systems, Minneapolis, USA) were used according to the manufacturer´s instructions.

2.4.3. Microscopic platelet count

After PRP production, platelet isolation or incubation, manual platelet counts were performed. Blood and PRP each were anticoagulated with 20 µl/ml (volume/volume [v/v]) EDTA (Lot-No.: BZBV5052; Sigma-Aldrich, St. Lewis, USA) and diluted 1:100 (v/v) in

ThromboCount fix (servopax GmbH, Wesel, Germany) for PRP and 1:5 (v/v) for whole blood according to the manufacturer´s instruction. Afterwards platelets were counted in Neubauer-chambers (Marienfeld, Germany) via light microscopy (VWR, Radnor, Pennsylvania, USA).

Platelet recovery rate was calculated at Recovery = $n_{ThrombocytesPRP}$ / ($n_{ThrombocytesBL}$ x 3.5).

2.4.4. Small blood counts and coagulation analysis

Small blood count, Quick, PTT and the International normalized ratio (INR) were measured by the central facility for clinical chemistry of the university hospital Ulm. For a small blood count, samples were anticoagulated by adding 20 µl/ml EDTA (Lot-No.: BZBV5052; Sigma-Aldrich, St. Lewis, USA). For coagulation parameters, samples were transferred into sodium citrate containing microtubes (Lot-No.: 0072121; Sarstedt, Nümbrecht, Germany).

2.4.5. Rotational Thrombelastometry (ROTEM)

ROTEM analyses were used to obtain more specific insights on intrinsic or extrinsic coagulation pathway. For sample preparation, whole blood was drawn into a neutral tube, prepared with FPX for a final concentration of 8 µg/ml. Specimens were transferred into sodium citrate containing micro tubes immediately after processing. In order to reactivate coagulation in the specimen, blood was recalcified using star-tem 20 (Ref-No.: 503-10; Tem Innovations GmbH, Munich, Germany) adhering to the manufacturer's instructions. For assays of the intrinsic coagulation pathway, in-tem (Ref-No.: 503-02; Tem Innovations GmbH, Munich, Germany) was added, while ex-tem (Ref-No.: 503-05; Tem Innovations GmbH, Munich, Germany) was used for testing the extrinsic pathway. After addition of the respective reagents and citrated blood into plastic cups (Ref-No.: 200011; Tem Innovations GmbH, Munich, Germany) each mixture was analyzed by ROTEM delta (Serial-No.: 5022; Tem Innovations GmbH, Munich, Germany). Read-outs of interest were clotting time (CT), clot formation time (CFT), α angle (α), maximum clot firmness (MCF) and lysis index (LI) of respective minute count (e.g.: LI60 = Lysis index at minute 60).

2.4.6. Flow cytometry (FC)

2.4.6.1. Analysis of platelets in citrated whole blood

In order to obtain detailed data of platelet surface proteins, flow cytometry was performed. For more accurate measurement, the flow rate must not exceed 600 events per second. To ensure this, each sample was diluted to a final platelet count of 20 Giga/L .Considering an average platelet count of 250 Giga/L in whole blood, citrated whole blood was diluted

1:12.5, using 1150 µl of Hank´s buffered Saline Solution (HBSS) -/- with a preadjusted pH of 7.4 and heated to 37°C. 40 µl of this dilution were added to either 10 µl of PBS or respective stimulus, which was provided in a 0.5 ml tube. For stimuli 5 µM ADP (Cat-No.: A-2754; Sigma-Aldrich, St- Lewis, USA), 50 µg/ml Histones or 25 µl/ml Jonosteril with a pH of 1 were used. After addition of the agents, each sample was incubated at 37°C for 10 minutes in a heat bath. Antibodies were used as follows: CD41 (Integrinα2b, Cat-No.: 303710, Clone: HIP8; BioLegend, USA) at a final concentration of 0.75 µg/ml, CD62P (P-Selectin, Cat-No.: 304904, Clone: AK4; BioLegend, USA) at a final concentration of 4 µg/ml and CD 284 (TLR-4, Cat-No.: 304904, Clone: AK4; BioLegend, USA) at a final concentration of 8 µg/ml. As isotype-controls for CD41 (Cat-No.: 400120, Clone: MOPC-21; BioLegend, USA), CD62P (Cat-No.: 400108, Clone: MOPC-21; BioLegend, USA) and CD284 (Cat-No.: 553456, Clone: G155-178; BD Biosciences, USA) immunoglobulins of the same subgroup, with the same label and the same concentration as the respective targeted antibodies were used. Antibodies were added to the solutions and incubated for another 5 minutes at 37°. After this final incubation step, all specimens were transferred into 2 ml of 4°C HBSS -/- with a preadjusted pH of 7.4. In order to obtain a lower flow rate and therefore a more sensitive analysis, 500 µl of the dilution samples were added to 500 µl of 4°C, 7.4 pH HBSS -/- that was provided in 5 ml polystyrene round-bottom tubes (Ref-No.: 352052; Corning Science Mexico S.A. de CV., Mexico).

Analysis of platelets in FPX anticoagulated PRP

After production of PRP, the product was either directly diluted 1:12.5 in HBSS (-/-) or transferred into an MPC-coated tube and incubated for 30 minutes. Following this incubation step, samples were processed in the same way. 40 µl of this dilution were added to either 10 µl of PBS or respective substances, which was provided in a 0.5 ml tube. For platelet activation 5 µM ADP (Cat-No.: A-2754; Sigma-Aldrich, St- Lewis, USA) was used. After addition of the agents, each sample incubated at 37°C for 10 minutes in a heat bath. In order to assess platelet activation, antibodies for CD62P (P-Selectin, Cat-No.: 304904, Clone: AK4; BioLegend, USA) at a final concentration of 4 µg/ml were added. The respective isotype was used accordingly (Cat-No.: 400108, Clone: MOPC-21; BioLegend, USA). After addition of the staining antibodies the solutions were incubated for another 5 minutes at 37°. Following this final incubation step, all samples were transferred into 2 ml of 4°C HBSS -/- with a preadjusted pH of 7.4. Subsequently, 500 µl of the dilution samples were added to 500 µl of 4°C, 7.4 pH HBSS -/- which was provided in 5 ml polystyrene round-bottom

tubes (Ref-No.: 352052; Corning Science Mexico S.A. de CV., Mexico). Each condition was tested at a low flow rate, not exceeding 600 events/ second.

2.4.6.2. Platelet isolation reagents

Table 3: Buffers used for platelet isolation

CPD buffer (citrate-phosphate-dextrose buffer)	16 mM citric acid (Lot-No.: SLBR3765; Sigma-Aldrich, St. Lewis, USA) 90 mM sodium citrate (Lot-No.: STBF5310V; Sigma-Aldrich, St. Lewis, USA) 16 mM NaH_2PO_4 (Lot-No.: 1.06346.0500, Merck KGaA, Darmstadt, Germany) 142 mM dextrose (Lot-No.: SZBD2810V; Sigma-Aldrich, St. Lewis, USA).
Platelet wash buffer	10 mM sodium citrate (Lot-No.: STBF5310V; Sigma-Aldrich, St. Lewis, USA) 150 mM NaCl (Lot-No.: 1.06404.1000, Prod-No.: K50705604843; Merck KGaA, Darmstadt, Germany) 1 mM EDTA (Lot-No.: BZBV5052; Sigma-Aldrich, St. Lewis, USA) 1% (w/v) dextrose (Lot-No.: SZBD2810V; Sigma-Aldrich, St. Lewis, USA)
HEP buffer (HEP refers to HEPES in the buffer)	140 mM NaCl (Lot-No.: 1.06404.1000, Prod-No.: K50705604843; Merck KGaA, Darmstadt, Germany) 2.7 mM KCl (Lot-No.: BCBK6676V; Sigma-Aldrich, St. Lewis, USA) 3.8 mM HEPES (Lot-No.: SLBH4761V, Sigma-Aldrich, St. Lewis, USA) 5.0 mM EGTA (Prod-No.: 324626; Merck KGaA, Darmstadt, Germany)

Tyrode's buffer	134 mM NaCl (Lot-No.: 1.06404.1000, Prod-No.: K50705604843; Merck KGaA, Darmstadt, Germany) 12 mM $NaHCO_3$ (Lot-No.: 1.06329.0500, Prod-No.: K39347429; Merck KGaA, Darmstadt, Germany) 2.9 mM KCl (Lot-No.: BCBK6676V; Sigma-Aldrich, St. Lewis, USA) 0.34 mM Na_2HPO_4 (Lot-No.: 1V009252; AppliChem GmbH, Darmstadt, Germany) 1 mM $MgCl_2$ (Lot-No.: 030M00602V; Sigma-Aldrich, St. Lewis, USA) 10 mM HEPES (Lot-No.: SLBH4761V, Sigma-Aldrich, St. Lewis, USA)

All buffers were adjusted to reach a pH of 7.4 and were stored at 4 °C. Before usage, all buffers were warmed to room temperature.

2.4.6.3. Platelet isolation protocol

Blood was drawn into a 9 ml neutral tube supplemented with 1/10 volume of CPD buffer and mixed gently with the anticoagulant by inverting the container manually. PRP was created by spinning the container at 150 G for 15 minutes. 45 µl of PRP were transferred into a new tube. HEP buffer was added at a 1:1 ratio. Also 1 µM of Iloprost (Lot-No.: SML 1651-1MG; Sigma-Aldrich, St. Lewis, USA) was added to disable platelet activation. The three reagents were mixed gently by hand. Samples were spun at 100g for 15 minutes. Afterwards platelets were retrieved as pellet. The supernatant was discarded, and the pellet rinsed with platelet washing buffer. Platelets were then carefully resuspended into 1 ml of Tyrode´s buffer containing 3 mg/ml of freshly added BSA.

2.4.6.4. Analysis of isolated platelets

After platelet isolation, 40 µl of platelet solution was added to 10 µl of PBS supplemented with or without a stimulating agent. Samples were incubated for 10 minutes at 37°C in a heat bath. After the first incubation step, antibodies were added. To stain platelets for TLR2 (Cat-No.: 309721, Clone: TL2.1; BioLegend, USA) and TLR4 (Cat-No.: 312805, Clone: HTA125; BioLegend, USA), antibodies were added at a final concentration of 1.9 µg/ml. In the same concentrations, isotypes of the TLR4 (Cat-No.: 400211, Clone: MOPC-173;

BioLegend, USA) and TLR 2 (Cat-No.: 400253, Clone: MOPC-173; BioLegend, USA) antibodies were added for control samples. In order to assure binding to the respective antigen, samples were incubated for another 5 minutes, before being transferred into fluorescent absorbance cell sorting (FACS) falcons spiked with 1 ml of ice cold HBSS -/- with a preadjusted pH of 7.4.

