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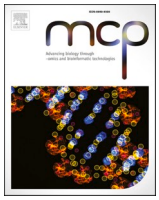

Decrease of exosomal miR-21-5p and the increase of CD62p+ exosomes are associated with the development of sepsis in polytraumatized patients

Birte Weber [a,*], Dirk Henrich [a], Ingo Marzi [a], Liudmila Leppik [a]

[a] Goethe-University, University Hospital Frankfurt, Department of Trauma-, Hand- and Reconstructive Surgery, Frankfurt am Main, Germany

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ABSTRACT

Sepsis as a severe systemic inflammation leads oftentimes to organ dysfunction and subsequently to death. In polytrauma patients, septic complications represent with 45% the predominant cause of late death and are responsible for extremely high costs in the healthcare system. Therefore, clinicians have to detect as early as possible the begin of sepsis to improve the patient's outcome. One new promising diagnostic tool to diagnose septic complications in polytraumatized patients are exosomes.

Plasma samples from polytraumatized patients (Injury Severity Score (ISS) ≥16) which developed sepsis (n = 10) and without sepsis (n = 10), were collected at emergency room (ER), 24h and 5 days after trauma. The EVs subpopulations were investigated by a bead-based multiplex flow cytometry measurement of surface epitopes and were compared with plasma EVs from healthy controls (n = 10). Moreover, exosomal cytokine concentrations were measured via high-sensitive ELISA and were correlated with systemic concentrations. For miRNA cargo analysis, we analysed the miRNAs miR-1298-5p, miR-1262, miR-125b-5p, miR-92a-3p, miR-93-5p, miR-155-5p and miR-21-5p and compared their exosomal concentrations by means of RT-qPCR.

CD62p + exosomes were significantly increased in septic polytrauma-patients ($p \leq 0.05$), while CD40+exosomes, as well as CD49e + exosomes were diminished ($p \leq 0.05$). Furthermore, we observed that the exosomal IL-6 concentration reflects the systemic IL-6 concentration ($r^2 = 0.63$) and did not significantly alter between patients with and without sepsis. The exosomal IL-10 concentration seemed to be constant in all patients and healthy controls. We observed that a decrease of miR-21-5p in exosomes was associated with the development of sepsis ($p \leq 0.05$), while exosomal miR-93-5p, miR-155-5p and miR-92a-3p were not specifically altered in septic patients.

Taken together, the present study in polytraumatized patients demonstrated that the development of sepsis is associated with an increase of CD62p + exosomes. Furthermore, the exosomal cargo was changed in septic patients: miR-21-5p was diminished.

1. Introduction

Sepsis is a life-threatening organ dysfunction, induced by a dysregulation of the host response to infection, which is a major public health concern, responsible for costs of >$20 billion of the total US hospital costs [1–3]. In patients with polytrauma, septic complications are the main cause of late death, accounting for 45% of cases [4]. To enhance the patient's survival and mortality, clinicians must be able to distinguish as early as possible between the inflammatory response to trauma and the start of a sepsis. In recent years, the wide spectrum of extracellular vesicles (EVs) gained importance in studies focussing on sepsis and the early diagnosis of septic deterioration. The role of EVs from different types of cells is described as diverse and crucial. The research on sepsis discusses both the induction of sepsis by EVs as well as the therapeutic benefits of EVs derived from MSCs [5]. Exosomes (small extracellular vesicles) are defined as the smallest population of EVs (with a size between 150 and 300 nm), which are released from healthy cells through endosomal route [6–8]. Exosomes allow the selective transport of proteins, nucleic acids, lipids and small molecules while protecting them from enzymatic degradation by the environment and facilitating their intercellular uptake [9]. The relationship between exosomes and sepsis was initially demonstrated in a pig model of

* Corresponding author. Department of Trauma-, Hand- and Reconstructive Surgery, University Hospital Frankfurt, Goethe-University, Theodor-Stern Kai 7, 60596, Frankfurt am Main, Germany.
E-mail address: bi.weber@med.uni-frankfurt.de (B. Weber).

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endotoxemia-induced sepsis in 1998 [10]. Since then, the number of studies focused on exosomes as markers and mediators of sepsis increased steadily. Nevertheless, studies in septic patients are rare. In septic shock patients, leukocyte- and platelet-derived microparticles were shown to be significantly increased in patients requiring longer vasoactive support and mechanical ventilation [11]. The presence of leukocyte-derived EVs and low level of endothelial cells-released EVs were correlated with a poor prognosis and development of septic coagulopathy in those patients [12]. Next to the cellular origin of exosomes in sepsis, little is known about the exosomal cargo and the information which might be delivered by exosomal miRNAs or cytokines. Therefore, the aim of the present study is to investigate whether sepsis development is reflected by the surface markers and cargo content (especially exosomal miRNAs and cytokines) of plasma exosomes in polytrauma patients.

