Hemostasis and endothelial damage during sepsis

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THE 3 ORIGINAL PAPERS ARE:

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II. Johansen ME, Johansson PI, Ostrowski SR, Bestle MH, Hein L, Jensen AL, et al. Profound endothelial damage predicts impending organ failure and death in sepsis. Seminars in thrombosis and hemostasis. 2015;41(1):16-25.

III. Johansen ME, Jensen JU, Bestle MH, Ostrowski SR, Thormar K, Christensen H, et al. Mild induced hypothermia: effects on sepsis-related coagulopathy--results from a randomized controlled trial. Thrombosis research. 2015;135(1):175-82

INTRODUCTION

Sepsis, derived from Greek meaning "decomposition of animal or vegetable organic matter in the presence of bacteria", was first described by Hippocrates (460–377 BC) as the process by which flesh rots, swamps generate foul airs, and wounds fester [1]. Despite knowledge of the condition for more than 2.500 years, a common definition of the sepsis syndrome was not agreed on until 1992 [2]. Since then, despite an increased focus on diagnosis and treatment, results from interventional trials to lower the mortality have been widely unsuccessful.

EPIDEMIOLOGY OF SEPSIS

Sepsis is a frequent cause of death worldwide, with mortality rates exceeding 50% depending on disease severity, follow-up time and patient population [3-6]. Expenses related to the condition amount to approximately $15 billion annually in the United States alone, equivalent to roughly 40% of total intensive care unit (ICU) expenditures [7,8]. The exact incidence of sepsis remains unknown, although an estimated 18 million individuals worldwide are affected by sepsis each year, and incidences are growing [4,9-11].

THE SEPSIS PATHOPHYSIOLOGY

The sepsis syndrome represents a disease continuum including severe sepsis and septic shock. Severe sepsis is defined as sepsis complicated by acute organ failure, and ultimately refractory hypotension, the latter septic shock [2,12]. Organ failure and shock significantly increases risk of death during sepsis [13].

Sepsis is caused by the response of the immune system to infection - most commonly bacterial [10]. In bacterial sepsis, the condition is initiated due to virulent membrane components of Gram-negative (endotoxins e.g. lipopolysaccharide (LPS)) and Gram-positive bacteria (e.g. peptidoglycan, lipoteichoic acid) [10,14,15]. The virulent membrane components and toxins activate the immune response causing release of inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), into the bloodstream. TNF-α has a particularly important role in sepsis. Circulating levels of TNF-α are greater in septic patients compared with critically ill non-septic patients [16]. Furthermore, infusion of TNF-α produces symptoms similar to those observed in severe sepsis [17], and anti-TNF-α antibodies protect animals from lethal challenge with endotoxin [18].

The release of inflammatory mediators induces apoptosis [19,20] which occurs systemically in patients with sepsis [21,22]. Sepsis-induced apoptosis is initiated by two signaling pathways; the intrinsic (i.e. *the mitochondrial caspase-9 pathway*) and the extrinsic (i.e. *the death receptor caspase-8 pathway*) [23-25]. The intrinsic pathway is activated by number of stimuli including toxins, nitric oxide (NO) and free radicals due to ischemia/reperfusion injury. The stimuli result in mitochondrial dysfunction by affecting the inner mitochondrial membrane leading flux of Ca^{2+}, and relocation of cytochrome *c* [23]. Mitochondrial dysfunction is directly linked with poor patient outcome in sepsis [26]. The extrinsic signaling pathway involves transmembrane death receptors that bind ligands such as TNF-α and the Fas ligand [23,27]. The signal transduction of the intrinsic and the extrinsic pathway activates caspase-3 downstream which subsequently initiates a protease cascade resulting in death of the cell

[28,29]. Experimental studies have found that inhibiting apoptosis protect animals from organ dysfunction and death [30,31], suggesting that prevention of apoptosis may act as a potential therapeutic strategy in sepsis [32,33].

THE HEMOSTATIC SYSTEM DURING SEPSIS

The hemostatic system comprises coagulation and fibrinolysis, and thereby platelets, coagulation factors and endothelium throughout the microcirculatory system. Severe stages of sepsis induce marked alterations in the microcirculation with hypoperfusion and oxygen depletion [34,35], directly linked to organ failure and death [35-37].

Coagulopathy, resulting in formation of microthrombi, play an important role in microvascular alterations [38,39]. Pro-inflammatory mediators initiate sepsis-related coagulopathy characterized by hypercoagulation [40,41], impairment of natural anticoagulant [41,42] and hypofibrinolysis [43]. The hypercoagulant state leads to formation of microthrombi causing vascular occlusion and ischemia-reperfusion injury resulting in cellular apoptosis [38].The formation of microthrombi is thought to be important in development of microvascular breakdown and multi-organ failure [44]. In the late stages of sepsis, an uncontrolled consumption of platelets and coagulation factors occurs resulting in hypocoagulation [45,46]. Classically, sepsis-related coagulopathy is associated with Gram-negative bacterial infections, although it also occurs in Gram positive sepsis [47].

Clot formation in sepsis occurs predominantly via the extrinsic pathway of coagulation. Tissue factor (TF) is not expressed within the endovascular system under normal conditions [48]. However, stimulated by endotoxins [49], cytokines [42,50,51] and activated platelets [52], TF mediates thrombin generation and subsequent generation of a fibrin clot [40,41]. Additionally, impairment of natural anticoagulant mechanisms, such as the antithrombin and protein C systems [41,42,53,54] in the presence of hypofibrinolysis [42,43] seem to be of importance in the formation of microthrombi. Furthermore as the sepsis condition progress, erythrocytes lose their normal ability to deform within the microcirculation [55,56]. Together with the extensive clot formation throughout the microcirculation, the erythrocyte deformation contribute to the uneven distribution of capillary perfusion due to stopped or intermittently perfused capillaries resulting in the defect in oxygen extraction observed in sepsis [35,57-59]. However, in spite of the emerging evidence supporting a central role of coagulopathy and formation of microthrombi in the progression of sepsis, the exact extent and consequences are unknown [60,61].

The platelets

Hypocoagulation is considered to occur late in the course of sepsis, attributable to uncontrolled consumption of coagulation factors and platelets [45,46]. However, thrombocytopenia (platelet count < 150 x 10^9/L) is a frequent phenomenon throughout the entire sepsis continuum [62-64] and the condition is associated with prolonged hospitalization and reduced survival rates [65]. The incidence of thrombocytopenia in severe septic patients is 35–45% [62,63]. A platelet count of < 100 x 10^9/L is seen in 20–25% of patients, whereas 12–15% of patients have a platelet count < 50 x 10^9/L [62,63]. Most episodes of thrombocytopenia occur within the first 4 days after ICU admission [66].

The platelets are centrally involved in hemostasis and absolute platelet count ≤100 x 10^9/L increases risk of bleeding [62]. Within minutes after activation, platelet aggregation occurs via the binding of the glycoprotein IIb/IIIa receptor to Von Willebrand factor or fibrinogen [67].

Low platelet count may be a surrogate marker of immune activation or severe infection. In addition to being a cellular effector of hemostasis, platelets mediate interaction between inflammation and coagulation, and potentially modulate the inflammatory process [68]. Platelets are activated rapidly and deployed to sites of infection [69]. The platelet cells interact with leukocytes and secrete antimicrobial peptides [68,69]. Thus, a decrease in platelet count might protract clearance of infection [70]. Consequently, presence of sufficient numbers of well-functioning platelets may improve survival in critical illness by beneficially modifying the immune response [68].

Platelet consumption, sequestering in the spleen and microcirculation, peripheral destruction, and decreased production due to hemophagocytosis, all cause thrombocytopenia during sepsis [64,71,72]. In addition, several drugs administered to treat severe infection during ICU admission may induce thrombocytopenia due to bone marrow suppression or/and immune-mediated platelet destruction [64,73,74]. The prevalence of drug-induced thrombocytopenia is largely unknown, although observational studies suggest an incidence as high as 10 % [64]. A number of antimicrobials, including beta-lactams and fluoroquinolones, have been proposed to be implicated in induction of thrombocytopenia [75]. However, evidence supporting an association between antimicrobials and thrombocytopenia is largely based on case reports and laboratory studies, and testing for platelet-reactive antibodies is rare [75-77]. In the absence of a more reliable method, the gold-standard for diagnosing suspected drug-induced thrombocytopenia is the observation of a rise in platelet count after discontinuation of the drug [75,76,78]. As critically ill patients are implicitly vulnerable, the discontinuation of a potential lifesaving drug is difficult. Furthermore, empirical distinction between platelet count fluctuation due to resolution of treatment and resolution of the underlying illness remains uncertain. Thus, identification of possible causative agents based on the available evidence continues to be challenging, and further clarification on the contribution of specific antimicrobials frequently used in critically ill patients on risk of thrombocytopenia continues to be warranted [76,79].

The endothelium

The vascular endothelium is a dynamic cell layer involved in numerous physiologic functions including hemostasis and inflammation [80]. Under normal conditions, the endothelium contributes to balancing the hemostatic response [81,82]. During severe infection, pro-inflammatory cytokines damage the endothelium, inducing apoptosis and facilitating coagulation activation [83-85]. It has been proposed that the endothelium is a primary site of deterioration during sepsis [86,87] and that endothelial damage increases the formation of thrombi causing circulatory and organ failure and eventually death [60,83].

No is a vasoactive substance released by endothelial cells. Under normal condtions, NO is synthesized via endothelial NO synthase (eNOS) and participates in the regulation of blood flow. However during sepsis, inflammatory mediators such as LPS, interleukins and microphages, activates the inducible NO synthase (iNOS) [88].

This results in massive vasodilation which ultimatly may lead to hypotension. NO overproduction associates with severity of clinical outcome [26]. Furthermore, redistribution of fluid from intravascular to interstitial space play a role in sepsis-related hypotension as a consequence of increased endothelial permeability and reduced arterial vascular tone [89].

The luminal superficial endothelial layer formed by the glycocalyx is affected as the earliest during sepsis [90]. Stimulated by inflammatory mediators, syndecan-1, a constituent of the glycocalyx, is shed and released into the bloodstream [91,92], reflecting glycocalyx damage and hence superficial endothelial disruption. Beneath the superficial glycocalyx layer are the endothelial cells themselves. Thrombomodulin (TM) is an integral endothelial cell membrane protein [93]. Upon direct endothelial cell damage TM is enzymatically cleaved from the endothelial cells and released into the bloodstream, hereby reflecting profound endothelial cell damage [94-96]. Despite the emerging evidence that damage to the endothelium plays a considerable role in the progression of sepsis, the extent and consequences of superficial and profound endothelial damage in organ failure and death remains to clarified [60].

POTENTIAL TREATMENT DURING SEPSIS - MILD INDUCED HYPOTHERMIA

The keystone when treating sepsis-related coagulopathy is to treat the underlying infection using broad-spectrum antimicrobials and source control. In addition, supportive care is often required, aimed at respiratory and circulatory support as well as replacement of organ function. However, coagulopathy might progress, even after appropriate treatment has been initiated. In those cases, supportive measures to manage the coagulation disorder may be considered.

The increased insight into the mechanisms that play a role in development of sepsis-related coagulopathy has enhanced the incentive to acquire such supportive management strategies. Within the last couple of decades, several clinical randomized trials have been performed testing different anticoagulatory agents against sepsis-related coagulopathy [97-100]. However, all these trails have failed to identify any significant effect on mortality. Because none of the trials had a built-in analytic plan to probe the reasons for failure, it is still not clear why these trials failed to improve patient outcome. As severe sepsis and septic shock is characterized by an impaired microcirculation, one explanation for the therapeutic failure, may be the inability to achieve effective drug concentrations the peripheral microcirculation (i.e. the capillary system) [101]. Furthermore, the sepsis pathophysiology is complex and targeting only one anticoagulant molecule may perhaps be overly simplistic.

Mild induced hypothermia (cooling to 32-34°C for 24 hours, MIH) is independent of a well-functioning microcirculation and has been proposed as a potentially advantageous treatment in patients suffering sepsis [102,103]. MIH inhibits several deleterious effects of sepsis among them TNF-α- induced apoptosis [84], tromboxane A2-induced platelet aggregation and leukocyte adhesion, the latter two which in sepsis contribute to the formation of microthrombi [104,105]. Furthermore, the synthesis of coagulation factors is reduced at temperatures of 33◦C and below [104,106,107]. Correspondingly, studies have demonstrated increased tissue perfusion upon local cooling in animal models of sepsis [105].

Although, MIH is routinely used in the ICU [108], it is still not clear how coagulopathy is affected by hypothermia [109]. The possibility of MIH worsening sepsis-related coagulopathy is present, as hypothermia affects enzymatic coagulation reactions by inhibiting the kinetics of the clotting factors [110], while having minimal effect on their concentrations [111,112]. Thus, monitoring the coagulative response to hypothermia using conventional routine coagulation tests may not be sufficient.

Thromboelastography (TEG) presents a functional and dynamic approach of assessing whole blood coagulation. In healthy volunteers, it has been demonstrated via TEG that decreasing temperatures lead to a progressive attenuation of the blood coagulation system by delaying the initiation and build up of thrombus formation [113]. In patients with severe sepsis, hypocoagulability assed by TEG associates with increased mortality [114]. However, no studies so far have investigated the impact of hypothermia on sepsis-related coagulopathy.

