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Performance of microvesicles as biomarkers of clinical outcome in sepsis and trauma: A pilot study

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ABSTRACT

Sepsis remains one of the main causes of death in intensive care unit (ICU) worldwide, despite all technological and scientific advances. Microvesicles (MV) have become promising biomarkers for quick and accurate monitoring of several illnesses. The aim of this pilot study was to characterize and evaluate the performance of MV as biomarker of clinical outcome in septic and trauma patients. For this purpose, 39 subjects, both genders, aging from 18 to 85 years were included in three groups referred as Sepsis, Trauma and Healthy Control. Kinetic analysis of MV was carried out at four consecutive time points: admission (baseline)/T1, 24 h/T2, 72 h/T3 and outcome/T4 of discharge or death. At admission, an overall increase in total MV (Annexin V^+) was observed in Sepsis.MV $CD14^+$ (monocytes) was a putative biomarker to identify trauma patients, while MV $CD3^+$ (T-cells) and $CD41^+$ (platelets) were qualified to discriminated Trauma from Sepsis. Sepsis (Death) presented an increase in MV Annexin V^+, $CD45^+$, $CD16^+$, $CD14^+$, and CD41+ in comparison to Sepsis (Discharge). Moreover, Trauma (Death) presented an increase of MV $CD3^+$ and $CD235^+$ as compared to Trauma (Discharge). Analysing the ROC curve of specific MV evaluated according to performance, an accuracy of 100% was found to segregate the outcome in sepsis, and 95% in trauma. Our findings suggest that MV might be useful as a potential role in discriminating outcome in patients with sepsis/septic shock and trauma with high accuracy. However, further studies with a larger number of participants will be necessary to validate our findings.

1. Introduction

Sepsis remains one of the main causes of death in intensive care units (ICU) worldwide [1,2], despite all technological and scientific advances. In 2017, almost 50 million cases of sepsis were registered worldwide and 11 million deaths related to sepsis were reported [3]. The Latin American Institute of Sepsis (ILAS) released a 46% global lethality rate from sepsis in Brazil in 2016 [4].

The response to a systemic and acute inflammatory reaction to a series of aggressions, in particular bacterial infection (sepsis) and tissue damage (trauma) [5–7] leads to the activation of a series of mediators, including cytokines, leukocytes, and the coagulation cascade [8].

Abbreviations: CD, Cluster of Differentiation; ICU, Intensive Care Unit; MV, Microvesicles; PFP, Platelet-free plasma; PPP, Platelet-poor plasma; RNA, Ribonucleic acid; ROC, Receiver-Operating Characteristics; AUC, Area Under the Curve.

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Indeed, this uncontrolled immune response is expressed by exacerbated production of several inflammatory and anti-inflammatory mediators responsible for organs dysfunction and coagulation disorders [9].

The use of biomarkers is extremely important in the context of critically ill patients; they are indicators of normal biological processes that can be altered in the most diverse types of diseases and their use can help in diagnosis, prognosis, and clinical monitoring. In diagnosis, biomarkers identify a pathological process and are universally used in ICUs. In monitoring, they detect response to therapy and allow for adequate treatment. Their prognostic value is limited or still remain unclear and should be interpreted in the context of multiple clinical data [10]. Thus, numerous other markers have been evaluated in critically ill patients and their importance lies in the ability to assess responses to therapy.

In 2018, Raeven, P., et al. [10] indicated microvesicles (MV) as a promising biomarker for use in quick and accurate diagnoses, even avoiding the possibility of unnecessary interventions with the application of individualized approaches. Therefore, circulating MV can play a potentially crucial role, since the pathophysiological mechanisms of vascular hyporesponsiveness are poorly understood, and the origin and actual role played by MV in sepsis are not fully understood.

MV are small vesicles (usually < 1 μm in diameter) released from the plasma membrane of activated or apoptotic cells. They were first described in 1940 when human plasma or serum was observed to contain a subcellular factor that facilitated fibrin formation. In 1967, electron microscopy techniques demonstrated that this subcellular factor consisted of small vesicles, which were named platelet dust, as they presented procoagulant activity, similar to platelets [11]. MV are known to be present in the blood of healthy or sick individuals; the total of circulating MV composes a mixture of vesicles released from blood cells and those belonging to blood vessels; it is also assumed that they may be derived from tumours and healthy bone marrow progenitor cells. MV are formed from the plasma membrane of their cell of origin [12]. During the release of MV, the phospholipid phosphatidylserine is exposed on their outer membrane and reveals membrane antigens that indicate their cell of origin [11,13].

Therefore, interest in MV has increased substantially in recent years, especially after the demonstration of their increased blood concentration in clinical situations where thrombotic risk is high. Fonseca, et al., 2015 [13] described that no biological importance was given to MV until the 1990 s, and they were considered inert particles resulting from cell destruction or just markers of apoptosis; however, in 1996, Raposo, et al. [14] suggested the importance of MV in the adaptive immune response. Since then, several studies have shown the importance of these vesicles as vectors of intracellular exchange of biological information through the identification, characterization, and quantification of MV in various situations such as obesity, diabetes, heart attack, depression, cancer, HIV, and renal failure. According to Fonseca, et al., 2015 [13], healthy individuals and those with different diseases could have MV in their plasma.

Considering the above, there are gaps to be answered regarding the uncontrolled inflammatory condition in sepsis, as well as the behaviour of biomarkers in the evolution of this disease, including MV.

Therefore, the importance of researching biomarkers that can be used to monitor the severity of sepsis and differentiate it from other non-infectious inflammatory conditions is evident.

2. Objectives

The aim of this study was to characterize the profile of circulating MV as a biomarker of clinical outcome in sepsis and trauma.