2.4.6.5. Calculation of absorbance relative to isotype

Two replicants of isolated platelets each were incubated alongside 50 µg/ml histones or isovolumetric PBS as a control for 10 minutes. Afterwards, one of each was stained for TLR 4 (Cat-No.: 312805, Clone: HTA125; BioLegend, USA) while the other one was stained with the respective isotype (Cat-No.: 400211, Clone: MOPC-173; BioLegend, USA) in the same concentration of 1.9 µg/ml. Then, the geometric mean absorbance intensity for isotypes was set to 1 and values for its amplification of this were calculated by dividing the geometric mean absorbance intensity of the antibody by the geometric mean absorbance intensity of the isotype.

2.3. Statistics and analysis

For statistical testing as well as graphical depiction of the data, GraphPad Prism (Version 8.4.3, GraphPad Software, USA) was used. Conditions including multiple replicants were represented as their calculated mean ± 95% confidence interval (CI) in the graphs. For every data set, descriptive statistics were acquired. For statistical testing, one-way ANOVA (Analysis of variance) was performed with Tukey´s test as the post-hoc test for multiple comparisons. As sphericity could not be assumed, tests were performed utilizing the Geisser-Greenhouse correction. Values for $p < 0.05$ were considered statistically significant. Outlier testing was performed using the ROUT method set for Q = 5%.

3. Results

3.1. Platelet rich Plasma (PRP)

3.1.1. Comparison of FPX anticoagulated PRP and citrate anticoagulated PRP

To assess the effects of the defined stimulating agents on platelets and the coagulation system while excluding the impact of other blood cells, purified PRP was generated by a newly established centrifugation protocol. The manually obtained thrombocyte counts of the generated PRP samples showed a mean yield of 970 Giga/L (± 250), which was nearly 2.5-fold the concentration of the baseline (BL) at 345 Giga/L (± 58) (Figure 3A). Values are presented as mean ± standard deviation (SD). The Baseline here is defined as blood, freshly drawn from the vein. Complete blood counts from the routine laboratory of the University Hospital of Ulm confirmed that no blood cells other than platelets could be found in the generated PRP. Since platelets were exposed to 150 G and therefore highly stressed, the avoidance of activation by processing before stimulation had to be ensured. Therefore, commonly used soluble platelet activation markers such as sCD40L and vWF concentrations were determined before and after PRP generation for the evaluation of platelet activation. Since the common standard for PRP preparation is citrated blood, it was processed with the same protocol for the control group. There were no significant differences in the CD40L and vWF levels in the generated PRP compared to the baseline (Figure 3B).

Flow cytometric analysis of platelets in citrated whole blood compared to FPX based PRP showed an undefiled product of centrifugation with less than 1% of cells belonging to the manual set gate for other blood cells than platelets. Further, PRP samples before and after a 30-min incubation period were compared in terms of baseline activation and activatability by 5 mM ADP (Figure 3C). Both approaches showed no activation without the addition of ADP measured by superficial CD62P exposure on the platelets. In presence of the agonistic agent however, the measured density of CD26P on the platelet surface increased. As displayed exemplary below, the mean fluorescent intensity after the addition of ADP did not change after 30 min of incubation compared to fresh PRP.

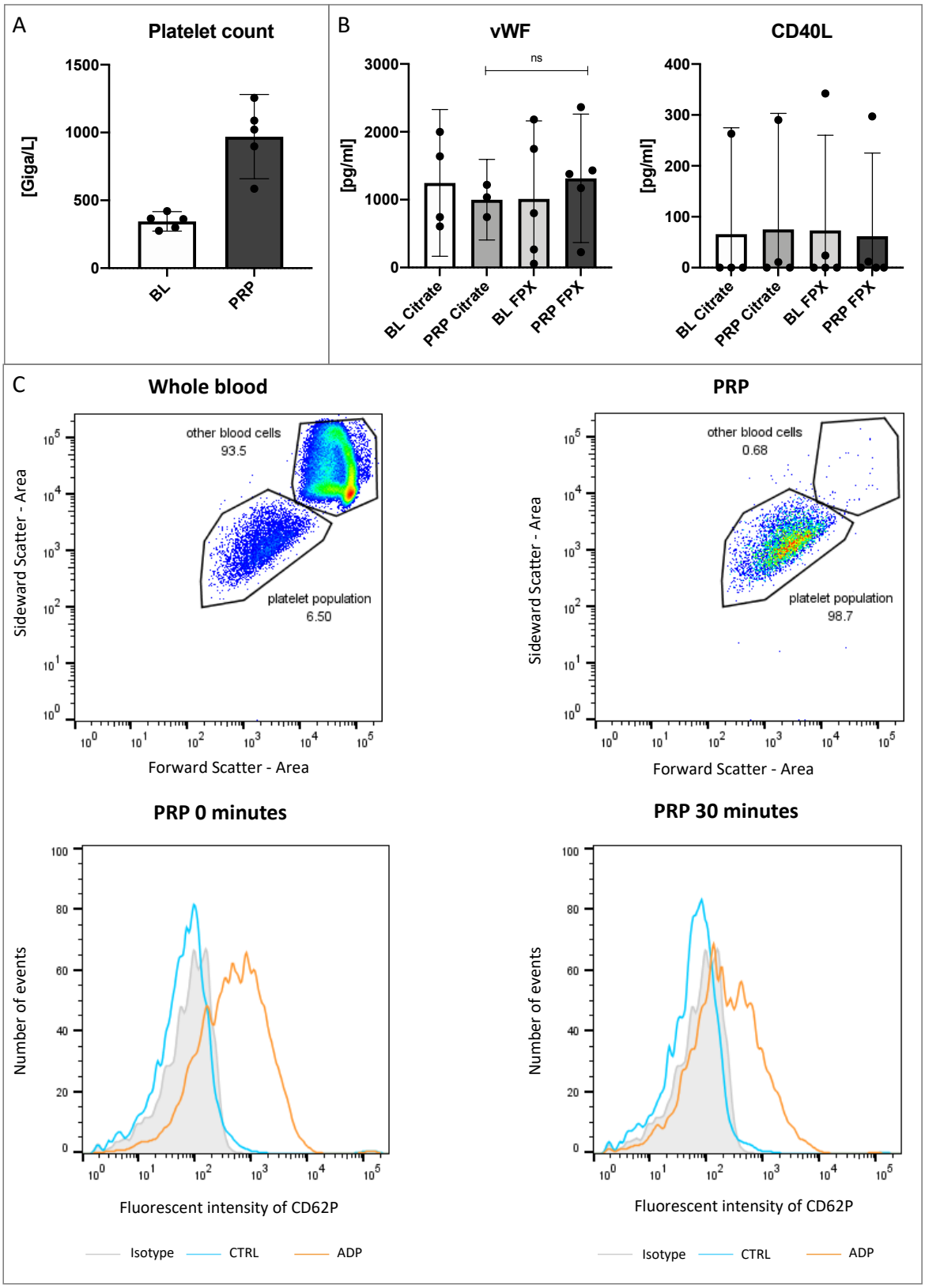

***Figure 2** PRP generation and analysis of platelet baseline activation and reactivity to ADP. Fondaparinux (FPX) anticoagulated whole blood was spun at 150G in order to obtain platelet rich plasma (PRP). (A) Microscopic platelet count taken before (BL=Baseline) and after (PRP) processing of the samples, showed a significant increase in platelet counts. (B) vWF and sCD40L concentrations of fresh venous blood (BL) treated with citrate or FPX in comparison to the concentrations after PRP preparation (PRP), respectively, showed no significant alteration by the PRP generation process. (C) Flow cytometric analysis of platelets in PRP showed a pure product containing less then 1% of cells exceeding the manual set platelet scatter. Also, increased superficial CD62p exposure could be seen on platelets upon stimulation with 5 mM ADP. ns=not significant. The experiments were repeated to an n = 5.*

3.1.2. Lethal triad simulation in platelet rich plasma

Hemodilution was simulated by replacing 30% of the sample with Jonosteril. For hypothermic conditions, samples were incubated at an ambient temperature of 34 °C. Acidosis was attained by adjusting the pH to 7.369 (min. 7.32 - max. 7.39) for a mild (LT°I) and 7.01 (min. 6.98 – max. 7.09) for a more severe (LT°II) LT condition (Figure 4C). Except for BL samples, all samples were incubated for 30 min before further processing. The concentration of platelets decreased upon dilution. The platelet counts for the control group (CTRL) was 676.0 Giga/L (± 184.8), while LT°I was 470 Giga/L (± 126.4) and LT°II was 456.5 Giga/L (± 170.5) reflecting an extent of dilution of about 70% (Figure 4A). In terms of routine laboratory coagulation parameters, PTT as measurable value for the intrinsic coagulation pathway, was not significantly impaired by the given conditions. PTT test showed mean clotting times of 38.8 s (± 5.8) for control, 40.6 s (± 6.0) for LT°I and 42.2 s (± 6.2) for LT°II. The Quick could be impaired significantly by both LT conditions, albeit stronger by the more acidotic environment. The mean Quick was 72.4 % (± 6.2) for control, 59.0 % (± 5.7) for LT°I and 55 % (± 5.8) for LT°II. Values are presented as mean ± standard deviation (SD).

No signs of platelet activation were found by analyzing plasma samples of each specimen for vWF and sCD40L. Although the average vWF concentration in LT concentrations seemed to be slightly elevated, it failed to reach statistical significance (Figure 4B).

