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Ph.D. Thesis

Platelet and platelet-derived microparticle studies in severe sepsis

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Abbreviations

ADP	**A**denosine-**d**i-**p**hosphate
ADR	**Adr**enaline
ATP	**A**denosine-**t**ri-**p**hosphate
BUN	**B**lood **u**rea **n**itrogen
CARS	**C**ompensatory **A**nti-Inflammatory **R**esponse **S**yndrome
COL	**Col**lagen
CRP	**C**-**r**eactive **p**rotein
GP	**G**lyco**p**rotein
ICU	**I**ntensive **c**are **u**nit
IL	**I**nter**l**eukin
LPS	**L**ipo**p**oly**s**accharide
MODS	**M**ultiple **O**rgan **D**ysfunction **S**core
MP	**M**icro**p**articles
NF-κB	**N**uclear **F**actor-**k**appa **B**
PCT	**P**ro**c**alci**t**onin
PMP	**P**latelet-derived **m**icro**p**article
PS	**P**hosphatidyl**s**erine
SAL	**Sal**ine, 0.9% NaCl solution
SIRS	**S**ystemic **I**nflammatory **R**esponse **S**yndrome
SOFA	**S**equential **O**rgan-**F**ailure **A**ssessment
SSC2008	**S**urviving **s**epsis **c**ampaign **2008**
TF	**T**issue **f**actor
TLR	**T**oll **l**ike **r**eceptor
TNF-α	**T**umor **n**ecrosis **f**actor-**a**lpha
VWF	**v**on **W**illebrand **f**actor

Introduction

The signs and symptoms of inflammation and sepsis are widely available in historical records [1], but the widely-accepted exact definition for the Systemic Inflammatory Response Syndrome (SIRS) and sepsis was only created in 1991 by the joint work group of the American College of Chest Physicians and the Society of Critical Care Medicine [2]. These definitions were renewed and accepted by the International Sepsis Definitions Conference in 2001 [3]. At least two criteria of the currently used diagnostic criteria must be fulfilled for the diagnosis of SIRS. These criteria are discussed in Table 1.1.

TABLE 1.1: Diagnostic criteria for the Systemic Inflammatory Response Syndrome.

Body temperature	$> 38°C$, or $< 36°C$
Heart rate	$> 90\,\text{min}$
Hyperventilation	respiratory rate $> 20\,/\text{min}$, or arterial partial oxygen pressure ($PaCO_2$) $< 32\,\text{mmHg}$
White blood cell count	> 12000 cell/µl, or ≤ 4000 cell/µl

According to the most recent definition, sepsis is the development of SIRS as a result of proved or suspected microbiological infection. The term severe sepsis is used for sepsis complicated with the development of at least one organ dysfunction. Septic shock is defined as hypotension regardless of appropriate fluid challenge and without any different known cause. Levy emphasises the importance of bedside clinical work in the diagnosis of sepsis. The 2001 sepsis consensus conference stated that in day-to-day work, the notion of the patient "look septic" should be considered more important than strict data.

The following aspecific clinical signs can help the diagnosis of sepsis [3]:

- Documented or suspected infection
- Fever (core temperature $> 38.3\,°C$) or hypothermia ($< 36\,°C$)

- Heart rate $> 90\,\text{min}$ or > 2 standard deviation above the normal value
- Tachypnoea
- Altered mental status
- Significant oedema or positive fluid balance ($> 20\,\text{ml/kg}$ over 24 hours)
- Hyperglycemia (plasma glucose $> 7.7\,\text{mmol/l}$), in the absence of diabetes
- Leukocytosis ($> 12\,000\,/\mu\text{l}$), leukopenia ($< 4000\,/\mu\text{l}$) or >10% immature forms
- Plasma C-reactive protein (CRP) > 2 standard deviation above the upper limit of the reference range
- Plasma procalcitonin (PCT) > 2 standard deviation above the upper limit of the reference range
- Arterial hypotension ($< 90\,\text{mmHg}$), mean arterial pressure $\leq 70\,\text{mmHg}$ or systolic blood pressure drop $\geq 40\,\text{mmHg}$ or >2 standard deviation below normal for age
- Central vein oxygen saturation $< 70\%$
- Cardiac index $>3.5\,\text{l/min/m}^2$
- Arterial hypoxia ($\text{PaO}_2/\text{FiO}_2 < 300\,/\text{mmHg}$
- Acute oliguria (urine output $0.5\,\text{ml/kg/h}$)
- Creatinine increase $> 44.2\,\mu\text{mol}\,\text{l}$
- Coagulation abnormalities (International normalised ratio > 1.5 or activated partial thromboplastin time > 60 seconds)
- Ileus
- Thrombocytopenia (platelet count $< 100\,000\,/\mu\text{l}$)
- Hyperbilirubinaemia (plasma total bilirubin $\geq 70\,\mu\text{mol/l}$)
- Hyperlactataemia ($> 1\,\text{mmol/l}$) or decreased capillary refill or mottling

The presence of organ dysfunctions can be early diagnosed by the above mentioned listing, or by altered laboratory values and by clinical scores. Multiple clinical scores,

including the Multiple Organ Dysfunction Score (MODS) or the Sequential Organ Failure Assessment (SOFA) were developed to assist sepsis outcome prediction and overall patient status description for clinical studies [4, 5]. Also, the current diagnostic criteria for severe sepsis recommend the use of the SOFA and MODS scores for the diagnosis of organ dysfunctions in adult patients [3]. As sepsis mortality is highly affected by the number of organ dysfunctions, the development of multiple organ failure is a serious complication of sepsis.

1.1 The epidemiology of severe sepsis

Diagnostic, therapeutic and organ-support therapy of sepsis rapidly evolved in the past decades, while sepsis and severe sepsis are still leading causes of in hospital mortality [6]. Angus et al. conducted extensive research on sepsis incidence and mortality in the United States of America. According to their results, in 1995, 192980 cases suffered from severe sepsis of total 6.6 million hospital discharges studied. The national estimate for the USA was 751000 cases annually (3.0 case / 1000 population), accounting for 20% of all Intensive Care Unit (ICU) admissions. Although incidence increases with patient age exponentially, severe sepsis has a small incidence peak in infants. While children in age between 5 and 14 have incidence of 0.2/1000 only, in elderly patients (85 years and above) incidence reaches 26.2/1000. The overall hospital mortality of severe sepsis was high, 28.6%, while the estimated sepsis-caused death rate (9.3%) was comparable to the mortality of acute myocardial infarction in 1995. According to Angus et al. mortality is slightly higher in adult male patients (male/female mortality: 29.3% vs. 27.9%) and has a good correlation with patient age. Of all cases studied by Angus et al. 73.6% had developed one organ dysfunction, while 20.7% of patients suffered from two organ dysfunctions. Only about 1% of patients had estimated mortality of 76.2% with the development of four or more organ failures. Most patient in this study suffered from respiratory (45.8%), circulatory (24.4%), renal (22%), or haematopoetic system dysfunction (20.6%) [6]. Angus also covered clinical studies regarding severe sepsis in his review and found a wide range of incidence. Most data assessed originated from tertiary academic centres and differed significantly by the patients geological location, which may biased the whole picture of sepsis incidence. The overall mortality based on different studies was 49.7%, but this result is also controversial because of different study inclusion

criteria [7]. A review by Linde-Zwirble emphasises, that differences between ICU staff experience and available equipment may highly affect the diagnostic and therapeutic success of sepsis [8]. Short-term patient survival is usually determined by liver status, haematological diseases in patient history, as well as inappropriate antimicrobial therapy. Following 48 hours of ICU stay, chronic alcohol abuse, congestive heart failure, shock and the number of organ failures are the main survival determinants [9].

In the year of 2005 and 2010, two opt-in audits were carried out in Hungary regarding sepsis incidence and mortality. Both studies included a two-week period of patient care, including 789 and 866 patients consecutively. Median age of included patients were: 64 and 66 years, while gender ratio was 62%/38% and 50%/50% (male/female). Patients with proved infections in 2010 (307), 64% of patients were diagnosed with severe sepsis, while 38% developed septic shock. Overall mortality was 33%, while severe septic patients had 39% mortality ratio and septic shock patients showed 55% mortality rate. Presence of multiple organ dysfunction decreased in the 5 years period with near 10%. 26% of patients had 2 or 3, while 17% developed 4 or 5 organ dysfunctions. Length of intensive care unit stay did not differ in these studies with a median of 8 and 7 days [10]. According to Annane et al. 30-50% of septic patients have Gram-positive bacterial infection, most commonly methicillin-susceptible *Staphylococcus aureus*. Gram-negative bacteria were detected in 25-30% of all cases, while fungi account for about 6%. Viruses and parasites share another 7% of frequency [11]. Of all patients treated with suspected infection in Hungary, regarding to the 2010 data (307 patients), community acquired infections were the leading cause, accounting for 59% of all cases, while in-hospital infections decreased to 23% from the former 42%. In the cause of infection, there was a slight shift in the 2010 data, as Gram-negative bacteria become the leading infection sources with 53%, while Gram-positive bacteria accounted only for 41%. Also, Bogár et al. reported a non-significant increase of detected fungal infections from 4% to 6% [10].

The treatment of sepsis is a high burden for the social system. Angus et al. reported an average 20 days length of stay, and about $22,100 cost per case. Non-survivor cases showed higher costs with almost $26,000/case. For comparison, in the same study, non-ICU patients cost $13,900/case.

1.2 The etiology and pathophysiology of severe sepsis

1.2.1 Early response and inflammatory mediators in sepsis

Sepsis is usually characterised as a result of a complex, but inappropriate response to pathogen-associated stress. Although our current view emphasise this inappropriate host defence, pathogens also clearly contribute to the development of sepsis through virulence factors. Bacteria harbour cellular elements and ligands to adhere to and colonise epithelial surfaces (including adhesins, flagella and fimbriae, bacterial protein secretion systems, ligand mimicry, biofilm production and anti-phagocytosis) [12]. The highly conserved innate immune system is responsible for the recognition of the "non-self" and "damaged-self" via pattern recognition receptors. Recognised pathogen-associated molecular patterns and damage-associated molecular patterns, are conserved and normally not expressed in healthy hosts. These patterns are present on both Gram-positive and Gram-negative bacteria, fungi and viral limiting membranes or walls [13, 14], or may develop as new compounds during cellular damage. Gram-negative bacteria can be recognised by wall component lipopolysaccharide (LPS) which usually binds to CD14 containing LPS binding complex. Peptidoglycans (Gram-positive bacteria wall components) and LPS may bind to Toll-like receptor-2 (TLR) and TLR-4 respectively. The TLRs were originally described as *Drosophila* dorsoventral pattern determinant signalling pathway proteins, their exact role in human pathophysiology was only recently elucidated. Medzhitov et al. demonstrated that TLRs in humans, particularly TLR-4 vastly contributes to LPS recognition through a nuclear-factor-κB (NF-κB) mediated pathway [15]. To date, at least 11 human TLR proteins are identified [16]. These receptors contains a leucine-rich extracellular and an interleukin (IL)-1-receptor associated intracellular part. Although TLR-1, -2, -4, -5 are recognised as bacterial and TLR-3, -7, -8 as viral pattern receptors, most of them also have their own endogenous ligands [17]. Increased expression of TLR-2 and TLR-4 in septic patients compared to controls is already described [18]. Activation through TLRs induce multiple intracellular signal-transduction pathways, including the PI3k-Akt, p38 and IκB/NF-κB pathways [18]. The degradation of IκB through protein phosphorylation leads to NF-κB activation. Following NF-κB transports inside the cell nucleus and induce various gene expressions, including inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6), acute phase proteins (C-reactive protein, LPS-binding protein), tissue factor (TF), and tissue

factor pathway inhibitor, adhesion molecules [19]. There is increasing evidence of the hypothesised role of apoptosis protein caspase-1 and caspase activated DNase in sepsis. The caspase activated DNase may induce DNA fragmentation and apoptosis. The importance of these mechanisms was confirmed in caspase-1 knock out mice, which are evidently protected from sepsis [20]. The nucleotide-oligomerization domain leucine-rich repeat proteins (capable to recognise bacterial peptidoglycans) and cytoplasmic caspase activation and recruiting domain helicases are also capable to initiate innate immune response [20, 21]. Leukocytes including monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, B-, T-, and NK cells as well as platelets also express TLRs. Leukocytes activated through TLRs release pro-inflammatory cytokines, including IL-1β, IL-6 and tumour necrosis factor alpha (TNF-α) which activates the innate immune system and surrounding cells leading to further cytokine release (cytokine storm, as referred earlier). Activated leukocytes may leave the circulation and migrate towards damaged tissues, while released pro-inflammatory cytokines induce the production of eicosanoids and platelet-activating factor, further increasing vascular permeability and platelet activation [11]. Also, complement system proteins C3a and C5a anaphylatoxin release from mast cells and basophils increase vascular permeability and smooth muscle contraction. The equilibrial cross-talk of endothelial cells and surrounding circulating cells is impaired during sepsis. Structural and functional changes of endothelial cells include swelling, blebbing, contraction, and detachment from subendothelial surface. Functional changes enhance leukocyte attachment, rolling a diapedesis, as well as the activation of the coagulation cascade [19, 22, 23]. Genetic polymorphism is a strong determinant of inflammatory cell activation. TNF-α gene promoter region, or TLR4 receptor polymorphisms may determine the levels of cellular activation, shaping the course of inflammation. An amino acid change (position 532) in IL-1 receptor associated kinase-1, which is present in 25% of Caucasian population may cause increased NF-κB activation leading to increased mortality rate [24].

Contrary to pro-inflammatory response, the release of anti-inflammatory substances and the development of Compensatory Anti-Inflammatory Response Syndrome (CARS) is also present in sepsis [11, 25]. Bone hypothesised, that an effective anti-inflammatory immune suppression is already developed in patients with severe inflammatory diseases, explaining the harmful effect of inflammation suppression therapies [26, 27]. CARS

itself seems not only the sings of amelioration following SIRS, but an existing pathophysiological phenomenon. There is evidence for anti-inflammatory IL-10, -4, -6, -13 and transforming growth factor-β and interferon-α production. Cellular contributors to immune responses are also affected by CARS. In the process of leukocyte reprogramming, neutrophil-driven inflammation limitation and neutrophil clearance is deteriorated through delayed apoptosis. This characteristic may sustain inflammation and organ injury [22]. Monocytes have diminished antigen presentation potential and cytokine production shifted towards anti-inflammation, while leukocyte anergy and decreased responsiveness to mitogenic stimuli is characterised in patients. Septic patients also show increased levels of Th2 T helper cells, which shifts the immunoresponse towards anergy [22, 25]. The antagonism with inflammatory response of novely identified factors, including macrophage inhibitory factor, an early acting factor in sepsis and high-mobility group box protein type-1, a late acting cytokine of inflammation may have high impact on disease progression and increase patient survival [22].

1.2.2 Development of organ dysfunctions in severe sepsis

The exact mechanism behind the development of sepsis-related organ failures is yet to be elucidated [22]. Although microcirculatory deterioration and intravascular clot formation are often addressed as a cause of sepsis-related organ dysfunctions, the anatomical appearance of organs from non-survivors, the lack of evidence for massive coagulation emphasises the role of inflammatory mediators, including TNF-α, IL-1α, nitric oxide and reactive oxygen species. On cellular level, these mediators may inhibit the respiratory chain in mitochondria, leading to intracellular hypoxia and reduced ATP availability. This may lead to DNA, protein and lipid membrane oxidation and damage. The development of mitochondrial impairment because of poly(ADP-ribose) polymerisation is a known cause of cell death, vascular hyporeactivity, endothelial and epithelial function degradation [28]. Altered vascular function is a key contributor to the development of sepsis-related organ failures. Own TLR receptors of the endothelial lining, as well as the presence of inflammatory cytokines activate endothelial cells, decreasing thrombomodulin concentration and increasing TF and plasminogen activator inhibitor-1 presence. Activated endothelial cells provide procoagulant surface and support the sequestration of polymorphonuclear cells. Increased TNF-α concentration supports swelling through endothelial cells, causing local oedema, deterioration of oxygen diffusion and loss of

intravascular fluid. Cardiac output is usually elevated in patients recently diagnosed with sepsis. This hyperdynamic circulation is maintained by heart ventricle dilatation (because of fluid therapy), while myocardial function is also affected by inflammatory cytokines (TNF-α, IL-1β, nitric oxide) and mitochondrial dysfunction. This functional impairment will be a leading cause of systolic and diastolic dysfunction. In the compensated phase, capillary refill is still maintained, clinically the patient present a warm skin temperature and increased metabolism. Fluid loss via vascular swelling, intravascular fluid redistribution through vascular tone loss, myocardial dysfunction and vasodilatation leads to hypotension. The first clinical signs of circulatory decompensation are: decreased turgor of the skin, the drop of systolic blood pressure $\leq$90 mmHg and increased lactate levels. During circulatory shock, mean arterial pressure $\leq$70 mmHg disturbs coronary circulation. Stagnant blood in vessels increase local intravascular pressure, supporting swelling and oedema. The vascular reactiveness to nitric oxide, or local levels of NO and metabolites, as well as the activation of vascular potassium levels and hormone changes (corticosteroids) together may result in these alterations. However, these vascular changes are not globally equal in the whole vasculature, leading to over and underperfused specific tissue areas [22].

