Platelet and platelet-derived microparticle studies in severe sepsis

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List of abbreviations

ADP: Adenosine-diphosphate, **ADR:** Adrenaline, **BUN:** Blood urea nitrogen, **COL:** Collagen, **GP:** Glycoprotein, **IL:** Interleukin, **MP:** Microparticle, **PCT:** Procalcitonin, **PMP:** Platelet-derived microparticle, **PS:** Phosphatidylserine, **SAL:** Saline, 0.9% NaCl solution, **SIRS:** Systemic Inflammatory Response Syndrome, **SSC2008:** Surviving sepsis Campaign 2008, **TF:** Tissue factor, **TLR:** Toll-like receptor, **TNF-α:** Tumor necrosis factor-alpha, **VWF:** von Willebrand factor

Introduction

Although the signs and symptoms of inflammation and sepsis are widely available in historical records, the exact definition of Systemic Inflammatory Response (SIRS) and sepsis were only created in 1991. At least two of the following diagnostic criteria are necessary for the diagnosis of SIRS: hypothermia or hyperthermia (<36°C or >38°C), increased heart rate (>90/min), hyperventilation (respiratory rate >20/min, arterial carbon dioxide pressure < 32 mmHg), white blood cell count > 12000 cell/ul or < 4000 cell/ul. 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.

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. 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, or may develop as new compounds during cellular damage. Leukocytes activated through toll like receptors (TLRs) release pro-inflammatory cytokines, including interleukin-1β (IL), 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. Also, complement system proteins C3a and C5a anaphylatoxin release from mast cells and basophils increase vascular permeability and smooth muscle contraction.

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.

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.

In severe sepsis, the classical components of Virchow's triad (increased coagulablity, endothelial injury and impaired blood flow) for altered haemostasis are all present (Remick 2007). 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.

Platelet activation and aggregation: 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 GPIb-V-IX and GPIIb-IIIa. While former is involved in platelet shear-stress based activation and in the binding of von Willebrand factor (VWF) and collagen, the latter is responsible for fibrinogen binding of activated platelets and in the formation of platelet aggregates. 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 α2β1 and GPVI, increasing platelet Ca^{2+} through a phospholipase Cγ2 mediated way, while VWF activates through GPIbα and α2bβ3 receptors. The initial binding cause a smaller Ca^{2+} peak in platelets, resulting in the release of adenosine diphosphate (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 α2bβ3, an increased Ca^{2+} surge precedes platelet aggregation.

The formation and role of microparticles (MPs): The first description of microparticles 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. During cell activation, apoptosis or increased shear stress the presence of phosphatidylserine (PS) in the outer leaflet is one of the first signs of this process. 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. Outer membrane composition of MPs in the circulation of mainly platelet origin consists of cholesterol (about 60%), sphingomyelin, phosphatidylethanolamine and PS. 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 are 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, platelet derived MPs (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. As reported by del Conde et al., monocyte/macrophage derived vesicles, are able to fuse with activated platelets, through a PS and P-selectin 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. 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. 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. 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.

Aims

Platelet aggregation in severe sepsis:

1, We aimed to evaluate the platelet aggregation alteration characteristics in severe sepsis.

2, We tried to assess the connection between reduced platelet count and measurable platelet function in severe sepsis.

3, We also aimed to assess the role of spontaneous platelet aggregation in severe septic patients.

Microparticle studies in severe sepsis:

1, We assessed the effect of different infectious agents on microparticle characteristics.

2, We aimed to provide further data regarding elevated microparticle levels in septic patients.

3, We evaluated the connection between developed sepsis-related organ failures and microparticle levels.

Methods

Our patient inclusion criteria were recently discovered severe sepsis (within 24 hours) with two or more sepsis-related organ dysfunctions. Criteria for sepsis included the abovementioned sepsis criteria and C-reactive protein levels above 10mg/l and procalcitonin (PCT) levels above 2 ng/ml, and 5 ng/ml in the fungal MP study. In our studies patients in moribund state or with any kind of haematological baseline disease such as myeloproliferative disorders like lymphoma or leukaemia, cytostatic treatment in the last 30 days, high dose prolonged steroid medication, patients with disseminated intravascular coagulation score >5, drugs known to alter platelet functions (i.e. acetylsalicylic acid), platelet transfusion during the study period were excluded. For the platelet aggregation study 3 × 2.7 ml Na_3-citrate anticoagulated blood was gathered from our patients, for the MP studies 2 × 2.7 ml Na_3-citrated blood was gathered. Platelet tests were carried out using the Carat TX4 light transmission aggregometer using adrenaline (ADR, 10 µmol), ADP (10 µmol), collagen (COL, 2 µg/ml) and normal saline (SAL) as inductors. Microparticles were isolated by multiple centrifugation steps, including a 10 minutes 18000 g step to pellet microparticles from platelet-free plasma. Particles surface PS was labelled by annexin V, cell-line specific antigens were labelled by fluorescent monoclonal antibodies. Constitutively expressed platelet fibrinogen receptor subunits, GPIIb-IIIa were measured by the CD41 and CD61 antibodies respectively. Activated platelet marker, fibrinogen binding form of α2bβ3 was assessed by the PAC1 antibody. Platelet adhesion receptor GPIb- V-IX has been tested with anti-IX (CD42a) labelling. Flow cytometry measurements and data analysis were performed on our FC 500 flow cytometer with CXP software. 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. For thrombocytopenia we utilised the criteria of the Surviving Sepsis Campaign 2008 (SSC2008) of 100000 cells/µl. For microbiological assessment we used standard blood cultures. Acute sepsis-related kidney injury was defined by the Injury category of the RIFLE criteria (serum creatinine normal × 2, >50% deteriorated filtration rate, or urine production <0.5ml/bwkg/h).