2. Material and methods

Study design: All analyses were performed with ethical approval given by the Local Ethics Committee of the University of Frankfurt (approval ID 89/19). In the present study plasma exosomes from multiple injured patients (PT) (Injury Severity Score (ISS)≥16, n = 10) were compared with plasma exosomes from polytraumatized patients, which developed sepsis (ISS≥16, n = 10) and healthy individuals (n = 10), The polytraumatized patients of both groups were ISS-matched (Mean ISS of both groups 22.7). In this study, sepsis was defined according to the third consensus definition as life-threatening organ dysfunction based on a blood stream infection. Inclusion criteria were: sepsis as diagnosis in the medical record; detection of bacteria in the blood and dysfunction of at least one organ (for example kidney or lung) [2,13]. The study included traumatized patients admitted from 2016 to 2020 to a German Level 1 Trauma Centre. Blood samples were taken at admission in the emergency room (ER), 1d and 5d after admission in the hospital. They were immediately kept on ice and plasma was gained by 15 min centrifugation at 3500*g* and 4 °C. The healthy plasma samples were further processed in the same way as patients' samples. Based on the patient's electronical record, outcome parameters (time in the hospital, ventilation time, time on ICU/IMC, blood transfusion, need for catecholamines etc.) are collected and summarized in Table 1.

Isolation and characterisation of exosomes: Exosomes were isolated from 100 μl polytraumatized patients- or healthy controls plasma by size exclusion chromatography (Exo-Spin TM, Exosome Size Exclusion Column, cell guidance systems, Cambridge, UK). Prior further investigations, characterization of the exosomes was conducted by nanoparticle tracking analysis (NTA) and Western blot analysis as described before [14]. EV protein concentration was measured by Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific, Rockford, IL, USA).

MACSPlex exosome analysis: 37 exosomal surface epitopes were quantified with the MACSPlex Exosome kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The exosomes (20 μg protein) from 10 samples of polytrauma patients, 10 samples of healthy control groups and 10 samples of polytraumatized patients developing sepsis were incubated with surface epitope-specific antibodies coupled with fluorescent-labelled beads according to the manufactory instruction and analysed by flow cytometry analysis. For each group, APC-A values were acquired with BD FACSCanto II cytometer (BD biosciences, Heidelberg, Germany) and FACS DIVA software (BD biosciences, Heidelberg, Germany) was used to analyse the data. For each sample the median signal intensity of the signals detected for the CD9, CD63 and CD81 capture beads were calculated and their geometrical mean was used as the normalization factor for each sample according to the manufacturer's protocol.

Systemic and exosomal Cytokine ELISAs: The systemic plasma concentrations of the cytokines IL-6 and IL-10 were detected at ER, 1 day and 5 days after trauma by using Quantikine ELISAs (Human IL-6: #D6050; Human IL-10: D1000B: #DTA00D, R&D Systems, Minneapolis, USA) according to the manufacturers' introductions. For exosomal cytokine ELISAs, the isolated EVs were lysed with M-PER® Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Massachusetts, USA) with Proteinase inhibitor Halt ™ (Thermo Fisher Scientific, Massachusetts, USA). Next, the exosomal cytokine concentrations were detected at the same time points by Quantikine high-sensitive ELISA (Human IL6: #HS600C; Human IL10: #HS100C, R&D Systems, Minneapolis, USA) following the manufactory's introductions.

miRNA Analysis: Isolation of miRNA was performed with 150 μL of exosomes isolated from plasma using the miRNeasy serum/plasma kit (Qiagen Inc., Hilden, Germany). For quality control of the RNA isolation and cDNA synthesis steps, *spike-in* control cel-miR-39 (miRNA spike-In Kit, for RT, Qiagen Inc., Germany) was added. Reverse transcription (RT) was performed with miRCURY LNA RT Kit (Qiagen Inc., Hilden, Germany). qPCR amplifications were done according to manufactory's instructions using miRCURY SYBR® Green PCR Kit (Qiagen Inc., Hilden, NRW, Germany). Real-time PCR was performed using a CFX96 Touch Real Time PCR Detection System (BioRad, Puchheim, Germany) with cycling conditions of 1 cycle with 3 min of 95 °C, 40 cycles with 10 s of 95 °C, 50 s of 56 °C followed by melting curve analysis. We decided to

Table 1
Clinical data.

	Healthy	Polytrauma			PT + Sepsis		
		ER	1d	5d	ER	1d	5d
Sex (male/female in %)	30/70	70/30			60/40		
ISS	0	22.7 ± 8.4			22.7 ± 8.4		
PT mechanism	0	10% work accidents 40% fall from great hight 50% traffic accidents			10% gunshot wound 30% traffic accident 60% fall from great hight		
Existing Comorbidities	0	50%			70%		
Infection					pneumonia (50%) urogenital infection (30%) intestinal perforation (20%)		
Leucocytes [/nl]	9.92–9.81	11.9 ± 3.1	9.3 ± 2.1	7.0 ± 1.8	16.0 ± 5.0	17.2 ± 7.3	11.0 ± 5.3
CRP [mg/dl]	<0.50	0.1 ± 0.1	2.9 ± 2.4	1.6 ± 1.3	1.3 ± 1.7	9.9 ± 5.0	13.6 ± 4.7
IL-6 [pg/ml]	0	167.7 ± 147.2	65.6 ± 54.1	21.3 ± 24.6	166.5 ± 238.5	11.3 ± 98.0	60.6 ± 40.6
Exosomal IL-6 [pg/ml]	0	0.5 ± 0.7	0.1 ± 0.1	0.1 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
IL-10 [pg/ml]	0	223.0 ± 252.3	8.0 ± 15.0	0.8 ± 0.9	91.5 ± 81.9	14.5 ± 19.4	4.7 ± 3.9
Exosomal IL-10 [pg/ml]	0.5 ± 0.9	0.3 ± 0.3	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.4 ± 0.6
Initial lactate [mg/dl]	4.5–14.5	23.4 ± 9.6			22.0 ± 11.0		
Death [%]	0	10			0		
Time on ICU [days]	0	8.6 ± 5.0			20.4 ± 14.2		
Ventilation time [days]	0	1.0 ± 2.2			11.7 ± 8.7		
Total time in hospital [days]	0	21.0 ± 9.9			28.7 ± 17.2		
Need of catecholamines [%]	0	30			70		