Loss of renal function affects about 50% of severe septic patients. Besides formerly discussed factors, the direct effect of nitric oxide, angiotensin and endothelin on renal tissue is hypothesised. Autoregulated renal circulation is impaired because of increased catecholamine levels, while endothelial damage of renal vessels support microaggregate formation and leukocyte diapedesis towards renal parenchyma, causing tubular cell dysfunction. The development of metabolic acidosis, increasing creatinine and blood urea nitrogen (BUN) levels, finally potassium level increase are the best clinical signs of deteriorating renal function. Acute sepsis-related renal function impairment is assessed by the RIFLE criteria (Table 1.2).

One of the most serious complication of sepsis is the development of acute lung injury and acute respiratory distress syndrome [30]. The exact mechanism behind the development of both syndromes are not fully elucidated. Destruction of the alveolo-capillary membrane, impaired gas diffusion and surfactant dysfunction are the main contributors. Capillary damage is characteristic to the previously discussed alterations, protein rich fluid, and activated neutrophils may reach the alveolar space through damaged endothelial cells. By the detachment of alveolar and bronchial epithelial cells

Table 1.2: The RIFLE (Risk, Injury, Failure, Loss of function and End-stage kidney) criteria for sepsis-related acute kidney injury [29].

Class	Glomerular filtration rate	Urine output
Risk	Serum creatinine × 1.5	$< 0.5\,\mathrm{ml/kg/h} \times 6$ hours
Injury	Serum creatinine × 2	$< 0.5\,\mathrm{ml/kg/h} \times 12$ hours
Failure	Serum creatinine × 3 or serum creatinine $\geq 354\,\mu\mathrm{mol/l}$ with an acute rise $> 44\,\mu\mathrm{mol/l}$	$< 0.3\,\mathrm{ml/kg/h} \times 24$ hours or anuria × 12 hours
Loss	Persistent acute renal failure = complete loss of renal function > 4 weeks	
End-stage kidney disease	End-stage kidney disease > 3 months	

a hyalin membrane fills the alveoli, while the cytokine production of local macrophages activate neutrophils. These activated cells release proteases, produce reactive oxygen species, increasing local tissue damage, while surfactant is inactivated by the protein rich fluid [31]. Reparation processes starts immediately following tissue damage. Type II pneumocytes proliferate to restore alveolar structure, while oedema leaves the alveolar space through active transport and protein components are phagocyted. The increase of alveolar closing capacity and the decrease of functional residual volume will cause the collapse of the alveoli. Unventilated alveoli increase the shunt circulation in the lungs, increasing the oxygen need of patients [31, 32].

The reaction of the neurohormonal system can be described as an "ebb and flow". The ebb phase is characterised by the stresshormone release, sympathetic system activation, impaired glucose tolerance and peripheral insulin resistance, increased lipid degradation. In the flow phase inflammatory cytokines develop the clinical picture of sepsis. Degraded proteins are mainly of skeletal muscle origin, further complicating the renal impairment. The direct effect of inflammatory cytokines and worsening hepatic and renal function will result in the development of neuropathies and sepsis-induced encephalopathy, increasing the ventilation period of patients [33, 34].

The contribution of coagulation in the process of organ dysfunction development may be due to the aforementioned vicious circle of bidirectional communication between inflammatory and coagulation pathways (Figure 1.1) [22, 25, 35].

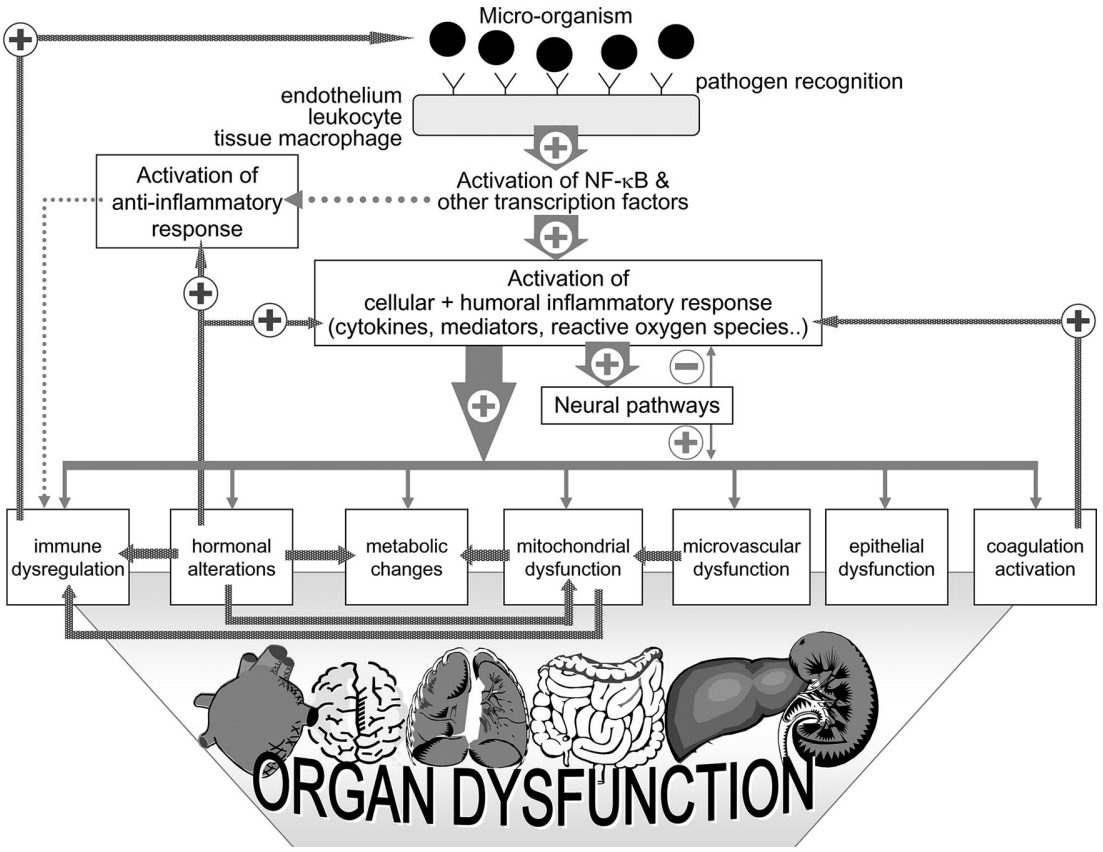

FIGURE 1.1: Development of sepsis-related multiple organ failure. [22] (with written permission from original author).

1.2.3 Altered coagulation status in sepsis

In severe sepsis, the classical components of Virchow's triad (increased coagulablity, endothelial injury and impaired blood flow) for altered haemostasis are all present [23]. Released cytokines and activated immune cells vastly contribute to altered coagulation state and shift towards procoagulation in sepsis. Coagulation proteases and anticoagulant proteins may bind to their cellular receptors, modulating cytokine release and cell activation. Endothelial cells may respond to and also release cytokines, while increasing expression of adhesion molecules and growth factors. These factors promote inflammatory response and contribute to tissue factor mediated thrombin generation, dysfunctional anticoagulation and deteriorated inhibition of fibrinolysis [19]. The formation of intravascular clots may deteriorate tissue perfusion causing local hypoxia. According to Nduka and Parillo, the complex bidirectional connection between coagulation and inflammation results in a vicious cycle of tissue injury, organ dysfunction and cell death (Figure 1.2).

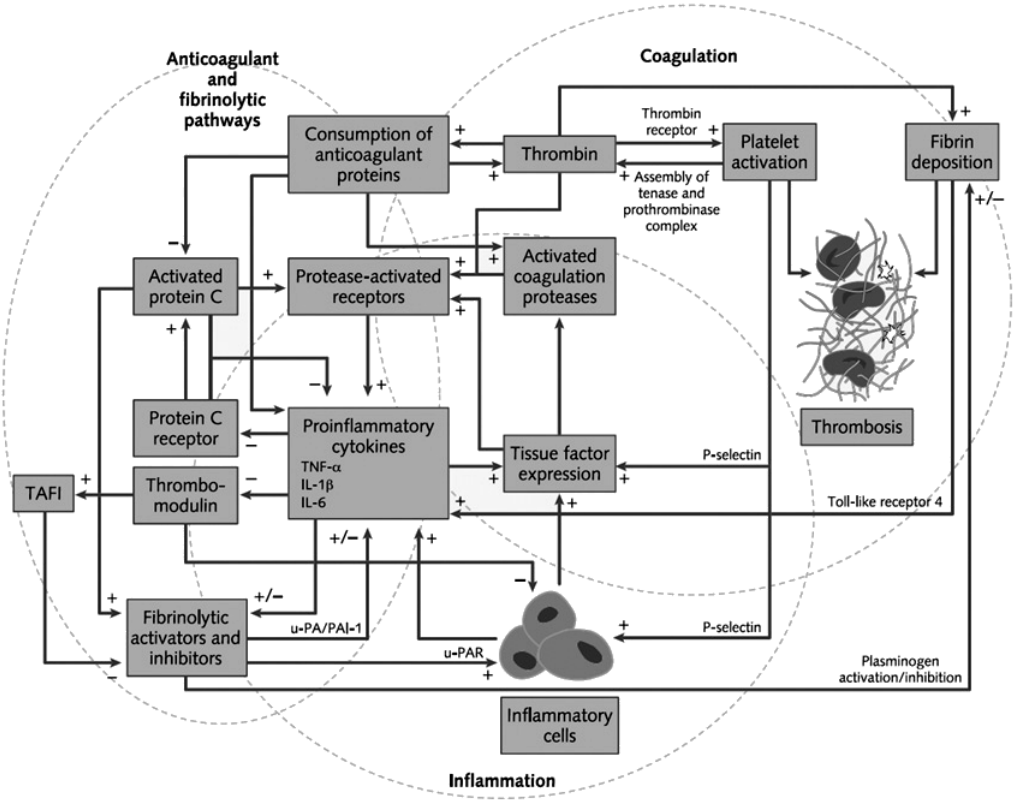

FIGURE 1.2: Major pathways involved in inflammation and coagulation (+ signs stimulatory effects, - symbol state for inhibitory effects). Abbreviations used: IL, interleukin; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activatable fibrinolysis inhibitor; TNF, tumor necrosis factor; u-PA, urinary plasminogen activator; u-PAR, urokinase-type plasminogen activator receptor [36] (with written permission from original author).

1.3 Therapy and organ support of severe septic patients

Although various sepsis biomarkers are of extensive research, regrettably a specific and sensitive marker of sepsis is yet to be identified [11]. However, widely used C-reactive protein (CRP) and procalcitonin (PCT) inflammatory markers can help in the differential diagnosis from other causes of shock [11, 37, 38]. Following the diagnosis of severe sepsis, initial goal-directed fluid therapy and prompt, broad-spectrum empirical antibiotic treatment (within 1 hour) are the current priorities of early therapy. The Surviving Sepsis Campaign 2008 guideline (SSC2008) recommends fluid resuscitation with clearly defined goals, based on the results of the study by Rivers et al. (Table 1.3) [38, 39]. Both crystalloids and/or colloids can be used for fluid therapy. Following initial steps, microbiological specimen should be collected as soon as possible, but this must not delay the administration of antimicrobial treatment [40, 41]. Microbiological sampling,

TABLE 1.3: The early goal-directed fluid treatment target parameters [39].

Central venous pressure	$8 - 12$ mmHg
Mean arterial pressure	> 65 mmHg
Urine output	0.5 ml/kg/h
Central venous (v. cava sup.) oxygen saturation	$\geq 70\%$

identification and antibiotics susceptibility testing are still the "gold standard" method. Currently the de-escalative use of antibiotics is supported. Wide-range empirical antimicrobial treatment should be the first choice treatment, based on the possible source of infection. Based on microbiology results and clinical picture, medication should be reconsidered and a single targeted treatment is preferable. According to the rational of the SSC2008, early narrowing of the antimicrobial effect may decrease the possibility of fungal (*Candida*), *Clostridium* or multiresistant *Enterococcus* infections [38]. Besides proper antimicrobial treatment, infection source identification and sanation are also important steps [42]. In case of interventional need for source control, the least invasive access is recommended. If clinicians are not aware of any possible infection source, the presence of systemic infection must be proved by microbiological sampling. This may include blood cultures, pharyngeal or tracheal specimen, intraluminar catheters, wound discharge and/or bronchoalveolar lavage fluid [38].

As patients usually develop arterial hypotension despite of fluid resuscitation, continuous vasopressor therapy is necessary to maintain mean arterial pressure of $\geq$65 mmHg [43, 44]. The actual guideline does not prefer norepinephrine or dopamine above the other, but recommends epinephrine in case of anergy against these medications. The use of corticosteroids is long debated in sepsis therapy [45, 46]. It is known that septic patients have deteriorated corticohormon levels, but the current guideline recommends substitution only in case of ineffective vasopressor therapy [38]. Formerly wide-accepted "renal protective" low-dose of dopamine is not proven by the meta-analysis of Kellum et al. [47] therefore the use of this therapy is not recommended.

Blood product transfusions may be necessary when haemoglobin levels reach < 70 g/l, in the absence of cardiac complications, while coagulation disorders should be resolved by the administration of fresh frozen plasma [48, 49]. Platelet transfusions should be considered if platelet count ranges in $5000 - 30\,000$ /l. Invasive procedures may require $\geq$50 000 /l, while patients below 5000 /l should receive supplementation regardless of bleeding complications.

Supportive therapy of sepsis is aimed to bridge the period of severe sepsis, while supporting the function of sepsis-affected vital organs. Patients developing acute respiratory distress syndrome may require mechanical ventilation. These patients should be ventilated with low tidal volumes (about 6 ml/kg) and plateau pressures ($\leq 30\,\mathrm{cmH_2O}$) and head of the bed elevated by 30°. To minimise pressure and volume related lung injuries, permissive hypercapnia is allowed [50, 51]. Patients must be sedated during the time of mechanical ventilation, while neuromuscular blockade seems not to be beneficial for patients. Glucose levels of patients should be controlled by the administration of short-acting insulin (or analogues), maintaining blood glucose levels < 8.3 mmol/l [52]. Continuous or intermittent renal replacement therapy should be considered in patients with renal impairment [53]. Deep vein thrombosis prophylaxis can be managed by the use of low molecular weight heparin and unfractionated heparin, but high-risk thrombosis patients should receive low molecular weight heparins [54]. For stress ulcer prophylaxis, histamine H2 receptor inhibitors or proton pump inhibitors can be used, but these medications may increase the risk of ventilation associated pneumonia [38, 55].
Utilising the recommendations of SSC2008, according to the review of Levy et al., overall mortality decreased by 5.4%, in centres following guidelines for at least 2 years. This review claims, the use of board spectrum antibiotics, proper microbiological sampling and treatment, as well as blood glucose control are responsible for the improved survival of patients [56].

1.4 Platelet activation and platelet aggregation

Platelets are megakaryocyte produced cellular fragments with the diameter of $2 - 5\,\mu m$ and thickness of about $0.5\,\mu m$. Their average lifespan in the circulation is 7-10 days. Aged platelets are removed from blood in the spleen. Platelets surface show an open canalicular system, connected "tunnels" of platelet membrane folds. Specific glycocalyx elements of platelet outer surface, called glycoproteins (GP) contribute to various platelet functions. Principle GPs involved in the main platelet function, haemostasis are GP Ib-V-IX and GP IIb-IIIa. While former is involved in platelet shear-stress based activation and in the binding of von Willebrand factor (VWF) and collagen, the later is responsible for fibrinogen binding of activated platelets and in the formation of platelet aggregates. These receptors are able to move horizontally in the membrane even inside the canalicular system. This movement enables to increase local receptor density in case of activation. Platelets harbour different types of inner vesicles of various physiological functions. The α-granules contain proteins synthesised in megakaryocytes, including: coagulation factor V, thrombospondin, P-selectin, and VWF. Dense bodies are comparably smaller than α-granules and are easily recognised in transmission electron microscope images by the electron dense inner sphere surrounded by an almost clear space inside the granule membrane. Dense bodies are rich in adenosine-triphosphate and -diphosphate (ATP and ADP), pyrophosphate, calcium, and magnesium. According to analytical electromicroscopy, the dense core is formed by calcium complexes of pyrophosphate and serotonin. Few lysosomes are also present in platelets, but their exact role are still debated. Commonly accepted view is they are simple remnants from megakaryocytes [57]. Platelet function is highly regulated by the surrounding endothelial lining and various factors. Nitric oxide, prostacyclin and the ecto-nucleotidase CD39 through an ATP-adenosine pathway (ADP cleavage to AMP) are all able to inhibit platelet activation and aggregation in physiological situations [58]. Nitric oxide inhibits platelet activation acting on guanilate cyclase, while prostacyclins and analogs increase cyclic-AMP through Gs-proteins. Classically, platelet aggregation is discussed in 3 consecutive steps: initiation, extension and perpetuation. The initiative activation may rise from exposure of collagen and VWF following vascular wall injury, gathering a monolayer of activated platelets lining the injury site. Collagen acts through $\alpha 2\beta 1$ and GPVI, increasing platelet Ca^{2+} through a phospholipase $C\gamma 2$ mediated way, while VWF activates through GPIbα and $\alpha 2b\beta 3$ receptors. The VWF

is crucial in platelet adhesion and rolling in high-shear situations, where VWF have a linear structure compared to low-shear where VWF has a globule like form [59]. The initial binding cause a smaller Ca^{2+} peak in platelets, resulting in the release of ADP. ADP can act on the $P2Y_1$ receptors of resting platelets, activating platelets locally in a positive loop in a Gi-protein coupled manner, decreasing intraplatelet cyclic-AMP levels. Following firm adhesion to VWF through $\alpha 2b\beta 3$, an increased Ca^{2+} surge precedes platelet aggregation. From various collagen receptors, GPIV plays a pivotal role in platelet activation and aggregation. Other possible form of activation is through PAR receptors in the presence of thrombin, usually reported in inflammatory and thrombotic diseases. Patients receiving heparin can have platelet activation through FcR-IIA, while pathogens (mainly bacteria and fungi) may act both on FcRs and TLRs. The extension phase is mainly a positive loop of platelet activation, initiated by the monolayer of activated platelets. Among many other molecules, these fragments secrete thromboxane A2, ADP and help local thrombin formation, activating resting platelets of the circulation. These activating substances induce phospholipase C activity in platelets, increasing internal Ca^{2+} levels and the presence of surface $\alpha 2b\beta 3$. The $\alpha 2b\beta 3$ complex changes its conformation following the binding of phosphorylated talin [60]. Binding of fibrinogen fibres and platelet plug formation is mainly developed through these receptors. In the perpetuation phase, G-protein coupled receptor signals are already faded, integrins and receptor tyrosine kinase signalling is responsible for platelet plug stability [58, 61]. Besides the classical role of platelets in haemostasis, their contribution to innate immune response is of high interest. The review of Beaulieu et al. summarises platelet reactions to pathogens and pathogen associated patterns. The activation of platelets through TLR-2 may induce PI3K-Akt-Erk pathway activation, causing platelet adhesion, aggregation, α-granule secretion and reactive oxygen species production, while TLR-4 activation and signal transduction through MyD88 cause cytoskeletal rearrangement, α-granule secretion and aggregation [62].