OVERALL USED METHODOLOGY

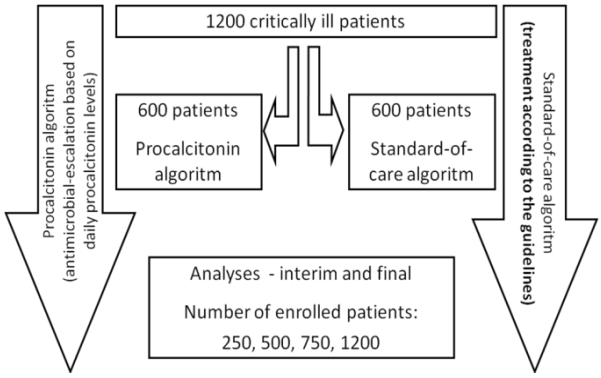
STUDIES

This thesis is based on data from two randomized controlled trials; The Procalcitonin And Survival Study (PASS) (*paper I and II*) and The Cooling And Surviving Septic shock (CASS) study (*paper III*).

The Procalcitonin And Survival Study (PASS)

Between 2006 and 2010 the PASS study randomized 1200 patients either to receiving antimicrobial treatment according to standard-of-care or standard-of-care supplemented with daily drug-escalation on the basis of biomarker increases [115] **(Figure 1)**. Nine mixed medical/surgical intensive care units in tertiary care public university hospitals across Denmark participated in the study. Interim analyses were performed after enrollment of 250, 500, 750 patients, respectively. The primary endpoint of the trial was a comparative mortality rate between the two randomized groups (overall 28-day mortality was 31.8%). Trial registration: NCT00271752

Figure 1. Overview of enrollment in the PASS study



To be enrolled, patients had to be ≥18 years, enrolled ≤24 hours of admission to the ICU and have an expected length of stay of ≥ 24 hours. Patients with bilirubin >40 mg/dl (>684 µmol/L) and triglycerides >1000 mg/dl (>11.3 mmol/L) were excluded. Patients were followed until death or day 28 and patient status along with biochemistry and drug therapy was collected daily and monitored according to Good Clinical Practice (GCP) [116]. Survival status and date of death was additionally determined at two single time

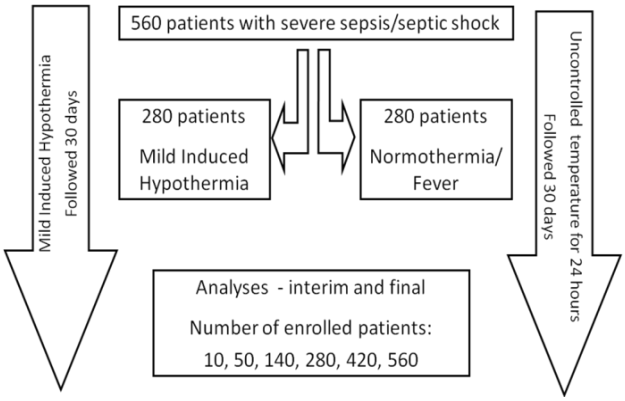
points, 90 and 180 days after enrollment for all patients. In addition, daily serum samples from all patients admitted to the ICU was collected during follow-up; blood was sampled consecutively daily, starting upon arrival, in serum tubes and frozen within 1 hour. The serum samples were stored at –80°C until unfrozen for analysis (*paper II*).

After analysis of the primary endpoint in the PASS study, it was confirmed that patients randomized to the intervention group (procalcitonin algorithm) had received considerably more broad-spectrum antibiotics compared with the control group (standard-of-care algorithm). Since platelet count and infection status at the time of randomisation as well as 28-day mortality were similar between the two groups, the study design could be used to investigate the effect of exposure to large amounts of broad-spectrum antimicrobials on platelet count in critically ill patients (*paper I*).

The Cooling And Surviving Septic shock (CASS) study

The CASS study is an ongoing randomized controlled trial investigating whether mild induced hypothermia (MIH) reduces mortality and organ-related complications in patients suffering severe sepsis or septic shock. A total of 560 patients will be enrolled in mixed medical/surgical intensive care units. Patients are allocated to either standard-of-care (the control group) or standard-of-care supplemented with MIH (the MIH group). During MIH the body temperature is lowered to 33°C (target temperature 32°C -34°C) for 24 hours. Subsequently, the patient is rewarmed and kept at normothermia (36-38°C) until 72 hours from time of study enrollment **(Figure 2)**. Interim analyses are to be performed after enrollment of 10, 50, 140, 280 and 420 patients, respectively.
Primary endpoint in the CASS study is 30-day mortality. Trial registration: NCT01455116.

Figure 2. Overview of enrollment in the CASS study



To date, 229 patients have been enrolled at seven different intensive care units in Denmark.
3 interim analyses have successfully been performed without any remarks from the Data and Safety Monitoring Board (DSMB).

To be eligible for enrollment in the CASS study, patients must be diagnosed with severe sepsis or septic shock (defined according to the ACCP-SCCM consensus conference on sepsis and organ failure [2]), have a mean arterial pressure < 70 mmHg or receive inotropics or vasopressors, and have indication for intubation. These events may not have lasted together more than 6 hours. Furthermore, patients must be ≥ 50 years of age, not have undergone surgery within 24 hours and may not be suffering from chronic bleeding disorder or uncontrolled bleeding (the latter is defined as a decrease in the hemoglobin concentration of 1.8 mmol/l (3.0 g/dL) in the preceding 12 hours or a requirement for at least 3 units of packed red blood cells during the same period).

DATA COLLECTION AND MANGEMENT

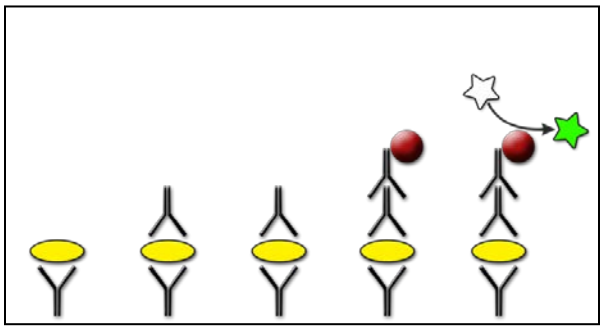
Clinical data

In clinical trials, three different case report forms (CRF) are usually required; a baseline assessment, a follow-up assessment and an assessment of other aspects requiring monitoring [117]. In both the PASS and the CASS study a standardised CRF was developed by the coordinating centre (i.e. the sponsor of the two studies) in order to collect the specific data needed to answer each research question. After study enrollment, an individual trial number was assigned to each patient, so that he or she remained anonymous throughout the rest of the trial. Only the trial number remained on the CRF.
In the PASS study the CRF was filled out by hand by the investigators. Next, the CRF document was sent to the coordinating center and typed into a central database. In the CASS study, all data are entered directly into an electronic database by local investigators. In both studies all collected data is monitored continuously according to Good Clinical Practice (GCP) [118] and the handling of the collected trial data is carried out by a specially trained database manager, ensuring high-quality data.

Enzyme-linked immunosorbent assay test

In the study underlying *paper II,* Enzyme-Linked Immunosorbent Assay (ELISA) was used analysing the levels of biomarkers of endothelial damage. ELISA is a type of immunoassay which involves an antigen (i.e. an "analyte") linked to an antibody whose activity can be determined serving as a quantitative estimate of the amount of the investigated antigen in a biological sample. The so-called sandwich ELISA technique was used in the study underlying *paper II*. When using the sandwich technique, the antigen to be measured must contain at least two antigenic epitope capable of binding to antibodies. The sandwich method is as follows; An antibody specific for an investigated antigen is coated to a 96-wells microplate. A subsequent secondary antibody is added binding the antigen, and any excess unbound antigen or secondary antibody is washed away. A substrate (e.g. streptavidin-horseradish peroxidise (-HRP)) is then added biding the secondary antibody. Lastly, a substrate, is added, yielding a colour change upon addition of a stop solution. The amount of visual colour change is directly proportional to the amount of specific antibody-antigen-antibody, and consequently to the concentration of antigen present in the biological specimen tested **(figure 3)**.

Figure 3. Overview of the sandwich ELISA method

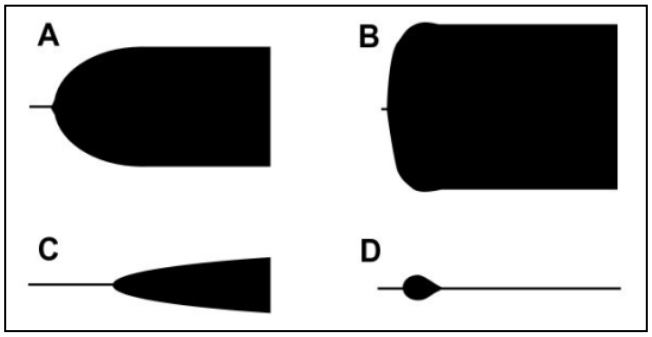


ELISA is a reliable method to quantify soluble proteins because of its generally high **specificity** (true negative rate) for a particular antigen [119]. The **sensitivity** (true positive rate) of the ELISA test is defined as the limit of detection (LOD) and is the lowest measurable concentration of a substance [120]. In *paper II* LOD of syndecan-1 was 4.94 ng/ml (syndecan-1) and 0.31 ng/ml (soluble Thrombomodulin). The **reproducibility** of an assay is the degree of error within each assay (intra-assay variation) and between the assays (inter-assay variation). The intra-assay variation is calculated from replicates analyzed on the same plate while the inter-assay error is calculated from replicates analyzed on different plates. In *paper II* the intra- and inter assay variation of the syndecan-1 kit were 3.9% and 9.8% and that of sTM were 6.2% and 10.2%, respectively.

Thrombelastography

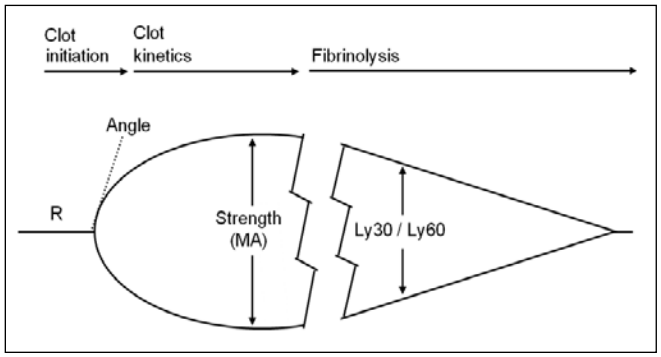
In the CASS study, thrombelastography (TEG) was performed on the first 100 patients enrolled at four different ICU's in the Capital region of Denmark. TEG is a viscoelastic test presenting a functional way of assessing the entire clotting process in whole blood. The TEG reports; R (reaction time), angle (α), the maximum amplitude (MA) and clot lysis after 30 minutes and 60 minutes (LY30/Ly60) **(Figure 4)**.

Figure 4. Schematic TEG trace indicating the commonly reported variables reaction time (R), alpha angle (α), maximum amplitude (MA) and lysis at 30 min and 60 min (Ly30/Ly60)



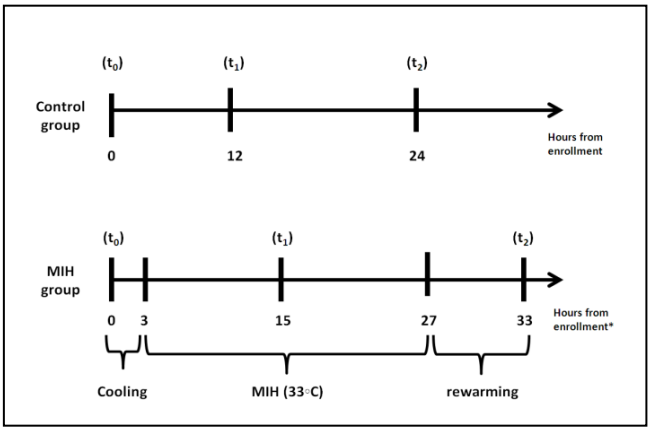
Typical TEG profiles observed in septic patients are normal, hypercoagulable, hypocoagulable and hyperfibrinolytic profiles **(Figure 5)**.

Figure 5. The various TEG profile observed during sepsis A) Normal, B) Hypercoagulable, C) Hypocoagulable and D) Primary hyperfibrinolytic



During the CASS study, three departments of clinical immunology/blood banks carried out TEG from the enrolled patients. All TEG profiles were blinded during the ongoing clinical trial. Thus, the laboratory technicians, the treating physicians and the investigators were unaware of the TEG results prior to unblinding of the results. TEG analyses were carried out three times after study inclusion in all the patients. For patients randomized to the control group TEG analyses were performed at study enrollment (t_0), after 12 hours (t_1) and 24 hours (t_2). For patients randomized to MIH group TEG analyses were performed at study enrollment (before initiating MIH) (t_0), 12 hours after target temperature was reached (t_1) and when the intervention was complete and normothermia (36-38°C) was regained (t_2) **(Figure 6)**.

Figure 6. Schedules of thrombelastography (TEG) sampling in the two randomized groups during the CASS study



***Approximate hours based on post calculated median results. Mild induced hypothermia, MIH**

Clot formation was assessed in 3.2% citrated whole blood sampled in 4.5-mL vacutainer tubes using a TEG 5000 Hemostasis Analyzer System (Haemonetics Corp, Braintree), according to the manufacturer's recommendations. The variables recorded were reaction time (R [3-8 minutes]; rate of initial fibrin formation), angle (α [55°-78°]; clot growth kinetics reflecting the thrombin burst), maximum amplitude (MA [51-69mm]; reflecting maximum clot strength), and lysis30 (Ly30 [0%-8%]; reflecting fibrinolysis). The day-to-day coefficient of variation percentage of TEG MA was less than 7% in the participating laboratories [121]. All TEG assays were run without heparinase and all samples were analyzed at 37°C and 33°C.

