Review

Extracellular Vesicles as Markers and Mediators in Sepsis

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Abstract

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. It remains a highly lethal condition in which current tools for early diagnosis and therapeutic decision-making are far from ideal. Extracellular vesicles (EVs), 30 nm to several micrometers in size, are released from cells upon activation and apoptosis and express membrane epitopes specific for their parental cells. Since their discovery two decades ago, their role as biomarkers and mediators in various diseases has been intensively studied. However, their potential importance in the sepsis syndrome has gained attention only recently. Sepsis and EVs are both complex fields in which standardization has long been overdue. In this review, several topics are discussed. First, we review current studies on EVs in septic patients with emphasis on their variable quality and clinical utility. Second, we discuss the diagnostic and therapeutic potential of EVs as well as their role as facilitators of cell communication via micro RNA and the relevance of micro-organism-derived EVs. Third, we give an overview over the potential beneficial but also detrimental roles of EVs in sepsis. Finally, we focus on the role of EVs in selected intensive care scenarios such as coagulopathy, mechanical ventilation and blood transfusion. Overall, the prospect for EV use in septic patients is bright, ranging from rapid and precise (point-of-care) diagnostics, prevention of harmful iatrogenic interventions, to using EVs as guides of individualized therapy. Before the above is achieved, however, the EV research field requires reliable standardization of the current methods and development of new analytical procedures that can close the existing technological gaps.

Key words: sepsis, inflammation, point-of-care, microparticles, exosomes

Introduction

Sepsis was recently redefined as life-threatening organ dysfunction caused by a dysregulated host response to infection. For decades, it has remained a highly lethal condition in which dependable diagnostics and therapeutic decision-making are far from optimal. Currently, sepsis incidence is estimated at 270 cases per 100,000 persons/year followed by an approximate 26% mortality rate [1,2]. Programs such as the Surviving Sepsis Campaign [3] and World Sepsis Day have increased awareness to the global burden of sepsis as well as the related diagnostic/therapeutic difficulties. Both the Surviving Sepsis Campaign and the most recent Sepsis-3 definitions stress a dire need for reliable biomarkers. The current Surviving Sepsis Guidelines are limited to a weak recommendation regarding the use of procalcitonin for shortening/discontinuation of ongoing antimicrobial treatment only and encourage evaluation of further markers, e.g., for renal dysfunction and coagulopathy [2,3]. An ideal sepsis biomarker should possess a diagnostic and predictive value (high sensitivity, high specificity), display low donor
variability, be measured in a rapid and cost-effective bedside assay and withstand validation in multicenter trials [4]. Establishing a biomarker of sepsis should not be merely limited to correlating its concentration in the blood (and/or other fluids) with a particular disease phase and/or clinical outcome. It should also govern further management, e.g., the initiation of further diagnostics, escalation or de-escalation of a specific antibiotic regimen and initiation or discontinuation of invasive and extracorporeal treatments.

Many compounds with biological impact to the host defense are detectable in body fluids at elevated levels during sepsis and other states accompanied by an acute inflammatory response. Several inflammatory cytokines (circulating peptides) like tumor necrosis factor (TNF)-alpha, interleukin (IL)-1-beta and IL-6 have been identified as markers of sepsis severity and outcome predictors, and were targeted in several clinical trials [5-8]. The failure of the trials targeting the “cytokine storm” was surprising and disappointing; it is now known that their design was based on a false assumption that deaths from sepsis were primarily attributable to the overwhelming synthesis of cytokines (i.e., hyperinflammation) [9,10]. The newest evidence shows that sepsis responses are multifactorial and fluctuate rapidly [9]. Thus, it is not the abundance of one particular cytokine but rather a specific complex inflammatory “signature” that accurately depicts a patient’s immune status as well as the probable prognosis and response to treatment [9,11].

Only recently, the role of exosomes in critical illness has been summarized [12]. This small population (size range 30-100 nm) of EVs derived from the endosomal system represents only a subset of the extracellular messenger pool. EVs are 30 nm to several micrometers in size, formed either by outward budding of the plasma membrane upon activation, during programmed cell death (i.e., apoptosis), or by exocytosis of multivesicular bodies [13]. They express epitopes on their membranes that are specific for their parental cells. A review on their function in health and disease has been published recently [14]. Systematic assessment of EVs in sepsis is an area of diagnostics that may support the developing concept of patient-tailored therapy by filling up the wide biomarker gap. Release of EVs represents an immediate cell communication mechanism that fulfills three major functions: a) transfer of an expression pattern to other cells, b) dissemination of the membrane-bound mediators within the liquid phase and c) rapid rearrangement of the cell surface. Like all messengers, EVs can immediately modify a phenotype of neighboring cells in a paracrine fashion but also bring forward an altered micro-milieu to other tissues by transferring the membrane composition and/or expression pattern to other regions of the circulation (endocrine release). Figure 1 illustrates the different types of EVs and basic modes of cellular release. The existing literature suggests that EV monitoring can effectively aid in the identification and characterization of specific pathophysiological blueprints of a given septic patient as well as in assessing the efficacy of various therapeutic regimens available to that patient. Over the last two decades, a large number of original studies investigating EVs in sepsis have been published. However, more is not always better, and a large number of experiments does not automatically ensure clinical progress.

This review has several objectives. First, we provide an analysis of the 40 existing studies on EVs in septic patients (summarized in Table 1) with a focus on their study design. This is important given that EVs have been suggested to serve as diagnostic biomarkers [15], while the methods for their detection and data interpretation in complex diseases such as sepsis are still under development. This translates into large variability in the quality, usefulness and/or comparability among the existing sepsis-related studies. Second, we focus on the role of EVs as biomarkers and mediators in sepsis as well as emphasize their potential utility to guide treatments and their role as potential targets for treatment. Third, we debate whether EVs are a meaningful part of the adequate evolutionary host response or also act as...
involuntary players of the inflammatory response [15]. Finally, we characterize the influence of EVs in the exemplary, most severe clinical manifestation of sepsis (i.e., disseminated intravascular coagulation, DIC) and common treatment routines (i.e., blood transfusion, mechanical ventilation) used in the intensive care unit (ICU). While a systematic analysis of all existing in vitro and preclinical studies was not the main scope of this review, several preclinical papers relevant to the discussed topics are also included.

**Heterogeneity versus standardization:**
**critical assessment of EVs studies**

In order to be useful, data from in vitro/ex vivo, preclinical (no patients involved) studies and clinical (patients-based) studies must meet several stringent criteria including: 1) best possible match between the research model and a given clinical disease scenario, 2) an adequate study design, sample sources and sampling time points, 3) adequate statistical power, 4) standardized detection assays and 5) reproducibility. In a complex disorder like sepsis, mediators like EVs dynamically fluctuate and those changes may play a different (if not opposite) role in various body compartments as well as in the local and systemic circulation.

The intensive care unit (ICU) patient populations in general, but especially those fulfilling sepsis criteria [2], are extremely heterogeneous. They present with variations in genetic background, age, gender, accompanying diseases, chronically prescribed medications, source of infection (medical or surgical) and pathogens involved. Heterogeneity has been one of the major hindrances in sepsis research, particularly affecting the development of therapies. On the one hand, studies performed in heterogeneous cohorts better reflect the entire population and are easier to power, making any novel findings more robust and applicable to a wider target population. Conversely, large patient cohorts can also mask potential benefits (and harms) of any novel treatment in defined, smaller patient sub-groups. Although retrospective analyses may enable post-hoc detection of such benefits, the investigative value of such findings is weaker. Below, we present several key elements that modify the perception and understanding of EV-based research in the clinical context. The “sepsis” diagnosis encompasses patients with a multitude of scenarios by which the microbial invasion develop. To our knowledge, only one recent study compared the role of EVs among subsets of septic patients based on the site of infection [16].