Results

Platelet aggregation study: Forty-five patients were included in our platelet aggregation study. The evaluation of platelet aggregation in septic patients compared to healthy controls (n=30) revealed a significant deterioration in the inducible aggregation among septic patients ($p<0.05$), while SAL based aggregation showed a significant increase in the platelet function of septic patients ($p<0.001$). 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 of SSC2008. 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 ($p<0.05$). ADR caused aggregations were lower in the 2nd, 3rd, 4th and 5th consecutive day ($p<0.05$) in the low platelet count group. COL induced aggregation was significantly lower on the 1st, 2nd, 3rd days following admission ($p<0.05$). There was no difference between the two groups based on the SAL aggregation. 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 3rd, 4th, 5th days and ADP induced aggregation on the 5th day ($p<0.05$). The low platelet group had significantly deteriorated aggregation levels with all inductors in all cases ($p<0.001$) 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 ($p>0.05$). All induced and saline aggregation measurement results, when compared to survivors were not significantly different in non-survivors (Figure 4.3). The spontaneous 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 1st, 3rd, 4th 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.

Microparticles in sepsis: 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). 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 showed non-significant difference compared to the septic, mixed fungal and non-fungal groups. 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. 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^+ MPs and $CD41^+$ 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^+ MP number throughout our study with a slight decrease on the 3rd day, the non-fungal septic patients showed constantly decreasing MP numbers during our study period. The elevation was significant in mixed fungal compared to non-fungal septic patients on the 1st study day ($p<0.05$). 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 1st day ($p<0.05$). CD61 results were statistically identical to CD41 results in all measurements. 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 ($p<0.05$). Mixed fungal septic patients presented a wide range of $CD42a^+$ particles. 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 1st and 5th study days compared to non-fungal septic patients. Non-fungal patients had low numbers of PAC1 positive PMPs ($p<0.05$). Receiver operator characteristic curves 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 resulted in 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 differ significantly throughout our study period.

After patient data and flow cytometry result reassessment, we included data from 37 severe septic patients from the 65 known cases in our next study. In this study we focused on sepsis outcome, the presence of various organ dysfunctions and the connection with microparticle profile. 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 ($p<0.001$). CD42a positive particles showed a constant elevation in severe septic patients ($p<0.001$). We also found elevated PAC1 positive particle numbers in severe sepsis, but a steady decrease is notable from day 1 towards day 5 ($p<0.05$). 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 ($p>0.05$).

Our results showed, that actual number of organ failures do not have a direct effect on total annexin V^+, $CD13^+$ or $CD14^+$ microparticle count. 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 ($p<0.05$). 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 ($p<0.05$) 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 blood urea nitrogen 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.

Discussion

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. 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. In concordance with Yaguchi's findings, our study found a similar decrease in aggregation among severe septic patients. 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.
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 cannot 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. Using saline as "inducer" in Born's optical method showed significant aggregate formation in a group of severely septic patients compared to healthy controls. 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. 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. The lack of lactate difference and steady decrease in both groups may rise from appropriate fluid and supportive therapy.

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 (Yeaman 2010). 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.

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, while in another paper Nieuwland et al., found increased vesicle numbers in meningococcal sepsis. 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. 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.

The results of our results are in concordance with Mostefai et al. who also reported increased MP numbers from septic patients compared to controls. Soriano also found elevated MP numbers in survivor septic patients, but no difference in PMP between patients and controls. 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. 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 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. 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.

Novel findings

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

Novel findings of the microparticle studies:
1, We provided further evidence on increased MP levels in severe sepsis.
2, 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.

3, Our novel approach revealed that there is no direct connection between the number of organ failures and MP numbers.
4, Data from our second study support the contribution of MPs in the development of sepsis-related acute kidney injury.