3. Material and methods

3.1. Study type and location

A prospective cohort study was carried out in an adult ICU and an emergency room at Hospital das Clínicas, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil. The study was performed from February, 2019 to March, 2020. Laboratory analysis was performed at Grupo Integrado de Pesquisas em Biomarcadores, Instituto René Rachou, Fundação Oswaldo Cruz, FIOCRUZ-Minas, Belo Horizonte, MG, Brazil.

3.2. Ethical aspects

The study was submitted and approved by the Ethical Committee of Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Written informed consent was obtained from the patient or from an appropriate surrogate if the patient was unable to provide consent. This study fulfilled resolution no. 466/2012 from the Brazilian National Health Council for research involving humans.

3.3. Study population

Thirty-nine subjects, both genders, aging from 18 to 85 years were included and categorized into three groups, referred as: Sepsis - sepsis/septic shock patients; Trauma - trauma victims and Healthy Control - reference group. The Sepsis group consisted of 10 patients admitted to the ICU. Patients with sepsis/septic shock who had been admitted for more than 12 h were excluded. The criteria used to diagnose sepsis followed the recommendation of the Surviving Sepsis Campaign 2016 [15]. All sepsis/septic shock patients received fluid resuscitation, use of vasopressors and broad-spectrum antibiotics, which were later adjusted according to culture results.

The Trauma group consisted of 19 critically ill non-septic patients from the same hospital, victims of multiple trauma with systemic inflammatory response criteria such as tachycardia, tachypnea, high levels of c-reactive protein and leukocytosis, with age and gender ratio similar to those of septic patients.

Sepsis/septic shock patients and trauma victims were followed until hospital outcome and further categorized according to discharge or death as follows: S(Discharge) or S(Death) and T(Discharge) or T (Death).

The Healthy Control group included 10 healthy individuals, employees of the hospital where the study was conducted, with similarity in gender and age to the study groups.

Exclusion criteria comprised: pregnancy, HIV infection, haematological diseases, bone marrow transplantation, patients undergoing chemotherapy or taking immunosuppressive medicine as well as patients with immune-mediated inflammatory diseases.

The Table 1 shows detailed demographic and clinical characteristics of the study population. The compendium of the study population is summarized in the Fig. 1.

3.4. Biological samples

Blood samples (five ml) were collected by venepuncture using sodium citrate as anticoagulant in a vacuum system (Vacutainer Blood Collection Tube, BD Medical, Franklin Lakes, NJ, USA). Samples from sepsis/septic shock and trauma patients were collected upon ICU admission/baseline (time 1-T1), 24 h (time 2-T2), 72 h (time 3-T3) and at outcome (time 4-T4). Samples from healthy controls were collected at a single time point. Training and instructions were given to all nursing professionals to ensure that blood sampling followed the standard protocol.

Samples were processed within four hours after collection to obtain the platelet-poor plasma (PPP) by centrifugation at 600 x *g* for 10 min at room temperature. Subsequently, the PPP samples were centrifuged at high speed (1500 x g) for 5 min to obtain platelet-free plasma (PFP). The PFP samples were divided into three one ml aliquots for storage in a freezer at −80 °C until MV analysis by flow cytometry.

Table 1
Demographic and Clinical Characteristics of the Study Population.

Parameters	Sepsis n = 10	Trauma n = 19	Healthy Controls n = 10
Age (mean, years)	59	34	39
Female, n (%)	6 (60)	6 (32)	6(60)
Comorbidities, n (%)			
Hypertension	4 (40)	2 (10.5)	–
Diabetes Mellitus	3 (30)	2 (10.5)	–
COPD	2 (20)	0	–
Infection*			
Pneumonia, n (%)	4 (40)	–	–
CAUTI, n (%)	2 (20)	–	–
Peritonitis, n (%)	1 (10)	–	–
Endocarditis, n (%)	1 (10)	–	–
Mediastinitis, n (%)	1 (10)	–	–
Osteomyelitis, n (%)	1 (10)	–	–
SAPS 3 (mean)	70	–	–
Death probability (mean)	65	–	–
Outcome, n (%)			
Discharge	7 (70)	16 (85)	–
Death	3 (30)	3 (15)	–

COPD: Chronic obstructive pulmonary disease, CAUTI: Catheter-associated Urinary Tract Infections; SAPS3: Simplified Acute Physiology Score III. * Among sepsis/septic shock patients, nine presented germ isolation: pneumonia (three isolation of *Morganella morganii* in hemoculture samples and one isolation of *Pseudomonas aeruginosa* in tracheal aspirate); CAUTI (one isolation of *Pseudomonas aeruginosa* and one isolation of *Escherichia coli* in uroculture); Peritonitis (one isolation of *Klebsiella pneumoniae* in uroculture); Endocarditis (one isolation of *Staphylococcus epidermidis* in hemoculture); Mediastinitis (one isolation of *Staphylococcus aureus* in hemoculture); Osteomyelitis (one isolation of *Enterobacter cloacae* in bone fragment culture).