For example, at least 19 of the analyzed studies (Table 1) were performed in cohorts with mixed sites of infection (i.e., pneumonia, intra-abdominal infection, urinary tract infection, necrotizing fasciitis, wound infection, meningitis, mediastinitis) and underlying pathologies and injuries (i.e., trauma, cancer, immunosuppression). The relatively small number of existing clinical EVs studies exacerbates the difficulty of any reliable cross-comparisons. It is currently unclear whether the origin of the infection focus in any way modulates the EVs characteristics and their signaling routes and/or diagnostic potential. Although different anticoagulants for blood samples were used, citrate predominated. This is appropriate as citrate is recommended for achieving stable EVs counts throughout the initial processing period [17]. In the context of potential EV utilization, the heterogeneity of the sepsis population remains one of the most significant hurdles, necessitating careful planning (e.g., only including patients with site-specific sepsis focus) and reliance on clear definitions and reporting.

Most recently, sepsis has been redefined as a dysregulated host response to infection and its gradation largely rests upon the state of organ dysfunction present in the patient [2]. The latter element partly overlaps with the earlier definition of severe sepsis [18] and can occur in the presence of underlying circulatory and cellular/metabolic abnormalities which are profound enough to additionally increase mortality (defined as “shock”). Given that sepsis and septic shock often develop from an initially non-life-threatening infection, the influence of EVs during this phase may be misinterpreted if patients enrolled for clinical studies are not meticulously selected based on the exact diagnosis and/or disease phenotype. In general, most EV studies were performed in patients suffering from severe sepsis (i.e., including organ dysfunction) according to the former definition, which is referred to as ‘sepsis’ according to the recent Sepsis-3 definitions [2] (see Table 1). In at least 14 studies, the septic patients were in the state of shock. It is unknown whether re-analysis of sepsis studies using the new Sepsis-3 definitions for enrollment (and thus redistributing groups) would change their conclusions; new studies selecting patients based on the new Sepsis-3 have been only recently emerging [16]. Interestingly, redistribution of patients according to the Sepsis-3 definition did not affect conclusions drawn in the recent study by Matsumoto et al. [19].

In patients, the exact time of the onset of a septic episode is typically unknown. Repetitive blood sampling from intravascular catheters at least once daily is a routine practice in the ICU. In the studies analyzed in Table 1, EVs were characterized based on only one sampling time-point in 23 studies, and on
multiple time-points (i.e., time course) in 17 studies. Additionally, marked variation existed regarding the time that elapsed between sepsis diagnosis and the draw of the first blood sample enrolled into the study. These factors related to timing of samples may hinder precise and/or protracted characterization of EV-related events in a rapidly fluctuating disease. Another critical factor is the choice of optimal and defined inclusion/exclusion criteria. EVs are ubiquitously detectable and have pleiotropic effects. Therefore, multiple preexisting disorders may confound the EV concentration in septic patients. The most frequent exclusion criteria were: a) diabetes mellitus [20], b) statin treatment [21], c) drugs altering platelet function [22,23], d) recent blood product transfusion [22,23], e) any hematologic malignancy, and f) other types of cancer [24]. As a result, a substantial part of average ICU septic patients was excluded, potentially rendering the population unrepresentative by amenable standardization procedures will directly enhance their clinical translatability.