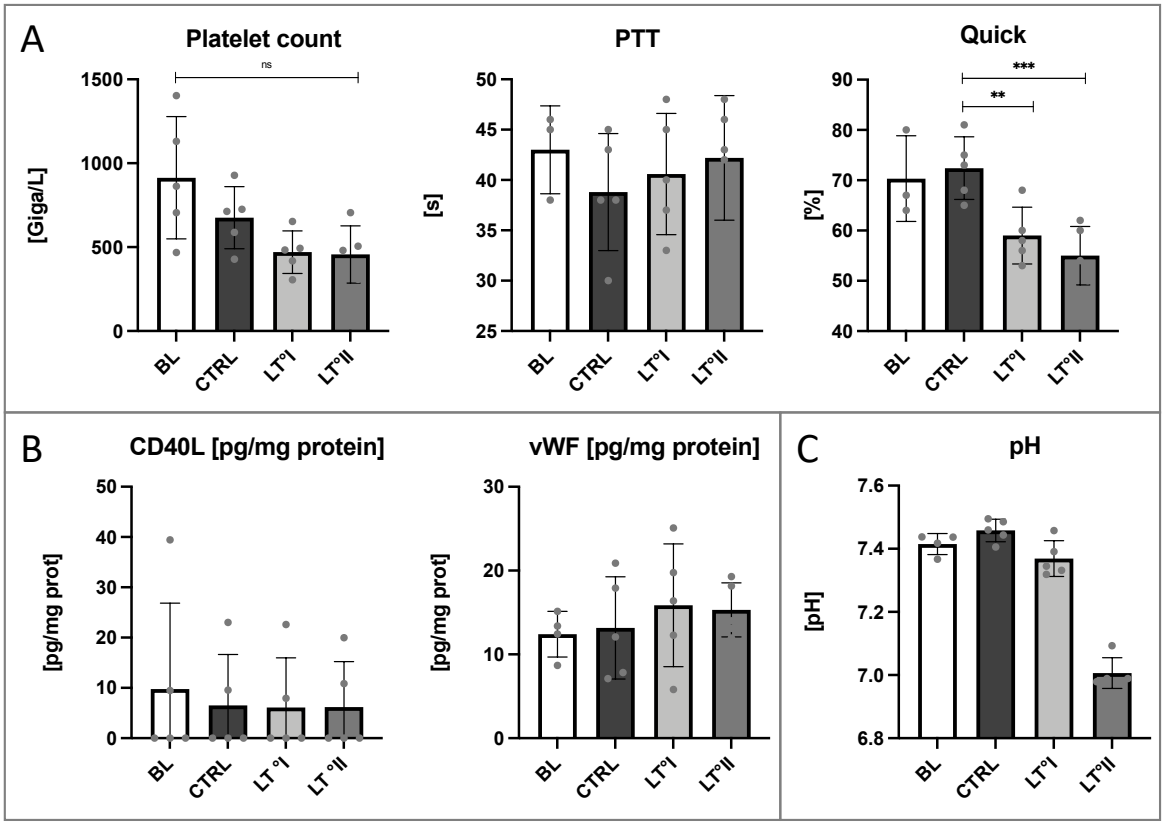

Figure 3 ***The impact of lethal triad on platelet counts, routine laboratory coagulation parameters and platelet activation markers in PRP.*** *(A) Platelet concentrations, Quick and PTT of freshly produced (BL) and 30-minute incubated PRP (CTRL) didn´t show any significant alteration compared to each other. Upon simulation of a mild (LT°I) or severe (LT°II) form of lethal triad, only Quick showed a statistically significant decrease. (B) vWF and sCD40L concentrations didn´t show platelet activation in any of the given conditions. (C) pH values for each replicant were tested via blood gas analysis. As these values only act as reference, they were not tested for significance. pH was 7.46 (7.41-7.49) for CTRL, 7.37 (7.32-7.39) for LT°I and 7.01 (6.98-7.09) for LT°II. * = p<0,05; ** = p<0,01; *** = p<0,001; **** = p<0,0001; ns=not significant. The experiments were repeated to an n=5. For coagulation parameters, two replicant´s values are missing due to technical difficulties.*

3.2. Impact of DAMPs on coagulation

3.2.1. The effect of the lethal triad and HMGB1 on laboratory coagulation parameters and platelet activation markers

To characterize the effects of pathological levels of the DAMP HMGB1 on coagulation and platelets, FPX anticoagulated whole blood was incubated for 30 min at 37°C with HMGB1 added to a final concentration of 500 ng/ml and without. The combined impact of LT and HMGB1 was also simulated. After incubation, samples were analyzed for platelet concentrations, vWF and sCD40L levels in plasma as well as for routine coagulation parameters. In the observed 30-min period, stimulation with HMGB1 in both whole blood and PRP did not show any statistically significant decrease in platelet counts or increase in platelet activation markers respectively (Figure 5A and B). ELISAs of PRP and whole blood samples did not show a statistically significant increase in vWF or sCD40L after addition of HMGB1, nor after the simultaneous admission of HMGB1 and LT. In contrast HMGB1 added under LT conditions led to significant decreases of platelet concentrations in both PRP and whole blood. While platelet counts decreased relatively moderately in PRP, some samples in whole blood appeared to attain platelet counts close to zero, which was mostly due to clotting of the sample. The mean platelet count in PRP was 513 Giga/L (± 93.0) for CTRL group, 503.9 Giga/L (± 84.5) in HMGB1 and 292.1 Giga/L (± 137.1) in HMGB1+LT°II displaying levels close to the ones expected by dilution. In whole blood the mean platelet count was 206.7 Giga/L (± 66.1) in CTRL group, 216.1 Giga/L (± 53.8) in HMGB1 and 59.3 Giga/L (± 68.4) in HMGB1+LT°II. Values are presented as mean ± standard deviation (SD).

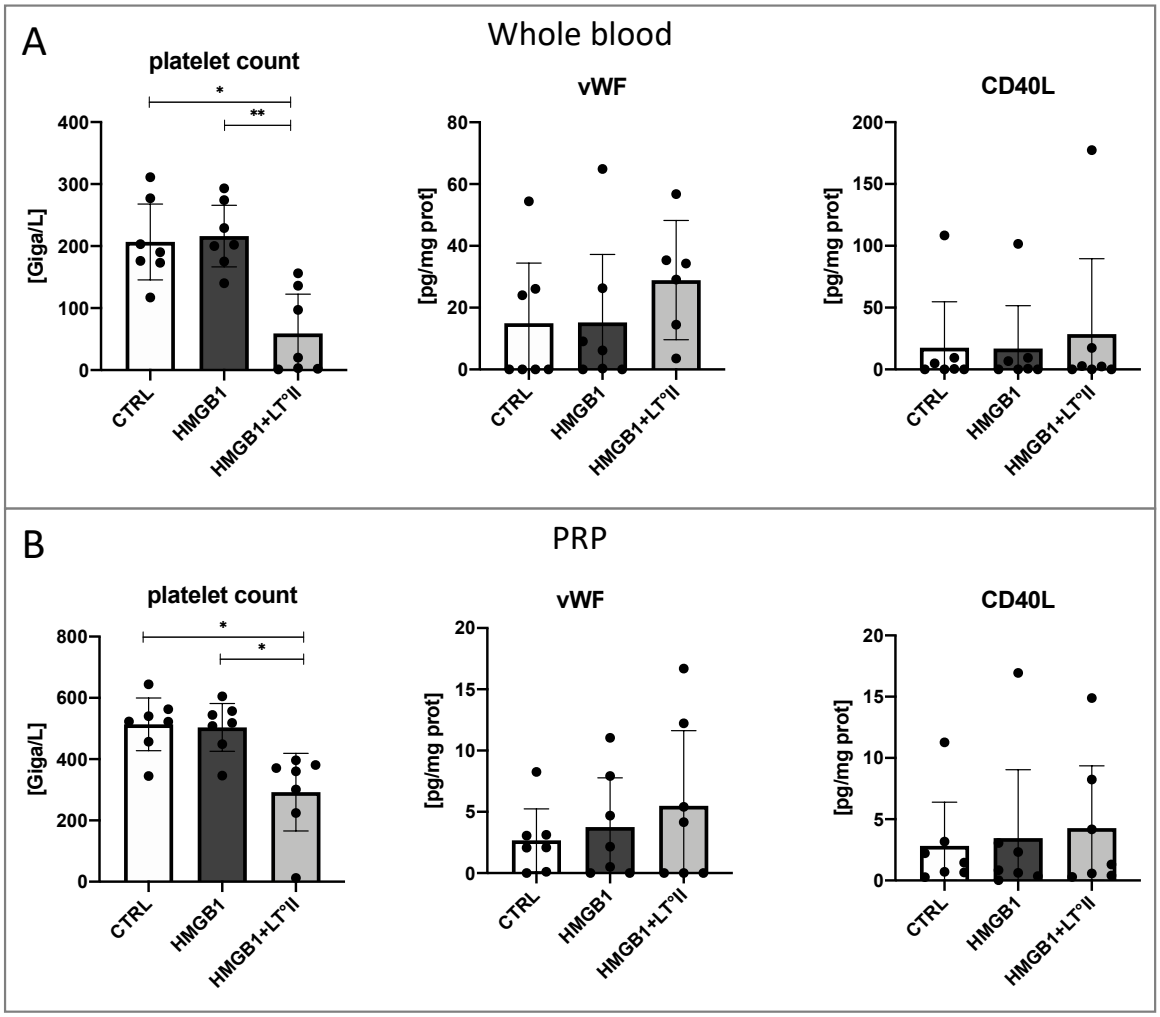

***Figure 4** The impact of HMGB1 in combination with lethal triad on platelet counts and platelet activation parameters in whole blood (A) and platelet rich plasma (PRP) (B).* *(A) Platelet counts, vWF and sCD40L concentrations after 30-minutes incubation in addition of 500 ng/ml HMGB1 (HMGB1) or the respective loading volume of PBS (CTRL) did not show any alterations. For samples in which lethal triad was simulated additionally (LT°II), a decrease in platelet counts was seen. vWF and sCD40L levels did not change in a significant manner. (B) Respective data in platelet rich plasma revealed similar findings. pH in whole blood was 7.35 (± 0.05) for CTRL and 7.25 (± 0.07) for LT°II, while in PRP it was 7.49 (± 0.04) for CTRL and 7.02 (± 0.08) for LT°II. Alterations in pH are displayed as standard deviation (SD). * = p<0,05; ** = p<0,01; ns=not significant. The experiments were repeated to an n=7.*

In terms of coagulation parameters, Quick and PTT were tested (Figure 6). The Quick showed a significant decrease in both whole blood and PRP, when incubated under LT conditions. The mean Quick in whole blood was 80 % (SD ± 9.9) in CTRL, 78.3 % (± 8.2) in HMGB1 and 45.7 % (± 25.0) in HMGB1 + LT°II. In PRP, the mean values for Quick were 73.9 % (± 4.7) in CTRL, 73.7 % (± 3.3) in HMGB1 and 57.7 % (± 7.0) in HMGB1 + LT°II. PTT appeared to be significantly prolonged in whole blood under LT condition while exposed to HMGB1 (Figure 6A). In PRP no changes regarding PTT were detectable (Figure 6B). Stimulation by HMGB1 on its own failed to impact coagulation parameters in any

significant manner. Yet, a slight shift towards higher PTT times was observable in whole blood. The pH in whole blood was 7.35 (± 0.05) for CTRL and 7.25 (± 0.07) for LT°II, while in PRP it was 7.49 (± 0.04) for CTRL and 7.02 (± 0.08) for LT°II. Values are presented as mean ± standard deviation (SD).