1.5 Microparticles in circulation

The first description of microparticles (MPs) came from Wolf, in 1967, who first noted them as platelet-dust in platelet free plasma. He proved their procoagulant activity and that this feature is removable by ultracentrifugation [63]. Although platelet-dust was known for more than four decades, the technical difficulty of MP measurement withheld their extensive analysis until the last decade. Nowadays we are aware that hypothetically all eukaryotic (and some prokaryotes) cells are capable of forming MPs, even though in the circulation of healthy individuals small amounts of MPs are present, most of them harbouring platelet-specific proteins (>80%) [64].

There was and still is a confusion in the naming of different bodily fluid vesicles. Throughout my thesis, I aim to use the phrase microvesicles for MPs and exosomes together, while the definition used for MPs is the widely accepted, $100-1000\,\text{nm}$ vesicular fragments produced by cell membrane shedding. Exosomes are $<100\,\text{nm}$ doughnut-shaped vesicles, generally produced during exocytosis from multivesicular bodies, or also by membrane blebbing [65].

1.5.1 Production and elimination of microparticles

Generally thought MPs are formed during the loss of membrane phospholipid asymmetry. Under normal resting conditions phosphatidylserine (PS) and phosphatidylethanolamine is located mainly in the inner leaflet of the membrane, while phosphatidylcholine and sphingomyelin are abundant in the outer leaflet [66, 67]. This asymmetry is sustained by the flippase enzyme transferring aminophospholipids (PS and phosphatidylethanolamine) into the cytoplasmic membrane layer. During cell activation, apoptosis or increased shear stress the presence of PS in the outer leaflet is one of the first signs of this processes [68]. Besides random phospholipid movement, the activation of floppase(s) (an enzyme capable to direct aminophospholipids towards the outer leaflet) and scramblase (enzyme catalysing random movement of phospholipids) accelerate the appearance of PS in the outer membrane. According to the "classical" view of MPs production, intracellular Ca^{2+} increase is the main determinant of MP formation. Calcium-induced degradation of cytoskeleton by calpains and the transient mass difference between membrane leaflets support the formation of MPs and (Ca^{2+}) influx following cell activation may inhibit flippase and activate floppase enzymatic

activity [69, 70](Figure 1.3).

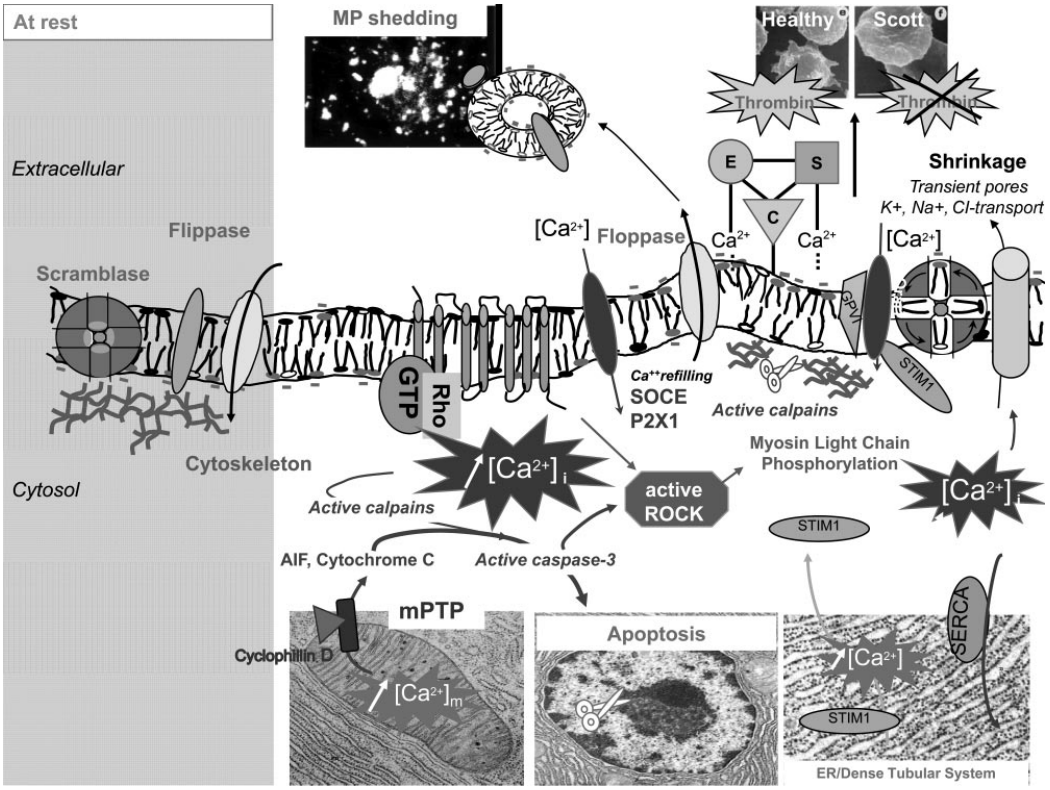

FIGURE 1.3: Cellular mechanisms of microparticle formation, by Morel et al. [71] (with written permission from original author).

Several signal transduction pathways are known to be involved in MP formation. Phosphorylation state of intracellular compartments as well as myosin light chain kinase, calmodulin and other calmodulin-related proteins are known to be responsible for platelet MP formation [72]. For platelets, stable Ca^{2+} levels are necessary to prevent platelet activation and particle formation. Ca^{2+}-dependent ATPases pump calcium ions back into the sarcoplasmatic and endoplasmatic reticuli, or out from platelets. Calcium concentration may increase by agonist-dependent pathways (store-operated calcium entry) or through the purinerg $P2X_1$ receptors [73]. The store-operated entry pathway causes a rapid PS exposure without receptor-ligand interactions. In human erythroleukaemic cell line this pathway is regulated by the RhoA GTPase, instead of ROCK [74]. The importance of the storage-operated pathway is hypothesised by the protection of collagen-induced arterial thrombus formation by the inhibition of Orai1 and STIM1 key proteins of this pathway. In platelets, STIM1 translocates from the dense tubuli into the membrane and co-localise with Orai1 upon store-depletion, to

facilitate extracellular Ca^{2+} uptake [75]. Another Ca^{2+} entry pathway is thrombin-induced. Even in the lack of Orai1 and STIM1 function, thrombin is still able to induce PS externalisation and thrombus formation [76, 77]. Also, cyclic adenosine monophosphate-dependent protein kinase activation reduced activation-dependent MP formation of platelets, while inhibition of this protein increased shedding. In HEL cells, the activation of ERK1 and ERK2 kinases by calcium ionophore A23187 induced PS^+ MP formation, while ERK inhibitors reduced MP shedding [78]. Endothelial cells tend to produce MPs following TNF-α activation, but this effect can be inhibited through p38 mitogen-associated protein kinase inhibition [79]. Also, IL-1 treated endothelial cells tend to produce MPs in the presence of thrombin. Cytoskeletal reorganisation can be triggered by the activation of caspases or calpain. Caspases are known to take part in cell apoptosis [80]. These proteins are able to cleave filamin-1, gelsolin, talin and also myosin [81]. The induced activation of caspases in platelets resulted in proportional PS exposure and membrane blebbing in the study of Schoenwaelder et al. Other vascular cell lineages are also producing MPs following caspase activation [82]. Research on Jurkat T-cells provided information regarding caspases and cytoskeletal reorganisation leading to MP formation. The cleavage of ROCK I kinase by caspase-3, or ROCK II activation by caspase-2 can both induce particle formation. ROCK I and II Rho kinases are responsible for myosin light chain phosphorylation which induce cell contraction and MP release during apoptosis. Regardless of the cause of PS^+ MP release, the presence of these particles may start a positive loop of further PS-MP release from previously resting endothelial cells and platelets [72, 83]. Although the above described mechanism is the currently accepted model for MP formation, certain data question the single role of Ca^{2+} concentration in MP shedding. Most of our knowledge based on the use of Ca-ionophores [84]. These compounds are able to increase intracellular Ca^{2+}, triggering membrane changes even without storage-operated pathway activation [85]. This effect of ionophore treatment is widely used in microparticle shedding assays, while the normalisation of cytosolic-cytoplasmatic Ca^{2+} levels may reduce particle numbers. Other research data implicates not only Ca^{2+}, but other electrolyte fluxes may cause the release of particles. Ca^{2+}-sensitive K^+ channels (Gardos channels) may cause the efflux of potassium, causing cell dehydration and PS exposure in platelets.

Outer membrane composition of MPs in the circulation of mainly platelet origin consists

of cholesterol (about 60%), sphingomyelin, phosphatidylethanolamine and PS [86], while synovial fluid particles (possibly originating from leukocytes) harbour phosphatidylethanolamine, sphingomyelin, phosphatidylcholine, lysophospholipids (20-25%) and PS [87]. Besides physiological membrane components Huber et al. found oxidised lipid compounds in endothelial MPs following oxidative stress, which were absent following ionophore activation [88]. Protein composition of particles generally follow their host cells, which can be used in particle origin characterisation. Platelet-derived MPs (PMPs) harbour glycoproteins (GP) and different proteins characteristic for resting platelets, as well as ones expressed in activated platelets (see Table 1.4). Besides PS bearing MPs an another group of MPs are PS negative. There is still a debate on the actual number and role of these MPs, as labelling of PS by annexin V is highly dependent on pH and the presence of Ca^{2+}. Characteristics of these vesicles are not completely clear. Some hypothesise that the loss of PS is due to interaction with other membrane vesicles, cell debris and lipophilic proteins [71].

Table 1.4: Constitutional and activation-dependent markers and representative antibody clones of platelets and platelet-derived microparticles, generally used in flow cytometry measurements [89].

Epitope	Antibody clones
Constitutional markers:	
glycoprotein Ib	6D268, N-19, C-20
glycoprotein IX	GR-P, Beb1, ALMA.16
glycoprotein IIb	5B12, VIPL3, HIP8
glycoprotein IIIa	F11, VIPL2
Platelet endothelial cell adhesion molecule-1 (also present on endothelial MPs)	CD31
Activation markers:	
glycoprotein IIb-IIIa fibrinogen binding conformation	PAC1
P-selectin exposure from α-granules	S12, AC1.2, AK-4
CD40 ligand	TRAP1
Factor Va binding	V237
Factor VIII binding	1B3

Our knowledge regarding the elimination of MPs is considerably limited. Rank et al. followed MP numbers after platelet transfusion in patients suffering from aplastic phase of haematological diseases. According to their data, transfused MP half-life was 5-6 hours. Phagocytosis by macrophages, as well as the role of lactadherin (binding and

phagocytosis by platelets and leukocytes) is hypothesised in the removal of MPs from circulation [90].

1.5.2 Role of microparticles in (patho)physiological processes

Vesicle formation is a conserved process of cells to exchange information with their environment [91]. Even by prokaryotes, outer membrane vesicles are used for communication, protection and for the horizontal exchange of genetic information. These vesicles are $50 - 100\,\text{nm}$ in size and may be considered as exosomes [92]. *Pseudomonas aeruginosa*, a human opportunist Gram-negative bacteria, commonly identified in sepsis, use membrane-derived vesicles in quorum sensing, the process of local population density perception. Removal of *P. aeruginosa* membrane-vesicles inhibited bacterial cell-cell communication, through the removal of Pseudomonas quinolone signal transmitted by vesicles [93]. Enterotoxic *Escherichia coli* strains produce membrane vesicles, containing heat-labile endotoxin. These vesicles are able to transport toxin into host cells, therefore take part in the development of traveller's diarrhoea [94]. The O157:H7 *E. coli* strain is able to produce vesicles containing DNA, RNA, phospholipids, LPS and various proteins. According to Yaron et al., these vesicles may fuse with *Salmonella enterica* to share DNA content and expressed in *S. enterica* genes from *E. coli* [95].
There is a growing evidence, that eukaryote cells also utilise vesicles in a wide variety of (patho)physiological processes.

In the last more than 20 years MPs were more and more extensively researched in the field of coagulation following the observation of Wolf. Key aspects of this effect is the distribution of TF in the circulation via small vesicles, cell activation and presentation of extensive negative PS surface for the haemostatic system. Following activation and shedding, PMPs (possibly following TF exchange with monocytes or monocyte-derived vesicles) express TF, surface receptors and ligands able to bind to various cells, including: macrophages, neutrophils and other resting platelets [96]. As reported by del Conde et al., monocyte/macrophage derived vesicles, are able to fuse with activated platelets, through a PS and P-selecting glycoprotein ligand-1 derived pathway. This fusion delivers TF, from TF-rich monocyte-derived particles into TF-sparse platelets, resulting in TF-harbouring platelets. These activated platelets are hypothetically capable to provide all factors of haemostasis [97]. Nagy et al. provided further evidence on the presence of MPs

in patients undergone percutaneous coronary intervention and associated MP production with increased stent restenosis [98]. Binding to resting cells can cause activation, as well as membrane fusion of MPs and recipient cells. Activated neutrophils are able to produce vesicles rich in Macrophage antigen-1, which can further activate still resting platelets. TF is considered as a key factor of coagulation, the binding of TF to factor VIIa can start the whole haemostasis cascade, therefore TF bearing MPs may play pivotal role [99]. Also, endothelial MPs harbour ultra large VWF molecules, able to stabilise aggregates [100]. Hrachovinová et al. assessed the effect of P-selectin in an in vitro model. According to their data, the production of TF positive MPs is increased in a dose dependent manner in the presence of P-selectin [101]. The importance of MPs in coagulation is emphasised by the Scott syndrome, a rare congenital disease characterised by the inability to rearrange platelet membrane components, resulting in bleeding disorder [102]. According to the observation of Koppler and Gasser, MPs released in early phases of inflammation express inhibitory effects through transforming growth factor beta-1, IL-10 and attenuation of IL-8 and tumor TNF-α release, but have pro-inflammatory function later. This pro-inflammatory state is maintained by CCR3, CCR4 delivery and IL-6 release stimulation. Also, abundant fibroblast-derived vesicle numbers were observed in arthritis patients. These particles induced matrix metalloproteinase (zinc-dependent, extracellular matrix endoproteases) release in arthritis patients [103–105]. Matrix metalloproteinases are responsible for neovascularisation, but may excert deleterious effect in septic patients [106]. MPs also contribute to vascular tone dysfunction, as besides procoagulant attributes, PMPs may bear superoxide anion and NADPH oxidase, able to induce stress response in endothelial and smooth muscle cells. In experimental settings reactive nitrogen species were also observed in MPs, which could modulate nitrogen oxide pathways in smooth muscle cells, impairing response to vasoconstrictors. Mortaza et al. observed hypotension in rats, induced by pooled MPs of septic rats (coecal ligature and puncture model), while control rat MPs were not effective [107–110], but sporadic data support the hypothetical effect of MPs on heart muscle contractility too. RANTES transfered to endothelial cells by PMPs help in the recuritment of leukocytes (mostly monocytes) and diapedesis [111].