Of the collected TEG parameters (R, α, MA and lysis30), only R and MA were unblinded and used in the analyses included in *paper III*. R and MA analyzed at 37°C were used for all patients at time of study enrollment (t_0). In the primary analyses samples analyzed at 37°C at t_1 and t_2 were used for patients randomized to the control group and for patients randomized to the MIH group samples analyzed at 33°C at t_1 and 37°C at t_2 were used. Secondary, the robustness of these primary results were tested in sensitivity analyses replacing R and MA at t_1 with the TEG profiles obtained at 37°C instead of 33°C and t_2 with the TEG profiles obtained at 33°C instead of 37°C.

DEFINITION OF EXPLANATORY VARIABLES AND ENDPOINTS

Sepsis

In *paper I-III* sepsis was defined according to the ACCP-SCCM consensus conference on sepsis and organ failure [2].

Absolute and relative thrombocytopenia

In *paper I* thrombocytopenia was defined as absolute (one platelet count ≤ 100 x 10^9/L) or relative (≥20 % decrease in platelet count from study enrollment). In a healthy population, thrombocytopenia is usually defined as a single platelet count measurement ≤ 150 x 10^9/L [122] but up to half of the patients in the ICU has a platelet count ≤ 150 x 10^9/L on admission [62,63,123,124]. As the aim of the study underlying *paper I* was to detect a potential development of thrombocytopenia when patients were exposure to antimicrobial agents administered during the stay at the ICU, a cut-off of platelet count ≤ 100 x 10^9/L was chosen defining absolute thrombocytopenia. Defining relative thrombocytopenia a ≥20 % decrease in platelet count from study enrollment was chosen. This definition was preferred in order to include as many patients as possible in the analyses and at the same time avoid that a decrease in platelet count was due to measurement variability.

Syndecan-1 and thrombomodulin

In *paper II*, biomarkers of endothelial glycocalyx and endothelium cell damage (syndecan-1 and soluble thrombomodulin (sTM), respectively [96,125]) were measured retrospectively using stored serum samples collected at time of study enrollment. ELISA test were conducted according to the manufactures' recommendations by commercially available immunoassays (Diaclone SAS, Besancon, France). In healthy volunteers normal range of syndecan-1 and sTM is defined as (means ±SD) 51 ±12 ng/ml and 4.5 ±0.8 ng/ml, respectively [126].

***Estimated Glomerular Filtration Rate* equation**

Estimated Glomerular Filtration Rate (eGFR) used in *paper I-III* was calculated using the modification of diet in renal disease (MDRD) formula. The MDRD formula was chosen as the equation has been validated extensively in adult Caucasian populations with impaired renal function (eGFR < 60 mL/min/1.73 m^2) [127].

Inotropic score

The dose of inotropic and vasopressor agents used in *paper II-III* was expressed as (dopamine dose x 1) + (dobutamine dose x 1) + (adrenaline dose x 100) + (noradrenaline dose x 100) + (phenylephrine dose x 100). The maximum daily dose of the specific inotropic drug was used. Inotropic score was first developed as a marker of disease severity in paediatric patients [128,129] but has later been validated in adult critically ill patients [130].

STATISTICAL METHODS

In *paper I* analyses were divided in two; univariable models comparing the two groups of the clinical trial and multivariable models combining the two groups into one group and treating it as a cohort. Cox and Poisson regression models were performed with the risk of death or thrombocytopenia as the outcomes, respectively. Mixed effects models were employed to assess the response in platelet count according to selected explanatory variables. Platelet count showed a normal distribution and was modelled in the raw scale. The following time-fixed and time-updated variables were included in the multivariable models; randomisation group (standard-of-care (SOC) group vs. high-exposure group), age (≥65 vs. <65 years), gender (male vs. female), Acute Physiology and Chronic Health Evaluation II (APACHE II) score (≥20 vs. <20), severe sepsis/septic shock at randomisation (yes vs. no), type of patient (surgical vs. medical), BMI (≥30 vs. <30) and chronic disease (Charlson score ≥2 vs. <2). Use of antimicrobials was fitted using time-updated variables. Current use of Cefuroxime was used as the comparator in all multivariable analyses.

In *paper II* Spearman´s rho statistic was used to assess the correlation between biomarkers and continuous endpoints at time of study enrollment. Survival analysis of the time to single organ failure, multiple organ failure and death was performed. Specifically, 90-day mortality was assessed. Kaplan-Meier curves were employed to compare survival times according to the quartiles of biomarkers of endothelial damage. A multivariable Cox regression model was used to control for potential confounding variables; age, gender, type of patient (surgical versus medical), body mass index, and chronic disease (defined by Charlson score), alert procalcitonin (inclusion procalcitonin (PCT)>1.0 ng/ml or an insufficient decrease within first 24 hours (PCT day 2 >0.9 x PCT day 1) [131]), at study enrollment. These were chosen a priori as likely to be associated with both antimicrobial use and risk of clinical progression. For the mortality outcome the multivariable model further included current values of the markers of organ dysfunction; bilirubin, eGFR, inotropic score (all fitted as continuous time-dependent covariates in the log10 scale) and mechanical ventilation at study enrollment. Lastly, multivariable Cox models were used to investigate the risk of single and multiple organ failure during the 28 days of follow-up. Patients who had already been diagnosed with organ failure within the first 24 hours of enrollment were excluded from these analyses. Besides adjusting for the potential confounders mentioned above, the models assessing predictors of developing each type of organ failure were also adjusted for failure at study enrollment of organs different from the one under investigation.

In *paper III* correlations between TEG parameters and biomarkers and severity scores at study enrollment were assessed using Spearman´s correlation statistic reported as rho and p value. Spearman´s correlation statistic was also applied when investigating the correlation between TEG parameters at study enrollment and the change in the same TEG parameter at two time points during follow-up in the two randomized groups (high-exposure group vs. SOC group).

Survival analyses: Unadjusted estimates

Time to the various definitions of thrombocytopenia (*paper I*), organ failure (*paper II)* and mortality (*paper I-II*) was investigated using the Kaplan-Meier method stratified according to different covariates at study enrollment. Survival curves were calculated by the Kaplan-Meier method displaying the cumulative probability of reaching the endpoint (thrombocytopenia/organ failure/death) at any time after study enrollment. Differences between groups stratified according to randomisation group (*paper I)* or biomarker levels (*paper II)* were compared by the non-parametric Wilcoxon test (*paper I*) or log rank test (*paper II*). Wilcoxon test was used in *paper I* instead of the more commonly used log-rank test because it was felt to be important to give more weight to events at early time points, given the fact that a large fraction of patients' follow-up was censored at late time points.

Both the Wilcoxon test and the log rank test compare differences between the observed number of events (thrombocytopenia/organ failure/death) in each group at each of the event times and the numbers of expected events, under the null-hypothesis (i.e. no differences in outcome between the groups). The log rank test is based on the assumption of proportional hazards within the groups and that the survival of censored and remaining subjects is similar (i.e. no change in hazard ratio over time). The Wilcoxon test does not require a consistent hazard ratio, but does require that one group consistently has a higher risk than the other.

Survival analyses: Adjusted estimates

Cox (*paper I and II*) and Poisson (*paper I*) regression models were used to estimate hazard ratios (HRs) and rate ratios (RRs), respectively. HR and RR are relative rates comparing risks in different groups, rather than the absolute risk, of an outcome. A multivariable Cox model was used in *paper I* and *II* to test the independent effect of explanatory variables (risk factors) when other risk factors in the model were held constant, for example at the value registered at study enrollment (i.e. adjusted analyses). The Cox model is based on the assumptions of a constant hazard risk relation over time (proportional hazards within groups) and that the effects of risk factors are additive (contribute independently to the relative hazard) and linear on the log risk scale. In contrast, Poisson regression (*paper I*) is a parametric model that does not assume proportionality of the hazards. Poisson regression assumes the response variable Y has a Poisson distribution, and assumes the logarithm of its expected value can be modelled by a linear combination of the parameters. For rare events it mimics a binomial distribution.

In *paper I and II* we excluded patients who already met the definition of the investigated endpoint at enrollment. Thus, for example, patients with platelet counts > 100 x 10^9/L at study enrollment were excluded from the analyses investigating risk of absolute thrombocytopenia in *paper I* and in *paper II* patients who already meet the criteria of the investigated organ failure were excluded from the analysis.

Correlation analyses

Spearman's correlation coefficient (rho) (reported in *paper III)* is a nonparametric statistical measure of the strength of a monotonic relationship between paired data. Interpretation of the test is easy; the closer rho is to ±1 the stronger the monotonic relationship between the two variables is. However, in the context of observational studies, a strong correlation does not imply causality. The calculation of Spearman's correlation coefficient and its significance testing, requires two variables that should be measured on an ordinal**,** interval or ratio scale. Importantly, rho=0 does not mean absence of correlation but only implies no monotonic correlation between the variables.

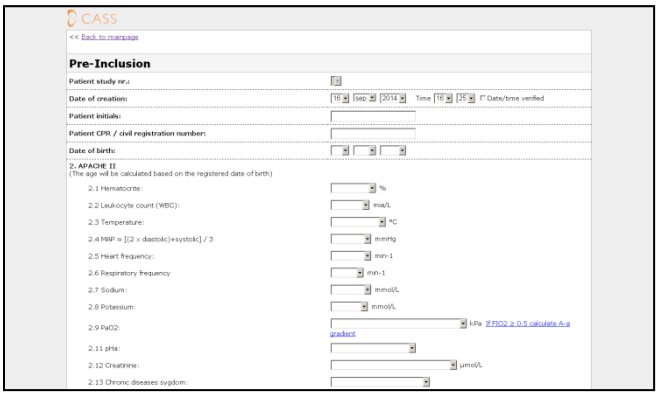
Randomized controlled trials, bias and confounding

The studies underlying the papers included in this thesis are based on two randomized controlled trials (RCT). A RCT is considered the gold standard to test the efficacy or effectiveness of various types of medical intervention within a patient population [132,133]. This is mainly due to the fact that, in a RCT, the randomized groups are balanced for both known and unknown prognostic factors. The only differences between the care received in the two groups, for example, in terms of procedures, tests, follow-up care ect. are those intrinsic to the treatments being compared. Indeed, participants are enrolled and randomly allocated to receive one or other of the alternative treatments under study (mechanism can be complex, but conceptually, the process amounts to tossing a coin). Provided that the balance by randomisation is maintained and there is no loss to follow-up, RCT can provide a reliably unbiased estimate of treatment effects.

When designing a RCT one of the most important steps is to generate an unpredictable random sequence of allocation of participants (the randomisation sequence) [134]. This randomisation sequence gives each participant a known, and usually equal, chance of being assigned to any of the groups. The unpredictability is necessary to insure concealment of allocation (i.e. once an individual is eligible for enrollment in the trial, the treating physician has to be blind to what the next allocation is in order to avoid selection bias).

During the PASS and the CASS study, a computer generated random sequence of allocation was used. When enrolling a patient, local investigators signed on the study web page, filling out a "pre-inclusion formula" (including patient initials, date of birth, the civil registration number and a preliminary APACHE II score), in order to get the randomisation result **(Figure 7)**. Thus, the investigator had no chance of knowing the allocation group prior to enrollment.

Figure 7. Screen print of the randomisation web page used in the CASS study



In addition to generating the randomisation sequence, two other important factors need to be considered when designing a RCT; one is a potential imbalance in group size and the other, is the chance that randomisation can create imbalances in baseline characteristics [135]. Especially, in multicentre trials, a potential difference in the number of enrolled patients at each site along with different catchment areas needs to be accounted for. Therefore, in many RCT´s balance of important characteristics is achieved, without sacrificing the advantages of randomisation, by combining two methods; restricted randomisation and stratification, the latter most commonly by centre and other prognostic variables (e.g. age and disease severity) [136].

Restricted randomisation controls the probability of obtaining an allocation sequence with a balance in size between the randomized groups [137]. Block randomisation was first described by Hill in 1951 [138] and is a form of restricted randomisation where a set of permuted blocks is generated for each combination of

prognostic factors. By sequencing participant assignments by block, the probability that each group will contain an equal number of participants is increased. Thus, restricted randomisation strives for unbiased comparison groups, while also striving for comparison groups of about the same size throughout the trial. The latter is especially helpful when performing interim analysis.

Stratified randomisation is used to ensure that an equal number of participants with a specific characteristic, thought to affect the response to the intervention, are allocated evenly among each comparison group. Stratification means having separate randomisation schemes for each combination of characteristics ("stratum"). These "stratums" are generated when planning the trial. However, stratification without restriction accomplishes nothing [136]. Thus, random permuted blocks within strata are the most common form of stratification [139].

In both the PASS and the CASS study restricted randomisation combined with stratification was used. The specific block sizes used in the two randomized trials were decided during the design phase by the trial statistician and the database manager. The size of the blocks in both the PASS and the CASS study changed during the two trials to retain unpredictability and remained concealed as long as the trials were running (i.e. still concealed in the CASS study). In both trials, patients were stratified according to age, APACHE II score and the ICU in which the patient was enrolled. The variables to which patients were stratified were entered in the "pre-inclusion formula" prior to randomisation **(figure 7)**.