analyse the following miRNAs based on a detailed literature research. The following commercially available primers were used: cel-miR-39-3p; hsa-miR-93-5p; hsa-miR-92a-3p; hsa-miR-21-5p has-miR-155-5p (miRCURY LNA™ miRNA PCR Assay). For relative quantification of target miRNA, the delta Ct method ($2^{-\Delta Ct}$) was used. Normalization was conducted by using the Ct values of miR39. Furthermore, the sample volume for miRNA isolation was standardized.

Statistical analysis: All statistical analysis were conducted with Graph Pad-Prism 9 (Dotmatic, San Diego, CA, USA). The values are expressed as mean ± standard error of the mean (SEM). Whether the data follow a normal distribution was tested by using the Kolmogoroff-Smirnow-Test. Normally distributed data were analysed by means of one-way ANOVA followed by Dunnettes multiple comparison test. For statistical analysis of two groups, a T-test was applied. For the correlation analysis, linear correlation (r) was assessed with the Pearson test. Results were considered statistically significant when $p \leq 0.05$ and were marked with a '*'.

3. Results

Patients collective: The present study was conducted in 20 polytraumatized patients (Mean ISS 22.7 ± 8.4, Table 1) divided in two groups based on the sepsis development (each n = 10). The first group are 10 patients with no sepsis (PT). The second group are the patients, which developed sepsis during the time in hospital (PT + sepsis), either due to pneumonia (50%), urogenital infection (30%) or intestinal perforation (20%). Both groups (PT and PT + sepsis) were compared with samples from healthy volunteers (Healthy, n = 10).

Distribution of cell-specific epitopes in plasma exosomes: In order to verify, if sepsis development affect the plasma exosomes' profile, the exosomal surface markers were analysed with MACSPlex approach. The ratios of specific exosomal subpopulations and their change over the time were graphically demonstrated in Fig. 1. We discovered that all investigated populations of exosomes could be found in all groups. CD42a + exosomes constituting the largest population, with a count of 22.2 % (PT 24hr) up to 33.11% (PT + sepsis 5d). Another large population of exosomes were CD41b+ (10.4% in PT 5d and 16.21 % in PT ER) and $CD29^+$ exosomes (9.6% in PT SR and 15.1% in healthy controls). Also prominently presented were the $CD31^+$, $CD40^+$ and CD49e + exosomes, which account for ≈ 2–6% of all measured plasma exosomes. These major populations of exosomes are of platelet origin.

Differential expression of exosomal surface proteins: More detailed analysis showed, that relative to healthy controls, $CD44^+$ exosomes were significantly decreased in polytrauma patients (1 and 5 days after trauma), but not in septic patients (Fig. 2A). At the same time, $CD209^+$ exosomes were significantly increased in polytraumatized patients (days 1 and 5), and in PT-patients + sepsis (ER) (Fig. 2B). At day 1 after polytrauma, the ratio of CD146+ exosomes were significantly higher in PT patients (Fig. 2C) as in controls. Some of the investigated subpopulations were specifically altered in PT-patients + sepsis at ER: CD62p + exosomes were significantly increased (Fig. 2D), and $CD40^+$ exosomes (Fig. 2E) and CD49e + exosomes (Fig. 2F) were significantly decreased.

Exosomal and systemic IL-6 and IL-10 concentrations: Next to the subgroups of exosomes, we evaluated the difference in cargo content (cytokines and miRNAs) of plasma exosomes isolated from both groups of patients and healthy controls. For cytokine analysis we focused on IL-6 and IL-10 cytokines, which are commonly used as systemic inflammation-response markers in polytrauma patients. As compared to healthy controls, exosomal IL-6 was significantly increased only in patients with sepsis at ER (Fig. 3A), while systemically IL-6 was significantly increased in both groups of polytrauma patients at this time point (Fig. 3 B) and in PT-patients with sepsis 24h after trauma. Exosomal IL-10 concentrations does not show any significant difference among the groups at any time point, whereas systemically IL-10 was highly upregulated in polytrauma patients at ER (Fig. 3C and D). Correlation analysis identified a moderate correlation ($r^2 = 0.63$, $p < 0.0001$) between the systemic and the exosomal IL-6 concentrations (Fig. 3E), while systemic and exosomal IL-10 did not correlate ($r^2 = 0.18$, $p = 0.1$; Fig. 3F).