### Table 1. Characteristics of clinical patient studies investigating the role of extracellular vesicles (EV) in sepsis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Diagnosis</th>
<th>Mortality</th>
<th>Time to first sample</th>
<th>Sampling time points</th>
<th>Anticoagulant</th>
<th>Cohort size</th>
<th>EV sources</th>
<th>Summary of findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Larsson (1996) [151]</td>
<td>Gram-negative sepsis on ICU</td>
<td>Unknown</td>
<td>At day of ICU admission</td>
<td>Day 1, 2, 4 and 6</td>
<td>Citrate</td>
<td>Sepsis (n=4)</td>
<td>Platelets</td>
<td>3 out of 4 septic patients showed high PEV count (no statistics).</td>
</tr>
<tr>
<td>2. Lundahl (1996) [152]</td>
<td>Mixed ICU population Meningococcal septic shock</td>
<td>Unknown</td>
<td>Unknown</td>
<td>&lt;24 h</td>
<td>Citrate</td>
<td>ICU patients (n=19), healthy (n=20)</td>
<td>Platelets</td>
<td>Increased PEV (no statistics) in 2 out of 8 patients who died shortly after sampling.</td>
</tr>
<tr>
<td>4. Joop (2001) [28]</td>
<td>Mixed severe sepsis</td>
<td>4 survivors, 5 non-survivors</td>
<td>At day 28</td>
<td>Citrate</td>
<td>Sepsis (n=14), SIRS (n=12) healthy (n=12)</td>
<td>Platelets</td>
<td>PEV/platelet count higher in sepsis vs. healthy, no difference trauma vs. sepsis.</td>
<td></td>
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<tr>
<td>5. Ogura (2001) [92]</td>
<td>Mixed sepsis, trauma &gt; SIRS, all with C-reactive protein &gt;10 mg/dl</td>
<td>Unknown</td>
<td>2-7 days post trauma</td>
<td>Once</td>
<td>Citrate</td>
<td>Sepsis (n=14), SIRS (n=12) healthy (n=12)</td>
<td>Platelets</td>
<td></td>
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<tr>
<td>8. Soriano (2005) [30]</td>
<td>Mixed severe sepsis</td>
<td>51.4% at day 28</td>
<td>24-48 h after organ failure</td>
<td>At admission, day 1 and day 2</td>
<td>Citrate</td>
<td>Sepsis (n=35), ECs, platelets healthy (n=45)</td>
<td>EEV, not PEV and EEV-monocyte conjugate count was higher in septic patients on day 1 vs. healthy controls. EEV-monocyte conjugates at all time points were higher in non-survivors vs. survivors.</td>
<td></td>
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<tr>
<td>9. Gambim (2007) [155]</td>
<td>Mixed septic shock</td>
<td>Unknown</td>
<td>&lt;24 h after diagnosis</td>
<td>Citrate</td>
<td>Sepsis shock (n=12), healthy (n=10)</td>
<td>Platelets</td>
<td>EV from human platelets were similar to septic patient-derived PEV after in vitro exposure to NONOate and LPS, but not to TNF-alpha or thrombin, generated superoxide and nitric oxide. ECs incubated with EVs underwent caspase-3 activation/apoptosis, inhibited by ROS</td>
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<tr>
<td>Study</td>
<td>Diagnosis</td>
<td>Mortality</td>
<td>Time to first sample</td>
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<td>Summary of findings</td>
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<tr>
<td>10. Azevedo (2007) [156]</td>
<td>Septic shock</td>
<td>Unknown</td>
<td>48 h after diagnosis</td>
<td>Once</td>
<td>Heparin</td>
<td>Septic shock (n=55), healthy (n=12)</td>
<td>Platelets</td>
<td>Exosomes from septic patients decreased maximal DT and positive Δt/Δmax in rat papillary muscle preparations and positive Δt/Δmax in isolated rabbit hearts pre-exposed to LPS. Exosomes from septic patients showed intrinsic NO production and induced myocardial NO content. No effects of exosomes from septic patients on isolated rabbit heart not pre-exposed to LPS and rat aortic ring contractility.</td>
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<tr>
<td>11. Huisse (2008) [157]</td>
<td>Severe sepsis</td>
<td>14% in-hospital mortality</td>
<td>Unknown</td>
<td>Once</td>
<td>Citrate</td>
<td>Severe sepsis (n=14), heat-stroke (n=18), healthy (n=18)</td>
<td>Total EVs, platelets, monocytes, granulocytes, ECs</td>
<td>Severely septic patients showed lower PEVs, increased GEVs and MEVs and similar EEVs vs. healthy volunteers.</td>
</tr>
<tr>
<td>12. Mostefai (2008) [105]</td>
<td>Mixed septic shock</td>
<td>28% at day 28</td>
<td>10 h post ICU admission</td>
<td>Once</td>
<td>Citrate</td>
<td>Septic shock (n=36), ICU non-septic (n=18)</td>
<td>Total EVs, platelets, ECs, leukocytes, monocytes, granulocytes, erythrocytes</td>
<td>Total EVs, PEVs, EEVs, L-Selectin+EVs and P-Selectin+EVs were increased, LEVs were decreased, and GEVs, MEVs, EryEVs and AnnV+ EVs were not different in septic vs. non-septic ICU patients.</td>
</tr>
<tr>
<td>13. Pérez-Casal (2009) [158]</td>
<td>Severe sepsis, rhAPC treatment</td>
<td>36% at day 28</td>
<td>At start of rhAPC infusion</td>
<td>Days 0, 1 and 4 of rhAPC infusion</td>
<td>Citrate</td>
<td>Severe sepsis with rhAPC (n=4), severe sepsis without rhAPC (n=4)</td>
<td>EPCR+ EVs</td>
<td>Co-localization of EPCR and APC on EVs from septic patients during, but not before, rhAPC infusion. APC on EPCR+ EVs was higher during vs. before (individual controls) rhAPC treatment and vs. non-rhAPC-treated controls.</td>
</tr>
<tr>
<td>14. Forest (2010) [159]</td>
<td>SIRS, mixed sepsis or septic shock</td>
<td>0% in all SIRS patients, 0% in &lt;50 y sepsis, 26% in ≥75 y sepsis</td>
<td>Within 1 h of hospital admission</td>
<td>Once</td>
<td>Citrate</td>
<td>&lt;50y SIRS (n=26), ≤50y SIRS (n=31), ≥75y SIRS (n=27)</td>
<td>ECs, erythrocytes, platelets</td>
<td>Lower EEVs, but the same EV procoagulant activity in older vs. younger patients. Lower EEVs in sepsis vs. SIRS in young patients. Lower EV procoagulant activity in sepsis vs. SIRS in both old and young patients. Elderly sepsis non-survivors had higher EEVs vs. elderly survivors.</td>
</tr>
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<td>15. Rank (2011) [160]</td>
<td>Sepsis</td>
<td>Unknown</td>
<td>Before conditioning</td>
<td>Twice a week for 30 days, thereafter once a week until discharge</td>
<td>Citrate</td>
<td>hematopoietic stem-cell transplantation (n=19), incl. infection/sepsis (n=15)</td>
<td>Erythrocytes</td>
<td>EryEVs level was affected only by development of graft-versus-host-disease but not conditioning therapy, total body irradiation, high-dose chemotherapy, in vivo T-cell depletion, aplasia or engraftment in uncomplicated patients, infectious complications and/or sepsis.</td>
</tr>
<tr>
<td>16. Pérez-Casal (2011) [70]</td>
<td>Severe sepsis (Pneumonia or intraabdominal infection), rhAPC treatment</td>
<td>36% at day 28</td>
<td>Start of rhAPC infusion</td>
<td>Before rhAPC infusion and at days 2, 3, 4, 5 and 6</td>
<td>Citrate</td>
<td>Severe sepsis with rhAPC (n=25) or without rhAPC (n=25), healthy (n=6)</td>
<td>EPCR+EV, APC+EV, myeloid cell line</td>
<td>Increased circulating EV carrying EPCR and APC expression after rhAPC infusion. Decrease of endothelial permeability by APC+EVs via PAR-1.</td>
</tr>
<tr>
<td>17. Prakash (2012) [101]</td>
<td>Septic peritonitis, mixed ICU with suspected ventilator-associated pneumonia or lung donors</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Once</td>
<td>Citrate</td>
<td>Septic peritonitis (n=3), control abdominal lavelage fluid from non-septic laparotomy (n=4), ICU suspected pneumonia BAL (n=33), control BAL from lung donor (n=2)</td>
<td>Granulocytes, platelets, ECs, erythrocytes</td>
<td>GEV only present in inflamed foci. THP-1 cells were activated by phagocytosis of GEV.</td>
</tr>
<tr>
<td>18. Woth (2012) [55]</td>
<td>Mixed severe sepsis</td>
<td>21%</td>
<td>At admission to the ICU</td>
<td>At admission, day 3 and day 5</td>
<td>Citrate</td>
<td>Septic shock (n=33), healthy (n=20)</td>
<td>Platelets</td>
<td>Elevated AnnV+ EV and PEV in septic vs. healthy on admission. Higher AnnV+ EV and PEV in fungal vs. non-fungal septic patients on day 1. CD42+ EV increased in fungal vs. non-fungal sepsis at all times and PAC1+ EVs at day 1 and day 5. GEV 5.6 fold higher in serum and bacterial-EV aggregates larger with serum of bactremic patients vs. healthy. Increased total, CD41+, CD42a+, and PAC1+ EVs in septic vs. healthy. Increased total, CD41+, and CD13+ EVs on admission in septic patients with renal dysfunction. Negative correlation of CD42a+ PEVs with blood urea nitrogen and creatinine concentrations in sepsis.</td>