Despite being considered the highest level of scientific evidence, randomisation is not always used in trials. RCT's are expensive, labour intensive, and time consuming, and the results apply only to the enrolled participants with relative few question addressed. In addition, ethical aspects may be present making it impossible to investigate certain types of exposures and the question to be addressed may not always fit a randomized set-up. Therefore, often decisions are based on observational evidence with the potential of having unmeasured confounders yielding erroneous conclusions.

At least three key issues that need to be addressed when conducting clinical research: 1) "The file drawer problem" (i.e. publication bias), 2) possibility of bias and confounding and 3) inadequate study size [140].
"The file drawer problem" is a continuingly recurring problem in medical research and is attributed to both sponsors, investigators and the medical research journals [141-143]. A systematic review stated that failure to publish study results is a non-random event profoundly influenced by the direction and strength of the research findings; manuscripts reporting statistically significant (i.e. positive) results are published preferentially over manuscripts with non-significant (i.e. negative) results [143]. One of the major initiatives to tackle this problem is the requirement of registration in a public database before trial initiation [144]. The clinical trials underlying *paper I-II* (The PASS study) and *paper III* (The CASS study) are indeed registered at www.ClinicalTrials.gov, an international registry of clinical trials run by the United States National Library of Medicine at the National Institutes of Health. Furthermore, new academic journals have sprung with the aim for publication and discussion of negative results [145]. Other journals encourage publication of study protocols [146].

Bias and confounding are systematic errors that may be encountered in the collection, analysis, or interpretation of research data. Whereas bias creates an association that is not true, confounding describes an association that is true, but potentially misleading. Bias can arise in both clinical trials and epidemiological studies and can occur through structural deficiencies in a study. Even though considered to be the highest level of scientific evidence, the RCT's cannot see themselves totally free of bias.
Selection bias is the error introduced when the study population does not represent the target population. It can be caused by narrow inclusion criteria or because some potentially eligible participants are selectively excluded from the study, because the investigator knows the group to which they would be allocated if enrolled [147,148]. In clinical trials, the latter can be avoided by generating an unpredictable random sequence of allocation of participants [134], as described earlier. Selection bias in the form of a study population not representing the target population is sometimes referred to as **sample selection bias** [149]. More in general, selection bias can be introduced by conditioning on a common effect or a common effect of causes of both exposure and outcome. In the two clinical trials underlying *paper I-III* the study inclusion criteria's were relatively wide in order to represent a common heterogeneous patient composition at a general mixed ICU. In addition, concealment of allocation was in place. Thus, the study results are unlikely to be affected by selection bias and are applicable to a general ICU population.
Besides selection bias, **information bias** (i.e. misclassification) may occur when investigators are aware of the treatment group to which patients have been allocated. Information bias occurs when the information collected during the trial is subjected to errors. Biological tests such as blood samples are considered to be objective. However, information such ad "Primary cause of ICU admission", are more likely to be, "observer dependent". The studies underlying this thesis (*paper I-III*) where based on use of antimicrobials, blood samples (i.e. platelet count, biomarkers of endothelial damage and TEG parameters) and mortality. Thus, both exposure and response variables must be considered highly objective, with only little potential for the occurrence of information bias.
Ascertainment bias occurs when the results of a trial are systematically distorted by knowledge of which intervention each participant is receiving. The best way to protect a trial against ascertainment bias is by blinding, meaning that all people involved in the trial are unaware of which treatment patients are receiving. Blinding helps to prevent selection bias and protects the randomisation after the interventions are given to study participants [150].
Both the PASS and the CASS study may be regarded as open-label trials (i.e. not blinded). Because of the nature of the interventions (different antimicrobial-strategy guided by a biomarker (the PASS study) and mild induced hypothermia (the CASS study)) it was not possible to blind neither the patients, the investigators nor the staff to the allocation group.
However in the PASS study, all procalcitonin measurements in the control group were blinded during the trial, so that the treating physicians would not include procalcitonin levels in their assessment or treatment of patients. In the CASS study, all TEG profiles were blinded for all patients over the duration of the trial. Thus, the laboratory technicians, the treating physicians and the study investigators were unaware of the TEG results and no adjustment in treatment was performed based on the TEG results.

In *paper I* we assess **channelling bias** (i.e. **confounding by indication**). This type of bias may arise when certain antimicrobial agents were prescribed preferably to patients with certain characteristics and different prognosis (e.g. worse prognosis). Because the use of specific antimicrobials was not directly randomized, it is possible that some unmeasured characteristics were imbalanced between groups. In addition, in survival analysis, bias may arise when a patient can experience an event different from the event of interest. For example, a patient may have died prior to experiencing thrombocytopenia. If the cause of death is not unrelated to the probability of thrombocytopenia, competing risks occurs. In paper *I,* the potential presence of confounding by indication was addressed by defining a composite endpoint of "thrombocytopenia (relative or absolute) or death". Our results showed that piperacillin/tazobactam and ciprofloxacin were associated with absolute thrombocytopenia *only* when using the composite endpoint including risk of death. Furthermore, in *paper I* we tested if there were any interactions between the subsets of patients with or without severe sepsis/septic shock at study enrollment (i.e. if the effect on the platelet count of any of the investigated antimicrobials was consistent across different strata).

In *paper I-II* we have tried to account for potential **confounders**. A confounder is a variable that is independently related to both the exploratory variable of interest and the outcome, without being on the causal pathway between the two, and whose presence may (partly or entirely) explain the association between these. In *paper I-II*, the Poisson and Cox models were adjusted for time-fixed variables and stratification according to the potential confounding factors (i.e. severe sepsis/septic shock) were used in *paper I*. The study underlying *paper III*, was entirely based on randomization. The strength of the RCT is that it distributes confounding factors (both known and more importantly unknown factors) equally between the randomisation groups.Thus, no adjustment was used.

SAMPLE SIZE AND POWER CALCULATION

In both observational studies and randomized trials adequate statistical power is needed to avoid type II errors, and this is why a calculation of power or sample size should always be performed [151].

In *paper I,* a chi-square test for the randomized groups with a significant level at 5% and a power of 80% was used. Using a premise of the endpoint occurring in 20% of patients in the SOC group and 1147 patients were included in the analysis, a detection limit (two-sided) for relative risk of 1.5 in the high-exposure group was established.

In *paper II* Cox regression was used with a detection limit (two-sided) for hazard ratio of 1.5, the summed squared correlations (Σrho^2) to the risk of the endpoint was calculated to 0.09 and the significance level was set to 5%. Using a premise of 30% risk of mortality in the lower biomarker quartile (Q1) and the frequency of the exposure was set at 25% a power of 95% was established with 1103 enrolled patients.

In *paper III* the sample size calculation was performed based on a cohort of patients suffering severe sepsis or septic shock [114]. By visual assessment (using a histogram) and by performing the Kolmogorov-Smirnov test, we established that the distribution of maximum amplitude (MA) in the cohort was approximately normally distributed. Student's t-test was used in the sample size calculation with the significant level at 5% and the power at 90%. To be able to detect a 15% difference in MA between the two randomized group, 45 enrolled patients were needed in each randomized group. In order to make sure that we reached a minimum of 90 enrolled patients with total TEG data during follow-up and assuming a drop-out rate of 30% (either due to death, discharged or trouble with TEG analyses) we calculated that we needed to measure TEG on a total of 117 participants.

Sample size and power calculations were performed using StudySize 2.04, CreoStat HB, Sweden.

PAPER I: THE POTENTIAL OF ANTIMICROBIALS TO INDUCE THROMBOCYTOPENIA IN CRITICALLY ILL PATIENTS: DATA FROM A RANDOMIZED CONTROLLED TRIAL [152]

RATIONALE AND OBJECTIVE

Thrombocytopenia is common among patients in the ICU and can be caused by sepsis [64,71,72] or by antimicrobials prescribed in relation to sepsis [73,74]. Despite commonly referenced, the exact incidence of antimicrobial-induced thrombocytopenia is unknown [64,75-77]. The aim of this study was to determine whether exposure to broad-spectrum antimicrobials increases the risk of thrombocytopenia in septic patient.

METHODS

Data from a randomized controlled trial [115], that per design lead to an experimental separation of exposure to antimicrobials proposed to cause thrombocytopenia, was used. Analyses were divided into two parts. In the first part, a randomized design investigating time to thrombocytopenia among the two groups (SOC group vs. high-exposure group) was used. In the second part, the two randomized groups were pooled into one cohort investigating the association between current drug use and the response in platelet count. Follow-up was defined as death or day 28, and thrombocytopenia was defined on the basis of platelet count as either absolute (platelet count $\leq 100 \times 10^9$/L) or relative (≥20% decrease in platelet count).

RESULTS

Of the 1147 patients with platelet data available, 18% had absolute thrombocytopenia within the first 24 hours of admission to ICU and an additional 17% developed absolute thrombocytopenia during follow-up. Furthermore, 57% developed relative thrombocytopenia during follow-up. Absolute and relative thrombocytopenia day 1-4 was associated with increased mortality (HR: 1.67 [95% CI: 1.30 to 2.14]; 1.71 [95% CI: 1.30 to 2.30], P<0.0001, respectively).

Table 1. Patient characteristics at study enrollment

Characteristic	Randomization arm			
	SOC (n=571)	High-exposure (n=576)	Total (n=1147)	p-value*
Gender, n (%) Male	317 (50.0)	318 (50.0)	635 (55.4)	0.9165
Age, years Median (IQR)	67 (59-75)	67 (58-76)	67 (58-75)	0.4337
>65	320 (56.0)	325 (56.4)	645 (54.9)	0.8965
Body Mass Index, kg/m^2 Median (IQR)	24.7 (22.2-27.8)	24.8 (22.5-28.1)	24.7 (22.2–27.8)	0.3683
>30	96 (16.8)	104 (18.1)	200 (17.4)	0.5795
Severe sepsis/septic shock	196 (34.3)	225 (39.1)	421 (36.7)	0.2408
APACHE II Median (IQR)	18 (13-24)	18 (13-24)	18 (13-24)	0.5218
≥20, no. (%)	232 (40.6)	215 (37.3)	447 (39.0)	0.2518
Surgical patient, no. (%)	166 (29.1)	158 (27.4)	324 (28.2)	0.5374
Platelet count (x 10^9/L) Median (IQR)	204 (132-301)	202 (117-295)	203 (126-298)	0.2408
PC ≤100 no. (%)	93 (16.1)	118 (20.0)	211 (18.4)	0.5692
Charlson score Median (IQR)	1 (0-2)	1 (0-2)	1 (0-2)	0.2631
>1	207 (36.3)	193 (33.5)	400 (34.9)	0.3298

In the high-exposure group, patients received more antimicrobials including piperacillin/tazobactam, meropenem and ciprofloxacin, compared with the SOC group. Cefuroxime was prescribed more frequently in the SOC group **(Figure 8)**.

The median platelet count did not differ significantly at any time day 1-28 between the two randomized groups (p ≥ 0.08).

Furthermore, no difference in the occurrence of absolute and rela tive thrombocytopenia was observed between patients in the SOC group and in the high-exposure group during follow-up **(Figure 8)**. Furthermore, daily decrease in platelet count (x 10^9/L) did not differ when comparing the SOC vs. high-exposure group (day 1-7; -1.1 [95%CI:-2.5 to 4.6], p=0.5613 and day 1-28; -1.7 [95%CI:-3.8 to 0.5], p=0.1403, respectively).

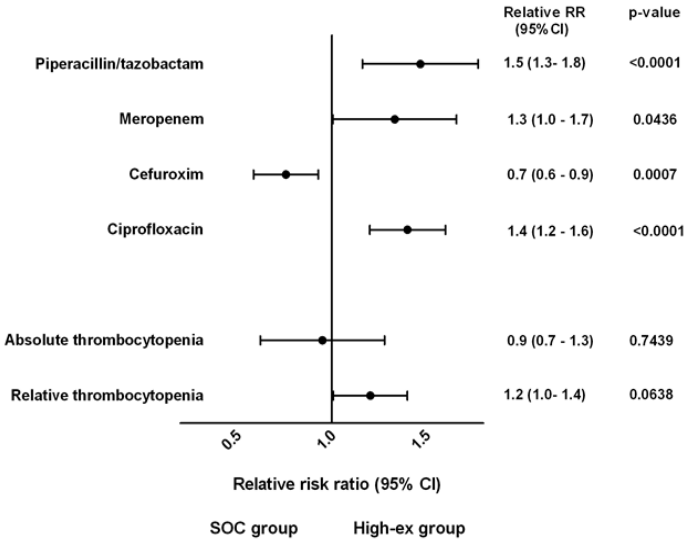
When combining the whole cohort, no association was observed with regard to absolute thrombocytopenia and any of the investigated antimicrobial agents **(Table 2)**. However, current use of either ciprofloxacin or piperacillin/tazobactam increased the risk of relative thrombocytopenia **(Table 2)**.