Exosomal miRNAs: For miRNA cargo analysis, we decided to analyse miRNAs miR-1298-5p, miR-1262, miR-125b-5p, miR-92a-3p, miR-93-5p, miR-155-5p and miR-21-5p based on literature research and compared their exosomal concentrations by means of RT-qPCR. No

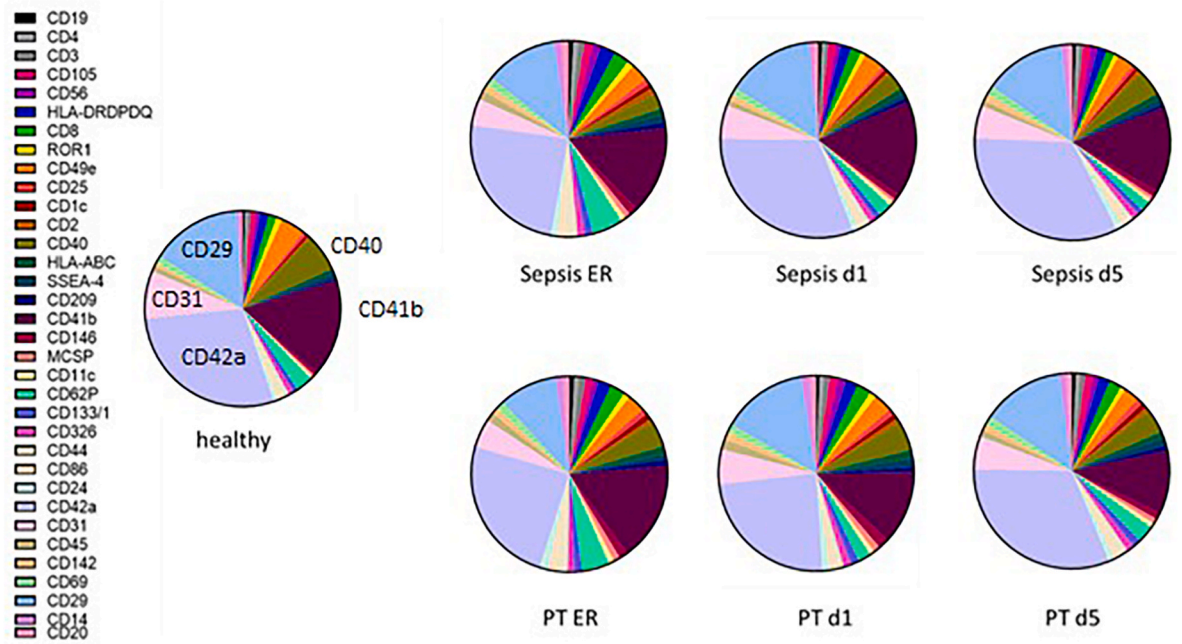

Fig. 1. Pie charts showing the distribution of cell-specific epitopes in plasma exosomes isolated from healthy controls and patients at different time points after trauma. Polytrauma patients (PT) n = 10; Polytrauma patients with sepsis (PT + sepsis) n = 10; healthy volunteers n = 10. Emergency room (ER), day 1 (d1) and day 5 (d5) time points of analysis.