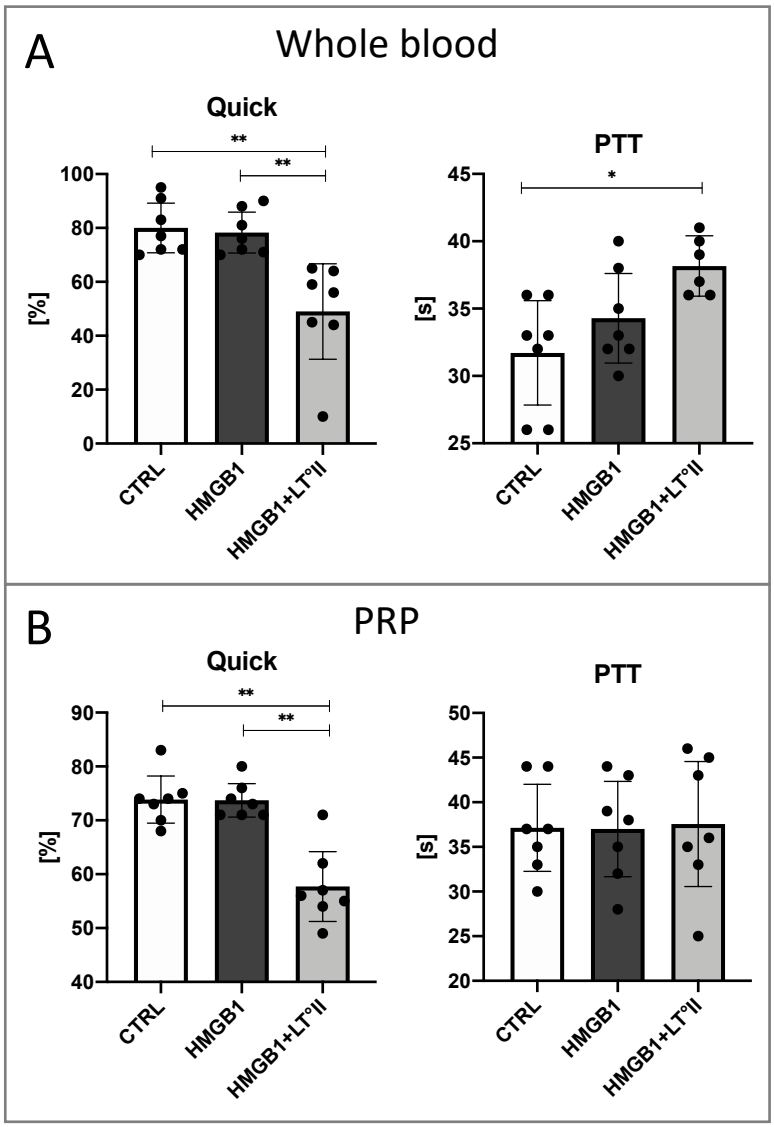

***Figure 5** The impact of HMGB1 and lethal triad on laboratory coagulation parameters in whole blood (A) and PRP (B).* *(A) Quick and PTT after 30-minutes incubation of whole blood containing 500 ng/ml HMGB1 (HMGB1) compared to control samples (CTRL) failed to induce statistically significant alterations in Quick and PTT. Specimen in which lethal triad was simulated (LT°II) showed altered coagulation parameters. (B) Respective data in platelet rich plasma revealed similar results regarding Quick, while PTT wasn´t impaired by any condition. * = p<0,05; ** = p<0,01. The experiments were repeated to an n=7.*

3.2.2. The effect of Histones in combination with the lethal triad on platelet counts, activation markers and coagulation parameters in platelet rich plasma

The effects of histones and LT on the hemostatic system was assessed in platelet rich plasma. PRP samples were stimulated by 50 µg/ml histones in final concentration alone or under additional mild or severe LT conditions and incubated for 30 min. While platelet counts were reduced significantly upon incubation alongside histones, vWF as well as sCD40L concentrations in the plasma of respective samples increased accordingly. When samples were exposed to the DAMPs under LT conditions, histones induced an even stronger effect (Figure 7A).

Mean platelet counts were 1154 Giga/L (SD = 327.7) for CTRL, 193.9 Giga/L (SD = 131.1) for Histones, 24.6 Giga/L (SD = 16.0) for Hist + LT°I and 7.5 Giga/L (SD = 6.1) for Hist + LT°II. Platelet activation as displayed by vWF reached plasma levels of 13.2 pg/mg protein (SD = 11.3) for CTRL, 41.4 pg/mg protein (SD = 12.5) for histones, 55.4 pg/mg protein (SD = 22.0) for Hist +LT°I and 54.68 pg/mg protein (SD = 22.1) for Hist + LT°II. Concentrations of sCD40L in plasma was 6.3 pg/mg protein (SD = 6.0) in CTRL, 17.0 pg/mg protein (SD = 8.8) for histones, 16.4 pg/mg protein (SD = 9.1) for Hist + LT°I and 17.4 pg/mg protein (SD = 10.1) for Hist + LT°II.

Histones on their own failed to change PTT in a statistically significant manner. Under this condition the Quick value decreased. Samples in which histones were incubated under LT conditions showed strongly impaired coagulation parameters (Figure 7B). A Trend, that shows a stronger impact on coagulation in severe LT than in the mild one could be observed, however, this trend did not end up being statistically significant.

Quick values were 80.3 % (SD = 6.3) for CTRL, 87.4 % (SD = 7.1) for Histones, 32.4 % (SD = 28.2) for Hist + LT°I and 10 % (SD = 0.0) for Hist + LT°II. Partial thromboplastin times were 34.7 s (SD = 6.9) in CTRL, 34.7 s (SD = 9.9) for Histones, 101.1 s (SD = 74.2) for Hist + LT°I and 163.7 s (SD = 37.3) in Hist + LT°II.

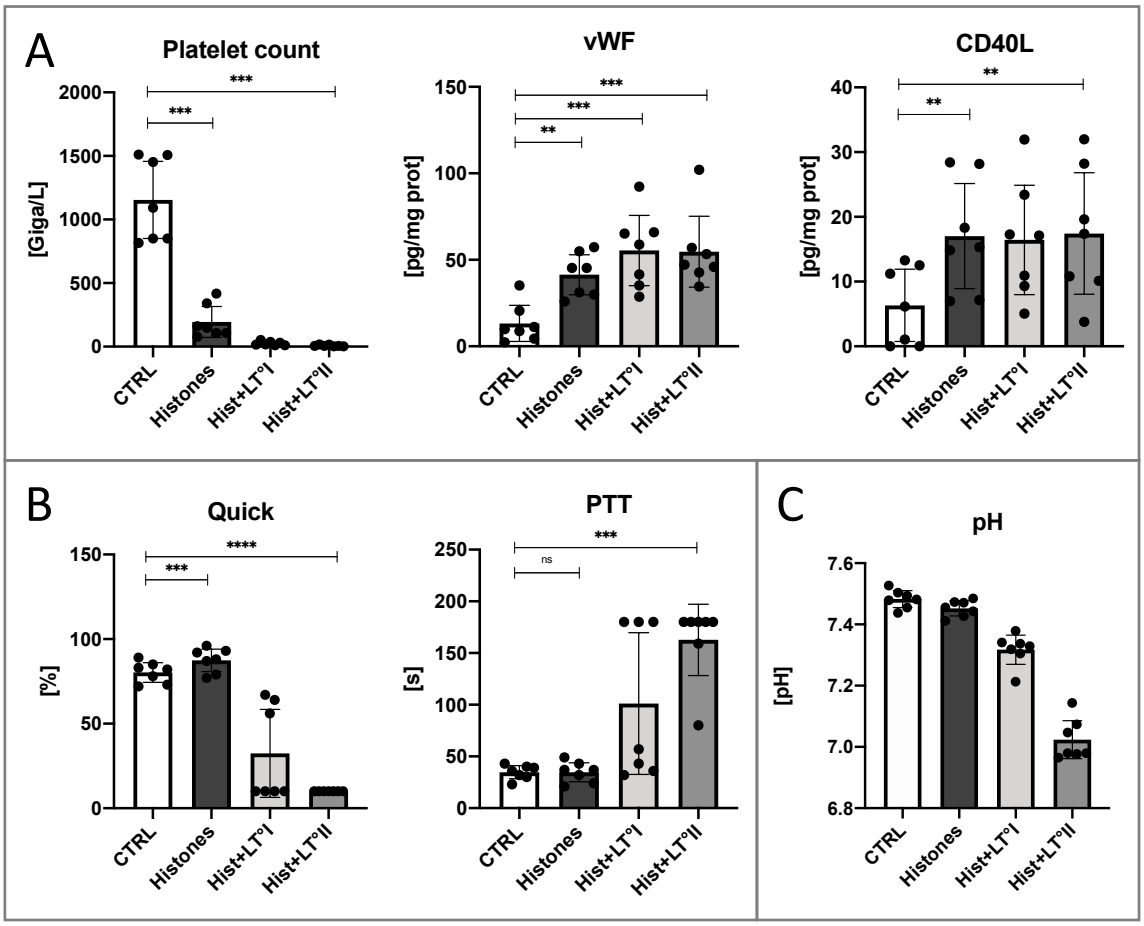

***Figure 6** **Coagulation parameter variation and platelet activation markers in PRP after stimulation with Histones and Lethal triad.** (A) Platelet, vWF and sCD40L concentrations in samples showed a rapid decrease of platelet counts after addition of 50 µg/ml histones (Histones/Hist). This effect was also amplified by additional simulation of a mild (LT°I) or severe (LT°II) form of lethal triad. Simultaneously, vWF and sCD40L concentrations increased in respective samples. (B) Quick and PTT of the conditions described earlier worsened in a similar pattern as the platelet counts. (C) pH of respective samples was determined by BGA for quality assurance. As these values only act as reference, they were not tested for significance. pH was 7.48 (+- 0.03) for CTRL, 7.32 (+-0.05) for LT°I and 7.02 (+-0.07) in LT°II. Alterations in pH are displayed as standard deviation (SD). * = p<0,05; ** = p<0,01; *** = p<0,001; ns=not significant. The experiments were repeated to an n=7.*

3.2.3. Concentration-dependent decrease of platelet counts in human whole blood and PRP by Histones

To investigate the concentration-dependency of the effect of histones on FPX anticoagulated whole blood and FPX anticoagulated platelet rich plasma, respective material was added by histones at a final concentration of 10, 20, 40, 80 or 120 µg/ml and incubated for 30 min in MPC-coated tubes. For the experiments each sample type, whole blood and PRP, were used to examine the effects of the DAMPs in either default or LT conditions. Results showed a concentration-dependent reduction of platelet counts induced by histones in both PRP and whole blood (Figure 8A and B). Platelet counts dropped even more when samples were incubated under LT conditions. The decrease in platelet concentrations overall appears to be more severe in PRP than in whole blood.