3.5. Phenotypic analysis of MV by flow cytometry

Analysis of MV in plasma was performed by flow cytometry as previously described by Bode and Hickerson (2000). PFP samples stored in a − 80°C freezer were thawed and diluted in a citrate buffer solution containing heparin (1 μg/ml) and centrifuged at 1500 x g for 90 min at room temperature. The MV-rich sediment was resuspended in commercially available Annexin V buffer (25 mM CaCl2 solution in 140 mM NaCl and 10 mM HEPES, pH 7.4; BD Bioscience, San Diego, CA, USA) to obtain the MV suspension. Aliquots of 100 μL of MV suspension were transferred to tubes containing 2 μL of monoclonal antibodies labelled with distinct fluorochromes to identify: CD45 (APC, clone HI30), CD16 (PE, clone 3G8), CD66b (PE, clone G10F5), CD14 (PerCP, clone M5E2), CD3 (PE, clone UCHT1), CD235a (PE-Cy5, clone GA-R2/HIR2), CD41 (PerCP, clone HIB8), and CD51 (PE, clone 23C6); and 2.5 μL of Annexin V-FITC, followed by incubation for 30 min in the dark at room temperature. Internal autofluorescence control was included in each trial run, in which an aliquot of MV suspension was incubated in the absence of monoclonal antibodies and Annexin V-FITC (All purchased from BD Biosciense, San Diego, CA, USA). Aliquots of monoclonal antibodies and Annexin V-FITC, incubated in the absence of MV, were used as internal controls. In the end, 300 μL of Annexin V buffer was added to each tube and the samples were acquired in a flow cytometer (Cytoflex S, Beckman Coulter, Brea, CA, USA) with volume control aspirated/minute. The Cytoflex S has a volumetric sample injection system that allows absolute particles counting. The sample flow rate was 30 μL/min and the sample acquisition was made during 2 min per sample. Calibration microbeads (Gigamix beads, Stago Co, Marseille, France) with standard size of 100 nm and 900 nm were used to identify MV of specific sizes. The protocol steps are summarized in the Fig. 1. Different gating strategies were used to analyse the MV phenotypic features as illustrated in the Fig. 2.

3.6. Statistical analysis

Statistical analysis was performed using the software Stata SE ® version 14.0 (College Station, TX). Initially, data were described using median, maximum and minimum values or mean and standard deviation, depending on the distribution of variables. The baseline fold changes in MV (T2/T1; T3/T1; T4/T1) were calculated to assess the kinetics over time in the Sepsis and Trauma groups and subgroups according to clinical outcome. Data analysis between pairs of groups or subgroups was performed by Mann-Whitney test. In all cases, significant differences were considered at $p < 0.05$.

Receiver Operating Characteristic (ROC) curve analysis [16] was carried out to assess the performance of MV as prognostic biomarkers of clinical outcome in patients with sepsis/septic shock and trauma. ROC

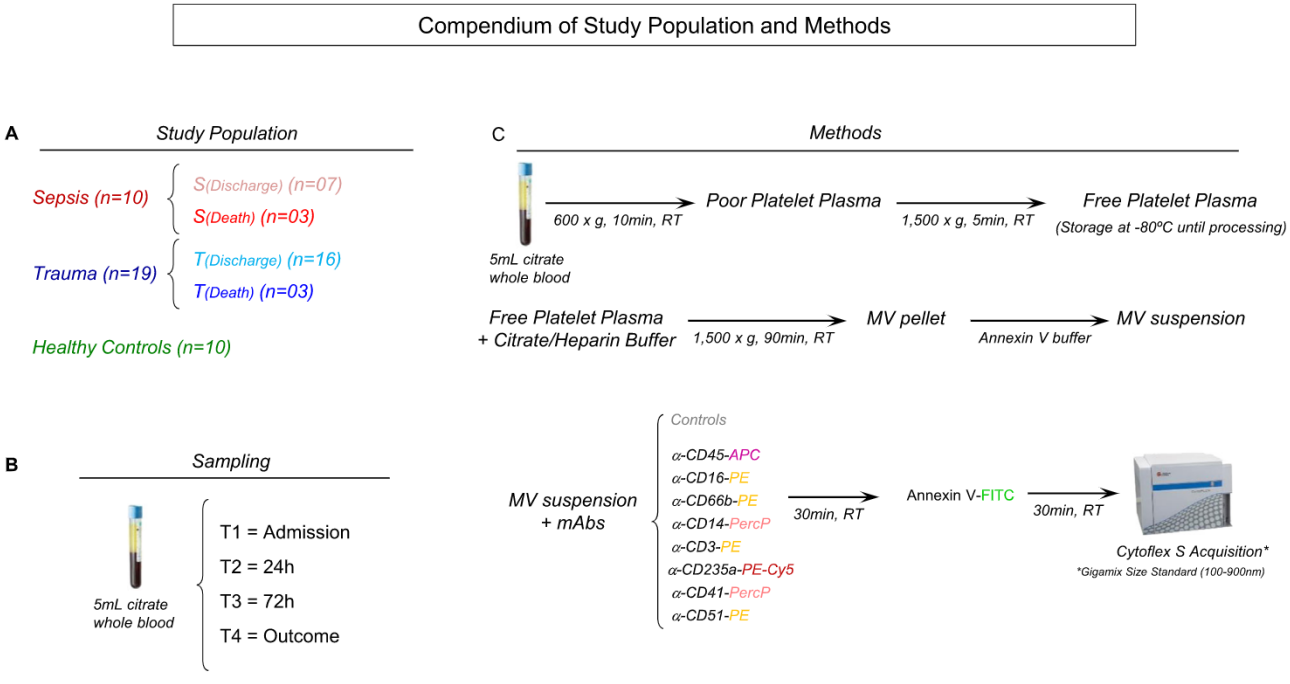

Fig. 1. Compendium of Study Population and Methods.