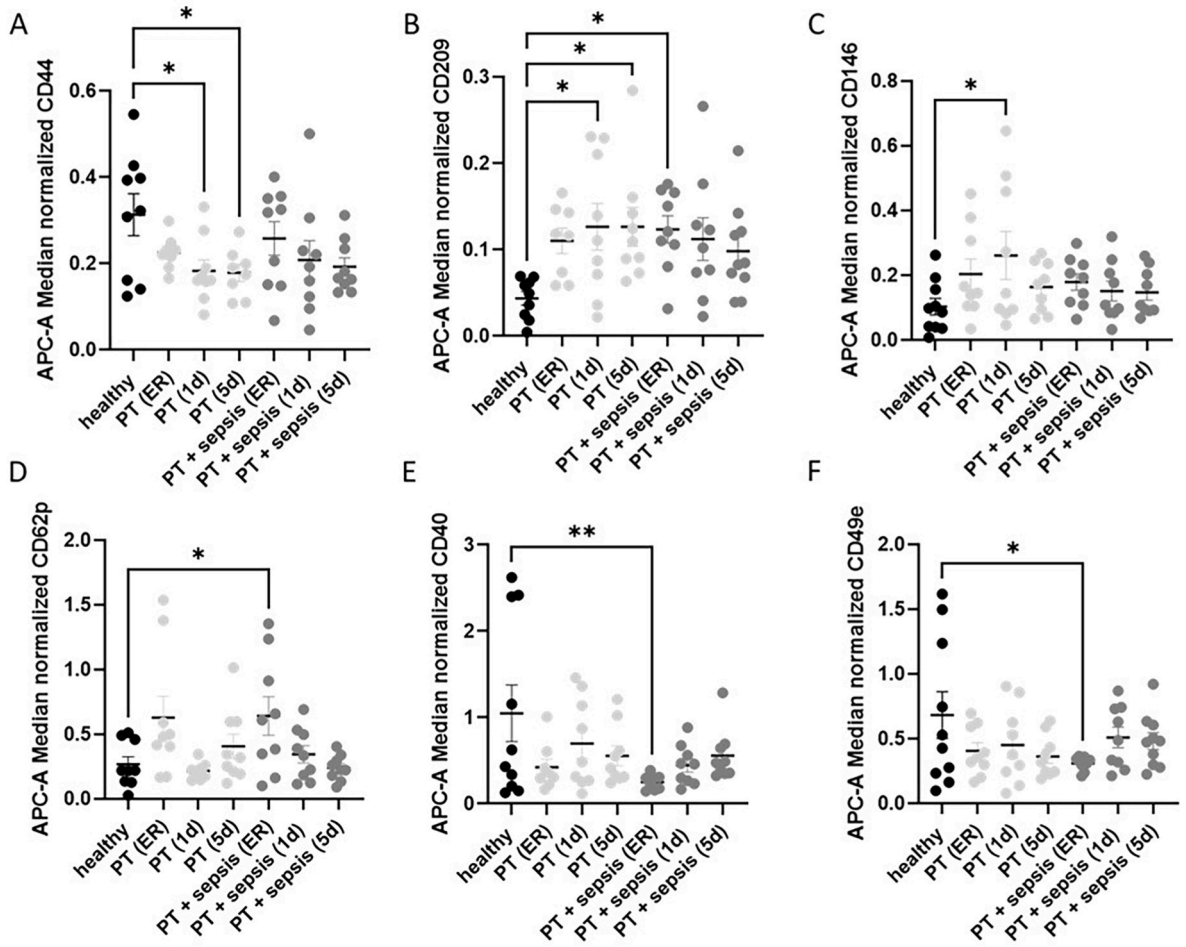

Fig. 2. Differential expression of exosomal surface proteins in polytraumatized patients (with and without sepsis) and healthy controls. A) The proportion of $CD44^+$ exosomes was significantly reduced in polytrauma patients at day 1 and day 5 of analysis as compared to healthy controls group. B) The ratio of $CD209^+$ exosomes was significantly increased in polytrauma patients at day 1 and day 5, as well as in septic patients at the Emergency room (ER) as compared to healthy controls. C) $CD146^+$ exosomes were significantly increased in PT patients at day 1 as compared to healthy controls. In polytrauma patients with sepsis at ER time point the ratio of $CD62p^+$ exosomes (D) was increased and the ratios of $CD40^+$ (E) and $CD49e^+$ (F) exosomes were significantly decreased as compared to healthy controls. *$p \leq 0.05$ and **$p \leq 0.01$. PT n = 10; PT + sepsis n = 10; healthy volunteers n = 10.

difference in exosomal expression of miR-1298-5p, miR-1262 and miR-125b-5p among the groups and time points was found (data not shown). At the same time, we found that amount of miR-92a-3p was greatly reduced in exosomes from polytrauma patients at day 1 (Fig. 4A) as compared to controls. Also, miR-93-5p was significantly reduced in polytrauma patients at day 1, but also in septic patients at day 5. (Fig. 4B). The miR-155-5p was only altered in polytraumatized patients without sepsis at day 1 and 5 (Fig. 4D). Interestingly, miR21-5p was significantly reduced relatively to controls only in septic patients (Fig. 4C).

4. Discussion

In a clinical context, early diagnosis of septic complications is crucial to improve patient's outcome. Especially in polytrauma patients, septic complications occur often and could be responsible for the high mortality [4]. Therefore, early markers of septic deterioration are necessary as they could provide the clinicians enough time for a proper treatment. In the present study, we hypothesized that plasma exosomes populations and cargo reflect the development of sepsis in polytraumatized patients and therefore could be suggested as an early marker of septic complications.

In previous study, the potential role of extracellular vesicle in discriminating outcome in patients with sepsis and trauma was suggested. For example, $CD3^+$ (T cell-derived EVs) and $CD41^+$ (platelet-derived EVs) were described to discriminate trauma patients from the septic ones [15]. In our patients, neither $CD3^+$, nor $CD41^+$ exosomes differed between the polytrauma patients with and without sepsis. This fact could be explained by differences in study design (patients with trauma + sepsis versus septic patients) and technical approaches (analysis of other EV subpopulations like microvesicles). Moreover, some authors declared that the source of inflammation/bacteria (pneumonia or urogenital infection) might influence the EV- release during sepsis. Patients with pneumonia-induced sepsis were characterised by higher level of microvesicles as compared to fecal peritonitis and healthy controls [16]. Mixed fungal septic patients showed significantly elevated annexin V and $CD41^+$ microparticles on day 1 as compared with non-fungal septic patients [17]. In the present study, patients developed sepsis during the hospital stay, based on either pneumonia (50%), urogenital infection (30%) or intestinal perforation (20%). Nevertheless, we did not observe a connection between the sepsis origin and special subgroups of exosomes or the number of exosomes.

In the present study, we discovered that all populations of exosomes could be found in all individuals and that CD42a + exosomes constituted with up to 30% the largest population (Fig. 1). Another large population are the CD41b+ and $CD29^+$ exosomes. Also prominently presented are the $CD31^+$, $CD40^+$ and CD49e + exosomes, which account for ≈ 2–6% of all analysed exosomes. These major populations of exosomes are of