Protective role of MPs and their possible role in cellular "waste-management" was demonstrated by Abid-Hussein et al. They found caspase-3 in vesicles released from viable endothelial cells, while the inhibition of caspase-3 containing vesicle release resulted

in subsequent cellular apoptosis. This finding suggests the pivotal role of vesicles in cell survival following stress [112, 113]. Others found, that cells may release chemotherapeutics, oxidised lipid compounds, drugs into the environment via vesicle shedding. The exchange of nucleic acids between cells was also found in eukaryotes. Valadi et al. showed that exosomes may contain mRNA or microRNA sequences. In this set of experiments, mouse exosomes transferred successfully genetic material into human mast cells, resulting in foreign protein expression [114]. Increased microparticle levels from different bodily fluids were reported in a large variety of diseases, but the exact role of vesicle formation and presence is still not completely elucidated [91].

The above description of various pathways involved in MP production emphasise the place of MPs in the crosstalk of inflammation and coagulation, two major players of sepsis pathophysiology. Considering the above discussed pathophysiology of sepsis and microparticles, one may not wonder on the growing evidence regarding the controversial, most likely deleterious effect of microparticles originating from sepsis-activated or damaged cells [115].

Aims

1. **Platelet aggregation in severe sepsis**, to elucidate our hypothesis whether microvascular flow deterioration can be a result of increased platelet aggregability:
    - We aimed to evaluate the platelet aggregation alteration characteristics in severe sepsis.
    - We tried to assess the connection between reduced platelet count and measurable platelet function in severe sepsis.
    - We also aimed to assess the role of spontaneous platelet aggregation in severe septic patients.
2. **Microparticle studies in severe sepsis**, as microparticles are mainly of platelet or megakaryocyte origin in the blood and other sepsis-affected cell lines of the circulation are also producing microparticles via activation and apoptosis, we aimed to follow the microparticle profile in severe septic patients:
    - We assessed the effect of different infectious agents on microparticle characteristics.
    - We aimed to provide further data regarding elevated microparticle levels in septic patients.
    - We evaluated the connection between developed sepsis-related organ failures and microparticle levels.

Patients and Methods

3.1 Patients

Recently diagnosed severe septic patients were enrolled from our multidisciplinary university intensive care unit of the Department of Anaesthesiology and Intensive Therapy, University of Pécs. Our studies were carried out in accordance with the ethical guidelines of the 2003 Declaration of Helsinki and we obtained the permission of the Regional Research Ethical Committee of University of Pécs (Ethical Committee reference numbers: 2406/2005 and 4234/2011). All patients provided a written informed consent following detailed information regarding the study protocol and blood sampling. In case of a consciousness disorder we obtained consent from the next of kin as per national law. Our inclusion criteria were recently discovered severe sepsis (within 24 hours) with two or more sepsis-related organ dysfunctions. Criteria for sepsis included: fever ($\geq$38°C) or hypothermia ($\leq$36°C), tachycardia ($\geq$90/min), tachypnoea, altered state of consciousness, positive fluid imbalance, hyperglycaemia, leukocyte count ($\geq$ 12000 cell/µl or $\leq$ 4000 cell/µl), elevated C-reactive protein (CRP)($\geq$10 mg/l), serum PCT (PCT) level ($\geq$2 ng/ml, and 5 ng/ml in the fungal MP study). In our platelet aggregation study patients in moribund state or with any kind of haematological baseline disease such as myeloproliferative disorders like lymphoma or leukaemia were excluded. For MP measurements we extended our exclusion criteria, with certain known factors which can significantly alter MP amounts. Besides criteria addressed for the platelet study, the following were introduced: cytostatic treatment in the last 30 days, high dose prolonged steroid medication, patients with disseminated intravascular coagulation score $\geq$5 [116], drugs known to alter platelet functions (i.e. acetylsalicylic acid), platelet transfusion during the study period.

We aimed to follow our patients until the 5^{th} study day, or death during research period. In case of death, patient data collected until the last platelet aggregometry or flow cytometry measurement was assessed. If the patient or family withdraw consent we did not used collected patient data. The diagnostic and treatment procedures of severe sepsis were conducted by strictly following the most recent SSC guidelines (2005 and 2008 guidelines for the platelets and 2008 guidelines for the MP studies) in both study and non-study patients [38, 117]. As detailed in before, sepsis therapy included: fluid resuscitation with crystalloids and colloids, early broad spectrum antibiotic treatment (most commonly carbapenems with or without aminoglycosides), in case of haemodynamic shock norepinephrine with or without dobutamine, for control of glucose levels controlled infusion of human insulin, in oliguria or anuria furosemide with haemodialysis, stress ulcer prophylaxis with pantoprazole or famotidine, thrombosis prophylaxis with enoxaparine or fraxiparine, morphine and propofol were used for analgesia and sedation respectively during mechanical ventilation at appropriate doses. No patient received drotrecogin alfa or antiplatelet drugs. Laboratory parameters including C-reactive protein, PCT, lactate concentration, blood cell count, electrolyte concentrations, blood gas parameters and organ function specific parameters were registered. Laboratory measurements were carried out in the Department of Laboratory Medicine, University of Pécs. To follow-up the clinical status of severe septic patients, MODS and SOFA scores were calculated every single day during the whole study period and Simplified Acute Physiology Score II was calculated 24 hours following admission [4, 5, 118]. For the assessment of various organ failures we used the reference values of the currently accepted clinical guidelines, including the results of the RIFLE study (criteria summarised in Table 1.2), as well as the SSC and our local laboratory reference values [29, 38]. All data regarding organ dysfunctions were reassessed upon blood collection.

3.2 Platelet aggregation in severe sepsis

3.2.1 Blood sample collection

Arterial, 3×2.7 ml Na_3-citrate (0.129 mmol) anticoagulated blood was gathered from our patients for platelet aggregation studies. Blood samples were collected daily from our patients once upon admission (1^{st} day) and once for the following four consecutive

days. Venous blood samples from antecubital venipuncture with a 21 gauge needle were collected from 30 healthy controls. We avoided arterial blood sampling from control volunteers due to possible bleeding complications. Furthermore, a pilot study showed that platelet aggregation of venous and arterial blood is significantly not different [119].

3.2.2 Platelet light transmission aggregometry

Light transmission ex vivo aggregometry was described by Born in the 60's and became a wide used method for platelet aggregation and aggregation inhibition studies [120]. Our measurements were carried out using the Carat TX4 (Carat Diagnostics Ltd., Budapest, Hungary) 4-channel light transmission aggregometer. The instrument trans-illuminate test samples with infrared spectrum light, registering the intensity decrease because of scattered light. A platelet poor plasma sample is used to calibrate the equipment and determine the minimum optical density of the measured sample (maximum aggregation). Following, 4 platelet rich samples can be tested simultaneously. The equipment continuously registers optical density changes during measurement. In platelet aggregation studies, ADP is used in a wide concentration range of $1 - 10\,\mu$mol. Adrenaline (ADR) is used in $5 - 10\,\mu$mol doses, but according to the literature is not a completely reliable inducer. Nonsteroid anti-inflammatory drugs, antihistamines, antibiotics and many other compounds may alter platelet reaction to adrenaline. One of the most effective classical inducers, collagen is usually used in $1 - 5\,\mu$g/ml concentration and produce a two-wave aggregation. The first wave represents the adhesion and early activation of platelets on collagen fibres, while the second phase (after an about 1 minute lag phase) is the actual aggregation [121]. As different inducers acts on their own receptors, we were able to determine the activity of various aggregation pathways. As formerly mentioned, ADP-induced platelets are activated through $P2Y_1$ and $P2Y_{12}$ receptors, while ADR acts on platelet α_{2a} receptors. COL acts via different GP receptors, namely: GPIb and GPIIb-IIIa (both in high shear condition via VWF), GPIa-IIa and GPVI (in low shear-stress conditions). According to our knowledge, 0.9% NaCl solution (SAL) itself is not able to induce platelet aggregation [122].

Blood samples were centrifuged with 150 $\times$ g, 5 minutes and platelet rich plasma was separated into four test cuvettes (450 µl each). The remaining blood sample was centrifuged for further 10 minutes with 2500 $\times$ g to obtain platelet poor plasma (500 µl)

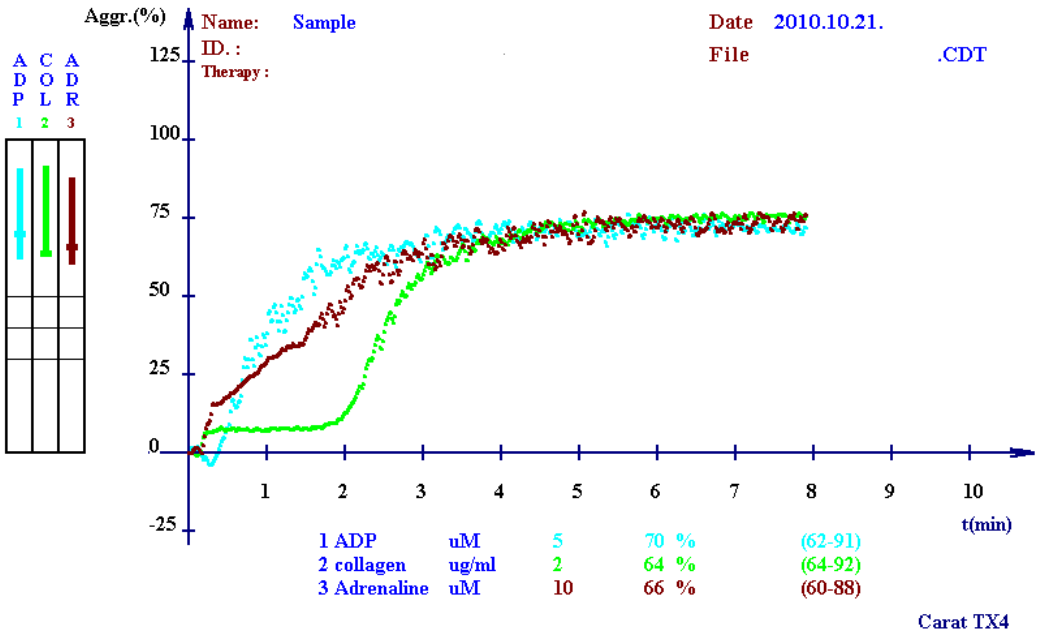

FIGURE 3.1: An illustration of analysed normal induced platelet aggregation results using the Carat TX4 aggregometer (Abbreviations used: ADR: adrenaline, ADP: adenosine-diphosphate, COL: collagen).

for minimum optical density determination. During aggregometry, agonists and saline were introduced separately into platelet rich plasma samples. We used 50 µl of SAL to measure the spontaneous and the in the same final volume 10 µmol ADP, 2 µg/ml collagen (COL), 10 µmol ADR (Theracont TA-3 inductor kit, Carat Diagnostics Ltd.) to evaluate the inducible aggregation. We assumed spontaneous platelet aggregation to be higher than 10%. Our opinion suggests that 1-3% aggregation could be an artifact. If the spontaneous aggregation is higher and consecutive in the series of measurements in one patient, it has to be analysed as an objective result. This is why we chose the 10% value as a reference due to the absence of data from literature. The inducible platelet aggregation was chosen $\geq$ 60% measured by the TX4 aggregometer. This value was verified in a study which was performed in our University [123]. We formed a low platelet count a normal platelet count group, based on the inclusion blood platelet count of our severe septic patients and thrombocytopenia criteria of the SSC2008 [38].

3.2.3 Statistical analysis

In our first study statistical analysis was carried out using SPSS 17 for Windows software. Data are presented with median and interquartiles. Kruskal-Wallis-test was used with

$p<0.05$ considered significant. The number of patients required was calculated by power analysis according to spontaneous and non-spontaneous platelet aggregation results from our previous pilot studies performed on similar population. Therefore, with type I a = 5% and type II (power) of 90% we needed 40 patients at minimum.

3.3 Microparticle studies in severe septic patients

3.3.1 Blood sample collection

For MP studies, additional 2 × 2.7 ml arterial blood samples were drawn on inclusion (1^{st}), and on the 3^{rd} and 5^{th} days with a 21 gauge cannula into a closed system blood sampling tube with Na_3-citrate (0.129 mmol) as anticoagulant. Samples were processed within maximum 30 minutes following collection. Volunteers provided blood for flow cytometry measurements on one occasion. Blood collection from volunteers was carried out through antecubital vein puncture without strangulation with a 21 gauge needle into closed system and processed the same way as patient samples. Upon blood collection, the first 3 ml arterial or venous blood was discarded to prevent mixture with infusion fluids or venipuncture induced activated thrombin. Both microparticle studies are based on the same patient pool, our second analysis took place after patient data reassessment and inclusion of new patients in our study. Twenty, age and gender matched apparently healthy volunteers were invited to determine the baseline values of our measurements. Patient exclusion criteria were also applied for our volunteer group.

3.3.2 Microparticle isolation and flow cytometry measurement

Flow cytometry is based on the measurement of scattered and absorbed-emitted light. With the utilisation of vacuum pumps, the cytometer takes small amounts of usually diluted samples (below 1 ml total volume) and appropriate sheath fluid in a stabilised flow rate. Sample material is centred using hydrodynamic focusing, in a flow cell (sheath fluid focusing) or nozzle tip (free air focusing). Hydrodynamic focusing is achieved by two parallel lamellar fluid streams. The outer sheath fluid stream and the narrowing part of the flow cell cause the same "narrowing" of the sample fluid, focusing one thin fluid line in the centre of the laser stream. Ideally, the centralised sample material

contains only 1 cell (or particle) in line with the focused exciting laser beam. In case of the Beckman-Coulter Cytomics FC 500 cytometer (Beckman-Coulter Hungary Ltd., Budapest, Hungary) a standard argon ion laser (488 nm, 20 mW) is used for light scatter and colour cytometry measurements, while an additional 637 nm (20 mW) HeNe or diode laser can be installed for 5 colour cytometry. Forward scatter detector measures diffracted light, while side-scatter detector measures refracted and reflected photons. Forward-scatter is proportional to the size, while side-scatter is proportional to the granularity and internal complexity of the illuminated event (according to Snell's law). The forward scatter detector of the FC 500 has a nominal size limit of ≥ 500 nm, therefore particles smaller than this cannot be reliably distinguished from light and electrical noise. Fluorochromes are excited by the primary (Ar-ion) or secondary (HeNe or diode) laser, elevating an electron of the fluorochrome to a higher energy state. Without the energy of the exciting light, the electron is unstable in the new state. Inside the new elevated energy state, it releases small amounts of energy in form of heat to reach the lowest energy of the excited state. Following it returns to its ground level, emitting a characteristic photon, which has different frequency because of the released heat. The emitted light is filtered through low- and high- wavelength pass optical filters and dichotomous mirrors to direct filtered light into photodetectors [124]. The evolving technical background of flow cytometry and the introduction of new cytometers boosted MP studies significantly. New cytometers are able to dissociate MP sized events from background noise. Our FC 500 equipment is able to measure events as small as 500 nm in forward and >200 nm in side-scatter mode (according to instrument description). Regrettably, no standardised method was available during the planning phase of our studies, but the International Society of Thrombosis and Haemostasis introduced a standardisation recommendation, which nowadays considered as a gold standard of measurements [125]. Our methods largely overlap with the presented recommendations, although we used different (custom made) beads for instrument setup.

3.3.2.1 Microparticle isolation and staining protocol

Flow cytometry measurements were carried out from fresh samples. Following collection, blood was centrifuged at 800×g for 20 minutes at room temperature to obtain platelet-rich plasma. The 1.5 ml supernatant was transferred into a new test tube and centrifuged at 1500×g for further 20 minutes to obtain platelet-poor plasma. The 1 ml volume of

platelet-poor plasma was further centrifuged at 1500×g 20 minutes in a new polystyrene tube to obtain cell-free plasma. The top 500 µl of cell-free plasma was transferred into an Eppendorf tube and pelleted at 18000×g for 10 minutes. The supernatant was carefully removed leaving 250 µl of MP rich plasma and pellet at the bottom of the Eppendorf tube. MPs were suspended with gentle vortexing over 20 seconds in 1.0 ml Apo-binding buffer (10 mmol/l HEPES, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 136 mmol/l NaCl, pH=7.4. HEPES was obtained from Sigma-Aldrich Ltd., Budapest, Hungary, other analytical grade reagents were obtained from Reanal Ltd., Budapest, Hungary) without $CaCl_2$. All antibodies and annexin V were titrated in preliminary experiments to determine the optimal labelling concentrations. Labelling concentrations were defined by antibody staining of samples and sample-free buffers in the presence or absence of $CaCl_2$. Labelling was considered optimal if $CaCl_2$ labelled sample measurement events were clearly distinguishable from background, $CaCl_2$ free staining, as well as from isotype controls. Isotype controls, fluorescein isothiocyanate, cychrome 5 or phycoerythrin conjugated mouse immunoglobulin G1 were used (Becton Dickinson Hungary Ltd., Környe, Hungary).

TABLE 3.1: Specificity, clone and manufacturer of antibodies used for microparticle labelling. Abbreviations used: Cy5: Cychrome5, PerCP: Peridinin chlorophyll protein, FITC: fluorescent isothiocyanate, PE: phycoerythrin, Ig: immonuglobulin.