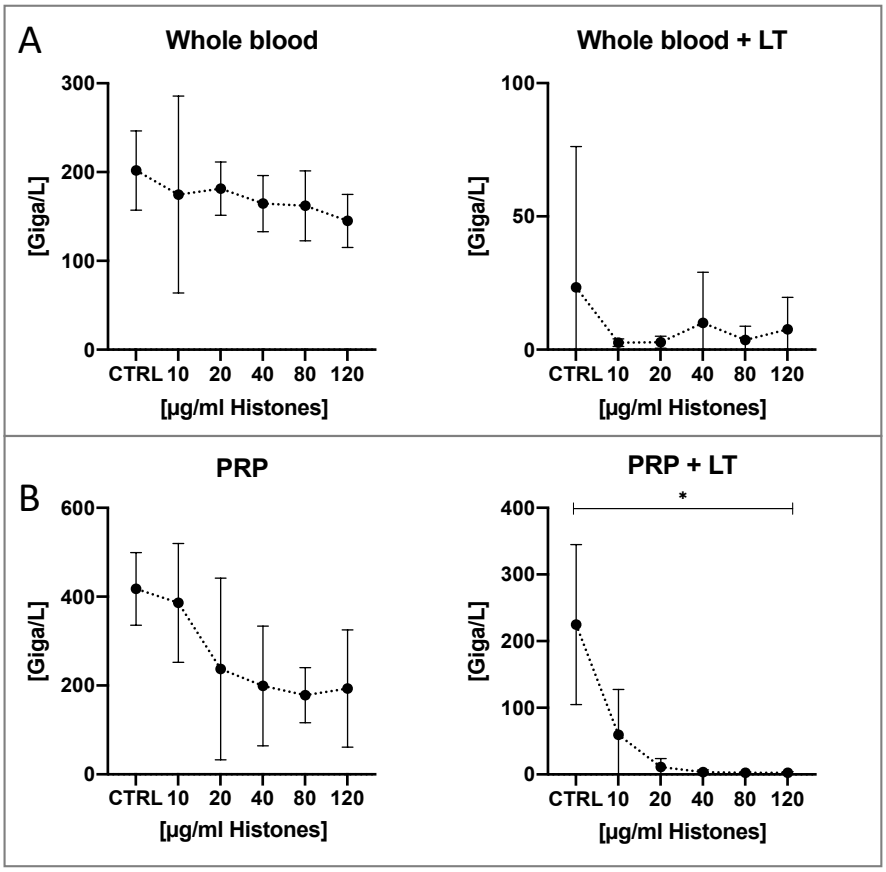

***Figure 7** concentration-dependency of the effects of histones on platelet concentrations in whole blood and PRP with (+LT) and without Lethal triad. Histone concentrations of 10, 20, 40, 80 and 120 µg/ml, respectively, were tested. Lethal triad was simulated as described earlier under LT°II. (A) Whole blood samples showed a trend to a dose-dependent reduction of platelet counts by histones. In combination with lethal triad, most samples were completely free of platelets due to clot generation. (B) in PRP similar effects as in whole blood were seen. However, the effect of histones in combination with Lethal traid were displayed more clearly. * = p<0,05. n=5/condition.*

Next, the coagulation parameters Quick and PTT were tested in the replicants of whole blood. Also, ELISAs were also performed in order to obtain CD40L and vWF concentrations in plasma samples yielded from each specimen (Figure 9A). The results revealed a trend towards higher vWF and CD40L levels as concentrations of histones increased.

While the Quick and thereby INR only showed a stronger impairment of coagulation as the concentration of histones increased, PTT changed significantly from 20 µg/ml compared to 80 µg/ml or 120 µg/ml (Figure 9B). PTT was 39.8 s (SD = 3.3) for CTRL, 38.2 s (SD = 2.8) for 10 µg/ml, 35.8 s (SD = 4.0) for 20 µg/ml, 39.0 s (SD = 10.0) for 40 µg/ml, 48.2 s (SD= 6.0) for 80 µg/ml and 60.6 s (SD = 8.8) for 120 µg/ml.

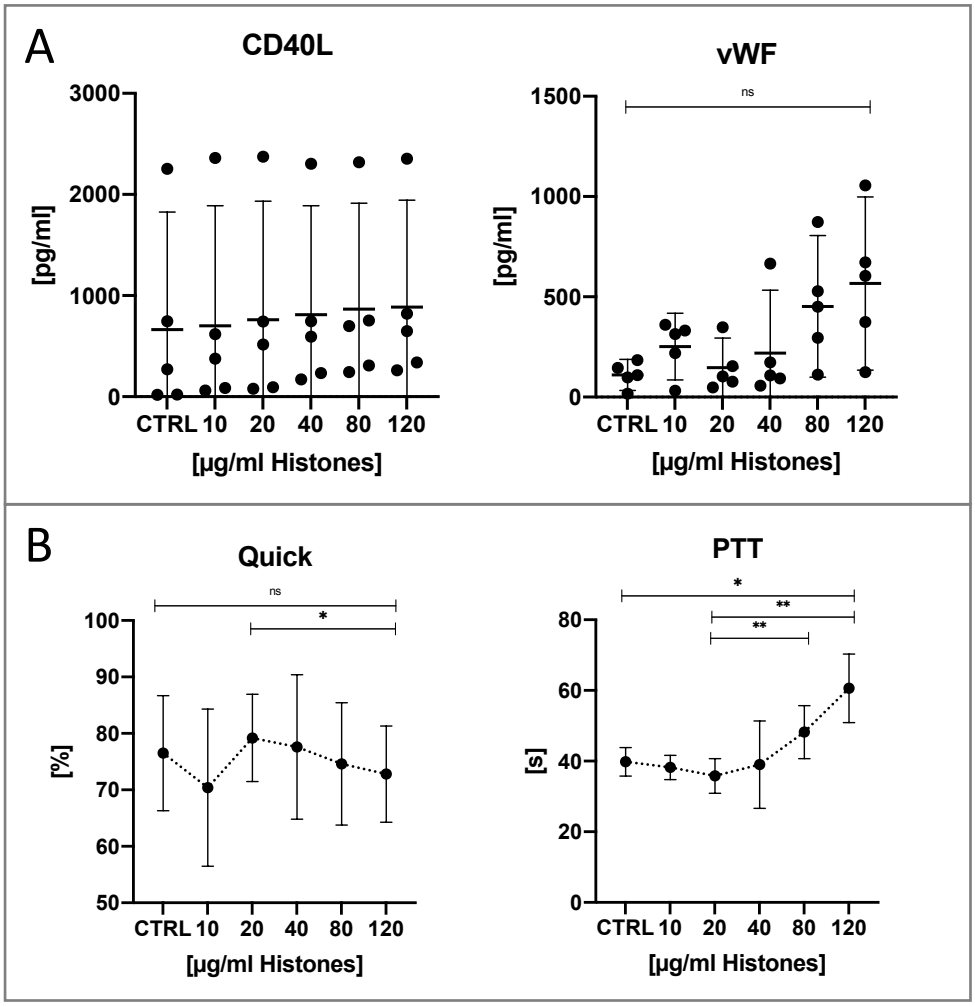

***Figure 8** Dose dependent effect of histones on PTT and Quick as well as sCD40L and vWF concentrations in whole blood. Histone concentrations of 10, 20, 40, 80 and 120 µg/ml were tested. (A) Concentrations of sCD40L and vWF in plasma samples showed a tendency towards higher levels of the platelet activation markers as histone concentrations increased. Even though this trend appears to be more pronounced for vWF, (B) While Quick didn´t change significantly when compared to control samples, PTT was prolonged by Histone concentrations higher than 80 µg/ml. * = p<0,05; ** = p<0,01; ns=not significant. The experiments were repeated to an n=5.*

3.2.4. Impairment of intrinsic coagulation as well as maximal clot firmness by histones

To validate previous results of histone-induced PTT impairment, histone concentrations of 120 µg/ml were added to FPX-anticoagulated whole blood, which was citrated either immediately or after a 30 min incubation at 37°C. The equivalent volume of PBS was added to whole blood from the same donor and processed in the same way to serve as a control. 30-min samples were added into citrate tubes after the incubation period. Each sample was analyzed for intrinsic (INTEM) and extrinsic (EXTEM) coagulation in ROTEM. Clotting time (CT) in INTEM but not EXTEM showed a significant increase of 297.4 s (SEM = 10.5) in control compared to 2185 s (SEM = 458.4) without incubation upon histone stimulation. After 30 min, however, the effect was attenuated, showing a CT of 220.8 s (SEM = 19.3) for control and 975.5 s (SEM = 358.6) upon histone stimulation (Figure 10). Alongside CT, clot formation time (CFT) was also prolonged in samples containing histones in INTEM. Matching that, the alpha angle also showed a slower generation of the clot in samples contained histones in INTEM. In EXTEM, only the CT of the 30-min incubated histone sample showed an impairment. CFTs in EXTEM appeared to be longer in the presence of histones than without, but this effect was not statistically significant.