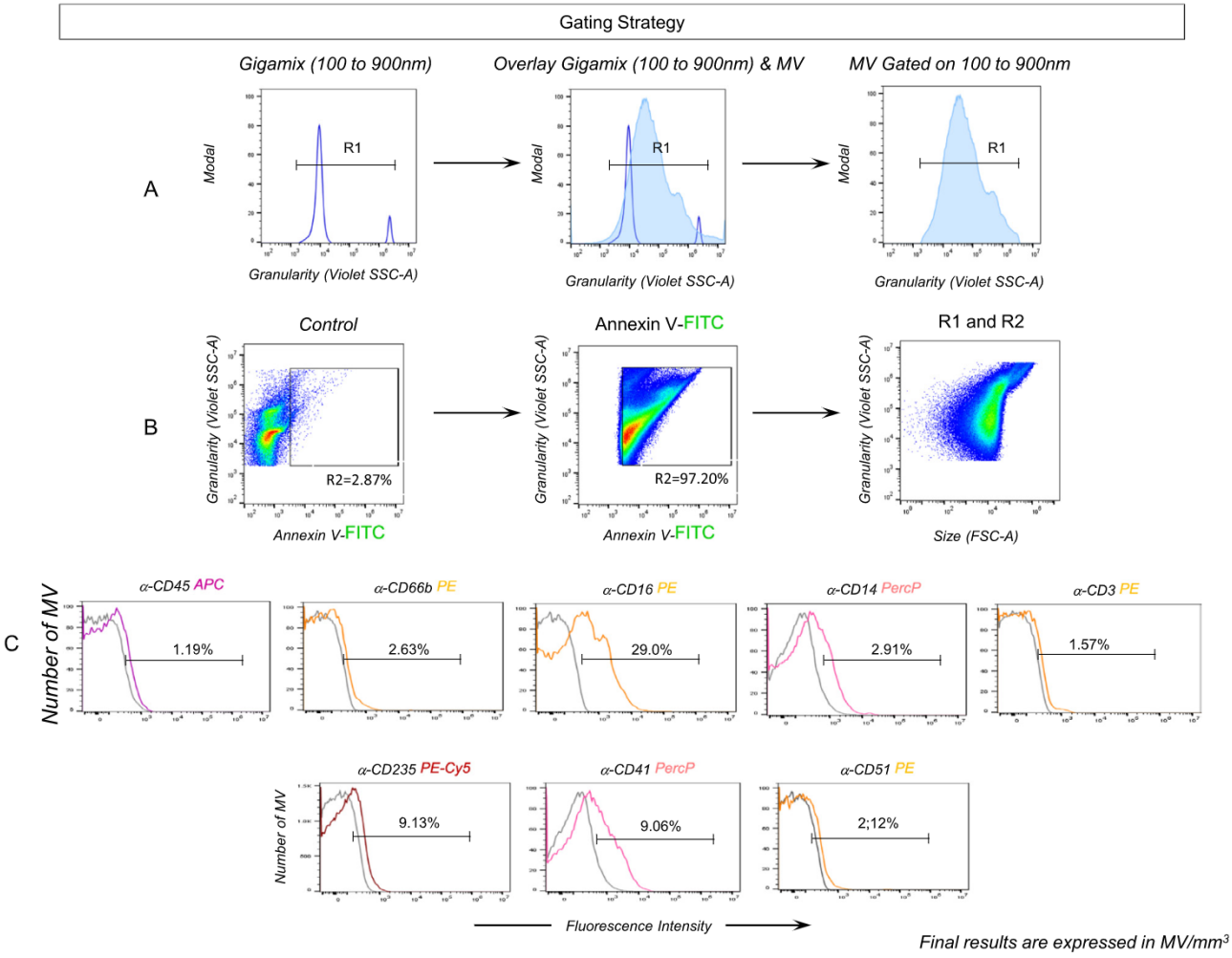

Fig. 2. *Analysis of strategies for the phenotypic characterization of MV by flow cytometry*. (A) One-dimensional granularity histograms (violet SSC-A) used for the selection of gigamixes ranging in size from 100 to 900 nm (R1). Overlapping one-dimensional Gigamix and MV histograms allow the selection of MV ranging in size from 100 to 900 nm. (B) Two-dimensional plot of Annexin V-FITC vs granularity (SSC-A violet) with MV point distribution of size between 100 and 900 nm in the control tube allows positioning of the positivity region for Annexin V-FITC (R2) with a lower threshold at 3%. The R2 region is applied to all immunophenotyping tubes. The combination of R1and R2 regions is used for the subsequent phenotypic analysis. (C) One-way fluorescence intensity histograms are used for the quantification of phenotype-specific MV in percentage using the control tube (gray curve) to position the positivity threshold marker. The final result is expressed in MV number/mm^3, considering the aspirated volume in 2 min of acquisition.

curve data were used to define cut-off points for each MV evaluated. Performance of selected MV (sensitivity, specificity and likelihood ratio - LR) were calculated at specific cut-off and the area under the curve (AUC) considered as an indicator of global accuracy.

4. Results

4.1. Panoramic profile of plasma microvesicles in critically ill patients at baseline

The Fig. 3 shows an overall baseline profile of plasma MV in Sepsis and Trauma as compared to healthy controls. Data analysis showed that Sepsis presented higher number of MV Annexin V$^+$ (total MV), while Trauma showed higher number of MV CD14$^+$ (monocytes) as compared to controls. An increase in MV CD66b$^+$ (neutrophils) and CD16$^+$ (neutrophils) was observed in both Sepsis and Trauma in comparison to controls. The levels of MV CD3$^+$ (T-cells) and CD41$^+$ (platelets) were higher in Trauma as compared to both, Sepsis and controls. Together, these findings at baseline demonstrated that neutrophil-derived MV did not discriminate Sepsis from Trauma. However, MV Annexin V$^+$ (total MV) appear as biomarker to identify Sepsis. On the other hand, MV CD14$^+$ (monocytes) was a putative biomarker to identify trauma patients, while MV CD3$^+$ (T-cells) and CD41$^+$ (platelets) were qualified to discriminated Trauma from Sepsis (Fig. 3).

4.2. Profile of baseline plasma microvesicles in critically ill patients categorized according to clinical outcome

The Fig. 4 shows the baseline profile of MV in Sepsis and Trauma categorized according to the clinical outcome and compared to the controls. Data analysis showed an increase of MV CD16$^+$ and CD66b$^+$ (neutrophils) in both S(Discharge) and T(Discharge) as compared to controls. There was an increase in MV Annexin V$^+$ (total MV) in S (Discharge) in comparison to controls. Conversely, an increase of MV CD14$^+$ (monocytes) and CD3$^+$ (T-cells) was observed in T(Discharge) as compared to controls. Moreover, the levels of CD235$^+$ MV (red blood cells) were higher in T(Death) as compared to T(Discharge), being able to differentiate the trauma clinical outcome. Although, the levels of MV CD41$^+$ (platelets) were higher in both trauma subgroups, it was selectively increase in S(Death) as compared to controls. Furthermore, there was an increase of MV CD41$^+$ (platelets) in T(Discharge) as compared to S(Discharge) (Fig. 4).