</tr>
<tr>
<td>19. Timár (2013) [103]</td>
<td>S. aureus bacteremia and fever</td>
<td>Unknown</td>
<td>Within 24 h after fever</td>
<td>Once</td>
<td>Unknown</td>
<td>Bacteremia (n=12), healthy (n=6)</td>
<td>Granulocytes</td>
<td>Septic EVs augmented histamine-induced contraction in human tissue-engineered vascular media. Septic EV treatment increased cyclo-oxygenase-1 and IL-10 expression of IL-10 (but not IL-1α, IL-1β, IL-6).</td>
</tr>
<tr>
<td>20. Tókés-Füzesi (2013) [22]</td>
<td>Severe sepsis</td>
<td>15% at day 28</td>
<td>24 h of diagnosis of severe sepsis</td>
<td>At admission, day 3 and day 5</td>
<td>Citrate</td>
<td>Septic shock (n=37), or non-septic ophthalmic patients (n=20)</td>
<td>Platelets, monocytes, myeloid cell line</td>
<td>Septic shock (n=16)</td>
</tr>
<tr>
<td>21. Mostefai (2013) [161]</td>
<td>Septic shock</td>
<td>12.5% at day 28</td>
<td>10h4 h after ICU admission</td>
<td>Once</td>
<td>Unknown</td>
<td>Septic shock (n=28)</td>
<td>Unknown</td>
<td>Septic shock (n=28)</td>
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<tr>
<td>Study</td>
<td>Diagnosis</td>
<td>Mortality (%)</td>
<td>Time to first sample</td>
<td>Sampling points</td>
<td>Anticoagulant</td>
<td>Cohort size</td>
<td>EV sources</td>
<td>Summary of findings</td>
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<tr>
<td>22. Van Ierssel (2013) [162]</td>
<td>Mixed severe sepsis or septic shock</td>
<td>14% at day 28</td>
<td>At admission (&lt;72 h of sepsis diagnosis)</td>
<td>Once</td>
<td>Citrate</td>
<td>Severe Sepsis (n=30), healthy (n=15)</td>
<td>ECs</td>
<td>EEV not different between septic and healthy.</td>
</tr>
<tr>
<td>23. Delabranche (2013) [122]</td>
<td>Mixed septic shock with DIC</td>
<td>29.3% at day 28</td>
<td>At diagnosis of septic shock</td>
<td>Unknown</td>
<td>Procoagulant EVs (prothrombinase assay), ECs, leukocytes</td>
<td>Septic shock (n=92), incl. (n=40) with DIC</td>
<td>CD11a+ and CD105+ EVs were increased in DIC at admission. Increased CD105+ EVs had higher and CD31+ EVs lower odds ratios for DIC.</td>
<td></td>
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<tr>
<td>24. Dalli (2014) [106]</td>
<td>Sepsis caused by CAP</td>
<td>Unknown</td>
<td>&lt;24 h from admission</td>
<td>Once</td>
<td>Unknown</td>
<td>Sepsis survivor (n=25), non-survivor (n=25), healthy (n=15)</td>
<td>Granulocytes</td>
<td>Alpha-2-macroglobulin+ EVs were higher in sepsis survivors vs. non-survivors and healthy volunteers. High alpha-2-macroglobulin+ EVs correlated with better outcome.</td>
</tr>
<tr>
<td>25. Hellum (2014) [132]</td>
<td>Meningitis with or without shock</td>
<td>61%</td>
<td>&lt;4 h from admission and symptoms &lt;72 h</td>
<td>Once</td>
<td>Citrate</td>
<td>Meningitis and septic shock (n=13) and meningitis (n=10), healthy (n=6)</td>
<td>PhtdSer+ EVs</td>
<td>Faster and more efficient thrombin generation by EVs in shock vs. no shock and strong correlation with LIPS level in shock.</td>
</tr>
<tr>
<td>26. Exline (2014) [110]</td>
<td>Sepsis</td>
<td>38% in-hospital</td>
<td>&lt;24 h admission</td>
<td>Unknown</td>
<td>Severe Sepsis (n=24), trauma SIRS (n=12), cerebral hemorrhage SIRS (n=6), healthy (n=23)</td>
<td>Total EV</td>
<td>Higher EV-derived caspase-1 activity on day 1 and day 3 in septic vs. non-septic patients. EVs from septic patients on day 1 induced lymphocyte apoptosis.</td>
<td></td>
</tr>
<tr>
<td>27. Woei-A-Jin (2014) [56]</td>
<td>Community-acquired febrile E.coli urinary tract infection</td>
<td>0%</td>
<td>&lt;24 h of symptoms</td>
<td>At admission and on the 3 days thereafter</td>
<td>Sepsis (n=34), non-infected critically-ill controls (n=16)</td>
<td>Febrile urinary tract infection (n=215), healthy volunteers (n=19)</td>
<td>Monocytes</td>
<td>Higher TF+ EV activity on admission in patients with higher APACHE II score categories, but weak correlation with soluble E-selectin, soluble vascular cell adhesion molecule, thrombin-antithrombin-complexes and procalcitonin. TF+ EV activity was higher in patients vs. healthy.</td>
</tr>
<tr>
<td>28. Matsumoto (2015) [125]</td>
<td>Mixed severe Sepsis + DIC</td>
<td>12.5% overall ICU mortality</td>
<td>&lt;24 h of diagnosis</td>
<td>Once</td>
<td>Citrate</td>
<td>Severe sepsis (n=24), trauma SIRS (n=12), cerebral hemorrhage SIRS (n=6), healthy (n=23)</td>
<td>ECs, T-lymphocytes, granulocytes, erythrocytes, leukocytes, monocytes, ECs, antiplatelets</td>
<td>The amount of PhtdSer+ EVs was not associated with mortality and organ dysfunction.</td>
</tr>
<tr>
<td>29. Hermann (2015) [27]</td>
<td>Sepsis (Gram- and Gram+, possibly polymicrobial infections, 62.5% pulmonary, 37.5% abdominal)</td>
<td>Sepsis 40%, ICU control 15%</td>
<td>Within 72 h of ICU admission</td>
<td>Once</td>
<td>Heparin</td>
<td>Sepsis (n=14), SIRS (n=9)</td>
<td>Granulocytes</td>
<td>CD11b+/CD18+ and CD11p+CD177+ EVs were higher in sepsis vs. control. Similar IL-6 plasma release by septic vs. control. Procoagulant activity increased, clotting time decreased, and bacteria aggregation activity higher in sepsis vs. control.</td>
</tr>
<tr>
<td>30. Zhang (2016) [23]</td>
<td>Mixed sepsis</td>
<td>Unknown</td>
<td>&lt;24 h of disease</td>
<td>Once</td>
<td>Citrate</td>
<td>Septic (n=15), healthy (n=10)</td>
<td>Platelets, ECs, monocytes</td>
<td>All PhtdSer+ EVs were increased in sepsis vs. healthy. EVs induced coagulation at PhtdSer-exposing sites in vitro; partly reversed by PhtdSer antagonism.</td>
</tr>
<tr>
<td>31. Trepech (2016) [163]</td>
<td>Mixed sepsis, severe sepsis, septic shock, survivor vs. non-survivors</td>
<td>36.7% ICU mortality</td>
<td>At sepsis diagnosis</td>
<td>Daily up to 7 days, then every second day up to day 13</td>
<td>Unknown</td>
<td>Severe sepsis (n=6), septic shock (n=20)</td>
<td>procoagulant (PhtdSer+) EV, plateaulets, monocytes, ECs, monocytes</td>
<td>All PhtdSer+ EVs were increased in sepsis vs. healthy. EVs induced coagulation at PhtdSer-exposing sites in vitro; partly reversed by PhtdSer antagonism.</td>
</tr>
<tr>
<td>32. Delabranche (2016) [133]</td>
<td>Mixed septic shock</td>
<td>Total 34.4%, without DIC 28.3%, with DIC 45.2%</td>
<td>&lt;6 h of septic shock diagnosis</td>
<td>Unknown</td>
<td>Procoagulant activity (PhtdSer+), clotting time (alpha-2-macroglobulin+), fibrinogen, platelets, monocytes</td>
<td>Septic shock (n=259), incl. with DIC at admission (n=61), with DIC within first 24h (n=32)</td>
<td>Increased CD105+ EVs and decreased CD31+ EVs were associated with DIC. Increased CD11a+ EVs/leukocytes supported leukocyte activation.</td>
<td></td>
</tr>
<tr>
<td>33. Lehner (2016) [29]</td>
<td>Septic shock</td>
<td>ICU-mortality 66.7%, hospital-mortality 70%</td>
<td>Median (25-75% interquartile range) 12.6 h (3.1-21) from diagnosis</td>
<td>Once</td>
<td>Citrate</td>
<td>Septic shock (n=30), healthy (n=18)</td>
<td>ECs, leukocytes, platelets</td>
<td>Low but increased counts of EEVs (CD144+, CD62E+, CD106+) and increased CD31+/CD41- EVs vs. healthy. Correlation between CD11a+/CD41- EVs and leukocytes. Increased levels of CD41+ EVs and CD31+/CD41-/AnnV- EVs in 84h non-survivors. CD144+, CD62E+ and CD106+ EVs not different between DIC and non-DIC. CD66+ GEV/ neutrophilic granulocyte count higher in septic shock with DIC vs. without DIC.</td>
</tr>
<tr>
<td>34. Stiel (2016) [164]</td>
<td>Septic shock with and without DIC</td>
<td>7-day mortality: septic shock with DIC 20%; septic</td>
<td>At admission</td>
<td>Once</td>
<td>Septic shock: with DIC (n=35), without DIC (n=65)</td>
<td>Granulocytes</td>
<td>All PhtdSer+ EVs were increased in sepsis vs. healthy. EVs induced coagulation at PhtdSer-exposing sites in vitro; partly reversed by PhtdSer antagonism.</td>
<td></td>
</tr>
</tbody>
</table>
### Diagnostic and/or therapeutic potential of EVs in sepsis