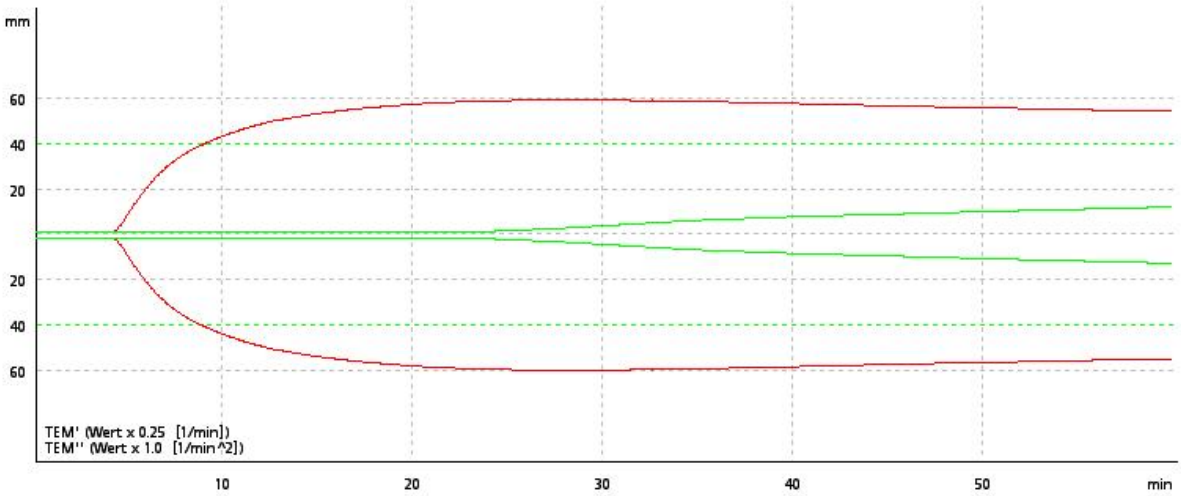

***Figure 9** **Representative image of a Rotem measurement**. Shown is INTEM of 0 min CTRL (red) vs. 0 min Histones (green). The y-axis describes the clot size in mm, while the x- axis shows time in minutes. Clotting time shows the time from the beginning of clotting, until the clot as reached 2 mm in size. Clot formation time is given as the time, it takes to generate a clot of 20 mm size. Nicely to see here is, that CT noticeably differs between control and histones. The histone samples seen here does not even attain a clot size of 20 mm. n=10*

***Table 4 The impact of histones on ROTEM parameters**. Samples were added to 120 µg/ml histones (Histones) or respective volume of PBS (CTRL). Measurements were done without incubation (0 min) or after a 30-min incubation period (30 min). Clotting time (CT), Clot formation time (CFT), Maximal clot firmness (MCF), Alpha-angle (Alpha) and maximum lysis (ML) are displayed. ANOVA performed with multiple comparisons found Changes in CT, MCF, Alpha and ML in INTEM, as well as MCF, and ML in EXTEM as affected statistically significant by histones compared to controls. Values are displayed as mean ± standard error of the mean. * = p<0,05; ** = p<0,01; *** = p<0,001; ns=not significant. The experiments were repeated to an n=10.*

		Groups				
		CTRL 0 min	CTRL 30 min	Histones 0 min	Histones 30 min	ANOVA
INTEM						
	CT [s]	297,4 (± 10,5)	220,8 (± 19,3)	2185 (± 458,4)	975,7 (± 358,6)	**
	CFT [s]	118,2 (± 7,7)	114,2 (± 11,8)	897,3 (± 336,0)	409,3 (± 98,2)	ns
	MCF [mm]	58,4 (± 1,3)	57,0 (± 1,8)	32,6 (± 7,9)	41,4 (± 5,2)	**
	Alpha [°]	66,8 (± 1,4)	68,1 (± 2,0)	30,0 (± 9,2)	34,1 (± 6,9)	***
	ML [%]	6,7 (± 1,5)	7,2 (± 1,1)	0,0 (± 0,0)	1,0 (± 0,5)	***
EXTEM						
	CT [s]	90,4 (±5,9)	82,0 (± 5,0)	88,9 (± 2,8)	118,9 (± 26,0)	ns
	CFT [s]	116,6 (± 20,6)	126,6 (± 16,6)	158,6 (±17,8)	168,9 (±27,5)	ns
	MCF [mm]	59,7 (± 2,5)	55,1 (± 2,9)	58,2 (± 1,7)	51,4 (± 3,2)	*
	Alpha [°]	68,2 (± 3,0)	66,4 (± 2,5)	65,6 (± 2,8)	62,3 (±3,5)	ns
	ML [%]	6,8 (±1,7)	7,3 (±0,8)	1,7 (± 0,8)	2,3 (± 0,8)	**

3.3. Effects of surface TLRs on platelets after histone stimulation

Citrated whole blood was analyzed by flow cytometry after a 15 min incubation at 37°C with stimulation by 5 µM ADP, 25 µl/ml Jonosteril set to the pH of 1, 50 µg/ml histones or without stimulation for the control group. CD41-positive gated cells were defined as platelets and tested for CD284 (TLR4). While the mean fluorescent intensity (MFI) increased slightly, however, still significantly from the control to those stimulated with ADP, it appeared to decrease at acidotic conditions. The MFI of histone-stimulated platelets was 30-fold higher than the other conditions and was found to be statistically significant in comparison to any of them. Interestingly, only 18% (min 14%- max. 22%) of the platelets in the control group were found to express TLR4 on their surface. Upon stimulation by ADP or during acidotic conditions, this fraction seemed to shrink even further. However, when incubated with histones almost all platelets became positive for CD284 (Figure 11).

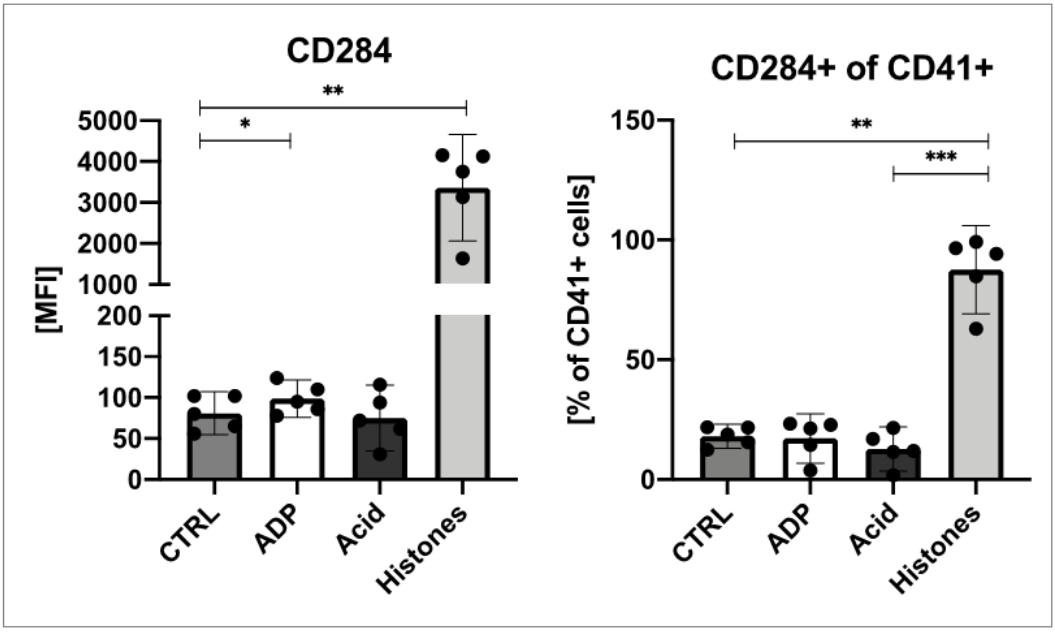

***Figure 10** TLR4 expression on platelets after stimulation with different agents. Stimulation by 5 µM ADP (ADP) was able to enhance mean fluorescent intensity (MFI) of a TLR4 (CD284) antibody. 50 µg/ml histones (Histones) were able to increase this parameter even 10-fold. In contrast to that, acidosis (Acid) seemingly decreased it. In comparison to control samples (CTRL), only Histones were able to raise the proportion of platelets bearing TLR4. When in control only 18.1% (+-4.0) of platelets were positive for CD284, after stimulation with histones this value increased to 87.5% (+-14.8). Variances among replicants are displayed as standard deviation. * = p<0,05; ** = p<0,01; *** = p<0,001. The experiments were repeated to an n=5.*

To ensure, that these effects were due to a direct impact of histones on platelets, isolated platelets were stimulated with 50 µg/ml histones too. After a total of 15 min incubation, samples were tested for TLR2 and TLR4 by flow cytometry (Figure 11 A and B). Like in whole blood, the total amount of platelets bearing both TLRs increased to almost 100%. The MFI also increased 10-fold for TLR4 and 1000-fold for TLR2.

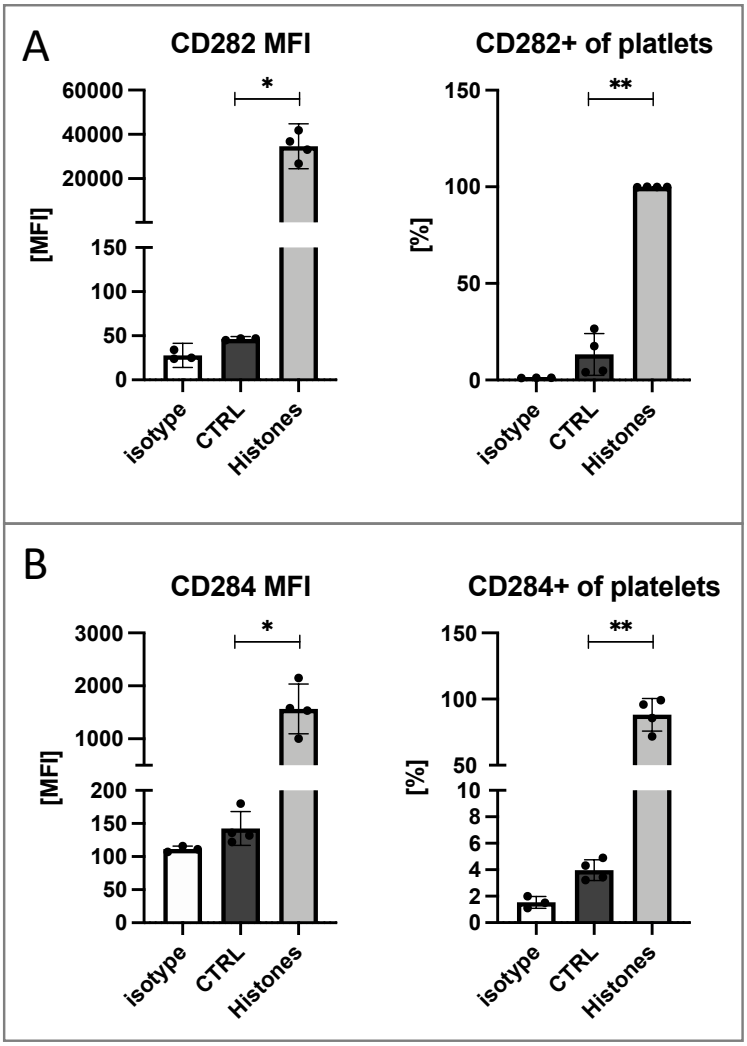

***Figure 11** **The impact of histones on TLR expression on isolated platelets.** (A) TLR 2 antibodies mean fluorescent signal on platelets increased near 1000-fold when incubated alongside histones. Also, 99.9% (+- 0.03) were found positive for this receptor after histone stimulation, while only 13.3 (+-10.8) were positive for it in control samples. (B) Similar findings regarding TLR4 as seen in TLR 2. MFI increased by 10-fold, while overall proportion of TLR4-positive cells increased from 4.0% (+- 0.78) in control samples to 88.2% (+- 12.32) in Histone stimulated platelets. Variances among replicants are presented as standard deviation. * = p<0,05; ** = p<0,01*