Table 2. Rate ratio of absolute and relative thrombocytopenia

	Absolute thrombocytopenia (95% CI)		Relative thrombocytopenia (95% CI)	
Antimicrobials	Unadjusted	Adjusted	Unadjusted	Adjusted
None	0.31 (0.17 to 0.54)	0.26 (0.15 to 0.48)	0.39 (0.28 to 0.53)	0.38 (0.28 to 0.53)
Piperacillin/ Tazobactam	0.93 (0.58 to 1.50)	0.86 (0.51 to 1.44)	**1.61 (1.24 to 2.10)**	**1.44 (1.10 to 1.89)**
Meropenem	1.10 (0.63 to 1.92)	1.09 (0.59 to 2.05)	**1.46 (1.07 to 1.99)**	1.36 (0.96 to 1.92)
Ciprofloxacin	1.62 (0.93 to 2.82)	1.59 (0.88 to 2.90)	**2.45 (1.76 to 3.41)**	**2.08 (1.48 to 2.92)**
Cefuroxime	1.00	1.00	1.00	1.00

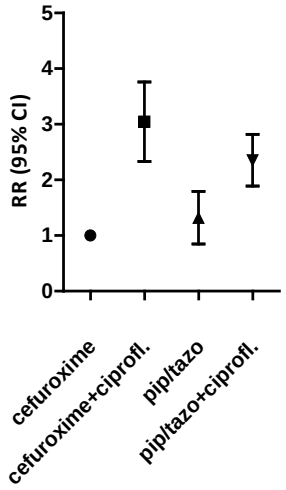
With regard to the combined endpoint of "relative thrombocytopenia or 28-day mortality", no association between current use of piperacillin/tazobactam and the combined endpoint was observed. However, current use of ciprofloxacin was associated with an increased risk of "relative thrombocytopenia or 28-day mortality" (RR: 1.85 [95% CI: 1.06 to 3.24]). Furthermore, piperacil-

Figure 8: Use of frequently prescribed antimicrobials and the occurrence of thrombocytopenia in the two randomized groups



lin/tazobactam in combination with ciprofloxacin increased the risk of relative thrombocytopenia compared to receiving piperacillin/tazobactam without ciprofloxacin **(Figure 9)**.

Figure 9: Rate ratio (RR) of relative thrombocytopenia of patients receiving single drug cefuroxime or piperacillin/tazobactam (pip/tazo) or drugs in combination with ciprofloxacin (ciprofl.).



DISCUSSION

Using a randomized comparison, no significant difference in risk of thrombocytopenia between patients with standard vs. high exposure to antimicrobials was observed. In subsequent analyses in a pooled cohort, a modest association between ciprofloxacin, and less so piperacillin/tazobactam, and thrombocytopenia was observed. Therefore, the findings suggest that antimicrobials only marginally affect platelet count during sepsis. Conversely the development of thrombocytopenia is possibly better explained by a host of other risk factors including severity of infection, increased age, prior surgery and comorbidity.

The knowledge of antimicrobial-induced thrombocytopenia is based on case studies [73,75,76]. The application of a randomized design contributes with novel information, allowing for quantification of thrombocytopenic risk associated with use of antimicrobials. Although statistically insignificant but consistent with the a priori hypothesis, a trend for excess risk of relative thrombocytopenia, a more sensitive marker of subtle changes in platelet count, was observed for the high-exposure group (p=0.06).

Using the entire cohort, the association of specific antimicrobials with thrombocytopenia was investigated. Consistent with the existing literature [76,153,154], current use of ciprofloxacin and/or piperacillin/tazobactam was associated with relative thrombocytopenia. None of the antimicrobials were however, associated with absolute thrombocytopenia. As certain antimicrobial agents may have been used more frequently in patients with deteriorating or worse prognosis, and thus higher likelihood of thrombocytopenia or death, confounding by indication cannot be altogether disregarded. Although regression analyses were adjusted for current prognostic factors, it is conceivable that other factors associated with disease severity (e.g. specific type of infection) and use of specific antimicrobials remained undetected. Plausibly, this is the most likely explanation as to why piperacillin/tazobactam and ciprofloxacin were found to be associated with absolute thrombocytopenia only when using the composite endpoint.

Since ciprofloxacin is often prescribed in combination with piperacillin/tazobactam, we hypothesized that the association between piperacillin/tazobactam and relative thrombocytopenia was driven by the thrombocytopenic induction of ciprofloxacin. Therefore, a model including agents in combination with or without ciprofloxacin was generated. The results indicated that such an effect was present and thereby underlining that use of ciprofloxacin in any combination may affects platelet count.

Time from drug exposure to thrombocytopenia has been reported to be between 1 day and up to 3 years [155]. However, as prior knowledge regarding timing of ciprofloxacin- and piperacillin/tazobactam-induced thrombocytopenia is derived from case reports, it remains impossible to reliably estimate the time course of the condition. Several reports have nonetheless reported decreasing platelet count and development of thrombocytopenia within 4 days after exposure to ciprofloxacin [153,156-158] and piperacillin/tazobactam [159-161], consistent with our observations. The unpredictable timing of drug-induced thrombocytopenia is undoubtedly due to variations in mechanism, [73,76], including possible pre-sensitization due to prior exposure and re-exposure [79].

Due to the non-significant result from the randomized analysis as well as the potential presence of residual confounding in the second part of the analysis, we can only speculate if the observed effect of ciprofloxacin on platelet count is true. Nevertheless, there could be several explanations for why ciprofloxacin in particular may affect platelets in critically ill patients. Firstly, the cumulative dose of ciprofloxacin might be increase in critically ill patients due to acute reduction in renal function [162] augmented by primary reduced glomerular filtration rate secondary to the age demographics [163].

Secondly, severe infection, common in our population, is known to independently induce platelet [72]. Despite the precise mechanism whereby ciprofloxacin might affect the platelets remains unknown, a structural link between fluoroquinolones and quinines has been purposed to explain the ability of the agent to affect the platelet count [153,164,165]. Thus, fluoroquinolones may induce drug dependent immune-mediated thrombocytopenia. However, it is important to stress that despite our findings indicating that ciprofloxacin may affect the platelet count, we can only speculate if these findings are based on a correct rejection of the true null hypothesis or if they are due to a type I error. The well-powered randomized comparison did not identify a significant difference in risk of thrombocytopenia between patients with standard vs. high exposure to antimicrobials.

Finally, we observed that a third of the patients suffered absolute thrombocytopenia and most episodes occurred within the first 4 days after ICU admission, consistent with prior findings [66]. Patients with absolute thrombocytopenia within the first few days after ICU admission had a 67% increased relative risk of death within 28 days. Absolute platelet counts ≤100 x 10^9/L increases the risk of bleeding [62]. In addition, bacterial infection

triggers platelet activation and secretion of antimicrobial peptides [69] and thus, a decrease in platelet count may protract clearance of infection [70].

Interestingly, a 20% decrease in platelet count predicted 28-day mortality; three-quarters of patients with relative thrombocytopenia never reached a platelet count below 100 x 10^9/L but still retained a 71% increased risk of death if the episode occurred within day 1-4 after study enrollment.
It is possible that the platelet count is a surrogate for immune activation or severe infection. Nevertheless, as the analyses were adjusted for severe sepsis/septic shock it seems that there is a separate effect of thrombocytopenia (absolute and relative) on risk of death which cannot be explained by the severe infection status. Thus, it could be that having a large number of well-functioning platelets assist in improving survival from critical illness and that a ≥20% decrease in the platelet count seems to influence prognosis during sepsis, even when the threshold for absolute thrombocytopenia is never reached [166].

STRENGTHS AND LIMITATIONS
The most prominent strength, in this paper, is that the primary results are based on a prospective randomized design, thereby avoiding a wide range of bias.
A power calculation was performed stating that the study was capable of detecting even a small effect difference in platelet count between the two randomized groups.
Highly sensitive definitions of endpoints were used including ">20% decrease in platelet count" and a response in platelet count in the raw scale.

In contrast, the second part of the paper was based on the pooled cohort. Despite, the analyses in this part of the study was adjusted for potential confounder and several sensitivity analyses was employed in order to test the robustness of the results, it is likely that the results are subjected to residual confounding and bias.
To exclude that initial sepsis severity had an effect on the main results, patients were stratified according to severity of infection at study enrollment (with or without severe sepsis/septic shock).
The results from the stratified analysis displayed consistency across these strata. Furthermore, even though the antimicrobial guidelines are more or less the same among the ICUs participating in the PASS study, there was a chance that a difference in empiric treatment would affect the results. Thus, when investigating the association between single agents and thrombocytopenia in the observational analyses, we adjusted for infection status and site of randomisation, and hence minimized a potential difference in empiric treatment.
When investigating the association between current use of antimicrobials and "risk of thrombocytopenia or death" the presence of confounding by indication cannot be dismissed if certain antimicrobials were used more in patients with worse prognosis. Although estimates were adjusted for current prognostic factors, it is conceivable that other factors associated with disease severity (e.g. specific type of infection) and use of specific antimicrobials was undetected. This is most likely the explanation as to why piperacillin/tazobactam and ciprofloxacin were found to be associated with absolute thrombocytopenia only when using the composite endpoint.
A major and important strength of the second part of the study was the fact that use of antimicrobials was fitted as time-updated variables (i.e. current use). This made is possible to distinguish between continued vs. intermittent use of an agent, making it possible to detect agent-specific effects on platelet count while patients were truly exposed to the specific agent.

In analyses investigating the specific effect of individual antimicrobials on platelet count, ciprofloxacin was observed to be the agent with the greatest association with thrombocytopenia.
To detect the individual effect of ciprofloxacin, a model was created comparing current use of an agent with the same agent prescribed in combination with ciprofloxacin. Although we cannot exclude the possibility of confounding by indication, we did observe similar differences between antimicrobial agents when using different approaches to the aforementioned analyses.

PAPER II: PROFOUND ENDOTHELIAL DAMAGE PREDICTS IMPENDING ORGAN FAILURE AND DEATH IN SEPSIS [167]
RATIONALE AND OBJECTIVE
Endothelial damage contributes to organ failure and mortality in sepsis, but the extent of the contribution remains poorly quantified.
The study aim was to assess the association between biomarkers of superficial and profound endothelial damage (syndecan-1 and soluble thrombomodulin (sTM), respectively), organ failure and death in sepsis.

METHODS
Data from a clinical trial comprising critically ill patients predominantly suffering sepsis was used. Syndecan-1 and sTM levels at study enrollment were determined. The predictive ability of biomarker levels on death and organ failures during follow-up were assessed in Cox models adjusted for potential confounders including key organ dysfunction measures assessed within 24 hours after ICU admission.

RESULTS
In total, 1.103 of the 1.200 randomized patients were included in these analyses (n=97 were excluded due to missing serum samples at time of study enrollment) **(Table 3).**

After rounding Syndecan-1 and sTM levels the following cut-offs for the four quartiles were set as follows: Syndecan-1 (ng/ml): <70 (Q1), 70-134 (Q2), 135-239 (Q3), > 240 (Q4) and sTM (ng/ml): <7 (Q1), 7-10 (Q2), 10-14 (Q3), >14 (Q4). Patients diagnosed with sepsis, severe sepsis or septic shock all displayed significantly higher median levels of biomarkers of endothelial damage compared with non-infected patients **(Figure 10)**.

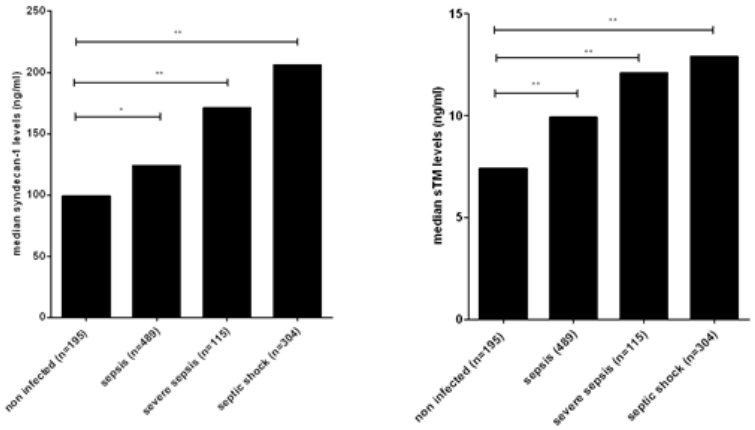
Table 3: Patients characteristics at enrollment (number and % or median and interquartile range (IQR)) by quartile (Q) biomarkers