Once prompted by activation and/or apoptosis, EVs that shed from the cell surface typically retain the membrane composition (or the main features thereof) of the cells they originated from. For example, the release of these vesicles from the endothelium and monocytes can be effectively simulated by incubation with endotoxins such as bacterial lipopolysaccharides (LPS), pro-inflammatory cytokines or TNFα [31]. Although extracellular vesicles differ in size, composition and biogenesis, some basic characteristics can be summarized. Irrespective of the dimensions, EVs represent spherical, subcellular compartments that are composed of a phospholipid bilayer and various membrane-bound or plasmatic cargo molecules (Figure 2). Depending on the EV release process, their membrane retains the pattern of the cell surface they originated from. Given that EVs are released from all known tissue types, the retained transmembrane molecules and phospholipids interact with countless cellular processes. The smaller EV fraction (which arises from the early endosome) and the multivesicular bodies display membrane features that resemble organelles of those cells but undergo additional fine-tuned wrapping mechanisms.

### Summary of findings

<table>
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<th>Study</th>
<th>Diagnosis</th>
<th>Mortality</th>
<th>Time to first sample</th>
<th>Sampling time points</th>
<th>Anticoagulant</th>
<th>EV sources</th>
<th>Summary of findings</th>
</tr>
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<tr>
<td>35. O’Dea (2016) [48]</td>
<td>Major burn injury vs. severe sepsis</td>
<td>16%</td>
<td>Within 24 h of injury</td>
<td>Citrate</td>
<td>Major burn injury (n=15), severe sepsis (n=12)</td>
<td>Leukocytes, monocytes, granulocytes, ECs</td>
<td>EVs increase on admission in burns vs. healthy. Only CD45+/CD14+ MEVs and CD66b+/CD11b+ GEVs increased in sepsis vs. healthy. CD45+/CD14+ MEVs and CD105+ EVs lower in sepsis vs. burns. CD45+ LEVs and CD66b+/CD11b+ GEVs increased in dying vs. surviving burn patients. No difference between dying and surviving sepsis patient.</td>
</tr>
<tr>
<td>36. Matsumoto (2017) [19]</td>
<td>Mixed SIRS vs. severe sepsis</td>
<td>16%</td>
<td>Within 24 h of injury</td>
<td>Citrate</td>
<td>Trauma patients (24), severe sepsis (n=25), healthy (n=23)</td>
<td></td>
<td>Monocytes</td>
</tr>
<tr>
<td>37. Panich (2017) [165]</td>
<td>Sepsis, septic shock</td>
<td>unknown</td>
<td>Day 0</td>
<td>Serum</td>
<td>Sepsis with AKI (n=79), sepsis without AKI (n=60), healthy (n=8)</td>
<td>Exosomes</td>
<td></td>
</tr>
<tr>
<td>38. Reithmair (2017) [66]</td>
<td>Sepsis, septic shock</td>
<td>unknown</td>
<td>Day 0 and day 4</td>
<td>Serum</td>
<td>Sepsis (n=22), healthy (n=25)</td>
<td></td>
<td>miRNA in serum, blood cells and EVs (exosomes, Tumor susceptibility gene 101+) by next generation sequencing</td>
</tr>
<tr>
<td>39. Lehner (2017) [73]</td>
<td>Severe sepsis or septic shock, medical ICU</td>
<td>33% ICU survival, 25% hospital survival</td>
<td>On average 7 h post hemofiltration</td>
<td>Citrate</td>
<td>Severe sepsis or septic shock (n=12)</td>
<td>Microparticles expressing platelet endothelial cell adhesion molecule (from platelets, leukocytes and/or ECs) and platelets.</td>
<td>Increase of CD31+/CD41+ EVs post-filter vs. pre-filter. Decreased total PhtdSer-exposing EVs and decreased TF+ EVs post-filter vs. pre-filter. No PhtdSer-exposing EVs and no TF activity measurable in ultrafiltrate.</td>
</tr>
<tr>
<td>40. Mashin (2017) [16]</td>
<td>Sepsis due to CAP or falcit peritonitis</td>
<td>50% in sepsis due to CAP, 50% in sepsis due to falcit peritonitis (selected based on survival)</td>
<td>Day 1 post ICU admission</td>
<td>Ethylene diamine tetraacetate acid</td>
<td>CAP (n=60), falcit peritonitis (n=40), healthy (n=30)</td>
<td>Granulocytes, monocytes, T-lymphocytes, platelets, erythocytes, and ECs</td>
<td>Circulating GEVs, MEVs, T-lymphocyte-derived EVs, and alpha-2-macroglobulin+ EVs (from all studied cell types) at day 1 were higher in CAP vs. falcit peritonitis and healthy volunteers. EVs only vs. healthy, EVs and EryEVs were not different. Alpha-2-macroglobulin+ EVs were higher in survivors of CAP, but not in falcit peritonitis.</td>
</tr>
</tbody>
</table>

AKI: acute kidney injury; Ann V +/-: Annexin V positive/negative; APC: activated protein C; BAL: bronchoalveolar lavage; CAP: community acquired pneumonia; CD: cluster of differentiation; DIC: disseminated intravascular coagulation; EC: endothelial cell; EVs: endothelial cell derived EVs; EPOR: endothelial protein C receptor; EryEVs: erythocyte-derived EVs; EVs: extracellular vesicles; GEVs: granulocyte-derived EVs; ICU: intensive care unit; IL: interleukin; LEVs: leukocyte-derived EVs; LPS: lipopolysaccharide; MEVs: monocyte-derived EVs; PEVs: platelet-derived EVs; PhtdSer: phosphatidylserine; rhAPC: recombinant human APC; ROS: reactive oxygen species; SIRS: non-infectious systemic inflammatory response syndrome; TF: tissue factor; TNF: tumor necrosis factor;
During endocytosis, vesicles retain their membrane integrity and are engulfed by invagination (Figure 3E). Aside from proteins and metabolites that directly provoke the transduction of signals, other content like miRNAs are capable of silencing the expression of genes (Figure 3F). By altering the posttranscriptional processing on an mRNA level, this interference not only affects the synthesis of signaling molecules but secondarily also the expression of transcription factors (Figure 3G). Finally, EVs also play a role in fundamental mechanisms of immunity, including cytokine synthesis and antigen presentation (Figure 3H).

Any compound to serve as a potential biomarker must be stable enough to enable a reproducible detection in the hospital setting. Availability of sensitive assays to quantify cellular and/or circulating compounds and metabolites (including their genomic or transcriptional fingerprints) has enabled us to identify “signatures” of potentially diagnostically relevant biomarkers in virtually any biological sample, such as the blood [32], urine [33,34], cerebrospinal [35] and nasal [36] fluid.

EVs represent cellular mediators that are released from all known cell types via a number of evolutionarily conserved mechanisms [37]. Sepsis triggers a multifaceted cellular activation including massive shedding of membrane vesicles from various cell subtypes [38]. Although other methods have been recently attempted [39], flow cytometry is currently the main technology for the high-throughput characterization of EVs. When EVs are used for further investigation, fluorescence-activated cell sorting, a specific form of flow cytometry by which cells are sorted based on their light scattering and fluorescence characteristics can be used. While flow cytometry by itself is reliable and sensitive, the standardization procedures for the detection of EVs are not. Currently, the utility/fidelity of EVs as biomarkers cannot be judged without defining clear standard operation procedures. Specifically, a reproducible detection and in-depth characterization of circulating EVs depends on two key variables: a) sample preparation (e.g., anticoagulation, storage time and temperature, agitation during manipulation or transport) [17,26] and b) precisely defined flow cytometry settings [40]. The international societies have been striving to reach an acceptable consensus on standardized sample preparation, documentation and measurement procedures [26,41-44]. Recently, the applicability of size-calibrated beads for the standardization of the EVs count analysis using flow cytometry was tested on platelet-free plasma from healthy volunteers by 44 laboratories using 14 different types of cytometers (with central collection and analysis of raw data). Although the test
demonstrated an acceptable variability among most commercial cytometers [40], these results need to be verified using clinical samples. Another important limitation of flow cytometry is that a large fraction of EVs remains under the lower limit of detection of the majority of available commercial cytometers (currently around 0.3 µm). This caveat should not be ignored given that the value of EVs as biomarkers may lie in recognizing changes in the ratios of specific EVs subpopulations and/or their presence/absence in a biological material. Such an EV-based approach has shown its value as a diagnostic support in the trauma setting [45].

From the technological standpoint, for EVs smaller than 0.3 µm, the method of choice is nanoparticle tracking analysis, which enables detection of specific antigens with fluorescence filter sets [46]. Although technologically advanced, this method has not yet been verified for EV-based diagnostics in septic patients. Regarding timing, a number of new point-of-care technologies that may deliver results within 2 hours are currently being studied. For example, Herrmann et al. used the aggregation of granulocyte-derived EVs (GEVs) and bacteria for the differentiation of infectious versus non-infectious inflammatory states using a microfluidic chip [47,48]. Another promising method is the lateral flow immunoassay: a one-step chromatographic immunoassay that has undergone continuous performance improvement by the use of modern labels such as gold nanoparticles, magnetic particles, carbon nanoparticles, colored latex beads, quantum dots, organic fluorophores, enzymes and liposomes. Oliviera-Rodriguez et al. have recently tested the value of this assay for the detection of (CD63 and CD9-positive) platelet-derived EVs (PEVs) in human plasma and identified gold nanoparticles as the optimal label [49,50]. Future studies testing the applicability of the lateral flow immunoassay for the early detection of sepsis and its complications using EVs derived from other sources are warranted. Table 2 identifies the main developmental directions needed for closing the current technological gap in EV research.