Further experiments showed that upon mixing histones and isolated platelets, all antibodies led to an immense fluorescent signal on the platelet surface. In order to identify the actual effect of platelets without the disruption of any antibody being bound on the platelet surface, isotypes and the respective antibodies` fluorescent intensities were

tested upon admission of histones or PBS for control. Control groups showed a 1.52-fold (CI_{95} = 0.62 – 2.23) and histone groups a 2.04-fold (CI_{95} = 1.17-2.91) increase in geometric mean intensity when analyzed for CD284. The calculation method was used as previously stated.

4. Discussion

4.1. The impact of the lethal triad on coagulation in FPX anticoagulated PRP

The Lethal triad (LT) impairs coagulation through a complex set of interactions with the hemostatic system [18], [29]. For example, acidosis and hypothermia each impair FVIIa activity on phospholipid surfaces, resulting in less FXa generation after TF admission [69]. While these findings indicate an effect of acidosis and hypothermia on the extrinsic tenase complex, the intrinsic tenase showed no sign of impairment by pH alterations as low as 6.8 [33]. ROTEM data comparing intrinsic and extrinsic coagulation indicate a selective affection of the extrinsic pathway by acidosis, while hypothermia was able to impair both pathways [29]. However, hypothermia of 33°C showed a significant impact in EXTEM CT, while INTEM CT temperatures as low as 30 °C were necessary to attain a coagulation attenuating effect [29]. Some findings even indicate that mild hypothermia is able to increase platelet aggregability [88]. Since LT was simulated at 34°C in the present study, a stronger impact on the coagulation parameters displaying extrinsic coagulation was anticipated. Detecting a selective impairment of Quick but not PTT when simulating LT conditions supports this theory. Hemodilution was not able to significantly impair intrinsic coagulation. A stronger reduction of the Quick value was detected in the severe acidosis group than in the milder one compared with the control group, while PTT on the other hand was not affected by pH change at all. These results indicate a pH, temperature and dilution-dependent impairment of the extrinsic, but not the intrinsic coagulation pathway in accordance with prior findings [18], [29], [69]. As the extrinsic pathway is often said to be the main initiator of the coagulation cascade it seems reasonable, that upon attenuation of the TF-pathway, hemostasis dysfunctions in a clinically relevant manner.

The impact of acidosis on platelets was also investigated earlier and did not show an alteration of surface markers such as vWF and CD40L [35]. However, both acidosis and hypothermia are known to change the platelet response to agonists [35], [87]. Soluble CD40L and vWF concentrations in plasma, which were used as platelet activation markers, did not show any alteration and thus there were no detectable signs of platelet activation in the LT setup.

To investigate hemostatic functioning and platelet activation ex vivo, anticoagulation is necessary. The presented assessment of hemostatic functions took place in an FPX-based model, which proved to be non-inferior to an enoxaparin-based setup. Whether the addition of any anticoagulant could induce minor alterations of coagulation or platelet function compared to the in vivo situation cannot be ruled out. Yet, it remains unlikely, since approaches based on different anticoagulating agents yielded similar results [18], [29].

4.2. The effect of DAMPs and the lethal triad on platelets and the coagulation cascade

DAMPs like histones and HMGB1 are often said to be associated with alterations in hemostatic functions [24], [36], [37], [91]. Both are known to induce or increase activation of the coagulation process in DIC [5], [9], [51]. Considering that most of the DIC-inducing DAMPs in trauma derive from NET formation due to immunological processes, injury per se and cell death caused by HS and associated TIC and in consequence LT-impaired coagulation, it seems logical to address the interaction of histones and HMGB1 and the LT condition from the outset [24], [111].

Regarding HMGB1, only a significant impairment of the plasmatic coagulation was seen in samples additionally exposed to LT simulation. This anticoagulant effect did not exceed the impact of the LT on its own, indicating no further amplification by HMGB1. Platelet activation was not detectably affected. It was previously described, that platelet activation markers of patients with the condition of DIC strongly correlate with HMGB1 levels [72]. This does not necessarily appear contradictory to the presented findings, as only contemporaneous presence of signs of platelet activation and HMGB1 but no interaction the both was displayed [72]. Supporting the present data, it was shown, that HMGB1 did worsen the mortality of rats in vivo when admitted together with thrombin but not on its own. While it did not change coagulation parameters in vitro it impaired the anticoagulant protein C pathway [51]. It seems that HMGB1 itself does not induce the activation of platelet or the coagulation cascade but can be seen as a mediator of hemostatic functions in settings of ulterior coagulation activation. A potential mechanistic explanation for this phenomenon can be found in previous research, indicating the binding of HMGB1 to platelets, but not having an impact on plasmatic coagulation [3], [103]. Rather, it is supposed, that the effect of HMGB1 on coagulation could be due to the platelet-immune

interaction through platelets receptor of advanced glycation end products (RAGE) [3]. Even though it can be stated that there was no sign of short-term procoagulant interaction of platelets or the humoral coagulation cascade and HMGB1 detectable in the observed 30-minute period, future research needs to analyse this complex and long-term interaction of platelets, HMBG1, the immune system and finally the coagulation cascade.

In contrast, histones are prominent promotors of coagulation and platelet activation [7], [37], [91]. While HMGB1 in both whole blood and PRP failed to impact clinical coagulation parameters, histones showed a clear effect. In the exhibited data they were found to deplete platelets and impair coagulation on their own. This effect was amplified when incubated under the condition of LT. These processes are mostly due to the consumption of coagulation agents as clots were found in respective replicants. Platelet activation parameters were not further elevated by the LT combined with histones compared to histones alone. However, this does not rule out further platelet activation beyond the detection limit of plasmatic platelet activation markers. In this regard, it is possible, that there could be a ceiling-effect of the analyzed parameters. As the amplification of histones´ impact on hemostasis and the LT has not been described before, these findings indicate a possible relevant interaction that needs to be addressed to further understand coagulation pathologies after severe trauma and deduce better management of dysregulated coagulation.

In regard to the mechanism of action, two main theories stand out. Firstly, the platelet could be “primed” by the LT and thus be more easily activated. Secondly, the concentration of free circulating histones increased due to reduced binding to red blood cells or plasma proteins as a matter of the dilutional effect. Supporting the second theory, there is evidence showing the binding of histones to both plasma proteins and red blood cells [78], [79], [90]. It has also been shown that the admission of binding agents like CRP, albumin or heparin induce attenuation of cytotoxicity and platelet activation by histones [1], [60], [78]. If this theory of histone interaction with LT proves true, early application of products like fibrinogen concentrates, fresh frozen plasma or human albumin could help reduce posttraumatic coagulation disorders in patients suffering from LT, which has already been seen in a clinical trial [30]. In this study histones induced a concentration-dependent reduction of platelet counts in whole blood, as seen previously in mice [37]. Considering the binding to blood cells mentioned earlier, it is not surprising that this effect was even aggravated in PRP and could also be amplified by simultaneous stimulation of LT. Even

platelet-derived vWF, which was only measured in whole blood, showed a concentration-dependent increase by exposure to histones. Taken together, these considerations support the second thesis of increased effect by higher levels of unbound histones.

Arguably, the concentrations used in these experiments exceeded the average amounts in patients with severe blunt trauma which is commonly stated at around 50 µg/ml [2]. However, there are also patients with histone concentrations surpassing 200 µg/ml and it could be argued, that these measurements only display the central blood levels, while the amount of histones at the site of actual injury or cell death could be much higher [2]. Also, histone levels in sepsis patients surpassing 75 µg/ml are associated with a worse outcome and therefore concentrations higher than that need in particular to be given more attention [4].

Even though histones induced a prolongation of CT, alpha angle and MCF in the presented ROTEM data, a previous study showed that fibrin polymerization and crosslinking is enhanced by histones in an FXIII-dependent manner leading to a faster and thicker clot generation than without the DAMP [66]. To implement the present findings alongside previous ones, it should be acknowledged, that in contradiction to the procoagulant roles known to date, histones apparently can also present an anticoagulant phenotype, which supports the findings shown here of a dose-dependent impairment of intrinsic coagulation both in PTT and INTEM [7], [37], [91]. Given the fact that selectively intrinsic coagulation appears to be affected, it seems likely that this effect is caused by the binding of histones to the negatively charged thrombomodulin used to trigger the contact activation pathway in those tests. Such interactions of histones and negatively charged proteins have been shown earlier and could play a major role in these findings [78], [79]. If histones had this impact on the intrinsic coagulation in vivo, they could on the one hand inhibit the thrombus formation leading to ongoing hemorrhage in places of vessel injury as the contact activating pathway is impaired, and on the other hand induce systemic clotting by activation of platelets and thrombin generation. In the setting of trauma and maybe even of LT, these effects could be amplified and therefore be especially hard to manage for the clinical practitioner. As well as these findings help further understanding the process of the hemostatic dysregulation after trauma, they also highlight new potential experimental approaches that need to be addressed in future research.

Impairment of clot lysis by histones was displayed by a strongly reduced ML in the here displayed findings and is in accordance with previous reports, which state that this effect

is partially due to the enhanced crosslinking of fibrin by histones in an FXIII-dependent manner, but also a competitive inhibition of plasmin by the DAMP [66], [67], [102].