	Patients	Syndecan-1					Soluble thrombomodulin (sTM)				
Charac-teristics	Total (n=1103)	Q1 <70 ng/ml (n=268)	Q2 70-134 ng/ml (n=283)	Q3 135-239 ng/ml (n=273)	Q4 >240 ng/ml (n=279)	p-value*	Q1 <7 ng/ml (n=279)	Q2 7-10 mg/ml (n=242)	Q3 10-14 ng/ml (n=286)	Q4 >14 ng/ml (n=296)	p-value**
Male, No. (%)	611 (55.4)	130 (48.5)	159 (56.2)	154 (56.4)	168 (60.2)	**0.0060**	144 (51.6)	133 (55.0)	163 (57.0)	171 (57.8)	0.1382
Age>75 years, no. (%)	274 (24.8)	71 (26.5)	70 (24.7)	68 (24.9)	65 (23.3)	0.6279	58 (20.8)	59 (24.9)	86 (30.1)	71 (24.0)	0.2346
Intervention, no. (%)	556 (50.4)	126 (47.1)	144 (50.9)	137 (50.2)	149 (53.4)	0.1351	133 (47.7)	114 (47.1)	153 (53.5)	156 (52.7)	0.2277
Sepsis (severe/ shock), no. (%)	413 (37.4)	57 (21.3)	94 (33.2)	111 (40.7)	151 (54.1)	**<0.0001**	47 (16.8)	72 (29.8)	134 (46.9)	160 (54.1)	**<0.0001**
APACHE II ≥25, no. (%)	270 (24.5)	46 (17.2)	57 (20.1)	59 (21.6)	108 (38.7)	**<0.0001**	39 (14.0)	40 (16.5)	79 (27.6)	112 (37.8)	**<0.0001**
Surgical pt., no. (%)	316 (28.6)	63 (23.5)	90 (31.8)	81 (29.7)	82 (29.4)	0.1191	60 (21.5)	86 (35.5)	100 (35.0)	70 (23.6)	0.1246
PC< 100, no. (%)	194 (17.6)	12 (4.6)	35 (12.4)	50 (18.3)	97 (34.8)	**<0.0001**	26 (9.3)	24 (9.7)	54 (19.9)	90 (30.4)	**<0.0001**
Charlson score >1, no. (%)	384 (34.8)	82 (30.6)	99 (35.0)	87 (31.9)	116 (41.6)	**0.0076**	77 (27.6)	69 (28.5)	112 (39.2)	126 (42.7)	**0.0002**
Bilirubin Median (IQR)	10 (5-17)	7 (4-11)	9 (5-15)	9 (6-17)	10 (8-33)	**<0.0001**	8 (4-12)	9 (5-14)	10 (5-19)	13 (7-26)	**<0.0001**
eGFR Median (IQR)	51 (27-82)	82 (41-92)	58 (35-92)	45 (27-71)	3 (19-64)	**<0.0001**	82 (59-109)	66 (46-194)	40 (27-63)	25 (15-41)	**<0.0001**
Mechanical vent., no. (%)	739 (67.0)	175 (65.3)	182 (64.3)	185 (67.8)	197 (70.6)	0.9334	190 (68.1)	169 (69.8)	202 (70.6)	179 (60.5)	0.9633
MAP <50mmHg, no. (%)	43 (3.9)	11 (4.1)	10 (3.5)	9 (3.4)	13 (4.7)	0.2155	11 (3.9)	4 (1.7)	15 (5.2)	13 (4.4)	0.8773
MOF, no. (%)	388 (35.2)	48 (17.9)	80 (28.3)	99 (36.7)	161 (57.7)	**<0.0001**	38 (13.6)	47 (19.4)	125 (43.7)	178 (60.1)	**<0.0001**
> 1 inotropics no. (%)	578 (52.4)	98 (36.6)	145 (51.2)	145 (53.1)	190 (69.6)	**<0.0001**	98 (35.1)	114 (47.1)	171 (59.8)	195 (65.9)	**<0.0001**
Syndecan-1, Median (IQR)	135 (72-242)	42 (29-56)	100 (85-117)	186 (164-214)	291 (262-342)	**<0.0001**	75 (37-142)	101 (60-178)	165 (93-249)	226 (157-289)	**<0.0001**
sTM, Median (IQR)	10 (7-14)	7 (5-10)	9 (7-13)	12 (8 -15)	14 (11-18)	**<0.0001**	5 (3-6.)	9 (8-9)	12 (11-13)	17 (15-20)	**<0.0001**

***Characteristics and biomarker levels at study enrollment were compared between participants with syndecan-1 <70 ng/mL (Q1) and those with syndecan-1 >240 ng/mL (Q4) using chi-square tests for categorical variables and Wilcoxon rank tests for continuous variables. ** Characteristics and biomarker levels at enrollment were compared between participants with soluble thrombomodulin <7 ng/mL (Q1) and those with soluble thrombomodulin >14 ng/mL (Q4) using chi-square tests for categorical variables and Wilcoxon rank tests for continuous variables. PC (platelet count (10^9/L), eGFR (ml/ min/1.72m^2), Bilirubin (µmol/l), syndecan-1 (ng/ml), TM (ng/ml), Multiple organ failure: MOF.**

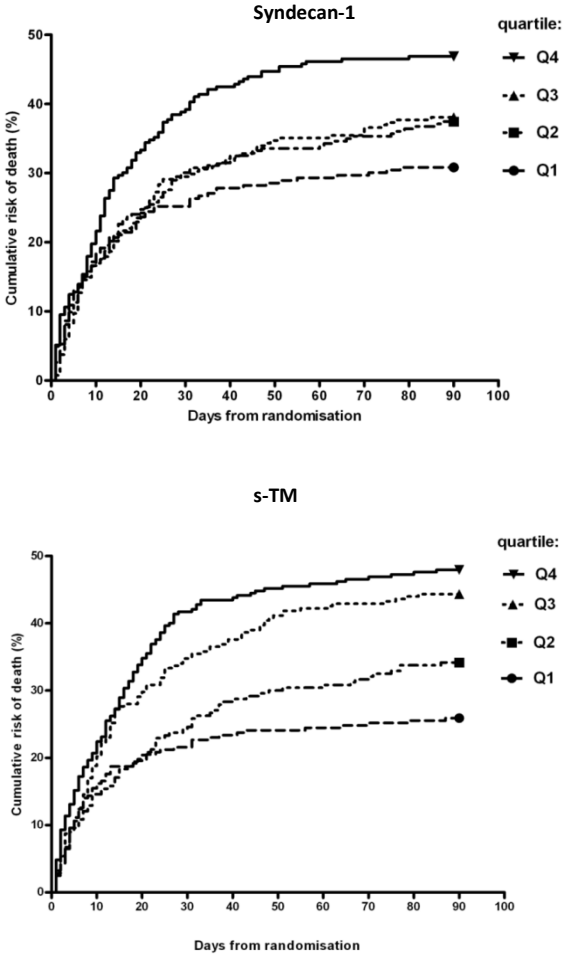
Figure 10. Median levels of syndecan-1 and soluble thrombomodulin at study enrollment stratified by infection status

Number of patients: Syndecan-1 (ng/ml) non-infected 99 (47-195); sepsis 124 (64-216); severe sepsis 171 (80-254); septic shock 192 (90-225) and sTM (ng/ml) non-infected 7.4 (4.7-11.6); sepsis 9.6 (6.5-13.3); severe sepsis 12.1 (9.0-14.9); septic shock 12.9 (9.6-16.9). Figure display median levels and p values *<0.05 and **<0.0001. Soluble thrombomodulin: sTM.



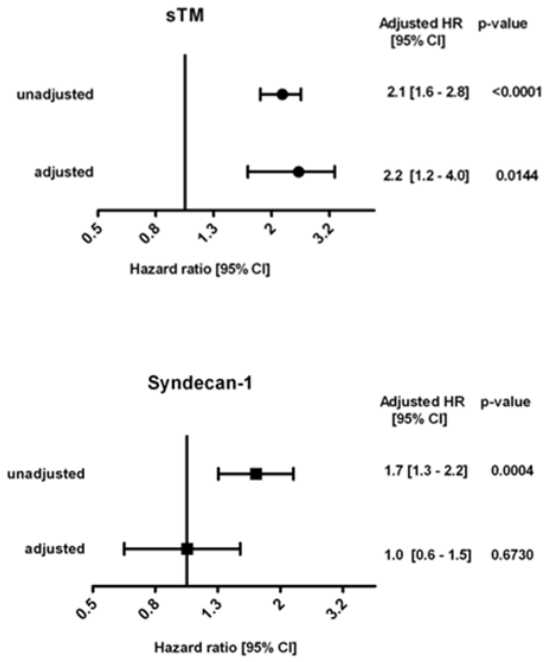
A total of 418 of 1103 (37.9%) patients had died by day 90, and the risk of death increased strikingly for higher quartiles of syndecan-1 and sTM levels at enrollment **(Figure 11)**.

Figure 11. 90-day mortality stratified by syndecan-1 and soluble thrombomodulin at study enrollment



In unadjusted Cox models, the hazard ratio (HR for Q4 vs. Q1) for syndecan-1 was 1.7 [95% CI: 1.3 to 2.2], P=0.0004 and 2.1 [95% CI: 1.6 to 2.8], P<0.0001 for sTM **(Figure 12)**.

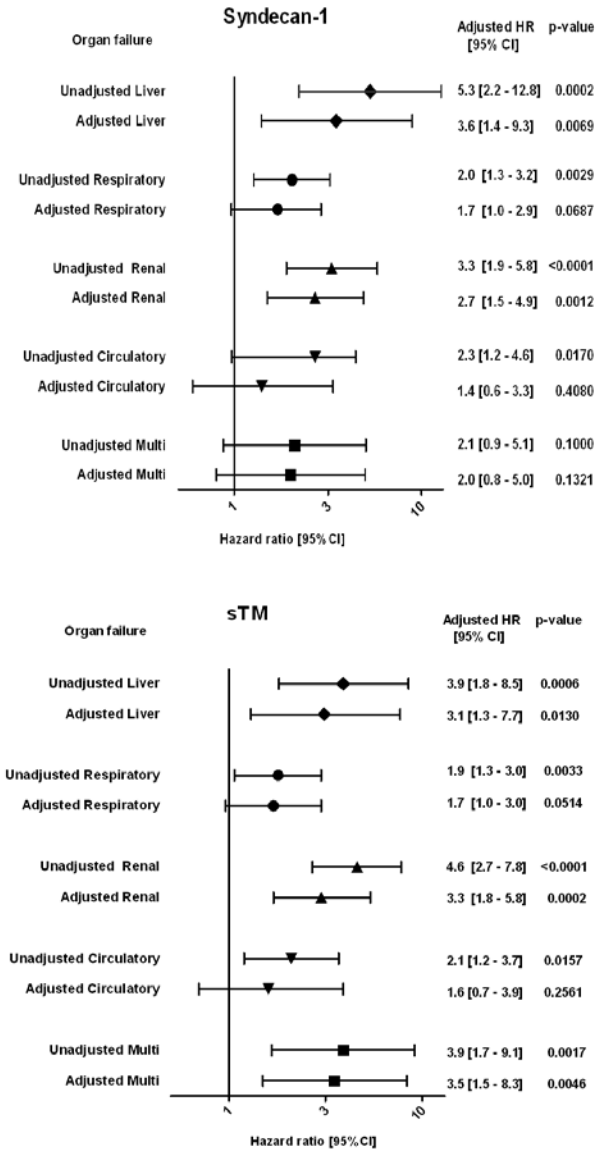
Figure 12. Syndecan-1 and soluble thrombomodulin levels at study enrollment and risk of 90-day mortality



Adjusting for potential confounders, the association between syndecan-1 levels at study enrollment and 90-day mortality was no longer evident, whereas sTM level remained an independent predictor of 90-day mortality **(Figure 12)**.

Levels of biomarkers of endothelial damage at time of study enrollment correlated with markers of liver and renal damage (syndecan-1 (Rho=0.32, p<0.0001 and Rho=0.26, p<0.0001, respectively) and sTM (Rho= -0.50, p<0.0001 and Rho= -0.60, p<0.0001, respectively). Furthermore, syndecan-1 and sTM levels at enrollment independently predicted impending liver failure and renal failure **(Figure 13)**.

Figure 13. Syndecan-1 and soluble thrombomodulin levels at study enrollment and association with risk of development of organ failure day 2-28



sTM but not syndecan-1 levels, were associated with the risk of developing multiple organ failure (≥2 single organ failure) **(Figure 13)**.

DISCUSSION

High levels of soluble thrombomodulin (sTM), a biomarker reflecting profound endothelial damage, at time of admission to the ICU independently predicted 90 day mortality and impending multiple organ failure in septic patients. In contrast, a biomarker of superficial endothelial damage (syndecan-1) did not possess the same predictive capacity, neither for death, nor multiple organ failure.

The fact that sTM but not syndecan-1 predicted development of death and multiple organ failure is likely explained by the biology of the two biomarkers of endothelial damage; syndecan-1 reflects superficial endothelial damage as it constitutes part of the glycocalyx (i.e. the superficial luminal endothelial layer) [92,168] whereas sTM is an endothelial cell transmembrane glycoprotein that is only released upon more profound endothelial disruption [94,95]. Thus, direct damage to the endothelial cell itself, reflected by high levels of sTM, is more likely to associate with death and severe organ damage compared with damage to the more superficial glycocalyx layer which is rapidly affected by infection [90], trauma [169] and ischemia-reperfusion injury [168].

As sTM is excreted by the kidneys [94], it could be that the predictive properties of sTM reflect accumulation in the same manner as the well-established biomarker creatinine [170]. However, the presented results demonstrate an association with mortality independent of eGFR, thereby rejecting accumulation as the sole explanation. Hence, these results support a "previously stated" hypothesis [171], that the relationship between profound endothelial damage and mortality seems not only to be mediated directly through development of organ failures. Instead, profound endothelial damage may be an independent pathogenetic factor in sepsis-induced organ failure and subsequent death. Thus, biomarkers of endothelial damage may identify organ failure associated mortality not detected by current used markers of organ dysfunction.

Previous studies have found correlations between syndecan-1 and sTM levels and clinical endpoints such as mortality [125,126,171-173]. However, these studies are characterized by relatively small sample sizes, without GCP controlled designs and predominantly lack of use of out-of-hospital mortality data. More importantly, our study is the first of its kind to employ a Cox proportional hazard model adjusted for organ dysfunction in identifying the independent contribution of endothelial damage as reflected by syndecan-1 and sTM to mortality in sepsis.

When organ failure complicates sepsis, the risk of death is increased [13,35]. Microvascular alterations, including disruption of the endothelium, have been proposed to play a significant role in the development of organ failure [35,60]. sTM levels demonstrated strong ability of predicting multiple organ failure even after adjustment for known factors associated with mortality. This was not true for syndecan-1 levels. This observation is key, not only as it adds to the understanding of the interplay between the endothelium and organ dysfunction during sepsis, but also as this implies that biomarkers of profound endothelial damage may be used in the risk assessment of individual patients predicting development of multiple organ failure.