### Table 2. The most relevant technological challenges in the field of EV.

<table>
<thead>
<tr>
<th>Detection of EVs below 0.3 µm (i.e., nanoparticles)</th>
<th>Precise receptor/cargo-based EV characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentiation between EV-associated and non-EV-associated miRNA in plasma</td>
<td>Development of quick point-of-care tests based on EVs</td>
</tr>
<tr>
<td>Development of EV-based vaccination techniques</td>
<td>Elimination of detrimental EVs from the bloodstream</td>
</tr>
</tbody>
</table>

The main portion of the EV pool under steady-state conditions is platelet-associated (i.e., resting/activated platelets, megakaryocytes) [51,52]. In pathological conditions, however, EVs are mostly derived from activated platelets, endothelial cells (ECs) and leukocytes. These EVs accumulate rapidly in the circulation and are readily detectable by flow cytometry [53,54]. The presence/absence, dynamic changes and/or composition of circulating EVs have a potential to be harnessed as fine-tuned diagnostic descriptors of ongoing pathophysiological inflammatory processes in sepsis (and beyond). In addition to the considerable role of EVs as markers of a systemic inflammatory response, an elevation of TF-bearing vesicles could be considered as an early indicator of a generalized (in some cases even focal) infection. TF-positive EVs could be used to guide further or more aggressive treatment with antimicrobials [55,56]. The mediator/effector role of PhdTSer/TF-expressing EVs in septic coagulopathy (blood clotting disorder) is detailed in the last subchapter in this review.

Besides analysis of EVs on the basis of vesicle surface antigens (the most important marker molecules displayed in Table S1), identification of cargo like micro-ribonucleic acid (miRNA) also appears to be an interesting research tool [57]. MiRNAs are short (19-25 nucleotides) noncoding sequences that are both actively and passively incorporated into EVs and that are capable of regulating gene expression by interfering with the translation of messenger RNA. In a preclinical sepsis study, miR-223 containing EVs released by mesenchymal stem cells attenuated cecal ligation and puncture-induced cardiac dysfunction in mice by reducing the Sema3a and Stat3 transcription factors [58]. Moreover, miR-223 is involved in the modulation of hematopoiesis and is seen as a promising indicator of immune response dysregulation [59,60]. Combined with the expression pattern of other miRNAs, miR-223 could potentially help to distinguish between non-septic and septic patients [61]. Studies in cancer patients have shown that miRNA phenotypes are consistent and reproducible among individuals and serve as a cluster of markers for a number of diseases [62,63]. Other miRNAs, such as miR-15a, miR-30d-5p, miR-30a-5p, miR-192-5p, miR-26a-5p, miR-23a-5p, and miR-191-5p appear to be capable of differentiating septic patients and those with non-infectious inflammation (also called systemic inflammatory response syndrome, SIRS) [64,65]. Some miRNAs are detectable in exosomes and serum only, whereas others are also present in multiple cell types [66]. Moreover, miR-21 has been shown to be an essential part of the protective effect of remote ischemic preconditioning in sepsis [67]. Another recent report demonstrated EVs as messengers that...
can suppress miRNA expression and activate downstream gene expression in the central nervous system during inflammatory states [68]. However, research on the utility of EV-derived nucleotide sequences is in its infancy. Amplification and detection of miRNAs is usually performed via reverse transcriptase polymerase chain reaction and next-generation sequencing. However, these methods do not adequately discriminate between circulating, Argonaute protein-bound miRNA and miRNA incorporated to EVs. Studies investigating miRNA in sepsis (not focused on EV detection) may in fact have measured EV-associated miRNA (and vice versa). Innovative detection technologies to bypass this limitation are urgently needed (Table 2). Given that there are no clear guidelines regarding EV-RNA analytics, any findings should be interpreted with caution [69].

In addition to their role as biomarkers, circulating EVs constitute a dynamic pool of messengers that allow an organism to rapidly respond to altered physiological conditions. Thus, in their mediator-like character, they are capable of conveying a multitude of information to even distant tissues within the body. In a short-range communication format, EVs released by various cells could affect other immuno-competent cells/tissues in their immediate surroundings modulating, for example, a focal inflammatory process. Additionally, EVs may also support cell communication over longer distances. For example, analogous to the soluble endothelial protein C receptor (EPCR), EVs expressing EPCR are able to bind activated protein C (APC) upon external administration of recombinant human protein C in septic patients in order to exert its effects at distant vascular sites [70]. This mode of mediation appears to be especially relevant in diseases involving endothelial activation and microvascular dysfunction (e.g., sepsis) given that they are accompanied by a robust release of EVs. Sepsis-induced acute kidney injury (AKI) is associated with an impairment of endothelial barrier function and the sequestration of (endothelial cell-derived) EVs is thought to be a substantial causative factor in AKI pathophysiology [71]. In the future, excessive EVs could be eliminated using continuous veno-venous hemofiltration [72] or be used for optimizing filtration fractions during hemofiltration [72,73].

![Figure 3. Modes of interaction of EVs with receiver cells.](http://www.thno.org)

EVs represent cellular shuttles that are capable of transferring a variety of compounds between cells. (A) Upon fusion, vesicles rapidly modify the membrane composition of the receiver cell by transferring phospholipids and transmembrane molecules. (B) Meanwhile, a variety of cargo molecules are directly ejected into the plasma of the cell. Compounds like growth factors, cytokines or other mediators are able to provoke an immediate metabolic response and to directly interfere with the receiver cell's signal transduction. (C) During states of systemic inflammation and cell activation, tissue factor and phosphatidylserine-bearing vesicles represent microcarriers for the dissemination of a procoagulant phenotype. (D) Any component of the vesicle membrane can function as a ligand for receptors at the surface of the receiver cell, thereby triggering a multitude of responses. (E) During endocytosis, vesicles retain their membrane integrity and are engulfed by invagination. (F) Aside from proteins and metabolites that directly provoke the transduction of signals, other content like micro ribonucleic acids (RNA) are capable of silencing the expression of genes. (G) By altering the posttranscriptional processing on a messenger RNA level, this interference not only affects the synthesis of signaling molecules but secondarily also the expression of transcription factors. (H) Finally, EVs also play a role in fundamental mechanisms of immunity, including cytokine synthesis and antigen presentation.
In summary, EVs appear to simultaneously serve as both markers and mediators in sepsis syndromes. This creates a multidirectional opening for their potential utilization: either diagnostic, therapeutic or both (depending on a specific sepsis scenario/phenotype and objective).

**EVs in sepsis: a friend or foe?**

EVs are involved in severe pathophysiological events such as infections and/or inflammatory processes. The existing literature [74] implies that EVs play an important role in sepsis and septic shock. Given that both the pathogen and the host appear to rely on EVs as their tool of either attack or defense, it needs to be precisely deciphered which EVs populations mediate and promote progression of sepsis and which support its resolution and propel healing. The most intuitive separation can be done based on the source of EV release.

Bacteria, viruses and fungi responsible for septic infections are all well-capable of producing and secreting varieties of membrane vesicles, yet without the specific surface lipid composition seen in eukaryotic microbes [75-81]. Gram-positive and Gram-negative bacteria, as the most frequent cause of sepsis [82], can release EVs (in Gram-negative termed outer membrane vesicles) that may carry toxins, adhesins, proteolysins and other virulence factors relevant for the development of the systemic inflammatory response [83-86] and/or subsequent dysfunction of organs and tissues. Incubation of *C. albicans*-derived EVs with bone marrow-derived macrophages resulted in synthesis of IL-10, IL-12, transforming growth factor-beta and nitric oxide, as well as increased expression of major histocompatibility class II and cluster of differentiation (CD)-86 on macrophages [87] - all suggesting a strong immunomodulating potential for fungal EVs during infections [80]. Similarly, development of sepsis, a leading cause of death in HIV type 1 patients [88], is promoted by exosomes from infected cells containing the accessory extracellular viral protein Nef, which was shown to induce apoptosis of CD4-T-cells [89]. Viruses are also capable of controlling the release and content of EVs produced by infected cells [90] to facilitate the viral spread. In this context, the foes could potentially become friends by protecting vulnerable cohorts of patients against specific hospital pathogens (e.g., *S. aureus* or *E. coli*) to prevent secondary infections. For example, bacterial protoplast-derived nanovesicles have been used to vaccinate mice against these germs in models of pneumonia and peritonitis [91]. In the context of the growing antibiotic resistance crisis, this could be a life-saving preventive option (Table 2).