Antibody	Fluorescent dye	Clone	Manufacturer
CD41	Cy5	HIP8	
CD42a	PerCP	Beb1	
PAC1	FITC	PAC1	Becton Dickinson
annexin V	FITC, Cy5	-	
mouse IgG1	FITC,Cy5, PE	X40	
CD61	FITC	RUU-PL7F12	Beckman-Coulter

Fluorescein isothiocyanate or cychrome 5 labelled annexin V (Becton Dickinson) in 12 µg/ml or 8 µg/ml concentrations respectively were used to identify MPs. Constitutively expressed platelet fibrinogen receptor subunits, GPIIb-IIIa were measured by the CD41 and CD61 antibodies respectively. Activated platelet marker, fibrinogen binding form of $\alpha 2b\beta 3$ was assessed by the PAC1 antibody. Platelet adhesion receptor GPIb-V-IX has been tested with anti-IX (CD42a) labelling (Table 3.1).

For sample labelling, 10 µl MP in Ca^{2+} free buffer was incubated in 100 µl Apo-binding buffer supplemented with 2.5 mmol/l $CaCl_2$ with total 10 µl antibody, previously diluted to optimal labelling concentration. Staining was incubated for 30 minutes at room

temperature in dark chamber.

Flow cytometry measurements and data analysis were performed on our FC 500 flow cytometer with CXP software (Figure 3.2). The MP gate was defined in order to distinguish the true events from electronic noise and background, using 0.3 µm, 0.5 µm and 1.0 µm FITC labelled microbeads (a kind gift of SoftFlow Ltd., Pécs, Hungary). Side scatter, forward scatter and fluorescence channels were set in logarithmic scale. MP size gate was determined between 0.5 µm and 1.0 µm size range. Events in the MP gate were further discriminated by labelling with annexin V. MPs were defined as annexin V positive events in the MP gate with fluorescence intensity above the isotype control. Figure 3.3 illustrates our cytometry gating strategy [126]. Subfigure A illustrates the MP size gate and counting beads for particle number estimation. Figure 3.3B shows annexin V staining in the presence of ethylenediaminetetraacetic-acid, to determine background of annexin V. 3.3C is the actual annexin V stained sample, while 3.3D is the isotype control for fluorescein isothiocyanate and 3.3E shows the PAC1-FITC stained vesicles. For determination of the MP number, known concentration (1×10^5 /ml) of 3 µm diameter microbeads (Becton Dickinson) was used. Microparticle measurements were carried out in the Department of Laboratory Medicine, University of Pécs. Intra-run variation coefficient range of our complete isolation and flow cytometry method, determined by total 25 measurements from 5 different controls is 15.4-19.2%.

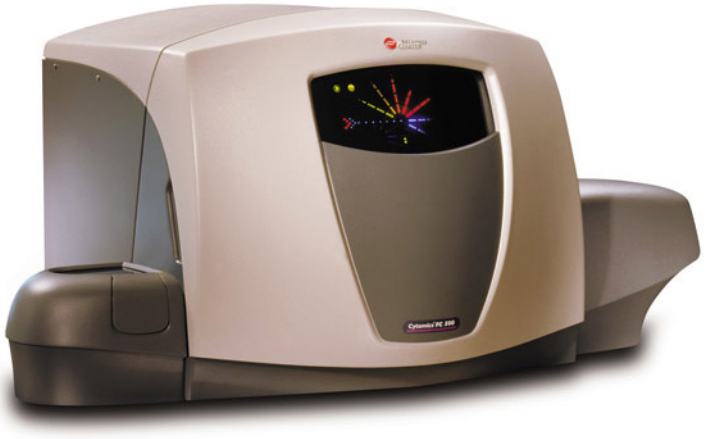

FIGURE 3.2: The Beckman-Coulter Cytomics FC 500 flow cytometer.

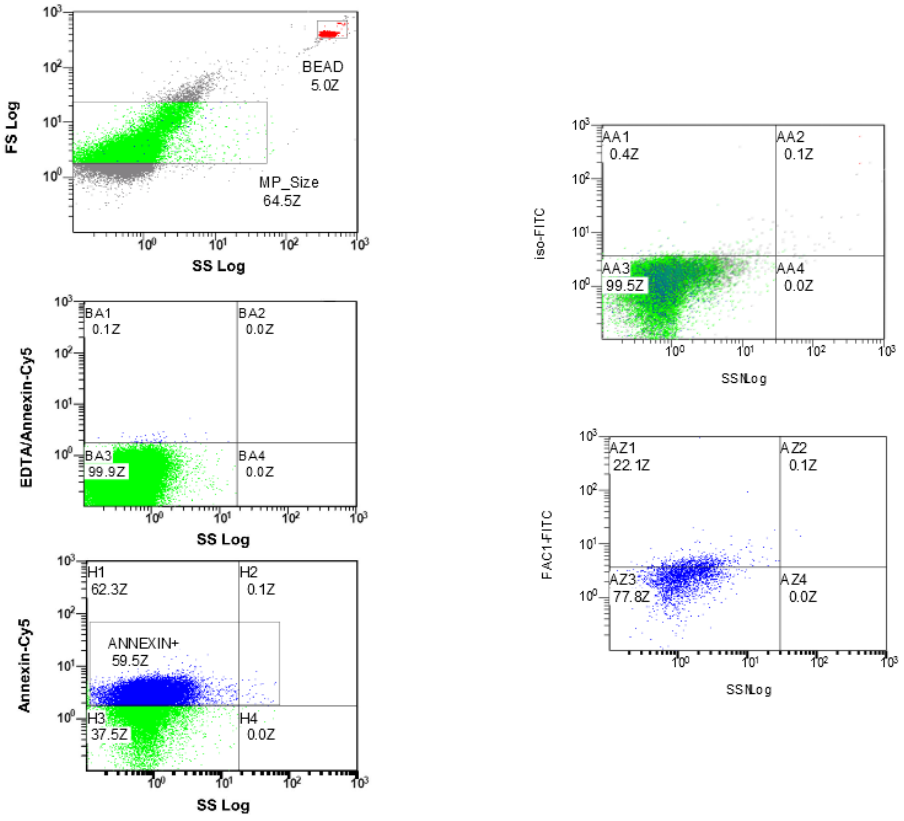

FIGURE 3.3: Flow cytometry gating strategy used for microparticle measurements. Abbreviations used: EDTA: ethylenediaminetetraacetic-acid, FITC: fulorescent isothiocyanate, Cy5: Cychrome5.

3.3.2.2 Microparticle measurement protocol controls

MPs isolated after stimulation of pooled platelet rich plasma from 5 healthy volunteers without platelet function altering medication with calcium ionophore A23186 (Sigma-Aldrich Ltd., 25 µmol/ml, 37°C, 10 minutes) were used as positive controls. 200 uL of pooled platelet rich plasma was mixed with the above mentioned A23186 concentration and incubated on room temperature for 30 minutes. MP isolation was continued from the 2nd centrifugation step accordingly. MPs stained with annexin V in presence of 2.5 mmol/l ethylenediaminetetraacetic acid were measured for negative controls.

3.3.3 Microbiological diagnosis and treatment of patients of the fungal infection - microparticles study

For the diagnosis of the microbial agent(s) in sepsis, haemocultures, urine samples and bronchial lavage fluids were gathered. Haemocultures were obtained through a fresh

vein or arterial puncture, prior to empirical antimicrobial medication, into BACTEC^{TM} haemoculture flasks (Becton Dickinson) from patients showing the signs of systemic inflammation response syndrome without recent microbiological results. Further microbiological sampling was carried out in case of continuous or elevated fever with shivering or overall patient clinical status deterioration including continuously elevated or increasing C-reactive protein and PCT results following the proper antimicrobial treatment. Microbiological identification was performed in the Department of Medical Microbiology and Immunology, University of Pécs. Empirical antibiotic therapy was started following microbiological sampling. The applied therapy generally consisted of carbapenems, respiratory fluoroquinolones, metronidazole and second or third generation cephalosporins. The selection of antibiotics was based on the clinical background and possible site of infection. Empirical antifungal therapy (mainly fluconazole) was administered in case of positive patient history for diabetes, immunocompromised state (i.e. tumours or steroid treatment), long-term antibiotic treatment, progressive sepsis without or with only slight PCT elevation accompanied by constant fever with shivers. Patients in our study were designated as fungal septic in case of positive blood culture results for fungal infection accompanied by the above mentioned clinical signs, following proper antibacterial treatment during our study period. Based on these criteria a fungal septic and a non-fungal septic group were formed.

3.3.4 Statistical analysis

For statistical analysis of our MP study data SPSS 19 for Windows software was used. For the comparison of septic and control patients Mann-Whitney U-test was carried out. For subgroup analysis of severe septic patients ANOVA test was used, corrected for multiple analysis. Correlation analysis was carried out using Spearman's rho. In calculations $p<0.05$ was considered significant.

Results

4.1 Platelet aggregation in severe sepsis

Forty-five patients were included in our platelet aggregation study during the study period of 2005 June - 2008 October. Main demographical data, MODS and SOFA scores calculated on the first, third and fifth admission day and sepsis-induced organ failures (day 1) of our study population are discussed in Table 4.1. We found no significant change of clinical scores in this patient population during our study period. As seen from our microbiological summary, only 36 of 45 patients had proved infection, while other sepsis criteria were met. Gram-negative bacteria were present in 26 patients, while Gram-positive infection was detected in 20 patients. The total number of infections is above our patient group size, indicating that some patients suffered from multiple infections. Renal function impairment (n=38), hypotension (n=35) and respiratory insufficiency (n=32) combined were the leading causes of patient admittance and inclusion. Besides the use of low molecular weight heparins (mainly enoxaparine) and corticosteroids in case of indication, patients did not received medications known to alter platelet function.

The evaluation of platelet aggregation in septic patients compared to healthy controls revealed a significant deterioration in the inducible aggregation among septic patients. The ADP, ADR and COL induced function measurements resulted in significantly lower levels for all the five study days ($p<0.05$) while SAL based aggregation showed a significant increase in the platelet function of septic patients (Figure 4.1, $p<0.001$). A slight increase is notable in ADP and ADR aggregation throughout our study period, but this increase does not reach control levels. COL aggregation was continuously low in septic patients. SAL aggregation was continuously elevated in severe septic patients

TABLE 4.1: Demographical, microbial and sepsis-related data of our platelet study patients and controls, data presented as median and interquartiles. Abbreviations: MODS: Multiple Organ Dysfunction Score, SOFA: Sequential Organ-Failure Assessment.

	Patients (n=45)	Controls (n=30)
Age	63 (51 - 71)	52 (49 - 67)
Gender (male / female)	29 / 16	19 / 11
MODS score (day: 1, 3, 5)	8 (6 - 10), 7 (5 - 9), 6 (3 - 8)	
SOFA score (day: 1, 3, 5)	9 (7 - 12), 8 (5 - 12), 6 (5 - 10)	
Gram-negative infection	26	
Gram-positive infection	20	
Fungal infection	11	
Patients with proved infections	36	
Renal failure	38	
Arterial hypotension	35	
Respiratory insufficiency	32	
Altered mental status	27	
Hepatic disorder	18	
Bone marrow insufficiency	8	

compared to controls, peaking on the 3^{rd} measurement day. Statistical comparison of the 1^{st} and 5^{th} day data shows no significant difference. On admission 19 patients were in the low platelet count range and 26 patients formed the normal platelet count group according to the thrombocytopenia criteria. We did not found significant difference in clinical and demographical data of these patient groups. ADP induced platelet aggregation was significantly deteriorated in patients with low platelet count in all 5 days ($p<0.05$), with an observable (but not significant) increase of aggregation in the normal platelet count group. ADR caused aggregations were lower in the 2^{nd}, 3^{rd}, 4^{th} and 5^{th} consecutive day ($p<0.05$) in the low platelet count group, with the same notable elevation of aggregation results in the normal platelet group. COL induced aggregation was significantly lower on the 1^{st}, 2^{nd}, 3^{rd} days following admission ($p<0.05$). COL aggregation of both groups did not changed notably during our study period. There was no difference between the two groups based on the saline aggregation, both groups kept the characteristic increase towards the 3^{rd} measurement day (Figure 4.2). Compared to our control group most aggregations measured in the normal platelet group were significantly lower than the control results with the exception of the adrenaline inducible aggregation on the 3^{rd}, 4^{th}, 5^{th} days and ADP induced aggregation on the 5^{th} day ($p<0.05$, data not shown). The low platelet group had significantly deteriorated

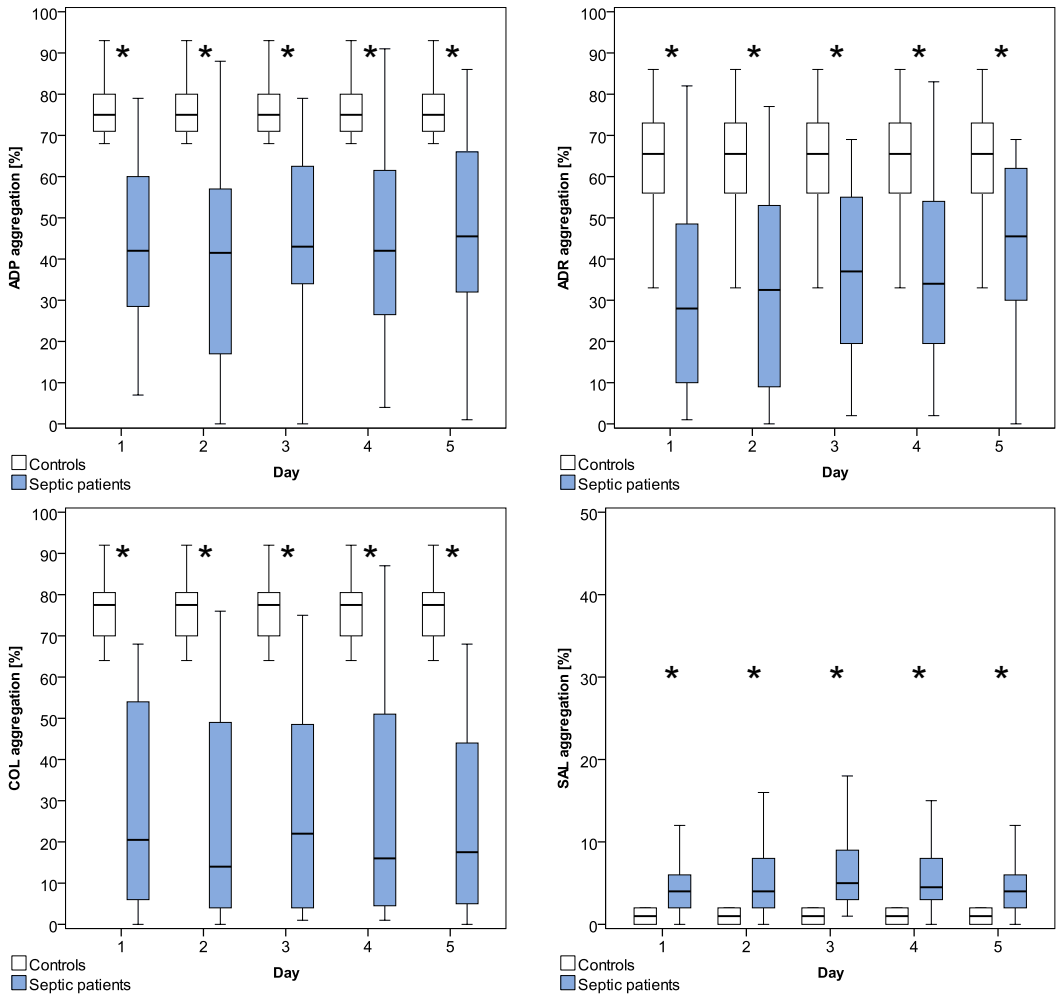

FIGURE 4.1: Platelet aggregation in severe septic patients and controls. Clear represents control patient data, shaded boxes represents severe septic patient data. *:p<0.05 between measurement points. Abbreviations: ADP: adenosine-di-phosphate, ADR: adrenaline, COL: collagen, SAL: 0.9% NaCl solution.

aggregation levels with all inductors in all cases (p<0.001, data not shown) compared to controls.