Syndecan-1 and sTM both demonstrated strong associations with development of liver and kidney failure in accordance with the large number of endothelial cells in both organs expressing syndecan-1 [174,175] and thrombomodulin [176,177]. However, surprisingly, despite biomarkers of endothelial damage being correlated with inotropic score at ICU admission, syndecan-1 and sTM only predicted impending circulatory failure when this endpoint was combined with death. Superficial and profound damage to the endothelium has been proposed to cause hypotension [83]. As both the glycocalyx and thrombomodulin play important

roles in maintaining the endothelial barrier and controlling hemostasis, deregulation and disruption may lead to microvascular dysfunction [82,178]. Although the use of inotropic score has been validated in patients suffering sepsis [179], we cannot rule out that our definition of circulatory failure (inotropic score> 20 or MAP < 50 mmHg), may be inadequate to describe this specific organ failure.

STRENGTHS AND LIMITATIONS

The major strength of this study is the large sample size, the long follow-up period and the inclusion of biomarkers reflecting both superficial and profound endothelial damage. In addition, all data collected during the trial was strictly monitored according to GCP guidelines. However, as the study is observational, it did not allow independent evaluation of the cause-effect relationship between endothelial damage and clinical outcome. Furthermore, the use of surrogate markers of organ failure is subject to uncertainties due to the possibility that other definitions, for example different cut-off values, might correlate differently with the exposure variable. However, an international consensus for definition of organ dysfunction does not exist, and as such, we chose currently often used definitions. Thus, the included endpoints were based on recognized classifications associated with prognosis in septic patients [130,180,181]. In the presented study, endothelial damage was defined as an increase in levels of two biomarkers (syndecan-1 and sTM, respectively). Different endothelial function tests have been developed within the last couple of years, among them reactive hyperaemia peripheral arterial tonometry (RH-PAT) [182], a simple and user-independent bedside technique measuring microvascular endothelial function. Despite being promising tools, endothelial function test are still far from being routine. The biomarkers of endothelial damage chosen for this study have been documented in in several well conducted studies to give a solid surrogate measure of endothelial function. In addition, serum biomarkers are a relatively cheap and easy method of assessing endothelial function.

PAPER III: MILD INDUCED HYPOTHERMIA: EFFECTS ON SEPSIS-RELATED COAGULOPATHY--RESULTS FROM A RANDOMIZED CONTROLLED TRIAL [183]

RATIONALE AND OBJECTIVE

Coagulopathy during sepsis is associated with poor outcome [35,39,114] and mild induced hypothermia (cooling to 32-34°C for 24 hours, MIH) has been proposed as a potentially beneficial treatment [102,103]. However, it is not clear how coagulopathy is affected by lowering of the body temperature [184], and the possibility of MIH worsening sepsis-related coagulopathy is present [110]. Thus, the aim of this study was to determine whether MIH worsens sepsis-related coagulopathy, as measured by thrombelastography (TEG).

METHODS

Interim data collected during the The Cooling And Surviving Septic shock (CASS) study was used. In this trial, patients suffering severe sepsis or septic shock were allocated to either the MIH- or control group (the latter, uncontrolled temperature). Thrombelastography (TEG) analyses were performed in 100 consecutive enrolled patients with a complete TEG profile three times during follow-up. All TEG profiles were blinded during data collection. Reaction time (R), maximum amplitude (MA) and patient characteristics were used in the analyses presented here.

RESULTS

One hundred patients (control n=50 and intervention n=50) with complete TEG during follow-up were enrolled **(Table 4)**.

Table 4: Patient characteristics at study enrollment reported as median (IRQ) is nothing else is specified

Variable	Unit	Control group (n=50)	MIH group (n=50)	Total (n=100)
Age	year	67 (58-76)	70 (60-80)	68 (58-78)
Male	no. (%)	28 (56)	31 (62)	59 (59)
BMI	kg/m^2	24.7 (22.5-27.8)	24.9 (22.2-27.7)	24.7 (22.2-27.8)
SOFA score		11 (9-14)	11 (9-13)	11 (9-13)
MAP	mmHg	64 (55-75)	62 (55-68)	63 (55-72)
Temperature	◦C	37.2 (36.9-38.2)	37.3 (35.6-38.1)	37.2 (36.5-38.1)
Dialysis	no. (%)	6 (12)	8 (16)	14 (14)
Mechanical ventilated	no. (%)	50 (100)	50 (100)	100 (100)
PaO_2/FiO_2	Mmhg	128 (77-184)	118 (84-206)	123 (84-194)
Inotropic score		20 (11-30)	22 (12-45)	21 (11-40)
Creatinine	µmol/l	86 (58-168)	131 (66-196)	106 (64-190)
Bilirubin	µmol/l	11 (7-21)	10 (7-18)	11 (7-20)
CRP	mg/L	163 (82-292)	176 (94-279)	173 (88-288)
Leukocytes	$X10^9/L$	14.6 (10.1-19.8)	14.7 (9.2-22.4)	14.6 (10.0-20-8)
Albumin	µmol/L	26 (23-30)	25 (21-29)	26 (21-29)
ALT	U/L	37 (27-60)	62 (22-65)	49 (22-82)
Lactate	mmol/l	1.6 (1.1 - 2.4)	1.6 (1.3-3.2)	1.6 (1.1 - 2.8)

At time of enrollment, 3%, 38%, and 59% had a hypocoagulable (MA<51 mm), normocoagulable, and hypercoagulable (MA>69 mm) TEG clot strength (MA), respectively **(Table 5)**.

Table 5: Patient coagulation status at study enrollment

Variable	Unit	Control group (n=50)	MIH group (n=50)	Total (n=100)
D-dimer	mg/L	2.7 (1.6-6.6)	4.9 (1.4-7.3)	3.4 (1.4-6.6)
aPTT	sek	36 (32-41)	39 (33-53)	37 (32-48)
INR	no. (%)	1.2 (1.0 - 1.4)	1.3 (1.1-1.8)	1.3 (1.1 - 1.5)
Platelet count	$X10^9/L$	194 (112-292)	194 (147-318)	194 (140-297)
Haemoglobin	mmol/l	6.4 (5.5-7.6)	6.6 (5.6-7.4)	6.5 (5.5-7.4)
R	min	6.9 (5.2 - 8.5)	7.2 (6.2 - 10.1)	7.2 (5.8 - 9.3)
MA	mm	71 (64 - 77)	74 (67 – 77)	72 (67 – 77)
Hypo-coagulable (MA<51 mm)	no. (%)	1 (2)	2 (6)	3 (3)
Normo-coagulable (MA=51-69 mm)	no. (%)	22 (44)	16 (32)	38 (38)
Hyper-coagulable (MA>69 mm)	no. (%)	27 (54)	32 (64)	59 (59)

At time of enrollment, R strongly correlated with INR (rho= 0.39, p<0.0001) and aPTT (rho=0.49, p<0.0001) and MA correlated with platelet count (rho= 0.53, p<0.0001) and d-dimer (rho= -0.55, p<0.0001). A statistical correlation was also observed between MA and SOFA score (rho= -0.30, p=0.0036), MA and bilirubin (rho= -0.33, p<0.0001) as well as MA and Pao2/Fio2 ratio (rho= -0.31, p=0.0019). Furthermore, R and MA correlated (rho= -0.44, p<0.0001).

In the MIH group, functional coagulopathy improved during the hypothermia phase, measured by R and MA, in patients with hypercoagulation as well as in patients with hypocoagulation. Similar results were not observed in the control group neither for R **(Figure 14)**.

Similarly, correlations between change in R or MA during follow-up (t_0-t_1) and R or MA at enrollment (t_0) were analysed; t_0 was defined as previously and t_1 was defined as 12 hours from study enrollment in the control group, and 12 hours after target temperature was achieved in the MIH group.
While a correlation was observed between change in R from t_0 to t_1 and enrollment R among both groups (MIH group rho= -0.46, p=0.0007 and control group rho= -0.31, p=0.0282), a significant correlation was only observed between the change in MA from t_0 to t_1 and enrollment MA for patients randomized to the MIH group (rho=-0.47, p=0.0005) (control group; rho=-0.15, p=0.3115).

In order to detect a potential influence of the TEG temperature adjustments, four sensitivity analyses were performed including only patients in the MIH groups. The above described correlation analyses were repeated by replacing R and MA at t_1 with the TEG profiles obtained at 37°C instead of 33°C and t_2 with the TEG profiles obtained at 33°C instead of 37°C. All results remained robust (all p<0.0005).

DISCUSSION

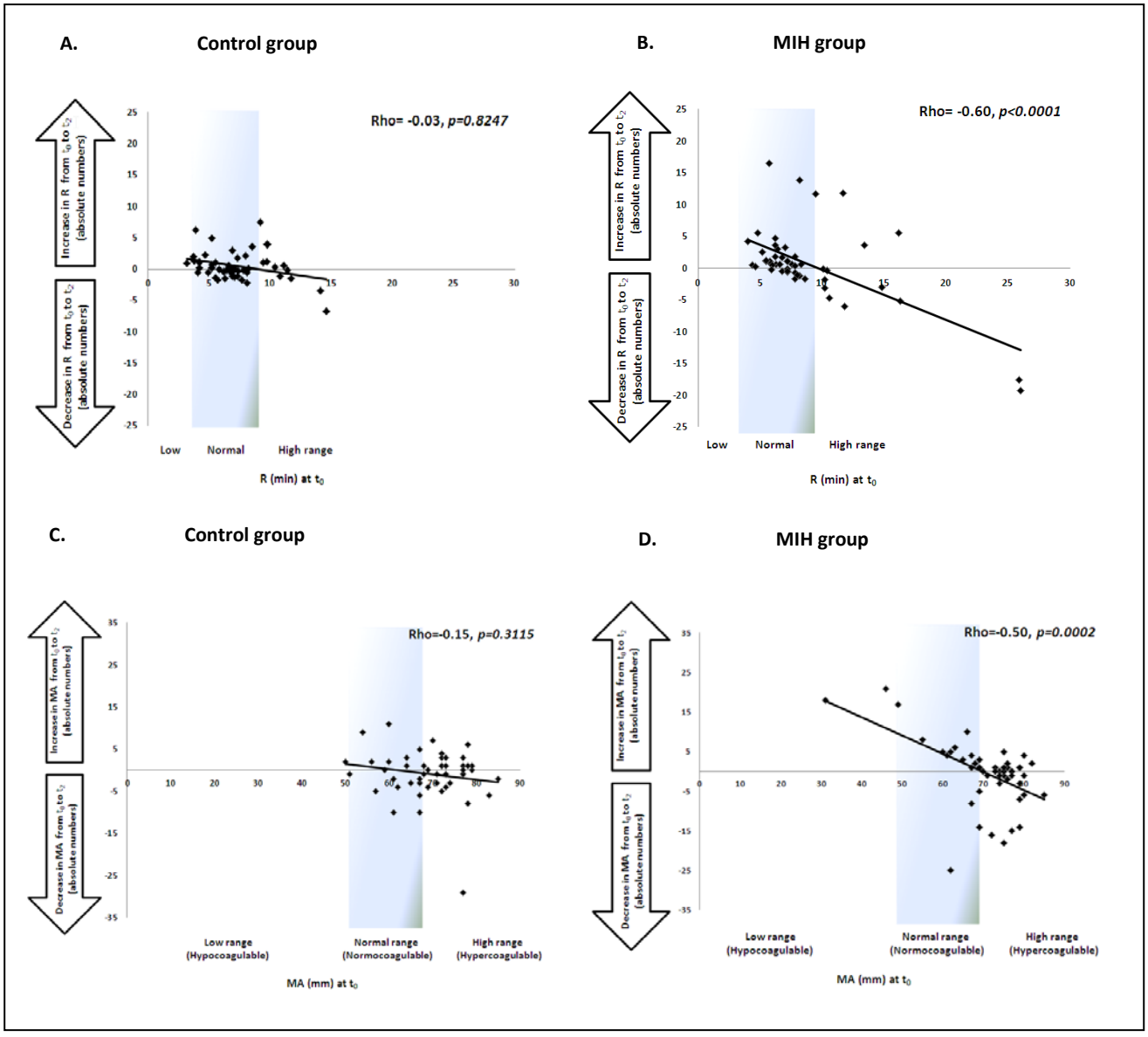
In a randomized controlled trial, MIH improved functional coagulopathy, in patients suffering severe sepsis or septic shock. Importantly, this normalization of the coagulopathy effect seemed to remain even after rewarming. The same pattern was not observed in patients randomized to the control group.
This is the first time that the effect of MIH on the haemostatic system in patients with varying levels of coagulopathy has been described. These results not only add to the understanding of the effect of hypothermia on the haemostatic system, but also indicate that MIH reduces sepsis-related coagulopathy assessed by a thrombelastography (TEG).

Sepsis-related coagulopathy progresses from a normal viscoelastic coagulation profile to hypercoagulability and hypocoagulability with increasing disease severity [185]. Hypercoagulability, as well as hypocoagulability, associates with fatal outcome in these patients [114,186,187]. Hence, it seems plausible that normalization of sepsis-related coagulopathy is beneficial to the patient.
We observed that a tendency towards normalization of the TEG profile was present in both patients with high or low TEG parameters (including the parameters within normal range). Thus, we hypothesize that MIH may reduce coagulopathy throughout the whole spectrum of sepsis-related coagulopathy.

As the presented results are the first of its kind, describing the effect of MIH on the haemostatic system in patients with sepsis-related coagulopathy, we can only speculate on the mechanisms underlying our results. Sepsis-related coagulopathy is due to an unbalance in the haemostatic system favouring either hyper- or hypocoagulation [41,43,45]. Because lowering of temperature inhibits enzymes involved in the haemostatic balance [104,105,107], we surmise that hypothermia affects the predominant factors the most. Thus, the determining factors resulting in either high or low TEG parameters would be inhibited resulting in trend towards normalisation of coagulation.