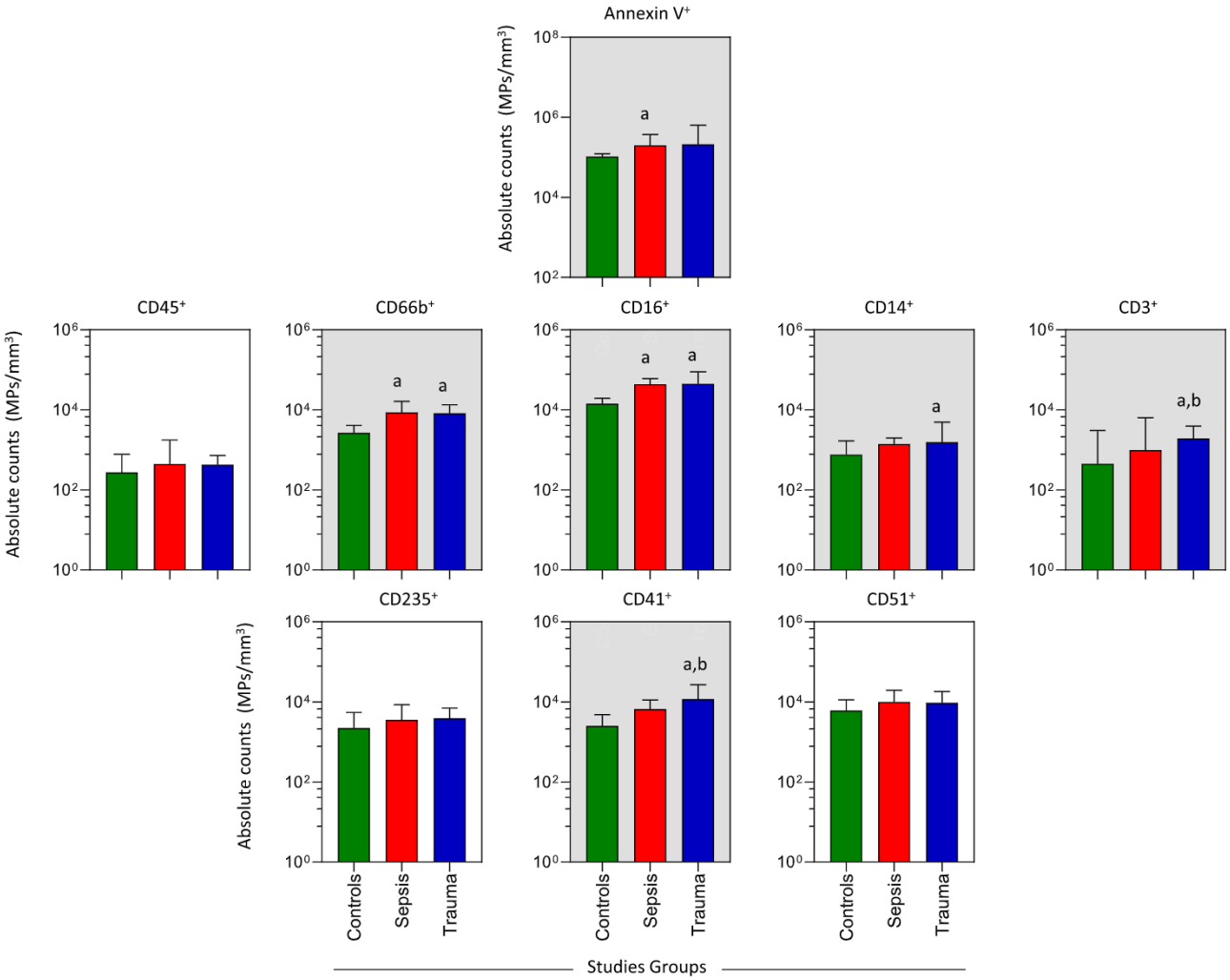

Fig. 3. *Overall profile of baseline plasma microvesicles in critically ill patients as compared to healthy controls.* MV levels were quantified in plasma samples collected at admission of sepsis/septic shock patients (Sepsis, n = 10), trauma victims (Trauma, n = 19) and healthy control subjects (Controls, n = 10), as described in materials and methods. The results are presented in bar charts, showing the median (interquartile range) of the absolute number of MV/mm^3 of plasma. Data analysis was performed by Mann-Whitney test. Significant differences were considered at $p < 0.05$ and underscored by letter "a" for comparison between Sepsis or Trauma with Controls. Comparative analysis between Sepsis and Trauma are highlighted by letter "b".

4.3. Kinetic profile of plasma microvesicles in critically ill patients categorized according to the clinical outcome

The Fig. 5 shows the kinetic profile of plasma MV in Sepsis as compared to Trauma and also in critically ill patients further categorized according to the clinical outcome. The kinetic analysis was performed upon admission/baseline (time 1-T1); 24 h (time 2-T2); 72 h (time 3-T3) and at outcome (time 4-T4). The panoramic snapshot was taken considering MV profile with higher median values at least in three points along the kinetic timeline. Based on this criterion, data showed that the Sepsis exhibited an increase of MV Annexin V$^+$ (total MV), CD45$^+$ (total leukocytes), CD66b$^+$ (neutrophils), CD16$^+$ (neutrophils), CD14$^+$ (monocytes) and CD51$^+$ (endothelium) as compared to Trauma. The analysis further demonstrated that S (Death) presented an increase in MV Annexin V$^+$ (total MV), CD45$^+$ (total leukocytes), CD16$^+$ (neutrophils), CD14$^+$ (monocytes), and CD41$^+$ (platelet) in comparison to S (Discharge). Moreover, T(Death) presented an increase of MV CD3$^+$ (T-cells) and CD235$^+$ (red blood cells) as compared to T(Discharge) (Fig. 5).