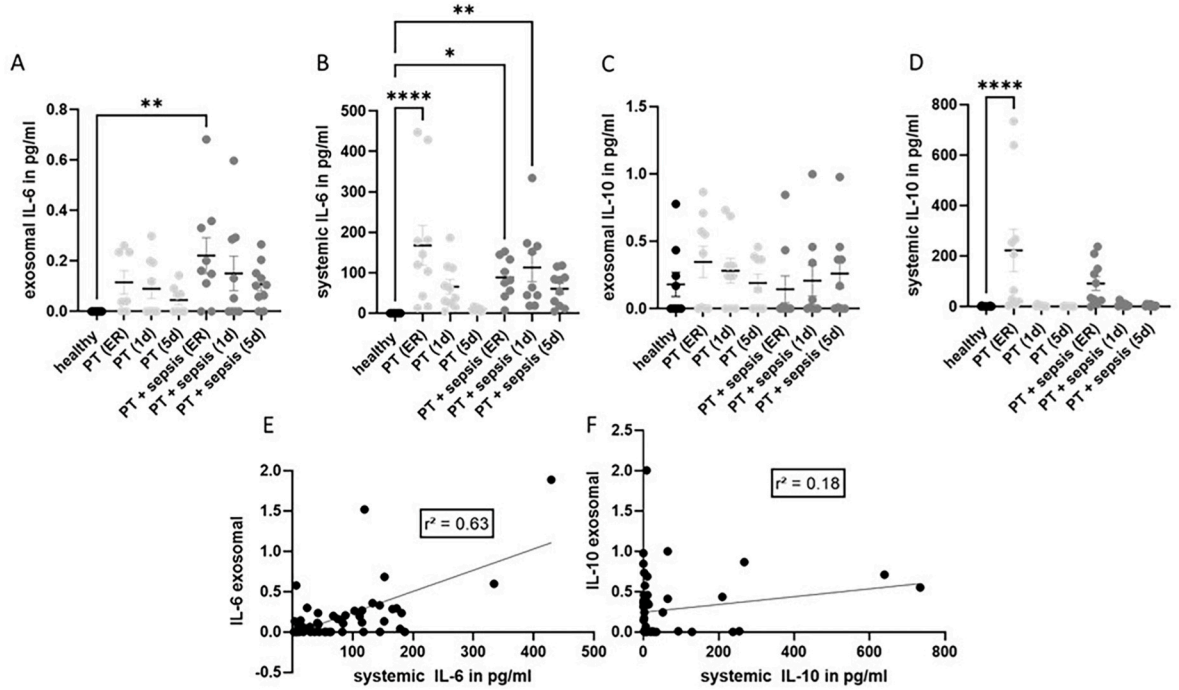

Fig. 3. Exosomal and systemic IL-6 and IL-10 concentrations in patients and healthy controls. A) Increase of exosomal IL-6 concentration was shown in septic patients at the first time point (emergency room (ER)). B) Systemic IL-6 concentrations are increased in patients with and without sepsis at the ER and 1 day after trauma time points. C) No differences in exosomal IL-10 concentrations was found among the groups at all time points. D) Systemically, IL-10 was significantly increased in polytraumatized patients without sepsis at the ER time point. E) Exosomal IL-6 concentration moderately correlates with the systemic ones ($r^2 = 0.63$). F) Exosomal IL-10 concentration das not correlate with the systemic IL-10 concentration in patients after trauma with and without sepsis. *p ≤ 0.05, **p ≤ 0.01, ***≤0.001, ****p ≤ 0.0001, PT n = 10; PT + sepsis n = 10; healthy volunteers n = 10.

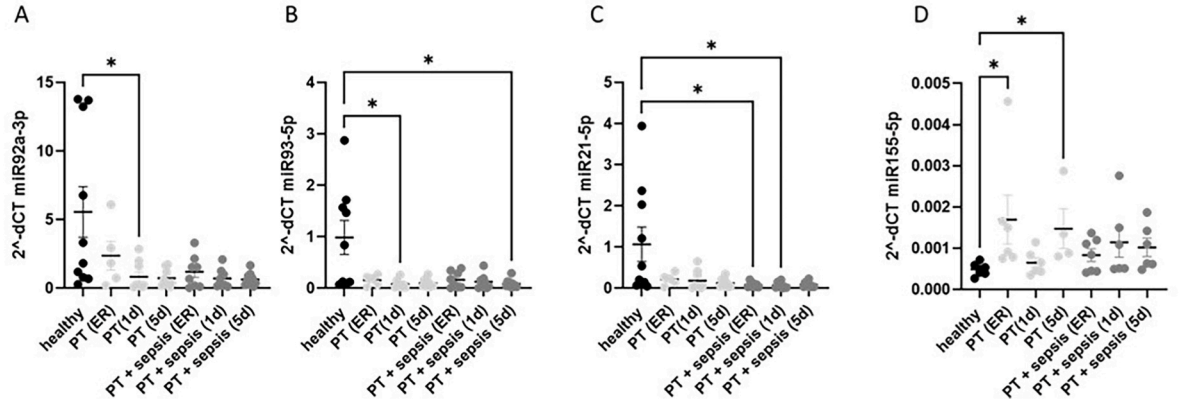

Fig. 4. Expression of exosomal miRNAs in polytrauma patients and healthy controls. A) Expression of miR-92-3p was significantly reduced in exosomes of polytrauma patients at day 1 after trauma. B) miR-93-5p exosome expression was significantly reduced at day 1 in polytrauma and at day 5 in polytraumatized patients developing sepsis. C) At the emergency room (ER) and 1d after trauma the expression of miR-21-5p in exosomes was significantly decreased in patients developing a sepsis as compared to healthy controls. D) miR-155-5p was significantly increased in polytrauma patients at the ER and after 5 days. *p ≤ 0.05, **p ≤ 0.01, ***≤0.001, ****p ≤ 0.0001. PT n = 10; PT + sepsis n = 10; healthy volunteers n = 10.

platelet origin. Platelet-derived EVs constitute the major fraction of EVs in the circulating plasma, previously considered to be up to 70–80% of the microvesicles, but this number has recently been revised to ~25% based on freeze-fixation electron microscopy of CD41-positive vesicles in plasma samples [18].