4.3. The impact of histones via TLR 2 and 4 on platelets

Interactions between the human immune system, platelets and coagulation are widely known [62], [92]. Regarding DAMPs, the procoagulant effects via TLR 2 and 4 on platelets as well as the importance of superficial TLRs on platelets in immunothrombosis were shown [10], [21], [91], [106]. Most of the TLRs detectable in platelets are not expressed on the anucleated cells' membrane but could rather be seen in permeabilized platelets and some of the subtypes of TLR exposed on the surface even rely on the plasma-derived protein CD14 to activate the cells with lipopolysaccharide (LPS) [23], [27]. As TLR agonists like LPS failed to induce an alteration in platelet response to common triggers, the question remains, whether the platelets' TLR household could be modulated by any agonist [54]. Especially as many more TLRs could be found in permeabilized platelets than there are expressed on their surface, assuming that this fact could have a physiological function appears reasonable [23], [94]. Therefore, this study tested, if any stimulus like acidosis, ADP (as a classical platelet activator) or even histones (as TLR agonists) themselves can induce changes in the amount or potency of TLRs on the platelet surface in FACS analysis [91]. ADP and acidosis failed to modulate the fluorescent signal intensity of TLR-detecting antibodies on platelets in comparison to histones when tested in FC. Also, the fraction of platelets expressing TLRs only increased when stimulated by histones.

However, it was noticed that histones themselves appear to bind any antibodies to the platelet surface, leading to a potentially false-positive fluorescent signal. In a follow-up experiment, comparing the specific antibody-binding relative to the isotype antibody, a slight but not significant effect on TLR expression on platelets was seen. As seen in all experiments, histones seem to impact platelets that do not even express TLR 2 or 4 on their surface in the first place, indicating that there has to be another way of histone-platelet-interaction and thereby supporting previous findings [91].

Neither an acidotic environment nor activation with ADP caused a detectable increment of surface expression of TLR 2 or 4 on isolated platelets. In contrast, high levels of histones may to some extent cause an increase in TLR density on thrombocytes, indicating a possible mechanism of interaction in settings of high histone blood concentrations, such as trauma and HS. Although these findings need to be interpreted with great precaution, they give impetus for further investigations concerning platelet TLRs.

4.4. Discussion of methods and limitations of the study

In this study, for the first time, the combined effects of DAMPs and the LT were assessed in human whole blood and PRP without citrate addition. Platelet activation and hemostatic functioning were looked at from many different perspectives. Parameters, used by clinicians on an everyday basis, like Quick, PTT, machinal platelet counts and ROTEM were investigated. Adding to these clinically established readouts, methods mostly applied in experimental settings like manual platelet counts, ELISAs and FACS analysis were used for more in-depth analysis and to determine platelet activation and consumption. Even though FACS analysis is commonly used in the clinical setting of hematological and immunological diseases nowadays, it does not yet find general use in polytrauma assessment. By using both kinds of tests for this investigation, the study was set up in an experimental way but could also be transferred into a clinical setting.

It is important to bear in mind, that most of the data arose from a newly established FPX-based ex vivo model for coagulation and platelet functioning assessment. Since it is based on another substance than other scientific models, where most data in coagulation and platelet functioning research arise from, it was very well quality controlled and many analyses were performed to proof this model in comparison to citrate or enoxaparin-based approaches beforehand. In this study, no significant drawback of an FPX based set-up compared to a Citate based set-up was seen when directly compared. However, one perspective does not reveal anything about the other dimensions and therefore different points of view are necessary to fully prove or deny a hypothesis. On the one hand, approaching the assessment of the hemostatic system from another angle could help to lead to as yet unknown findings, on the other hand alignment of the findings from this novel FPX based model alongside the results of classical models allows artificial effects of either added anticoagulant to be ruled out. Implementing the data arising from this newly established model with the findings from citrate-based models can further fortify earlier made observations and even lead to finding new ones.

Even though the time frame proved to be relevant considering the results, the lack of further time points could arguably be seen as a limitation of this study. Since the aim of the study was to address the short-term interaction of platelets, the coagulation system, the LT and DAMPs it was reasonable to focus on the first two hours of incubation. The decision

to perform incubation for 30 min was based on prior results comparing different time points which showed, that more than 30 min of incubation did not lead to further platelet or coagulation activation. However, additional or differential effects of the presented conditions at later time points cannot be fully ruled out. In particular, effects resulting from the crosstalk of a more delayed response by the immune system were not investigated, as this was not within the aim of this study. However, it should be specifically addressed in future research.

Overall, this study allowed the previously mentioned hypotheses to be addressed and due to the explorative set up even established hints towards newly and yet to be investigated approaches for future research. While several experiments led to findings of statistic significance, non-significant results could still be proven true in a differently set-up study. As some experiments were conducted with a small number of probands, non-statistically significant results may have been regarded as irrelevant due to type II errors. Therefore, further studies could help to unequivocally prove some of the observations of this work.

4.5. Conclusion

This study sheds new light on the interaction of early and late dysfunction of coagulation in trauma patients. Both, the LT and high levels of DAMPs in blood are seemingly a major cause of disruption in the hemostatic system but even more so when occurring simultaneously. The implication of the presented data in trauma-associated coagulopathies may lead to a better understanding of them and eventually to a better management of these conditions. The role of DAMPs in combination with the LT was displayed and evidence towards a newly discovered early anticoagulant function of histones was found, showing that early hemostatic dysregulation due to the LT-DAMP-interaction must be further studied.

In consideration of the hypothesis generated at the start of this investigation, the following conclusions can be drawn.

1) Although platelets are very fragile and susceptible to triggers like shear stress, PRP could be won by gentle centrifugation and cautious handling. Not only was the product free from other blood cells but it also displayed a very well perceived hemostatic function.
2) HMGB1 failed to show detectable signs of both platelets and coagulation activation in whole blood and PRP. In contrast, the role of histones as activating agents of platelets and coagulation could be further confirmed. However, an impairment of the intrinsic coagulation by histones was found.
3) Hemostatic function was impaired under the condition of LT. Histones added to whole blood or PRP under LT conditions appeared to impair hemostatic function more extensively than LT alone. The impairment of coagulation however seems mostly due to the consumption of both platelets and coagulation proteins. Therefore, the LT conditions in the presence of histones may even enhance the procoagulant phenotype of the DAMP instead of nullifying it.
4) The activation of platelets by histones leading to secretion of alpha granules was displayed. Furthermore, it was shown that upon incubation alongside the DAMP the quantity of TLRs 2 and 4 on platelets could potentially increase. Whether histones really modulate the quantity of TLRs on the surface of platelets and thus lead to an auto-amplified activation of immunothrombosis must be further evaluated.

5) A dose-dependent impact of histones on the coagulation cascade could be seen but the proteins did not activate the hemostatic system as expected. The DAMPS showed a concentration-dependent impairment of intrinsic coagulation in both ROTEM and laboratory coagulation parameters.

5. Summary

Polytrauma and related hemostatic disorders are still and will likely continue to be a serious burden for health care around the world. Coagulation in patients with severe trauma can initially be impaired by acidosis, hypothermia and iatrogenic hemodilution, altogether known as the lethal triad in trauma (LT). This condition is associated with substantial blood loss, trauma-induced coagulopathy (TIC) and eventually hemorrhagic shock (HS). Regarding the underlying pathophysiology, both damage-associated molecular patterns (DAMPs) and an overshooting systemic inflammatory response, systemic inflammatory response syndrome (SIRS), can cause an inadequate overactivation and dysregulation of hemostatic processes. This condition is known as disseminated intravascular coagulopathy (DIC). While the interaction of trauma and coagulation appears plausible, the underlying cause of this condition has only partially been discovered. This work aimed to gain insights into the pathophysiological mechanism of hemostatic dysregulation when HMGB1 and histones interact with human whole blood or platelet rich plasma (PRP) under the condition of LT. Additionally, the specific effects of histones on platelets and plasmatic coagulation were investigated.

Blood from healthy donors was anticoagulated with Fondaparinux (FPX) and either used directly or further processed to PRP for assessment in 2-methacryloyloxethyl phosphocholine (MCP) coated tubes. The lethal triad was modelled by replacing 30% of the volume with acidified Jonosteril while incubating the respective specimen at 34°C. Untreated control samples were incubated at 37°C. To address the impact of DAMPs in the setting of LT, either 50 µg/ml histones or 500 ng/ml HMGB1 were also added. In order to investigate a potential concentration-dependent effect of histones, both platelet counts, and coagulation parameters were analyzed after the addition of 10 - 120 µg/ml histones. All the samples were evaluated after a 30-minute period of incubation. For platelet activation markers, von Willebrand Factor and soluble CD40L levels were measured from frozen plasma samples and normalized to total protein concentration. Routine coagulation parameters, as well as cell counts, were determined by the central facility of the University Hospital of Ulm. Intrinsic and extrinsic coagulation analysis was performed by rotational thrombelastometry (ROTEM) with both citrated and primarily FPX treated whole blood added with 120 µg/ml histones. Platelets were tested for the expression of toll-like

receptor (TLR) 2 and TLR 4 as well as CD62P by flow cytometry (FC) after stimulation with 5 µM ADP, 50 µg/ml Histones or 25 µl/ml Jonosteril with a pH of 1.
Results revealed a significant impairment of coagulation by LT alone and LT combined with HMGB1. However, HMGB1 seemingly did not further impact the analyzed parameters. In contrast, histones were shown to be potent activators of coagulation. When added into the LT solution, histones led to an overshooting reaction of the whole hemostatic system resulting in an almost complete consumption of platelets and coagulation factors. A selective concentration-dependent attenuation of the contact activated pathway of coagulation by histones was found by intrinsic thrombelastometry (INTEM) and partial prothrombin time (PTT). When treated with histones, platelets even appeared to express substantially more TLR 2 and 4 on their surface than in control samples. However, these results could be at least partially displayed in an overexaggerated way as the natural stickiness of histones did bind any of the analyzed antibodies to their surface. When balanced relative to specific isotypes bound on the platelet surface, the actual antibodies did show a slightly, though insignificant, increased signal than in controls. Yet this study showed the first hints towards not only a qualitative but also quantitative change of TLRs on platelets when interacting with histones, which could have serious implications on further understanding platelet function and dysfunction in settings of sepsis and trauma.

Overall, this study showed a significant interaction between LT and DAMPs that is relevant for further understanding of the mechanism of DIC and novel treatment options after polytrauma.

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