4.4. Snapshot of baseline fold changes in plasma microvesicles in critically ill patients according to clinical outcome

The Fig. 6 shows the kinetic profile of baseline fold changes of MV in Sepsis as compared to Trauma and also in critically ill patients further categorized according to the clinical outcome. The baseline folds were calculated as the ratio of MV levels observed at 24 h (time 2-T2), 72 h (time 3-T3) and at outcome (time 4-T4) by those observed at admission/baseline (time 1-T1) represented as: T2/T1; T3/T1; T4/T1. Baseline fold changes data were analysed considering the following criteria: unaltered (=1), decreased (<1) or increased (>1) values throughout the kinetic timeline. Based on this criterion, the results showed that Sepsis showed an increase of MV CD45$^+$ (total leukocytes) in T2/T1, CD66b$^+$ (neutrophils), CD14$^+$ (monocytes) and CD235$^+$ (red blood cells) in T3/T1 along with CD235$^+$ (red blood cells) and CD41$^+$ (platelets) in T4/T1. On the other hand, Trauma showed a selective increase of MV CD45$^+$ (total leukocytes) in T4/T1. Further analysis demonstrated that S(Death) presented an increase of most MV analysed when compared to the S (Discharge). Conversely, regardless of clinical outcome, the trauma victims presented an increase of MV CD45$^+$ at time T4/T1 (Fig. 6).