Although coagulopathy is often associated with sepsis, the patients included in our study displayed neither hypo- nor hypercoagulability reflected in conventional coagulation tests such as international normalized ratio (INR), activated partial thromboplastin time (aPTT) or platelet count. Only d-dimer levels were increased beyond normal range.
Nevertheless, almost two-third of the patients presented a hypocoagulable or hypercoagulable TEG profile at study enrollment. Thus, as reported by others [188], the TEG-method detects sepsis-related coagulopathy earlier and more accurately than conventional coagulation tests.

Figure 14. Correlations between the change in reaction time (R) and maximal amplitude (MA) during the first day versus R and MA at study enrollment in the two randomized groups



R reflects mainly the enzymatic part of the haemostatic process and in accordance R at enrollment correlated with INR and aPTT. Furthermore, MA reflects clot strength, which is dependent on the thrombin burst, the number and function of platelets and its interaction with fibrin. As expected, we observed that MA highly correlated with platelet count and d-dimer at enrollment. In addition, MA correlated with disease severity at study enrollment; hypocoagulable MA were associated with higher median SOFA score. Based on our observations, we cannot comment on causality. However, late-phase sepsis is characterized by hypocoagulability and organ dysfunction [189].

As liver function declines and plasma bilirubin levels increase, the production of fibrinogen and other coagulation factors, and platelet count decreases, causing the strength of the blood clot (MA) to weaken. Thus, it could be hypothesized that MA to some extent reflects liver injury during sepsis, as supported by animal studies [190]. Furthermore, we observed that MA hypercoagulation was associated with respiratory failure (low PaO_2/FiO_2 ratio). Lung injury is often a fatal complication to sepsis, and is partly due to the formation of microthrombi and subsequent deposition of fibrin in the lung capillary system [191,192]. As a consequence, patients develope acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). Thus, the correlation between MA hypercoagulation and respiratory failure might reflect damage to the lungs caused by microthrombi.

STRENGTHS AND LIMITATIONS

The main strength of the study was the randomized study design, minimizing the degree of measured confounders and the high data quality due to a strict GCP monitoring.

A sample size calculation was performed in order to ensure that the study was well powered to detect relevant difference in MA between the two randomized groups. Despite the observed "normalization of coagulopathy" among patients randomized to the MIH-group, we cannot comment on the effect of MIH on prognosis. However, sepsis-related coagulopathy has previously been linked to increased mortality [114,186,187], and as such, a normalization could arguably be considered advantageous. Thus, it should be emphasized that clinical equipoise remains for use of MIH in sepsis.

A discrepancy in timing of obtaining the TEG result between the MIH- and the control group of 9 hours was present at t_2 (i.e. the primary analysis). Importantly, the primary result remained robust when comparing TEG results between the two randomized groups at t_1, where the difference in timing between the two groups was 3 hours. Despite being reassuring that the changes in R and MA were stable in patients over 3 - 9 hours, we cannot fully exclude that sampling variation could have an effect on the results.

We only included R and MA in this study. This was done in order to detect potentially detrimental effects on the coagulation, and at the same time maintain a high degree of blinding in the ongoing randomized trial. Data on fibrinogen levels at study enrollment was not collected, therefore it is not possible to correlate our results with the International Society of Thrombosis and Haemostasis disseminated intravascular coagulation *(*DIC) score. Thus, no comments can be made regarding correlations between the TEG parameters and DIC score.

OVERALL CONCLUSION AND PERSPECTIVES

Coagulopathy, ranging from subtle activation of coagulation to fulminant disseminated intravascular coagulation and hemostatic breakdown, occurs amongst virtually all patients developing sepsis. Within the last couple of decades it has become ever clearer that sepsis-related coagulopathy affects not only traditional coagulation factors but also platelets and the endothelium. Currently, functional testing of the hemostatic system has found application during critical illness. Thromboelastography (TEG) provides an overview of the hemostatic system allowing for an evaluation of interactions between coagulation factors and platelets.

The studies comprising this PhD thesis all investigate important aspects of disturbed hemostasis during sepsis. Together, the specific findings from the three studies increases the combined understanding of the sepsis-related coagulation disturbances and the possible influence of some of the treatments, we offer these patients. Thus, the findings presented in *papers I-III* provide important points pertaining to the general workings of the hemostatic system during sepsis.

Paper I: Thrombocytopenia during sepsis is a predictor of poor outcome. Sporadic reports of mainly casuistic nature have raised suspicion that antimicrobial agents cause sepsis-related thrombocytopenia. For the first time, this was explored, in the study underlying *paper I*, among 1103 ICU patients using the two randomized groups of the PASS study; no signal was evident. Thus, it was concluded that that the risk of antimicrobial-induced thrombocytopenia during sepsis in the general ICU populations does evidently not represent a substantial problem.

Paper II: The endothelium plays an important role in balancing the hemostatic system, and damage to the endothelium is associated with coagulopathy. In *paper II* it was observed, that during sepsis profound endothelial damage, as measured with the elevated levels of soluble thrombomodulin, results in long-term consequences for the patients including a high risk of mortality. The increased 90-day mortality rate in septic patients with profound endothelial damage at ICU admission cannot be solely explained by the presence organ failure, since the association between profound endothelial damage end mortality remained strong even after adjusting for frequently occurring organ failure. Thus, profound endothelial damage seems to contribute directly to mortality during sepsis. Furthermore, profound endothelial damage may represent a more severe problem than thrombocytopenia, as the marker of profound damage to the endothelium (soluble thrombomodulin) is more strongly associated with mortality compared with thrombocytopenia at ICU admission.

Paper III: Disturbances in the hemostasis during sepsis often results in coagulopathy, and coagulopathy during sepsis – in any form - is associated with increased morbidity and mortality. As a consequence, interventions tested to increase survival must seek not to worsen sepsis-related coagulopathy to such an extent that it cannot be compensated by the potential other benefits of the intervention.

Mild induced hypothermia has been proposed as a potential new treatment in severe sepsis and septic shock. Despite the potential of decreasing temperatures deteriorating an already fragile hemostasis, mild induced hypothermia in fact improves functional coagulopathy, measured by thrombelastography parameters. This was evident, also in using the thrombelastography results collected after rewarming. Thus, mild induced hypothermia may have a future in the sepsis treatment regime.

This thesis emphasizes both the complexity of the hemostatic system during sepsis and its determinant role in disease severity and prognosis. Despite increased focus on the hemostatic system during sepsis, continued research on restoring the disrupted hemostasis - including coagulopathy and endothelial damage – is needed. The role of the endothelium in the morbidity and mortality during sepsis cannot simply be explained by the presence of organ failure. Hence, reestablishing endothelial integrity and reducing coagulopathy appears to be required for improvement of sepsis outcomes.

A number of previous phase III trials have investigated the effects of different anticoagulatory agents on coagulopathy. However, clinical benefits have yet to be demonstrated. Methods for restoring the broken endothelium in patients suffering sepsis remain unexplored in clinical trials. Even so, reports have been published suggesting potential clinical benefits from different endothelial modulating therapies including mesenchymal stem cells and prostacyclin analogs in patients with severe, global endothelial damage, such as severe sepsis and septic shock.

Methods to investigate a potential beneficial clinical effect from endothelial modulating therapies, would include large randomized controlled trials designed to investigate relations between endothelial modulating therapies, morbidity and mortality. However, randomized controlled trials are labour intensive, time consuming, and expensive; and usually only one intervention is tested at a time. Hence, it could be advantageous for future trials to include a screening process for several potential endothelial modulating interventions using a phase II-like design with endpoints for markers of organ failure (creatinine, inotropic score ect.) as well as biomarkers of endothelial damage (i.e. soluble

thrombomodulin). Importantly, endpoints should be chosen based on their ability to predict phase III success; as such endpoints associated with poor prognosis in sepsis i.e. mortality would be preferable. Furthermore, use of repeated measurements would provide an insight into the time perspectives of endothelial damage.
Subsequently on the basis of the phase II trial, the most promising intervention should be tested in a well-powered phase III trial using clinical endpoints.

Mild induced hypothermia counteracts several deleterious effects of sepsis, and is associated with improvement of coagulopathy. Consequently, the intervention could be a promising treatment of sepsis-related hemostatic disturbance. The establishment of a bio-bank as part of the Cooling And Surviving Septic Shock (CASS) study, allows for testing of a potential modulating effect of mild induced hypothermia on the endothelium by analyzing biomarkers of endothelial damage. Accordingly, we await the results of the ongoing CASS study.

LIST OF ABBREVATIONS AND DEFINITIONS

95% CI: 95% Confidence interval is an interval estimate reflecting a statistical significance level of 5%
Absolute thrombocytopenia: One platelet count ≤ 100 x 109/L
ALI: Acute lung injury
Alert procalcitonin: Procalcitonin>1.0 ng/ml at study enrollment or an insufficient decrease within first 24 hours (PCT day 2 >0.9 x PCT day 1)
ALT: Alanine aminotransferase
APACHE II: Acute Physiology and Chronic Health Evaluation II
Apoptotic protease activating factor-1: APAF-1
aPTT: Activated partial thromboplastin time
BMI: Body mass index
CAM-ICU: The Confusion Assessment method for the ICU
CRP: C-reactive protein
DSMB: Data and Safety Monitoring Board
eGFR: Estimated Glomerular Filtration Rate
GCP: Good Clinical Practice
High-exposure group: Intervention group in the PASS study
ICU: Intensive Care Unit
INR: International normalized ratio
IQR: Inter quartile range
MA*:* Maximum amplitude
MAP*:* Mean arterial pressure
MDRD*:* Modification of diet in renal disease
R: Reaction time
PaO2/FiO2*:* Arterial oxygen pressure/fraction of inspired oxygen
RH-PAT: Reactive hyperaemia peripheral arterial tonometry
RR: Rate ratio
sTM: Soluble thrombomodulin
SOC group: Standard-of-care group in the PASS study
SOFA score: Sequential Organ Failure Assessment score
Relative thrombocytopenia: ≥20 % decrease in platelet count from study enrollment
TEG: Thrombelastography
ΔR: Change (decrease or increase) in R
ΔMA: Change (decrease or increase) in MA

SUMMARY

The sepsis syndrome represents a disease continuum, including severe sepsis and septic shock associated with high mortality. One of the main problems in severe sepsis and septic shock, resulting in organ failure and death, are disturbances in the hemostasis due to sepsis-related coagulopathy. Sepsis-related coagulopathy affects not only traditional coagulation factors, but also the platelets and endothelium. Functional testing of the hemostatic system has found application in critical illness. Thrombelastography (TEG) provides an overview of the hemostatic system allowing for an evaluation of interactions between coagulation factors and platelets. Additionally, the role of the endothelium during sepsis can be explored through testing of biomarkers of endothelial damage.
The three studies comprising this PhD thesis all investigate important aspects of the disturbed hemostasis during sepsis, including endothelial damage. Together, the specific findings from the three studies improve the existing understanding of sepsis-related coagulopathy, and the possible influences of some of the treatments offered these patients.
The first study investigates the occurrence of antimicrobial-induced thrombocytopenia among critically ill patients. In sepsis, thrombocytopenia is a predictor of poor outcome, and reports, of mainly casuistic nature, have previously hypothesized that specific antimicrobial agents could induce in sepsis-related thrombocytopenia. This hypothesis was tested using a randomized designed set-up, encompassing 1147 critically ill patients, and no significant difference in risk of thrombocytopenia was observed among patients receiving large amounts of antimicrobials vs. patients receiving standard-of-care. As a consequence, the risk of antimicrobial-induced thrombocytopenia in the general population of critically ill patients seemingly does not represent a substantial problem and thrombocytopenia during critical illness is most likely due to other factors such as infection severity.
In the second study of the thesis, the role of endothelial damage during sepsis was explored. Levels of biomarkers of superficial and profound endothelial damage (syndecan-1 and soluble thrombomodulin (sTM), respectively) were determined in a cohort of 1103 critically ill patients. The results showed that only high levels of sTM were associated with a markedly increased risk of 90-day mortality, as well as multi-organ failure. The finding suggests that profound damage to the endothelium is centrally involved in the pathogenesis of death in sepsis. Thus, the endothelium may be a target for new interventions against sepsis.
In the third study, we investigated, using a randomized controlled trial, how mild induced hypothermia (cooling to 32-34°C for 24 hours, MIH) influenced sepsis-related coagulopathy using TEG; functional coagulopathy improved in patients exposed to the intervention compared with the control group. This improvement of coagulopathy parameters during MIH persisted after rewarming. These results not only add to the understanding of the effect of hypothermia on the hemostatic system, but indicate that MIH reduces sepsis-related coagulopathy assessed by TEG.
Overall, this thesis emphasizes that the role of the hemostatic system during sepsis is not only complex, but centrally involved in disease severity and prognosis. The endothelium seems to play a central role in the morbidity and mortality of sepsis, which cannot be explained simply by the presences of organ failure. Thus, restoring the broken endothelium and reducing coagulopathy appears to be essential in order to significantly improve sepsis outcomes. MIH could be a promising intervention in sepsis, in part due to the improvement of the coagulopathy. Despite the increased focus on the hemostatic system during sepsis, it seems that continued research on restoring disrupted hemostasis - including endothelial damage- is needed.

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