Fourteen patients deceased during our study and 31 patients built up the survival group. Non-survivors showed no significant deterioration in platelet count (p>0.05, data not shown). All induced and saline aggregation measurement results, when compared to survivors were not significantly different in non-survivors (Figure 4.3). ADP aggregation showed a slight decrease on the 2^{nd} study day, but increased slightly in our study period. ADR aggregation increased throughout our study period in both groups, while COL aggregation does not shows elevation. SAL aggregation results represents the increase towards the 3^{rd} study day. In the survivor group we found four patients with notable aggregation levels ($\geq$50%) during the saline based aggregation tests. The spontaneous

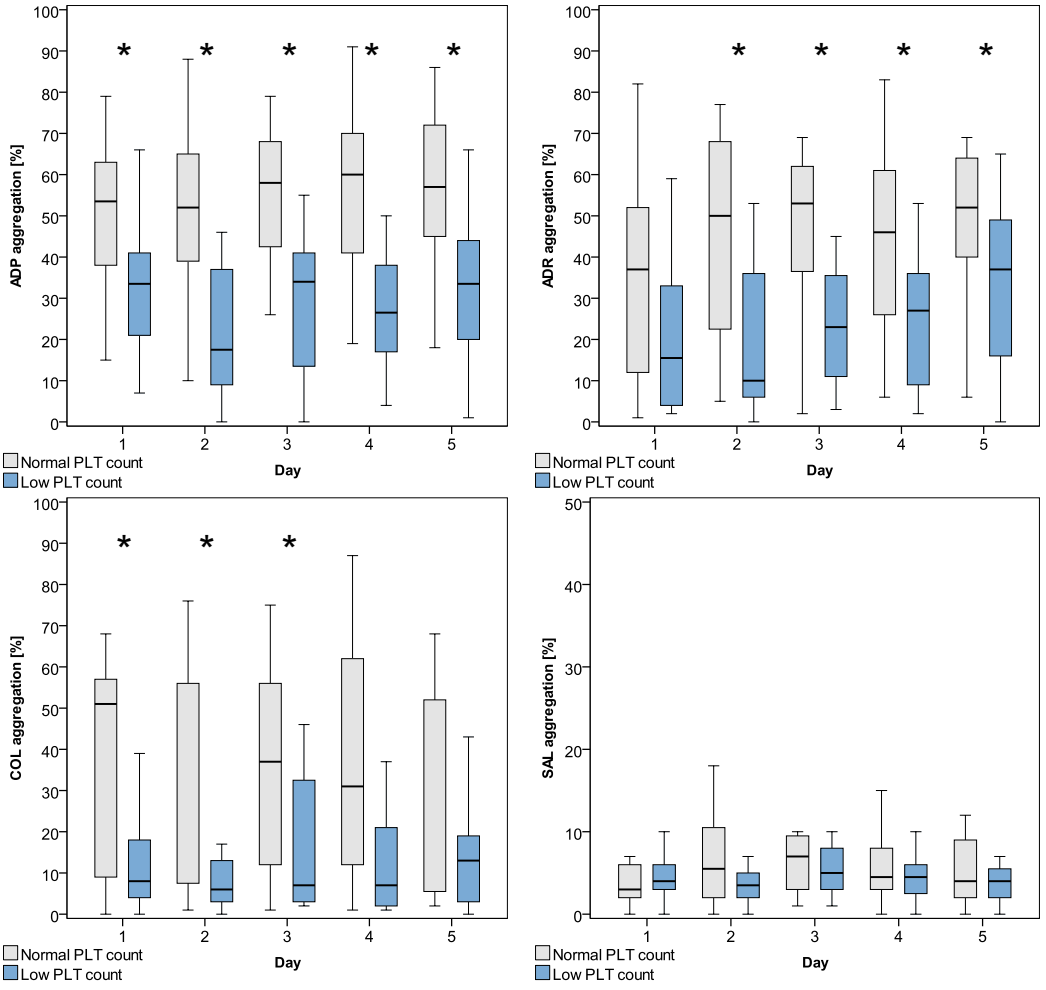

FIGURE 4.2: Platelet aggregation in severe septic patients with low (n=19) and normal (n=26) platelet count. Light shaded boxes represents normal platelet count patient data, dark shaded boxes represents low platelet count data. Data presented as median and interquartiles. *:p<0.05 between measurement points. Abbreviations: ADP: adenosine-di-phosphate, ADR: adrenaline, COL: collagen, SAL: 0.9% NaCl solution, PLT: platelet.

aggregation group (14 patients) revealed a non-significant difference in platelet counts compared to the non-spontaneous aggregation group. The non-spontaneous aggregation group showed a significantly higher, but constantly decreasing PCT levels on the 1^{st}, 3^{rd}, 4^{th} consecutive days. Lactate levels were also non-significantly lower in the spontaneous aggregative patients during our study (Figure 4.4). Also, we assessed if the presence of corticosteroids in patient therapy has any effect on platelet aggregation, but did not found significant difference in inducible or spontaneous aggregation results. Of all 36 patients with proven infections, only 7 patients presented Gram-negative bacteria only. According to our analysis, the presence of Gram-negative bacteria did not contribute to spontaneous aggregation significantly, although patients with spontaneous aggregation

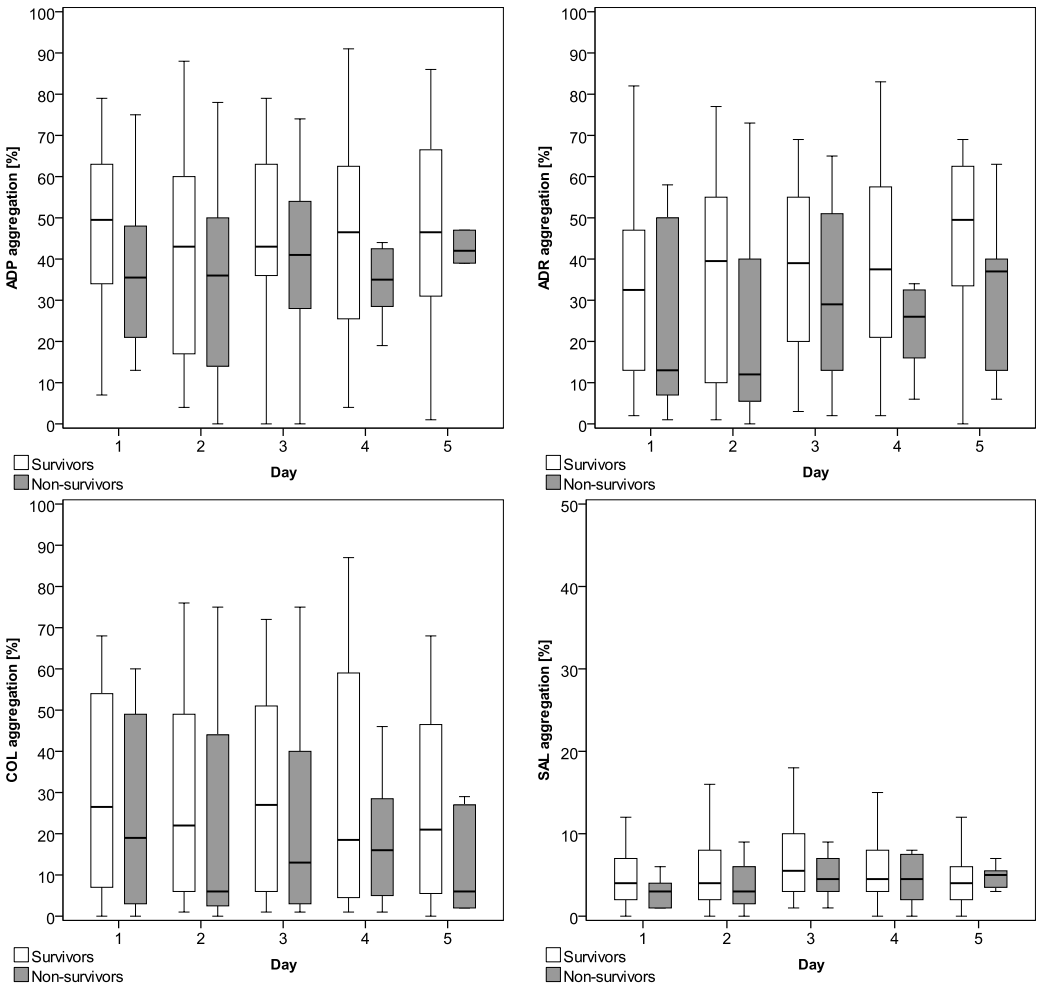

FIGURE 4.3: Platelet aggregation in survivor (n=14) and non-survivor (n=31) severe septic patients. White boxes represents survival patient data, grey boxes represent non-survival patient data. Abbreviations: ADP: adenosine-di-phosphate, ADR: adrenaline, COL: collagen, SAL: 0.9% NaCl solution.

and Gram-negative infection showed significantly increased aggregation levels compared to patients without Gram-negative bacteria.

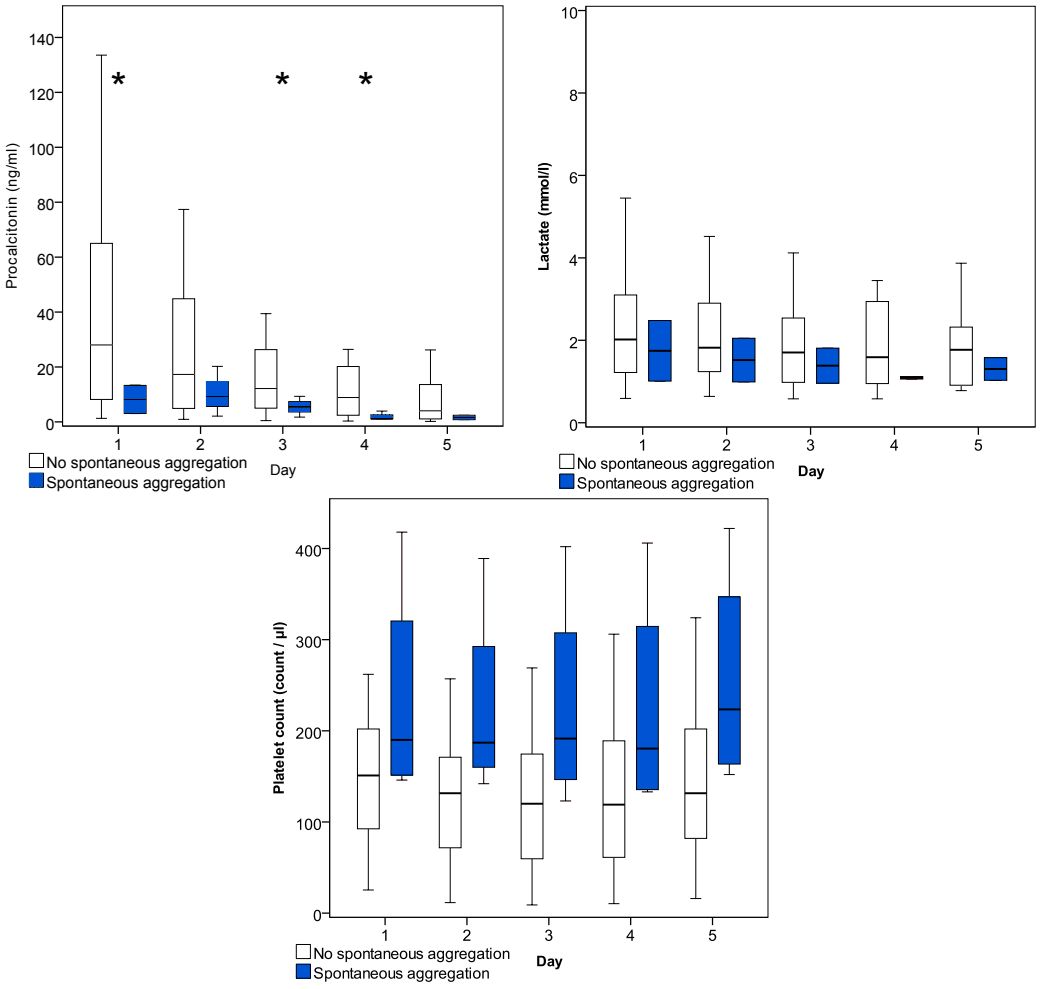

FIGURE 4.4: Procalcitonin, lactate and platelet count comparison of the spontaneous (n=14) and non-spontaneous (n=31) platelet aggregation groups. *:p<0.05 between measurement points.

4.2 Microparticle studies in severe septic patients

4.2.1 The effect of mixed fungal sepsis on microparticle profile

During our first microparticle study 57 patients were eligible according to our inclusion criteria, but only 33 patients gave an informed consent. Eight patients refused participation following detailed information about our study. 16 patients were excluded based on our criteria, after the reassessment of patient history (mainly because of platelet inhibitor use or prolonged steroid therapy). Our MP study took place from 2008 January, the first study assessment was carried out in 2010 January, the second assessment was done in 2011 December.

Basic patient characteristics and clinical data including clinical status scores are described in Table 4.2. Age, survival data, laboratory markers on admission and calculated clinical scores did not differ significantly between the mixed fungal and non-fungal septic patient groups. Also, age and gender characteristics of the volunteer group (n = 20, median age: 60 (56-65) years, 1:1 gender ratio) showed non-significant difference compared to the septic, mixed fungal and non-fungal groups. All enrolled severe septic patients presented with elevated PCT and CRP levels. Clinical data did not differ in mixed fungal and non-fungal septic patients. Also, clinical scores did not show significant change during our study period. The most common organ dysfunctions developing in our patients were: kidney dysfunction (oliguria or anuria), haemodynamic shock, consciousness disorder and respiratory failure. A large portion of bacterial and fungal infections identified in our patients were mixed bacterial or mixed bacterial-fungal infections. Six patients comprised the mixed fungal septic group and 27 patients were in the non-fungal septic group. Microbiological identification proved *C. albicans* species in all six fungal septic patients. Two patients of the mixed fungal septic group and 5 from the non-fungal septic group died during the study period.

Upon admission total annexin V positive MPs and CD41 positive PMPs were elevated in both the mixed fungal and in the non-fungal septic group compared to our volunteer group. Most MPs (above 60% average) were positive for constitutive platelet antigen CD41, therefore recognised as PMPs. While mixed fungal septic patients showed elevated annexin V positive MP number throughout our study with a slight decrease on the 3^{rd} day, the non-fungal septic patients showed constantly decreasing MP numbers

TABLE 4.2: Demographical and illness related data of our first sepsis - microparticle study. Data presented as median and interquartiles. Abbreviations used: S: survivor, NS: non-survivor, PCT: procalcitonin, CRP: C-reactive protein, MODS: Multiple Organ Dysfunction Score, SOFA: Sequential Organ-Failure Assessment, SAPS: Simplified Acute Physiology Score.

	All patients	Non-fungal septic	Mixed fungal septic
Demographic data:			
Number of patients	33	27	6
Age (years)	63 (53 - 72)	65 (54 - 72)	63 (54 - 75)
Outcome (S / NS)	26 / 7	22 / 5	4 / 2
Gender (male / female)	18 / 15	15 / 12	3 / 3
Clinical status:			
PCT (ng/ml, admission)	12.94 (5.11 - 69.805)	12.94 (5.11 - 70.39)	6.93 (4.89 - 65.91)
CRP (mg/l, admission)	200.94 (122.85 - 71.75)	185.77 (123.56 - 270.5)	156.34 (89.03 - 241.5)
Lactate (mmol/l, admission)	1.8 (1.3 - 3.32)	1.76 (1.29 - 2.55)	1.55 (1.12 - 2.04)
MODS (1^{st}, 3^{rd}, 5^{th} day)	8 (5 - 11), 7 (5 - 10), 6 (3 - 8)	7 (4 - 9), 7 (5 - 9), 6 (3 - 8)	7 (5 - 10), 7 (5 - 9), 4 (3 - 8)
SOFA (1^{st}, 3^{rd}, 5^{th} day)	9 (7 - 12), 8 (6 - 13), 7 (5 - 10)	8 (7 - 10), 8 (6 - 11), 7 (5 - 9)	7 (6 - 11), 8 (4 - 12), 5 (4 - 10)
SAPS II	50 (34 - 67)	49 (33 - 67)	50 (32 - 69)
Organ dysfunctions:			
Kidney dysfunction	28	23	5
Respiratory failure	26	22	4
Haemodynamic insuff.	25	20	5
Consciousness disorder	19	14	5
Hepatic disorder	11	8	3
Thrombocytopenia	8	7	1
Proved infections:			
Gram-negative	25		
(mixed)	(16)		
Gram-positive	18		
(mixed)	(17)		
Fungal infections	6		
(mixed)	(6)		

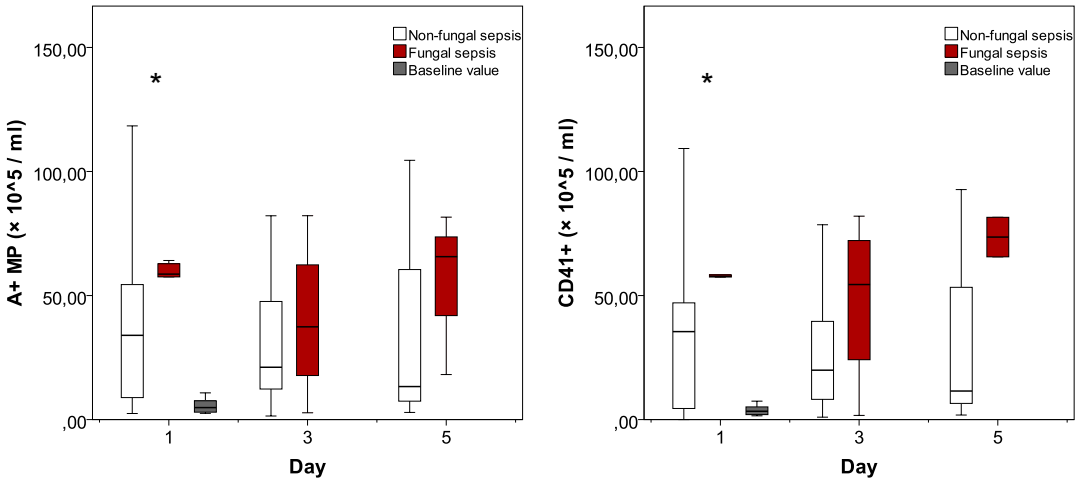

FIGURE 4.5: Annexin and platelet marker positive microparticles in mixed fungal (n=6) and non-fungal (n=27) septic patients. *: $p < 0.05$, comparing septic patient data