The second major pool of EVs stems from the host organism and can be equally bio-active as the EVs released by microorganisms. Key sources of host EVs during sepsis are circulating cells such as platelets and leukocytes [92,93], the latter being predominantly innate immune cells such as neutrophilic granulocytes, macrophages, dendritic and natural killer (NK) cells [94]. In sepsis, especially neutrophilic granulocytes show an increased activation but also higher apoptosis rates [95,96]—the two main conditions that trigger a robust EV release [97-100]. Unlike the microbial EVs with predominantly detrimental effects (as perceived based on the current literature), the action of the host-released EVs appears to be more heterogeneous. A number of protective EV-related mechanisms were recently implied. In a clinical study, GEVs were found in the infectious foci rather than in the circulation [101]. Due to the functional relationship to their parent cells, GEVs can exhibit antibacterial effects, support the production of inflammatory mediators [102-104] and/or protect from vascular dysfunction [103,105]. Selected danger-associated EV subsets (containing alpha-2-macroglobulin excreted by neutrophilic granulocytes) were shown to improve resolution of infection by enhancing bacterial clearance in a mouse sepsis model [105,106] and by interfering with leukocyte-trafficking in vitro [107]. Similarly, when THP-1 monocytes phagocytized GEVs which were previously isolated from peritoneal and broncho-alveolar lavages of patients with surgical sepsis, their general activity and phagocytic capacity significantly increased in vitro [101,105]. In septic rats, EVs produced by immature dendritic cells reduced mortality by diminishing TNFα and high mobility group box-1 release and supported engulfment of apoptotic cells [108].

In contrast, the emerging evidence demonstrates that some EVs released by leukocytes in sepsis can be harmful. For example, in a porcine model of endotoxemia, danger-associated populations of PEVs robustly increased in dying but not in surviving animals [109]. Furthermore, lymphocyte apoptosis, one of the major abnormalities observed in both pre-and clinical sepsis [110,111], was induced by caspase-1 released from circulating EVs in septic patients [110]. While the precise EV source was not determined in that study, peripheral blood monocytes are potential candidates given that they were shown to release EVs containing caspase-1 which induced apoptosis of smooth vascular muscle cells in vitro [112]. Blocking the release of EVs from bacteria-infected macrophages by pre-treatment with a sphingomyelinase inhibitor in septic mice had cardioprotective effects and prolonged survival [113].
It has also been suggested that EVs contribute to the development of sepsis-induced acute lung injury [94]. Intratracheal application of EVs released from LPS-primed alveolar macrophages to C57BL/6 mice provoked their alveolar macrophages to release EVs, which caused an increased expression of adhesion molecules, protein and neutrophilia [94]. In another example, dendritic cells activated by LPS released EVs that stimulated epithelial cells to secrete chemokines (e.g., IL-8 and Chemokine (C-C motif) ligand 5) which are important soluble components of a physiological innate immune response but can simultaneously fuel sepsis by excessive cytokine release [114]. Circulating EVs (without classification) containing reactive oxygen species (ROS) from LPS-stimulated mice induced vascular leakage and cardiac dysfunction in C57BL/6 mice [115]. The roles of exosomes in septic cardiac dysfunction have recently been reviewed [116].

Many other leukocyte subpopulations are capable of EV release but their role in sepsis has not yet been adequately investigated. NK cells are a suitable example. In contrast to other immune cells, NK cells produce EVs constitutively and independently of their parent cell activation status. NK cells contain so-called killer proteins such as Fas ligand and perforin that influence the tumor growth and immune system homeostasis [117]. Interestingly, NK cell-derived vesicles only act against the activated immune cells. This indicates that they could actually modulate septic processes by controlling the expansion and proliferation of stimulated immune cells. Another example is the potential function of EVs in adaptive immunity. Stimulated CD4+ and CD8+ T-cells can induce cytokine production (TNF-alpha, IL-1-beta, soluble IL-1 receptor antagonist) by human monocytes by either direct contact or by releasing EVs as messengers [118].

In summary, the existing evidence strongly suggests that EVs have both protective and detrimental roles, either locally or at distant sites. Whether EVs assume the shape of a friend or foe appears to partly depend on their origin (e.g., microorganism vs. host) and/or specific pathophysiological trait examined.

**Role of EVs in specific subsets of septic patients**

**Role of EVs in disturbed coagulation**

Although initiation of thrombotic pathways may inhibit dissemination of bacteria through the body, it may also lead to excessive activation and consumption of coagulation components. With their procoagulant potential, EVs are likely involved in the pathophysiology of DIC in septic patients [119]. The development of DIC is a severe sepsis complication; its manifestation is associated with doubled mortality rates and is characterized by the massive consumption of coagulation factors and platelets and the excessive infiltration of thrombi in the microcirculation. It is estimated that approximately 35% of sepsis patients develop DIC [120]. Currently, the management of sepsis complicated by DIC comprises combating the underlying infection by surgical source control, broad spectrum antibiotics and general goal directed therapy [121] with organ function support and/or replacement therapy. So far, other treatments have not shown to be beneficial in large prospective, randomized trials. In patients who do not present with DIC in the initial phase of sepsis, early assessment of the risk for developing DIC would be beneficial. Thus, determination of EVs may aid in assessing such a risk and in identifying possible targets for treatment. EVs may exhibit direct procoagulant properties via exposure of PhtdSer on their surface, a cell membrane phospholipid that supports the assembly of coagulation enzymes and TF, which is the main initiator of the coagulation cascade [38,122]. In sepsis, procoagulant (PhtdSer-exposing) EVs (typically identified by Annexin V staining and flow cytometry) are mainly released by platelets (PEVs), but also by endothelial cells (EEVs) and monocytes (MEVs) [28]. TF is the prime initiator of the coagulation cascade whereas PhtdSer serves as a catalyst for the activation of coagulation factors. Both functions make this vesicle subset a highly potent vector for the dissemination of a pro-coagulant phenotype throughout the circulation [23,123]. As discussed above, the main source of TF in plasma is PEVs [124]. Together with the up-regulation of TF on endothelial cells and monocytes, as well as the general activation of prothrombotic and fibrinolytic pathways, blood-borne TF-positive EVs are therefore also co-responsible for the prothrombotic milieu that underlies DIC [122,125]. This highly lethal complication of sepsis (associated with doubled mortality) is characterized by the massive consumption of coagulation factors and platelets and the excessive infiltration of thrombi in the microcirculation.

Several observations also support the more indirect role of EVs as ‘vehicle carriers’ that link inflammation to coagulation. Our lab recently showed that the release of EVs is a danger signal that eventually promotes the transition to a pro-thrombotic phenotype [126] and that IL-33 is involved in increased TF activity in EEVs [149]. The release of vesicles can occur downstream of multiple signal transduction pathways, depending on the

http://www.thno.org
initial receptor-activation by specific ligands. Sculpting and budding at the cell membrane or within the multivesicular bodies is mediated by highly conserved vesicular trafficking entities like the endosomal sorting complexes required for transport [127]. During inflammatory activation of cells, the increase in intracellular calcium results in the activation of calpains (calcium-activated neutral cysteine proteases) and scramblase and the loss of phospholipid bilayer asymmetry [128,129].

Furthermore, in vitro and in vivo endothelial and systemic activation by thrombin/CD40L and LPS enhanced EVs carrying matrix metalloproteinase 10 and CD40L. Elevation in circulating matrix metalloproteinase 10 and CD40L was associated with an increased mortality in septic patients who had enhanced thrombin formation [130]. Boisramé-Helms et al. showed that inoculation with EVs derived from septic rats into a cohort of healthy rats lowered their arterial pressure (not observed when ‘donor’ animals were treated with APC) [131]. Also blockage of calpains appears to exert therapeutic effects. Zafrani et al. studied the role of calpains in the development of DIC during sepsis in a clinically relevant animal sepsis model; they observed a survival benefit by attenuation of DIC after blocking calpains by overexpressing its endogenous inhibitor calpastatin [148].

EVs appear as promising markers of patients with DIC. For example, in meningococcal sepsis patients with multiple organ dysfunction, circulating EVs were mainly platelet-derived and their number was almost 15-fold higher compared to healthy controls [53]. Furthermore, EVs exposing TF were decreased in patients with sepsis and multiple organ dysfunction [28]. However, another study in patients with meningococcal septic shock demonstrated higher procoagulant activity of EVs compared to non-shock patients [132]. Overall, septic patients would greatly benefit from early markers that can reliably predict DIC, enabling in turn early and adequate treatment stratification. CD105+ EVs and CD31+ EVs are promising marker candidates that warrant further validation [122,133].