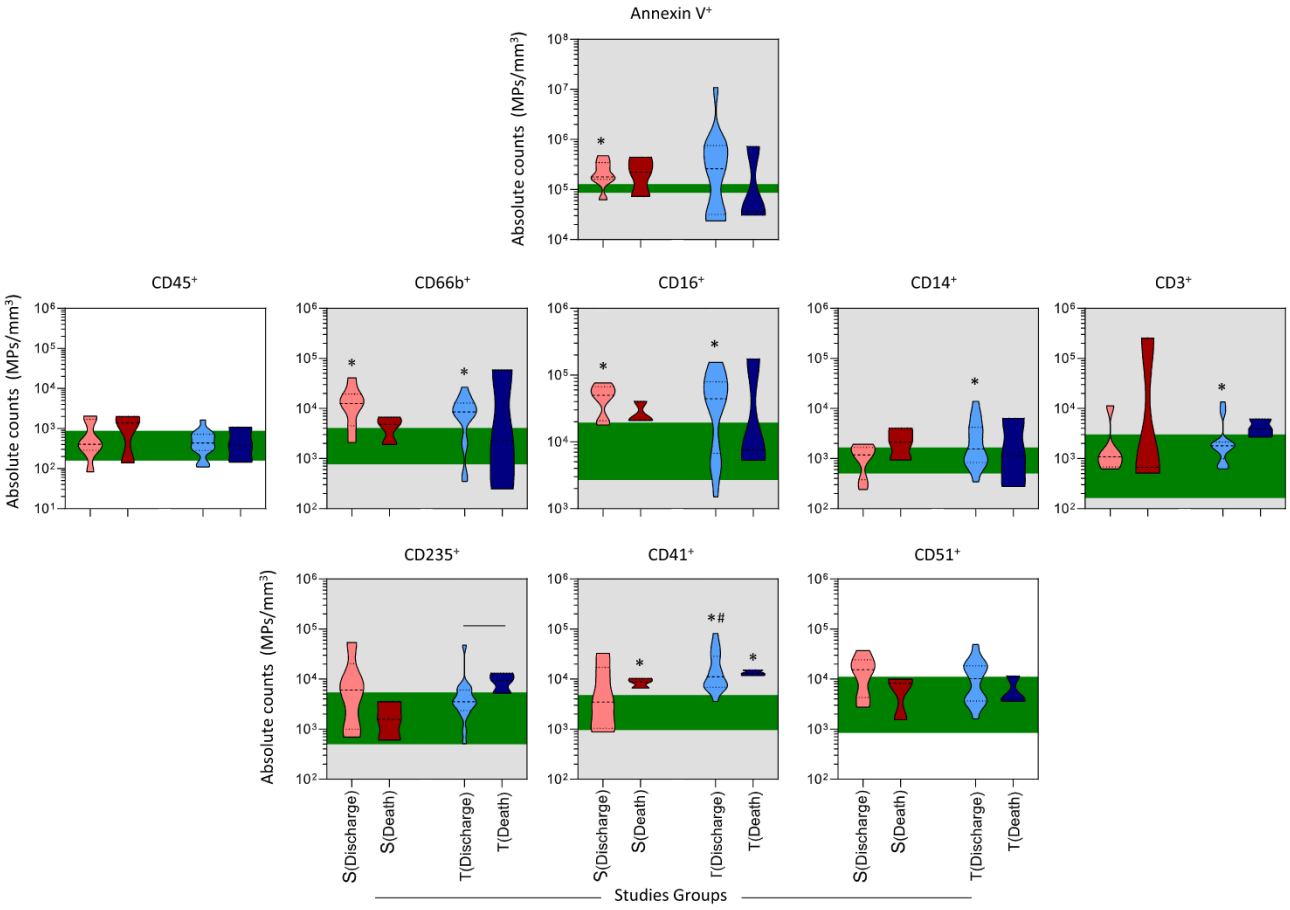

Fig. 4. *Overall profile of baseline plasma microvesicles in critically ill patients categorized according to the clinical outcome.* MV levels were quantified by flow cytometry in plasma samples from critically ill patients categorized according to the clinical outcome into subgroups named: sepsis/septic shock evolving to discharge [S (Discharge) ▭, n = 7], sepsis/septic shock progressing to death [S(Death) ▭, n = 3]; trauma victims evolving to discharge [T(Discharge) ▭, n = 16], trauma victims progressing to death [T(Death) ▭, n = 3]. Data were also compared with the interquartile range observed for healthy control group (▭, n = 10), as described in materials and methods. The results are presented in violin plots, showing the minimum and maximum values, the median (…) and interquartile ranges (…) of the absolute number of MV/mm^3 of plasma. Data analysis between pairs of groups or subgroups was performed by Mann-Whitney test. Significant differences were considered at $p < 0.05$ and underscored by asterisks " * " for difference between S(Discharge) vs controls, S(Death) vs controls, T(Discharge) vs controls and T (Death) vs controls. The symbol "#" was used to highlight the significant differences between S(Discharge) vs T(Discharge). The connecting lines was used to highlight significant differences between T(Discharge) vs T(Death).

4.5. Performance of plasma microvesicles as a prognostic biomarker in critically ill patients according to the clinical outcome

The Fig. 7 shows the performance of plasma MV as prognostic biomarkers in critically ill patients categorized according to the clinical outcome. The ROC curves were assembled to define the cut-off points for each selected MV and calculate the performance indices (sensitivity, specificity and likelihood ratio - LR), as well as the area under the curve (AUC) as an indicator of global accuracy.

In Sepsis, data analysis demonstrated that MV CD66b$^+$ (neutrophil) at T1, Annexin V$^+$ (total MV) at T2 along with Annexin V$^+$ (total MV) and CD14$^+$ (monocytes) at T3 showed moderate/high performance (Se=100% and Sp ranging from 71% to 100%) and elevated/excellent global accuracy (AUC ranging from 0.81 to 1.00) to discriminate S (Discharge) from S(Death) (Fig. 7A).

In Trauma, MV CD3$^+$ (T-cells) and CD235$^+$ (red blood cell) at T1 and CD66b$^+$ (neutrophils) at T4 showed low/moderate performance (Se=100% and Sp ranging from 53% to 81%) and elevated global accuracy (AUC ranging from 0.81 to 0.85) to discriminate T(Discharge) vs T(Death) (Fig. 7A).

Additional analysis using combined MV profiles was performed to classified the critically ill patients according to the clinical outcome. The discriminant analysis of "true" (clinical outcome) and "classified" (based on the proposed biomarkers) categories demonstrated a perfect accuracy (100%, 10/10) for the set of proposed MV [CD66b (T1), Annexin V (T2) and Annexin V and CD14 (T3)] for classification of Sepsis clinical outcome. Likewise, the set of biomarkers [CD3 and CD235 (T1) and CD66b (T4)] proposed for classification of Trauma clinical outcome also demonstrated high accuracy (18/19, 95%) with only one misclassification (Fig. 7B).

5. Discussion

The real role of microvesicles performance as biomarkers of outcome and prognosis evolution in patients with sepsis/septic shock and trauma is poorly understood in the current literature. This study reveals a promising scenario for the use of MV as a biomarker in sepsis to predict the outcome when the phenotypic assessment is longitudinally studied.

The results of this study reveal that patients in the sepsis/septic shock group showed an increase in the number of MV Annexin V$^+$ (total MV), however, selective MV were not found for specific phenotypes associated with sepsis, also found by Cloutier et al., 2013 [17]. In trauma patients, a greater amount of MV from T-cells (CD3$^+$), platelets (CD41$^+$) and monocytes (CD14 $^+$) were found, with no other data in the literature that could corroborate these findings so far.

Upon admission (T1), an increase in neutrophil-derived MV (CD45$^+$)