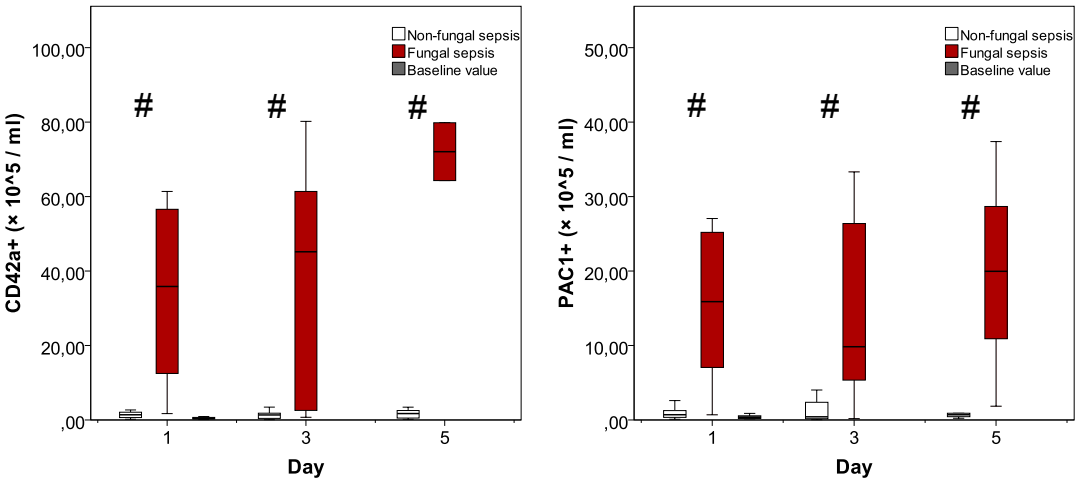

FIGURE 4.6: Activated platelet-derived microparticles in mixed fungal (n=6) and non-fungal (n=27) severe septic patients. #: $p < 0.01$, comparing septic patient data

during our study period. The elevation was significant in mixed fungal compared to non-fungal septic patients on the 1^{st} study day (Figure 4.5 $p<0.05$). The CD41 positive PMP numbers were decreasing until day 5 in non-fungal septic patients and were constantly elevated in the mixed fungal septic group. The elevation was statistically significant on the 1^{st} day (Figure 4.5 $p<0.05$). CD61 results were statistically identical to CD41 results in all measurements (data not shown). While $CD42a^+$ PMP numbers were negligible in the non-fungal group and in controls, mixed fungal septic patients showed significantly elevated numbers in all measurements with a steep elevation until day 5 (Figure 4.6 $p<0.05$). Mixed fungal septic patients presented a wide range of $CD42a^+$ particles.

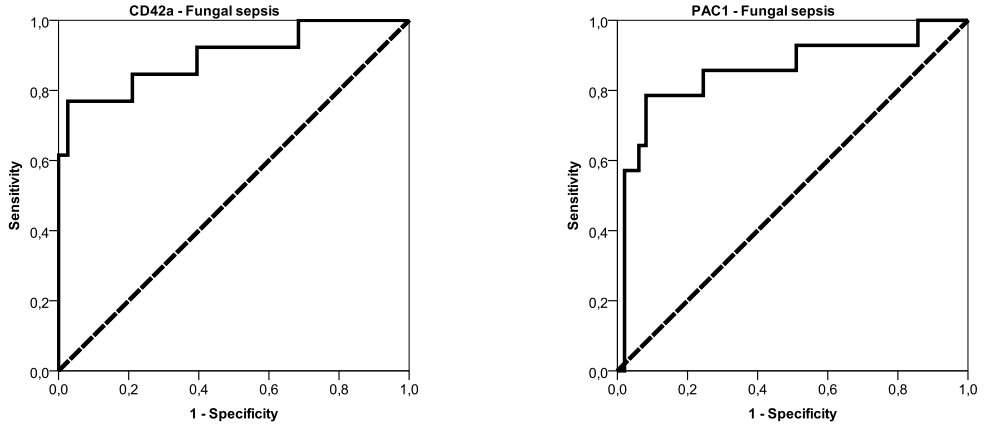

FIGURE 4.7: Receiver operator characteristics analysis of activated platelet markers in severe sepsis. (AUC_{CD42a}=0.857, AUC_{PAC1}=0.897)

Although PAC1+ PMPs numbers showed a slight decrease and marked variety until day 5 in fungal septic patients, this group of patients had significantly elevated numbers of PAC1 positive PMPs in the 1^{st} and 5^{th} study days compared to non-fungal septic patients. Non-fungal patients had low numbers of PAC1 positive PMPs (Figure 4.6 $p<0.05$). Receiver operator characteristic curves (Figure 4.7) calculated on the presence of mixed fungal sepsis in patients using all data of PAC1 and CD42a MP measurements, revealed an area under the curve of 0.857 and 0.897 respectively. The evaluation of different bacterial sources (Gram-negative or Gram-positive bacteria) in the non-fungal septic group revealed no significant differences of PMPs from various bacterial infection origins. Also, platelet counts in different groups (fungal, non-fungal or Gram-negative and Gram-positive) did not differed significantly throughout our study period.

Table 4.3: Demographical and sepsis related parameters of patients from our second microparticle study. Data presented as median and interquartiles

	Septic patients	Controls
Demographic data:		
Total number	37	20
Gender (male / female)	20 / 17	10 / 10
Age	63 (53 - 72)	47 (38 - 73)
7 days mortality (S/NS)	8 / 29	
28 days mortality (S/NS)	17 / 20	
Calculated clinical scores:		
MODS (1^{st}, 3^{rd}, 5^{th} days)	8 (5 - 10), 7 (6 - 10), 6 (4 - 8)	
SOFA (1^{st}, 3^{rd}, 5^{th} days)	9 (7 - 11), 9 (7 - 10), 8 (5 - 9)	
Specific markers on admission:		
PCT (ng/ml)	10.00 (4.45 - 44.98)	
CRP (mg/l)	185.77 (113.14 - 284.62)	
Lactate (mmol/l)	1.90 (1.23 - 2.62)	
D-Dimer (µg/l)	2342 (1043 - 4324)	
No of organ dysfunctions:	4 (3 - 5) in all patients	
Renal insufficiency	30	
Respiratory disorder	29	
Haemodynamic impairment	28	
Hepatic disorder	12	
Thrombocytopenia	14	
Impaired consciousness	21	
No of approved infections:		
Gram-positive	19	
Gram-negative	27	
Fungal infection	7	

4.2.2 Organ dysfunction, outcome and microparticles

After patient data and flow cytometry result reassessment, we included data from 37 severe septic patients in our next study. Although we gathered data from almost 65 patients, based on the exclusion criteria and lack of informed consent or patient data we were unable to include all patients. In this study we focused on sepsis outcome, the presence of various organ dysfunctions and the connection with microparticle profile. Summarised numbers of organ failures on inclusion accompanied by basic demographic and sepsis oriented laboratory data gathered are listed in Table 4.3. To take underlying baseline diseases into account, a new age and gender adapted control group was invited. Most patients suffered from renal disorder besides haemodynamic impairment on inclusion; therefore we focused our data assessment on this organ dysfunction. The control groups age and gender did not differ significantly from the septic patient group. The

measurement of microparticle numbers in severe septic patients compared to controls revealed a significant increase of annexin V and CD41 positive microparticles in severe septic patients during the whole study period (Figure 4.8, $p<0.001$). CD42a positive particles showed a constant elevation in severe septic patients (Figure 4.8, $p<0.001$). We also found elevated PAC1 positive particle numbers in severe sepsis, but a steady decrease is notable from day 1 towards day 5 (Figure 4.8, $p<0.05$). The presence of CD41, CD42a, and PAC1 positive particles showed no correlation with actual platelet numbers. According to our results, survivor and non-survivor patient results, assessing 7 days and 28 days mortality showed no significant difference in annexin V, or specific marker positive MP results (data not shown, $p>0.05$). Our results showed, that actual number of organ failures do not have a direct effect on total annexin V+ (Figure 4.8), $CD13^+$ or $CD14^+$ microparticle count (data not shown). The assessment of the effect of sepsis-related organ failures on microparticle numbers revealed, that patients with acute sepsis-related renal injury on admission have significantly different MP numbers compared to patients without renal impairment. To concentrate our assessment on the new onset renal injury in septic patients, we excluded 4 patients from the assessment of sepsis-related renal injury; as according to patients' history, they suffered from chronic, renal function altering illnesses. Total annexin V positive, as well as $CD41^+$ and $CD13^+$ particle numbers were significantly elevated in patients with renal injury on inclusion. Although patients presented a wide variety of total MP numbers, patients with renal injury showed significantly increased MP numbers on inclusion and a slight decrease of MP numbers on day 3. Patients without renal injury had a continuous elevation of MP numbers during our study period (Figure 4.9, $p<0.05$). Patients without renal injury showed a steady non-significant increase of $CD41^+$ particles, while sepsis-related renal injury patients had elevated CD41+ MP numbers on admission already. Also, CD13 harbouring particles were significantly elevated on admission in patients with renal impairment (Figure 4.9, $p<0.05$) and patients without renal failure showed elevating MP numbers. Summarised data from our study measurements showed, that the presence of $CD42a^+$ particles in patients with acute renal injury correlated negatively with measured BUN and creatinine concentrations (respectively: $p<0.05$, $r=-0.835$, $r=-0.569$). We carried out the same detailed assessment of other severe sepsis-related organ dysfunctions (arterial hypotension despite of fluid therapy, consciousness disorder, respiratory insufficiency, thrombocytopenia/blood marrow insufficiency, liver function), based on clinical assessment and laboratory data. Statistical analysis of these groups

revealed that MP, particularly PMP numbers do not show significant difference in these severe sepsis-related organ dysfunctions in our study setting (data not shown).

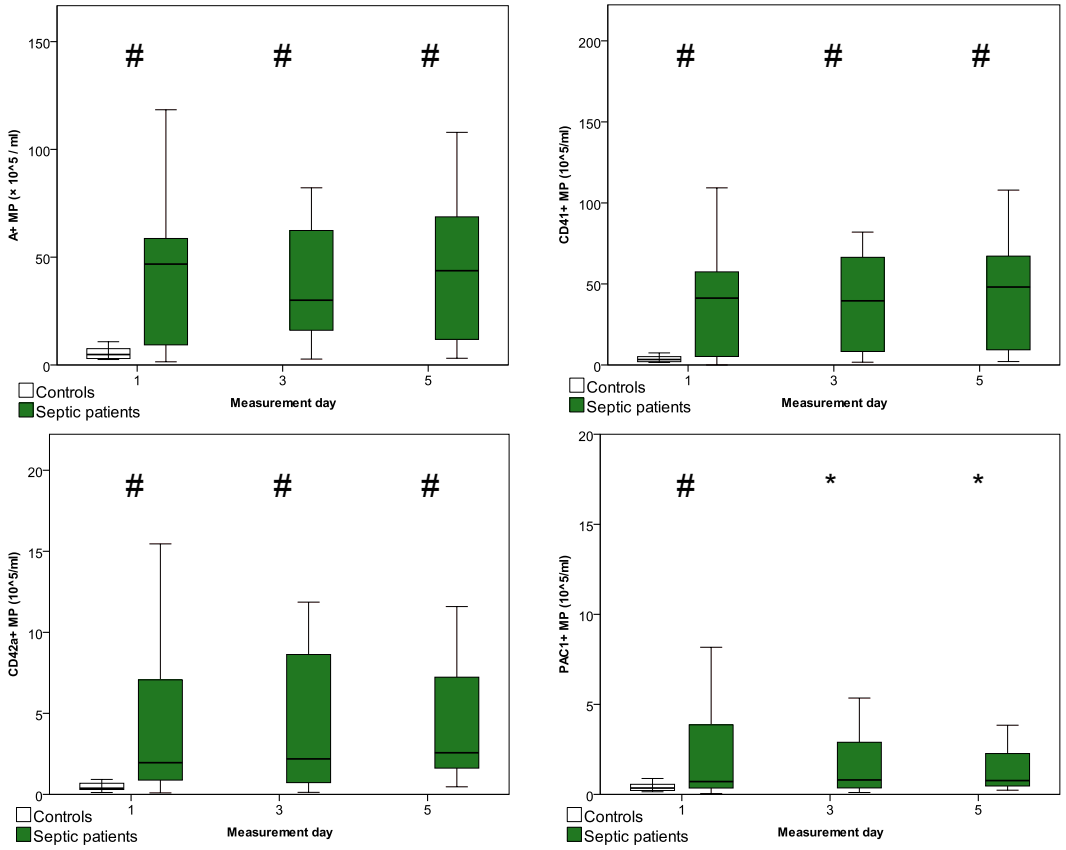

FIGURE 4.8: Microparticle count comparison of severe septic patients (n=37) and controls (n=20). (#: $p<0.01$, *: $p<0.05$)

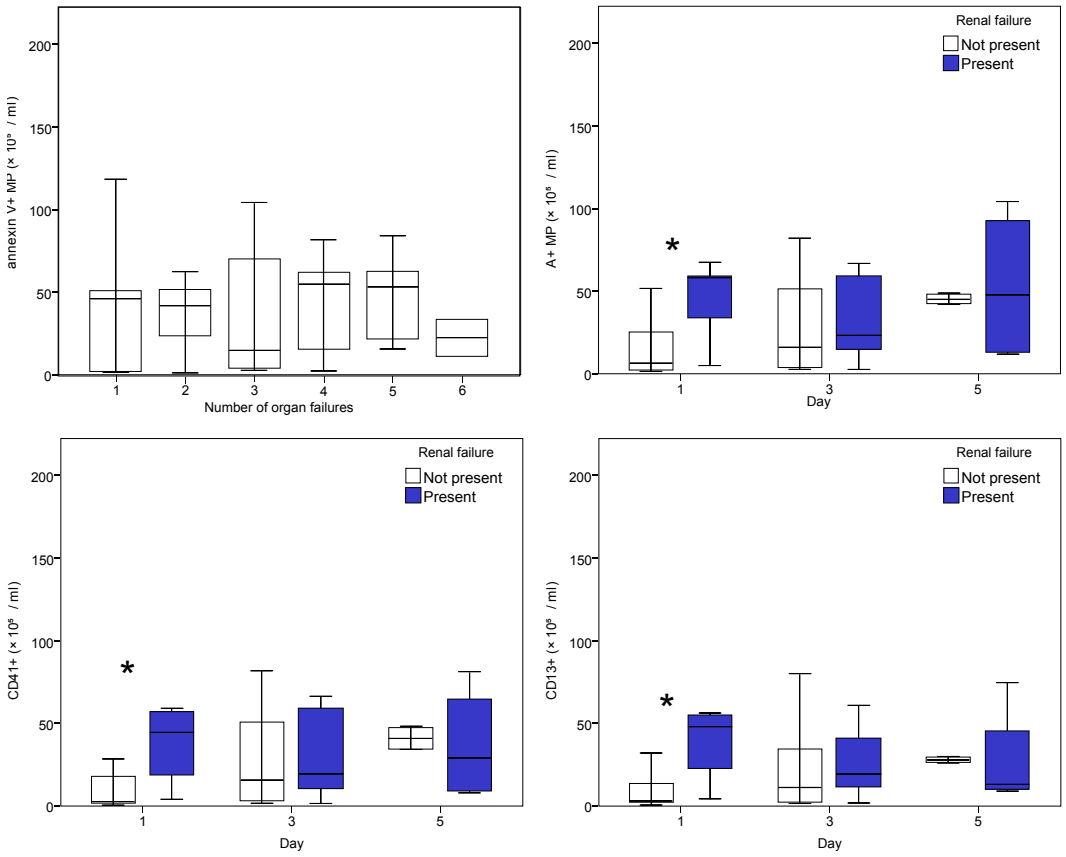

FIGURE 4.9: Microparticle count comparison of severe septic patients with multiple organ failure and presenting or without acute sepsis-related kidney injury. Subfigure 4.9A represents MP numbers and number of organ dysfunctions of severe septic patients. 4.9B-D shows data from patients with or without acute kidney injury. (#: $p<0.01$, *: $p<0.05$)