**Role of EVs in blood transfusion**

Septic patients regularly develop anemia, often necessitating transfusions of erythrocyte concentrates in case of insufficient oxygen delivery. Transfusion of blood components has been associated with adverse effects such as increased risk of infection [134]. However, the exact mechanisms of this phenomenon remain elusive. It has been recently suggested that EVs present in blood products trigger various inflammatory pathways [135-140]. Vlaar et al. showed a release of TNF-alpha, IL-6 and IL-8 after whole blood was incubated with the supernatant of red blood cell (RBC) concentrates, which was associated with the storage time of the RBC concentrate and the amount of EVs present in the supernatant [141]. Interestingly, no pro-inflammatory response was observed when the supernatant was depleted from EVs before incubation. The same authors showed that endothelial cells incubated with monocytes expressed more intercellular adhesion molecule-1 and E-Selectin only in the presence of EV-containing RBC supernatants. EVs were phagocytized by monocytes in a complement receptor 3-dependent fashion and elicited expression of Von Willebrand factor but not TF [142]. In murine transfusion models, EVs from RBCs affected the pulmonary endothelium via adhesion molecules [143] and thrombin-dependent complement activation [144]. The existence of such an activation effect is supported by pre-clinical evidence: hemorrhaged mice resuscitated with RBC and plasma rapidly accumulated neutrophil granulocytes in the lungs when the resuscitation fluids contained RBC-derived EVs [145]. Those EVs also induced increased neutrophilic CD11b expression after i.v. administration in mice and in vitro incubation with human neutrophilic granulocytes [145]. The occurrence of acute lung injury by blood product-derived EVs may be explained by the two-hit phenomenon: the clinical condition of the critically ill patient leads to polymorphonuclear granulocyte recruitment to the activated pulmonary microvascular endothelium (first hit) which is further aggravated by the activation of adherent PMNs by blood product transfusion (second hit). This can eventually lead to destruction of pulmonary ECs, capillary leakage and acute respiratory distress syndrome.

**Role of EVs in mechanical ventilation**

Mechanical ventilation may result in ventilator-induced lung injury. It appears that EVs can influence the pathophysiology of this and other lung-related conditions in the ICU patients. For example, increased EVs have been recently associated with a reduced risk of the acute respiratory distress syndrome [146]. More specifically, Mutschler et al. investigated the effects of mechanical ventilation upon EVs present in the broncho-alveolar lavage fluid of ventilated pigs and human patients at post-operative extubation [147]. They found that EVs are activated and adhere to neutrophil granulocytes in the pulmonary air-blood interface and suggested the use of EVs to guide the mechanical ventilation strategy. For example, a defined EV concentration could aid in deciding whether a patient with acute respiratory distress syndrome requires temporal...
extracorporeal membrane oxygenation in order to prevent any further lung injury from mechanical ventilation.

Few pre-clinical and in vitro studies further underline an active role of EVs in ventilator-induced lung injury. A pathological pulmonary EC stretching model (mimicking excessive tidal volume ventilation) induced EV generation which was counteracted by the caspase inhibitor Z-VAD [148] and was independent of co-treatment with thrombin, LPS, the Rho kinase inhibitor Y-27632 and calpeptin (a calpain inhibitor). In another study, high (in contrast to low) tidal volume ventilation in healthy mice increased systemic levels of pulmonary endothelium (CD31+)-derived EVs and decreased CD31 in the lung tissue. The same mechanism was investigated using a stretch model of human pulmonary EC which shed (Annexin V and CD31 positive) EEVs [149]. Those EEVs induced lung inflammation when instilled intratracheally into healthy mice. Subsequent proteomic analysis found multiple common molecules shared by the different EEV populations, e.g., CD31 [149]. The above examples strongly imply that disintegration of the pulmonary endothelial barrier (due to high volume ventilation) provokes shedding of EVs that express several adhesion molecules which likely exert strong systemic downstream effects upon inflammatory processes [150]. In a recent study, elevation of EVs in plasma was inversely associated with the risk for the development of acute respiratory distress syndrome in a group of critically ill patients with varying underlying pathologies. Interestingly, the closer post-hoc analysis revealed that this association was true in the septic patient cohort (representing less than half of the total studied population) but not in non-septic patients [146].

Conclusions and outlook

Within the last two decades, the multifaceted roles of EVs in inflammation have entered the focus of sepsis research. Given that both sepsis and EVs are two very complex fields and the existing evidence regarding the intricacies of EVs in sepsis is limited, no definitive connecting lines can be currently drawn between those two entities. However, a number of promising speculations pertaining to the role of EVs in sepsis are justified.

First, it is convincing that EVs have a strong role as mediators in sepsis. It has also become apparent that EVs can act both as friends and foes in systemic inflammatory reactions; their friendly or hostile character largely depends on the EV origins and cargo they carry. The latter element makes specific EV subsets potential candidates for drug delivery systems. In the future, the above EV properties could facilitate more precise and individualized treatment approaches.

The identification of specific EVs subsets remains fundamental for the isolation and application in diagnostic and/or therapeutic procedures. At the moment, however, it is clear that the restrictions of characterization (hence our biological understanding of EVs) are limited by the methodological isolation/detection boundaries. The gold standard for high-throughput detection of EVs based on surface antigens is flow cytometry with a current detection limit of well above 0.3 µm. To our knowledge, nanoparticle tracking analysis has not yet been tested in septic patients. Although size transition of EVs is fluent, discrimination of EV subsets is largely based on their dimension, a measure that ignores the biogenesis of the vesicles. Development of new methodologies to rapidly assess circulating miRNA and miRNA incorporated to EVs should have a high priority. Furthermore, quick point-of-care tests such as microfluidic chip tests and lateral flow immunoassays have a great potential to be used with plasma of septic patients, but need additional testing. Given their strong immunomodulatory capabilities and vast diversity of cargo molecules, EVs present a considerable therapeutic potential for the treatment of sepsis. It is not impossible to imagine that EVs could serve in the future ICU as new types of vaccines against secondary infections (Table 2).

Overall, the potential utility points for EVs in sepsis (and other ICU conditions) are multifaceted, reaching from preventive to diagnostic and therapeutic. Yet, before any of these points migrate into the clinic, basic methodological challenges in EVs detection have to be solved and any viable application of EVs must be first a) standardized for detection/description, b) evaluated for clinical applicability, c) validated in multi-center randomized clinical trials and d) thoughtfully adopted for clinical use.

Abbreviations

AKI: acute kidney injury; AnnV +/-: Annexin V positive/negative; APC: activated protein C; BAL: broncho-alveolar lavage; CAP: community acquired pneumonia; CD: cluster of differentiation; DIC: disseminated intravascular coagulation; EC: endothelial cell; EEVs: endothelial cell derived EVs; EPCR: endothelial protein C receptor; EryEVs: erythrocyte-derived EVs; EVs: extracellular vesicles; GEVs: granulocyte-derived EVs; ICU: intensive care unit; IL: interleukin; LEVs: leukocyte-derived EVs; LPS: lipopolysaccharide; MEVs: monocye-derived EVs; miRNA: micro ribonucleic acid; NK: natural killer;
PEVs: platelet-derived EVs; PhthSelSer: phosphatidylycerine; RBC: red blood cell; ROS: reactive oxygen species; SIRS: non-infectious systemic inflammatory response syndrome; TF: tissue factor; TNF: tumor necrosis factor.

Supplementary Material
Supplementary Table S1.
http://www.thno.org/v08p3348s1.pdf

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Competing Interests
The authors have declared that no competing interest exists.

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Susanne Drechsler obtained her diploma in veterinary medicine at the University of Veterinary Medicine in Vienna, Austria. She then enrolled for a doctoral program to study the effects of age and gender in a mouse model of posttraumatic sepsis in Marcin F. Osuchowski’s group at the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology and received her DVM degree from the University of Veterinary Medicine in Vienna, Austria. Susanne currently leads a FWF Hertha Firnberg project investigating the complement system in posttraumatic sepsis.