At the same time, we found that three other exosomal populations are specifically altered in patients with sepsis: CD62p + exosomes are significantly increased, while CD40+exosomes and CD49e + exosomes are diminished in the PT + sepsis samples collected at ER. In general, an increase of CD62p + microparticles in serum samples was previously shown in severely injured patients [19]. Our working group also demonstrated an association between CD62p + exosomes and traumatic brain injury [14]. CD62p + exosomes are released from endothelial cells, platelets and megakaryocyte [20] and therefore this release could reflect endothelial dysfunction and the disseminated intravascular coagulopathy (DIC) in septic patients [21].Although these studies provide a link between sepsis and the release of CD62p + EVs from endothelial cells and platelets, the role of these particles in septic endothelial dysfunction and DIC is yet unknown and requires further investigations.

As demonstrated in Fig. 2E, CD40+exosomes are significantly reduced in PT + sepsis patients. CD40 is a member of the tumour necrosis factor alpha receptor family, is expressed by B cells, professional antigen-presenting cells, as well as non-immune and tumour cells and is one of the best-characterized costimulatory molecules of the adaptive

immune response [22]. Under inflammatory conditions, CD40 binds its ligand CD40L, which is transiently expressed on T cells and other non-immune cells. Cellular and humoral adaptive immunity are initiated and progressed by CD40 engagement which leads to a wide range of molecular and cellular processes [23]. It was previously shown that during different inflammatory diseases e.g. sepsis, endothelial microparticles expressed CD40 on their surface. Also in vitro, it was shown that treatment of HBEC cells with a combination of TNF and interferon-gamma induced the release of $CD40^+$ microparticles [24]. Furthermore, when human endothelial cells were stimulated with a mixture of thrombin and CD40L to model the inflammatory/prothrombotic environment, the release of microparticles containing matrix metalloproteinase-10 and CD40L was described [25]. Given the crucial function of the CD40 receptors in the immunological response, the present observations suggest that the decrease in $CD40^+$ exosomes might indicate a dysregulation of the immune system during sepsis.

Next to $CD40^+$ exosomes also CD49e + exosomes were significantly reduced in patients with sepsis. CD49e, also called integrin alpha 5, is a part of the integrin alpha chain family and is expressed on the surface of thymocytes, T-cells, early activated B-cells, monocytes, platelets, fibroblasts, endothelial, and epithelial cells. Together with beta 1 subunit, alpha 5 integrin forms heterodimeric α5β1 fibronectin receptor [26]. Extracellular vesicles with α5β1 integrin receptors could interact with fibronectin and mediate fibroblast invasion (fibrosis) through activation of invasion-associated signalling pathways involving focal adhesion kinase and Src family kinase [27]. These data indicate that fibronectin is targeted to exosomes by binding to cognate cellular integrin receptors, and then secreted in adhesive form to promote adhesion formation and effective cell motility [28]. Overall, it was suggested that integrins in the exosomes could play roles in directing exosomal homing to tissues and remodelling of the homing niche; in horizontal transmission of the ability to activate TGF-β signalling and in cellular and exosomal cooperation (reviewed in Ref. [29])

Next to the surface epitopes/cellular origin of exosomes in septic patients, the present study also analysed the cargo of septic exosomes. In addition to other laboratory markers, systemic cytokines, particularly IL-6, are frequently utilized to forecast the onset of inflammatory complications as well as patient's outcome. Multiorgan dysfunction and mortality are associated with elevated levels of systemic cytokines such as IL-6 and IL-10 [30,31]. A significant correlation was found between the ISS and the levels of both IL-6 and IL-10 over 24hrs after trauma and significantly increased serum concentrations of IL-6 and IL-10 were shown in patients not surviving 30 days [32]. A reduced amount of systemic IL-10 was detected in patients suffering from sepsis after trauma [33]. However, little is known about exosomal cytokines in trauma or septic patients. During the early phases of sepsis, protein analysis in septic mice revealed that pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, and TNF-alpha were elevated in exosomes, whereas anti-inflammatory cytokines such as IL-10 increased during the late stages [34]. Additionally, it was demonstrated that the exosome inhibitor GW4869 significantly attenuated the levels of systemic cytokines in mice injected with LPS or exposed to CLP [35]. This observation might be associated by the fact that many methods measuring cytokines for example ELISA are not able to differentiate between the exosomal and the total amount of IL-6. In the present study, we observed completely different kinetics of exosomal IL-6 and IL-10. We found that the changes in systemic IL-6 concentration reflected the changes in exosomal IL-6 concentration, whereas the exosomal concentration of IL-10 was relatively constant over the time in all samples, while systemic IL-10 already declined. We hypothesize that these differences in the kinetics may be caused by the various tasks carried out during body homeostasis and trauma response by these exosomal cytokines.