Discussion

Platelet count disorders and thrombocytopenia have been already observed in 33-45% of septic patients, however the exact mechanism behind the development of sepsis-induced thrombocytopenia requires further investigation [127, 128]. Sigurdsson et al. using radioisotope-labelled autologous platelets provided evidence, that even though macroscopic pathology observation did not provided evidence of intravascular coagulation, non-survivor septic patients had platelet sequestration in the intestinal vascular system [129]. Also, Singer et al. showed platelet recruitment in terminal hepatic venules in a caecal ligation and puncture murine sepsis model [130]. It is commonly accepted, that platelets do not form aggregates in healthy individuals [131], while Eto et al. presented aggregates containing less than 100 platelets in patients with acute coronary syndrome using the high sensibility laser light scattering aggregometry method [132]. Also, patients with acute myocardial infarction have shown increased aggregation levels which correlate with the size of the necrotic tissue [133]. The primary objective of our first study was to evaluate the alterations of inducible aggregation in severely septic patients and determine its usefulness as a predictor of overall mortality. Former platelet function tests utilising flow cytometry presented the loss of platelet function in patients developing multiple organ dysfunction syndrome [134, 135]. Our data shows a significant deterioration in ADR, ADP, and COL inducible aggregation compared to the control group but no change was observed during the 5-day period. Yaguchi et al. showed a loss of inducible platelet aggregation in patients with severe sepsis in contrast with other studies suggesting increased platelet function in trauma based septic shock patients [136, 137]. In concordance with Yaguchi's findings, our study found a similar decrease in aggregation among severe septic patients. Interestingly, flow cytometry measurements showed decreased PAC1 and fibrinogen binding in septic patients, while unchanged CD62P avidity [136]. As resting platelets store CD62P in α-granules, the presence

of CD62P but lack of active α2bβ3 (measured by PAC1) may show unchanged platelet activation, but loss of aggregation function. According to Lundhal et al. more "active" platelets are activated and consumed in the early stage of sepsis therefore platelet function should decrease during critical illness [134]. Yaguchi formerly described, that patients with lower platelet count have more decreased inducible aggregation. Our data support this finding, as patients with low platelet count showed significantly deteriorated aggregation response to inducers. In our data survivor and non-survivor patient groups did not show significantly different inducible or spontaneous aggregation. As platelet sequestration may develop in the circulation of severe septic patients, we think that this result can not disprove or support the theory of Eisen, but prove that this platelet aggregation measurement could not be recommended for mortality prediction. Until now, studies have not reflected the use of saline as an alternative to inductors used for the measurement of spontaneous aggregation in severely septic patients, although saline was used to assess spontaneous aggregation in former studies [138, 139]. Using saline as "inducer" in Born's optical method showed significant aggregate formation in a group of severely septic patients compared to healthy controls. There is inconsistent data on the effect of LPS and Gram-negative bacteria on platelet activation, secretion and aggregation [140–142]. According to our data the presence of Gram-negative bacteria was not the sole cause of spontaneous aggregation, although patients with spontaneous aggregation and Gram-negative infections showed increased aggregation levels. There is a growing evidence of possible beneficial effect of anti-platelet therapy in the early phase severe sepsis. The review of Eisen discuss pathophysiological and pharmaceutical effects of acetylsalicylic acid therapy and conclude that platelet inhibition may be beneficial for patients [143]. Increased PCT levels in the non-spontaneous group hypothesise that members of this group may had a more severe state compared to the spontaneous group. Also, the cross-link between inflammation and haemostasis support the theory of marked platelet activation in severe sepsis [22, 25, 35]. Lactate levels did not differ significantly in the spontaneous groups. Although we are aware of the low sensitivity and specificity of lactate levels, we hypothesise that microcirculation and tissue oxygenisation was not significantly different in both groups [144]. The lack of lactate difference and steady decrease in both groups may rise from appropriate fluid and supportive therapy. A former study on rabbit platelets presented a dose-dependent inhibitory potential of methylprednisolone on inducible aggregation [145]. In patients who received corticosteroids during treatment, we did not find significant variations either in the

inducible platelet aggregation values or in spontaneous aggregation. Homogeneously, all patients received low molecular weight heparin treatment which could also slightly decrease platelet aggregation but we analysed and focused on the changes of the inducible aggregation [146]. Due to ethical concerns, the effect of other recommended and regular treatments (vasopressor and inotropic agents, infusions for fluid replacement, insulin, stress ulcer prophylaxis, analgesics, sedatives, antibiotics etc.) were not investigated in drug free patient subgroups.

Besides haemostasis and particularly aggregation, the role of platelets in early host defence and inflammation is long debated. Yeaman et al. discussed platelet reactions to endothelial injury and for the presence of microbial agents extensively [147]. Bacteria and fungi are known to activate platelets, following activation platelets can act directly by adhering to the endothelial wall or to the pathogen, forming aggregates. Also, antimicrobial proteins are released from platelets. These proteins, like thrombocidins are highly effective against certain bacterial and fungal strains while others successfully developed resistance against them. The increase of platelet activation markers and adhesive platelet markers on PMP surfaces may indicate platelets' contribution in early host defence [148–150]. Besides haemostasis and particularly aggregation, the role of platelets in early host defence and inflammation is long debated. Yeaman et al. discussed platelet reactions to endothelial injury and for the presence of microbial agents extensively [147]. Bacteria and fungi are known to activate platelets, following activation platelets can act directly by adhering to the endothelial wall or to the pathogen, forming aggregates. Also, antimicrobial proteins are released from platelets. These proteins, like thrombocidins are highly effective against certain bacterial and fungal strains while others successfully developed resistance against them. The increase of platelet activation markers and adhesive platelet markers on PMP surfaces may indicate platelets' contribution in early host defence [148–150]. Formerly, there was a notable inconsistency in MP data from severe septic patients. Joop et al. reported the decrease of MPs from various origins (platelet, erythrocyte, endothelial cells, granulocytes) in multiple organ dysfunction and sepsis, compared to healthy controls [151], while in another paper Nieuwland et al., found increased vesicle numbers in meningococcal sepsis [152]. Our data support the model of increased numbers of PMPs compared to volunteers in sepsis. We have shown, that compared to severe bacterial sepsis, severe sepsis with mixed

fungal infection contributes to more increased PMP levels in our multidisciplinary ICU setting. Although, formerly fungal sepsis was considered rare and according to current data fungi contributes only about 5% as a main pathogen of all infections, a current review by Lepak and Andes presented a 2-fold increase of *Candida* incidence from 1979 to 2000 with the estimated incidence of 25-30/100000 persons annually [153]. Most fungal infections are present as a superinfection or develop following proper antibacterial treatment [153]. In addition, a large portion of patients, who suffer from prolonged sepsis characterised by protein wasting, are prone to further infections, which combined further deteriorate overall outcome. Commonly used culturing methods are considered rather slow and not completely reliable, but these approaches support appropriate antimicrobial selection. The assessment of a conventional automated haemoculture system found, that opposed to current guidelines in sepsis (SSC2008, [38]), at least 3 cultures and 24 hours incubation time is necessary to reach an optimal infection detection rate of 95% [154]. According to the review of Annane et al. and Lepak et al., blood cultures may require more than 6 hours, while the detection rate could be as low as 30% [11, 153]. The identification of *Candida* fungal strains may need up to 5 days, while the detection rate may not increase above 67% [154]. Also, a recent study showed that most patients with yeast infections have inappropriate medication, but empirical, pre-emptive use of antifungal therapy is still questionable and not recommended [153]. Adverse effects of anti-fungals may vary from renal failure because of vasoconstriction (conventional amphotericin B) through visual disturbances, phototoxicity or skin rash (voriconazole) and hepatotoxicity (posaconazole, voriconazole). Itraconazole and other azole medications are well tolerated with only rare and mild cases of hepatotoxicity, but widely used fluconazole is prescribed for prophylaxis and treatment for a longer period, deteriorating its effectiveness. Hypersensitivity developed against an azole compound may trigger adverse reactions against other azoles. Also, administration of fluconazole is not recommended in non-albicans *Candida* infections. Besides novel laboratory techniques, multiple risk assessment scores were developed to help in the decision of whether to introduce anti-fungal therapy, but further validation is needed before introduction into wide clinical use [11, 155]. The above mentioned data emphasise the importance of the proper indication of antimicrobial treatment. Considering the above discussed shortcomings of generally used microbiological identification systems and risk assessment scores, we think our MP measurement based $PAC1^+$ and $CD42a^+$ PMP measurements can provide valuable additional information on mixed fungal sepsis

prone patients following the admission to the intensive care unit. According to our current knowledge, there is no evidence that fungal infections or antifungal medications alter platelet or PMP measurement results.

In our second MP study, we aimed to assess MP number and profile changes in severe sepsis and related organ dysfunction, while also aimed to provide further data regarding MP amounts in severe sepsis compared to controls. We found increased numbers of total MPs in severe septic patients compared to controls. Especially, platelet derived MP numbers were elevated throughout our study period. Our results are in concordance with Mostefai et al. who also reported increased MP numbers from septic patients compared to controls [156]. Soriano also found elevated MP numbers in survivor septic patients, but no difference in PMP between patients and controls [107]. Joop et al. as formerly discussed, observed lower MP numbers in septic patients compared to controls [151]. We and others showed significantly elevated annexin V+ MPs in sepsis [107, 156]. These discrepancies may result from differences in control group selection, sample handling and patient inclusion time frame. Differences may also originate from the isolation protocol, as well as different centrifugation protocol or including several washing steps, therefore losing large portion of MPs during sample processing [157]. Our main goal with strict control group assessment was to reduce the possible effects of age and underlying patient history on MP results. We assessed both 7 days and 28 days ICU mortality in our second MP study. Former findings of Soriano et al. showed that MPs harbouring activated endothelial marker (CD62E) was elevated in survivor severe septic patients, as well as PMPs were not significantly elevated in survivors. We also did not found significant difference between survivors and non-survivors during our study period [107]. We report the constant elevation of GPIX (CD42a) positive microparticles, which may contribute to platelet adhesion to damaged vessel walls and collagen, while activated α2bβ3 complex (PAC1 labelled), was decreased during study period. Based on the theory of Lundahl, some platelets have higher sensitivity toward activation, which may result in the early activation and consumption [134]. One may speculate that a group of platelets are activated earlier in sepsis. The consumption of these early-activated platelets may cause the decrease of PAC1 positivity, but constant CD41 and CD42a presence during our study. Another explanation for this change is the loss of antibody epitope, resulting from former fibrinogen binding by α2bβ3. In this case, PMPs may form aggregates,

deteriorating microcirculation. As stated before, a main goal in this study was to determine MP numbers in the presence of organ dysfunctions. We provide evidence that the overall number of organ failures have no measurable effect, neither on the total MP numbers, nor on amounts of various MP subgroups. After the assessment on the effect of various organ failures on MPs, patients with renal dysfunction on study inclusion showed overall MP, PMP and myeloid MP increase. The activation of the innate immune system and infiltration of the kidney by monocytes and macrophages contributes to sepsis-related renal failure [156]. Monocyte-derived microparticles are a main source of blood-born TF and carry high amounts of phosphatidylserine. Elevation of these microparticles can cause increased clot formation and obstructions in kidney vessels. CD13 supports monocyte/endothelial adhesion, therefore increased number of CD13+ particles may aid the trafficking of monocytes, infiltration of the kidneys [158] and local TF concentration increase. Blood flow may deteriorate in infiltrated tissue, increasing tissue hypoxia through local coagulation and cytokine release. Elevated CD42a PMPs are described in active vasculitis based acute kidney failure [159]. Negative correlation between BUN and creatinine concentrations and low levels of circulating CD42a PMPs may indicate attachment of platelets to the damaged renal endothelial surface, promoting vascular dysfunction resulting elevated BUN and creatinine concentrations. In our study most pronounced differences regarding renal dysfunction were observed on inclusion. Patients admitted to the intensive care unit receive extensive fluid therapy and a large variety of medications, which may explain the loss of the on-admission significant differences of various microparticle groups.

5.1 Limitations of presented studies

Due to the relatively small sample size, clinically our investigations should only be viewed as a pilot studies. All patients included in the mixed fungal septic group have suffered from *C. albicans* infection, therefore further (P)MP measurements are needed to assess our data on *Candida* strains different from albicans and non-*Candida* fungal infections to clarify the importance of (P)MPs as an early marker for mixed fungal sepsis.

5.2 Conclusions

Our hypothesis about the key role of highly inducible platelet aggregation in the development of microvascular flow insufficiency and patient survival could not be completely confirmed. Concerning aggregability we found no significant difference between survival and non-survival patients therefore we cannot recommend this test as a mortality predictor. Our results provided further evidence that platelet aggregation in severe sepsis can be altered by the actual platelet count. Our main finding in the platelet aggregation study is spontaneous aggregation in severe septic patients. The development of spontaneous aggregation may cause local microcirculatory disturbances therefore further investigations in the field are required.

Our data revealed that measurement of PAC1 and CD42a positive PMPs can provide useful and early information in the assessment for mixed *C. albicans* fungal sepsis, thus can improve treatment decisions during the care of severe septic patients. We plan further studies to confirm the clinical usefulness and biological background of similar PMP alterations in severe septic and multiple organ failure patients.

Although, according to our data MP numbers show no difference in survivor and non-survivor patients, our results support the model, that MPs contribute with increased procoagulant phospholipid surface, TF and monocyte sequestration in patients admitted to the ICU with severe sepsis and acute sepsis-related renal disorder. These patients showed negative correlation with serum BUN and creatinine concentrations and CD42a+ platelet-derived microparticles, suggesting the role of these particles in the development of renal failure. Although we have no firm causality between MP numbers and renal dysfunction, we would like to emphasise the importance of MPs in the pathomechanism of sepsis-related renal failure.

Novel findings

6.1 Novel findings of the platelet aggregation study

- Inducible platelet aggregation measurements cannot be recommended for severe sepsis mortality prediction.
- This study was the first to provide evidence on the presence of increased spontaneous platelet aggregation in severe septic patients.
- Our results provided further evidence on the direct proportion of platelet count and inducible platelet aggregation results.

6.2 Novel findings of the microparticle studies

- We provided further evidence on increased MP levels in severe sepsis.
- Our clinical study was the first which showed the MP profile difference in patients with severe sepsis complicated with fungal (*C. albicans*) infection. This finding could help the development of a future early diagnostic test based on MPs.
- Our novel approach revealed that there is no direct connection between the number of organ failures and MP numbers.
- Data from our second study support the contribution of MPs in the development of sepsis-related acute kidney injury.

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List of publications

Publications

The thesis is based on the following publications

- **G Woth**, A Varga, S Ghosh, M Krupp, T Kiss, L Bogár, D Mühl, Platelet aggregation in severe sepsis., J Thromb Thrombolysis. 2011;31(1):6-12. IF: **1.476**.
- **G Woth**, A Varga, M Krupp, K Jáksó, L Bogár, D Mühl., Thrombocytaaggregációs vizsgálatok súlyos szepszisben. Aneszteziol Intenz Ter. 2011;41(3):126-131. (article in hungarian) IF: **-**.
- **G Woth***, M Tőkés-Füzesi*, T Magyarlaki, GL Kovács, I Vermes, D Mühl., Activated platelet-derived microparticle numbers are elevated in patients with severe fungal (Candida albicans) sepsis., Ann Clin Biochem. 2012:49:554-560 (* marks equal contribution) total IF: 2.170, IF based on contribution (50%): **1.085**.
- **G Woth***, M Tőkés-Füzesi*, T Magyarlaki, GL Kovács, I Vermes, D Mühl., Microparticles and acute renal dysfunction in septic patients., J Crit Care. 2012 (in press, * marks equal contribution) total IF: 2.134, IF based on contribution (50%): **1.067**.

Summarised impact factor of publications discussed in this thesis, based on author contribution: **3.628**
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- D Mühl, **G Woth**, L Drenkovics, A Varga, S Ghosh, C Csontos, L Bogár, G Wéber, J Lantos., Comparison of oxidative stress & leukocyte activation in patients with severe sepsis & burn injury., Indian J Med Res. 2011;134:69-78. IF: **1.837**.
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Summarized impact factor of journal manuscripts: **3.971**
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Presentation and poster abstracts

Presentations and posters discussed in the thesis

- A Varga, **G Woth**., Súlyos szepszis és a thrombocyta aggregáció (Prospektív analízis), University of Pécs, Annual Student Researchers Conference, 2009.
- A Varga, **G Woth**, L Bogár, D Mühl., Súlyos sepsis és a thrombocyta aggregáció (prospektív analízis). Aneszteziol Intenz Ter. 2009;39:(Suppl. 1.):18.
- **G Woth**., Mikropartikulumok és a súlyos szepszis, University of Pécs, Annual Student Researchers Conference, 2010.
- **G Woth**, M Tőkés-Füzesi, T Magyarlaki, D Mühl., Microparticles in severe sepsis, Annual congress of the Hungarian Medical Association of America, 2010.
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- **G Woth**, M Tőkés-Füzesi, B Ernyey, I Vermes, D Mühl, L Bogár, GL Kovács., Microparticles in severe sepsis related organ failures. 56th annual congress of the Hungarian Society of Laboratory Medicine, 2012, Budapest.

Other presentations and posters

- Zs Tucsek, B Radnai, B Veres, T Dolowschiák, **G Woth**, J Pribér, M Schoenberg, F Gallyas jr., A ferulaldehid hatása LPS-indukálta gyulladásos folyamatokra egérben. 37. Membrán-Transzport Konferencia, Sümeg, 2007.

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- **G Woth**, A Varga, L Drenkovics., Oxidatív stressz súlyos szepszisben, prospektív analízis, University of Pécs, Annual Student Researchers Conference, 2009.

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- T Kőszegi, P Kustán, A Ludány, E. Györgyi, **G Woth**, D Mühl, GL Kovács. Urinary orosomucoid in sepsis. 56th annual congress of the Hungarian Society of Laboratory Medicine, 2012, Budapest.

- T Kőszegi, Z Horváth-Szalai, A Ludány, E Györgyi, **G Woth**, D Mühl, GL Kovács. Serum actin/gelsolin ratio: new biomarker in sepsis? 56th annual congress of the Hungarian Society of Laboratory Medicine, 2012, Budapest.

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