For the analysis of the exosomal miRNAs-cargo in our samples, we selected miRNAs, which were described earlier to be changed in exosomes during sepsis (reviewed in Ref. [5]). We decided to use RT-qPCR method for analysis, which enables detection of very small quantities of miRNAs [36]. By using qPCR to compare the expression of miRNAs in various samples, standardization and normalizing of the assay (for example by including reference genes) are required [36]. Different strategy for standardization and normalization of exosomal miRNAs analysis were suggested, however none of them fulfill gold-standard criteria and there are no established reference genes [37]. In our study, we used identical amount of sample material (plasma) for exosome and miRNAs isolation, standardized protocols and included synthetic cel miR-39 as spike in control in order to control the possible technical variations during the sample preparations. This is an often-used method in the literature about normalization of miRNAs. With this approach we have shown, that some of the investigated miRNAs (miR-1298-5p, miR-1262 and miR-125b-5p) did not differ in expression level among the groups, whereas expression of miR-21, miR-93-5p, miR-155-5p and miR-92a-3p differed significantly. Next to the normalization, there are unfortunately other limitations of our study. The sample size included in the present study, was quite small. We already included septic polytrauma patients over a time period of 5 Years, but unfortunately the number of septic patients meeting our inclusion criteria, who also consent to be part of such a study was low. This might be also the reason, why differences between polytraumatized patients and PT-patients with sepsis is small and did not reached significancy. We also analysed only a small selection of potential miRNAs from the literature und did not conduct a whole miRNome analysis. Future studies are needed to complete our analysis.

Reithmair et al. described in 2017 a downregulation of exosomal miR-21 in patients with sepsis, which was dependent on the severity of sepsis and the appearance of septic shock [38]. In the present study, we also observed a downregulation of exosomal miR-21-5p specifically in the PT + sepsis patients as compared to healthy controls. Exosomal miR-21 was described to lead to M2 polarization and therefore to have an anti-inflammatory effect, which was observed in EVs released from IL-1β-pretreated MSCs [39]. In a rat CLP model it was shown that sepsis-induced acute kidney injury could be alleviated by the treatment with endothelial progenitor cells-derived exosomal miR-21-5p, which inhibits RunX1 expression [40]. Downregulation of exosomal miR-21-5p in our sepsis patients could be result of the severity of their inflammatory symptoms and the absence of the proper anti-inflammatory response.

The other changes in the miRNA-profile of exosomes found in the present study (miR-93-5p, miR-155-5p and miR-92a-3p), were not specific for the patient's developing sepsis. In the present study, we observed that miR-93-5p was significantly reduced in both patients' groups (with and without sepsis). In sepsis-induced kidney injury, exosomal miR-93-5p was associated earlier with pyroptosis in renal epithelial cells [41]. While exosomal miR-93-5p derived from endothelial progenitor cells was associated with protection from sepsis-induced acute kidney injury reduction of TNF-alpha [42]. In the present study, miR-92a-3p was significantly reduced in polytraumatized patients at day 1 after trauma. We did not observe an association with the development of sepsis. Circulating serum miR-92a was described earlier as a risk factor for sepsis-induced ARDS [43]. Furthermore, miR-92a inhibition attenuated the adverse effects of LPS on ARDS through the Akt/mTOR signalling pathway [44]. In a mouse model of acute lung injury, miR-92a-3p modulated LPS-induced intrapulmonary inflammation, oxidative stress via endogenous A-kinase anchoring protein 1 [45]. Activation of NF-kB pathway after uptake of exosomes from alveolar epithelial cells by alveolar macrophages was further associated with miR-92a-3p exosomal transfer [46]. In addition, the miR-155 was associated with the development of ARDS. Exosomal miR-155 stimulated lung inflammation through NF-kB activation in macrophages resulting in TNF and IL-6 production [47]. In the present analysis exosomal miR-155-5p was observed in patients with polytrauma without sepsis.

In summary, our miRNA analysis showed that miR-215p-in exosomes might be associated with the development of sepsis, while exosomal

miR-93-5p, miR-155-5p and miR-92a-3p were not specifically associated with sepsis development.

5. Conclusion

Taken together, the present study in polytraumatized patients demonstrated that the development of sepsis is associated with the significant increase of CD62p + exosomes and significant decrease of $CD40^+$ and CD49e + exosomes. Furthermore, we observed that the exosomal IL-6 concentration reflected the systemic IL-6 concentration (correlation $r^2 = 0.63$) and did not significantly alter between patients with and without sepsis. The exosomal IL-10 concentration seemed to be constant in all analysed individuals. At least we observed that miR-21-5p in exosomes might be associated with the development of sepsis, while exosomal miR-93-5p, miR-155-5p and miR-92a-3p were not specifically associated with sepsis.

Declarations

Ethics approval/Consent for publication: All analyses were performed with ethical approval given by the Local Ethics Committee of the University of Frankfurt (approval ID 89/19). A written consent of all included patients was obtained.

Availability of data and materials

n.a.

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CRediT authorship contribution statement

Birte Weber: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Dirk Henrich:** Writing – review & editing, Validation, Supervision, Software, Project administration, Formal analysis. **Ingo Marzi:** Writing – review & editing, Validation, Supervision, Resources. **Liudmila Leppik:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2024.101954.

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