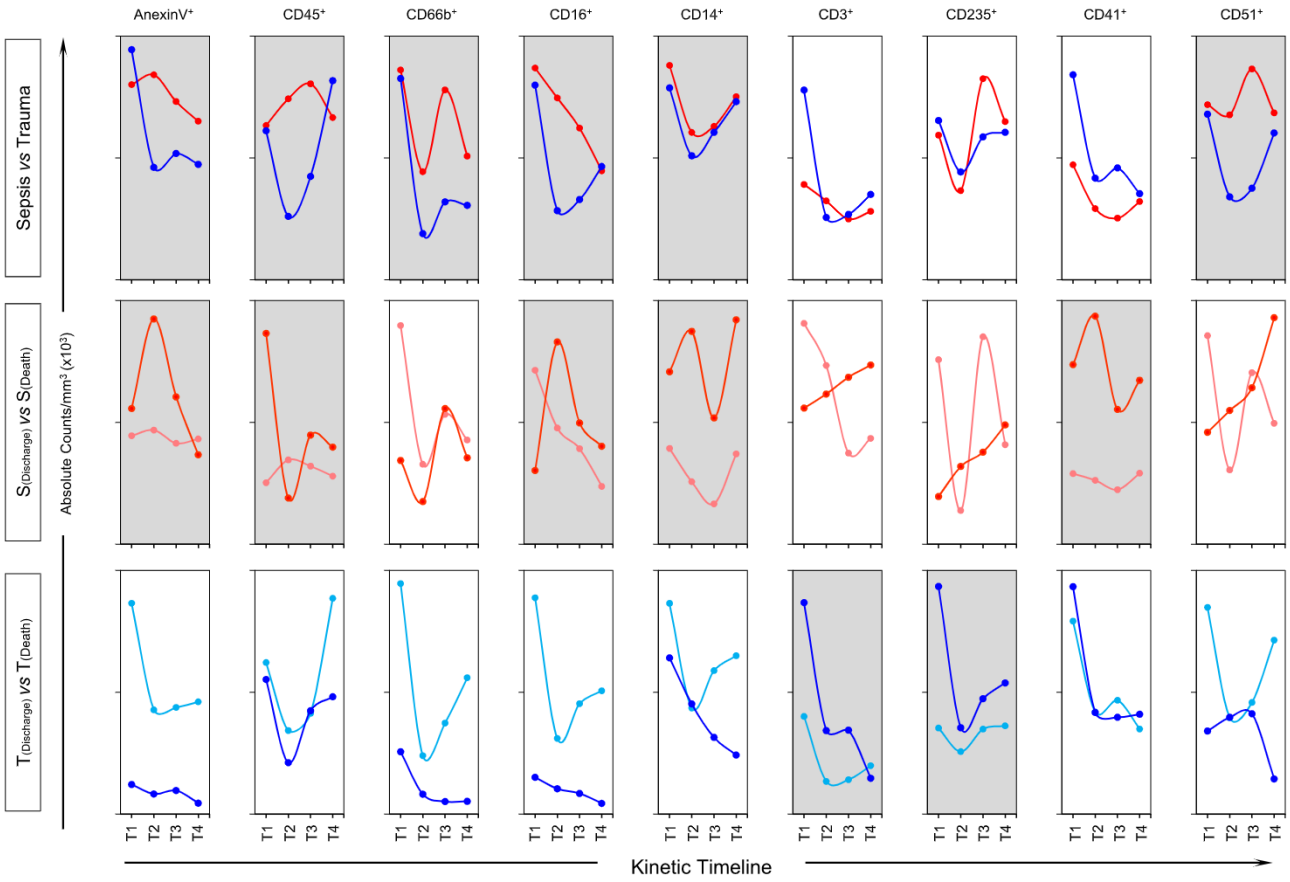

Fig. 5. *Kinetic profile of plasma microvesicles in critically ill patients categorized according to the clinical outcome.* MV levels were quantified by flow cytometry in plasma samples from critically ill sepsis/septic shock patients (Sepsis, n = 10) and trauma victims (Trauma, n = 19). Critically ill patients were subsequently categorized according to the clinical outcome into subgroups named: sepsis/septic shock evolving to discharge [S(Discharge), n = 7], sepsis/septic shock progressing to death [S(Death), n = 3]; trauma victims evolving to discharge [T(Discharge), n = 16], trauma victims progressing to death [T(Death), n = 3], as described in materials and methods. The results are presented in line graphs, showing the median values of the absolute number of MV/mm^3 of plasma samples collected at admission/baseline (time 1-T1); 24 h (time 2-T2); 72 h (time 3-T3) and at outcome (time 4-T4). Panoramic snapshot was taken considering the MV profile with higher values observed at least in three points along the kinetic timeline. Based on this criterion, the MV profiles able to discriminate Sepsis vs Trauma, S(Discharge) vs S(Death) and T(Discharge) vs T(Death) are highlighted by gray background.

was detected in both groups, which can be explained by the fact that they are pathophysiological inflammatory diseases. In patients in the sepsis/septic shock group, which is an acute infectious disease resulting from the presence of microorganisms, the predominance of MV resulting from neutrophil activation (CD66b$^+$ and CD16$^+$) can be explained. These findings corroborate the study of Mortaza S, et al. [18], who evaluated the MV profile in an animal sepsis model through the inoculation of MV derived from animals submitted to CLP in healthy animals compared to control. No difference was found in the total serum concentration of MV in animals submitted to CLP in this study, but there was an increase in MV derived from total leukocytes (CD45$^+$). However, this finding opposes what was described by Zafrani L, et al. [19], who also described an animal model for sepsis in which most of the MV found (85%) were derived from platelets (CD41$^+$), with a minority originating from endothelial cells (CD51$^+$) and monocytes (CD14$^+$). Overall, this study demonstrated an increase in monocyte-derived MV (CD14$^+$) in trauma, and an increase in platelet-derived MV (CD41$^+$) and T-cells (CD3$^+$) in both sepsis/septic shock and trauma at admission.

When analysing sepsis/septic shock and trauma concerning the outcome, an increase in neutrophil-derived MV (CD66b$^+$ and CD16$^+$) was demonstrated in both groups that were discharged. Findings related to the sepsis/septic shock group contrast with Soriano AO, et al. [20], who studied 35 patients diagnosed with sepsis and detected significantly higher levels of endothelial cell-derived MV (CD51$^+$) in patients who evolved to discharge when compared to those who progressed to death. In the trauma group, this study showed an increase in MV derived from red blood cells (CD235$^+$) in patients who died compared to those discharged. This finding can be explained by the intrinsic mechanical role of trauma, which is related to the destruction of red blood cells, or it may be related to transfusion events, which occurred more often in this group of patients. There is a lack of studies available in the literature to confirm these findings.

When evaluating the evolutionary profile of MV, patients who progressed to death in the sepsis/septic shock group presented a progressive increase in MV derived from Annexin V$^+$, total leukocytes (CD45$^+$), neutrophils (CD16$^+$), monocytes (CD14$^+$) and platelets (CD41$^+$) when compared to those who were discharged. This finding can be explained by the uncontrolled inflammatory reaction that occurs in sepsis, which may be related to the greater severity of these cases. This same profile of evolutionary increase in MV in sepsis was also described by Boscolo A, et al., [21]. In trauma patients who progressed to death, an increase in MV derived from T-cells (CD3$^+$) and erythrocytes (CD235$^+$) was observed as compared to patients who were discharged. Considering that sepsis is marked by a storm of biomarkers due to the presence of the infectious agent, a trend towards a progressive increase in MV in this group would be expected. In trauma patients, on the other hand, there was a tendency to decrease, which may be explained by the earlier resolution of the inflammatory process.

Regarding MV kinetics, differences in the MV kinetic profiles in sepsis/septic shock and trauma were observed when evaluating the

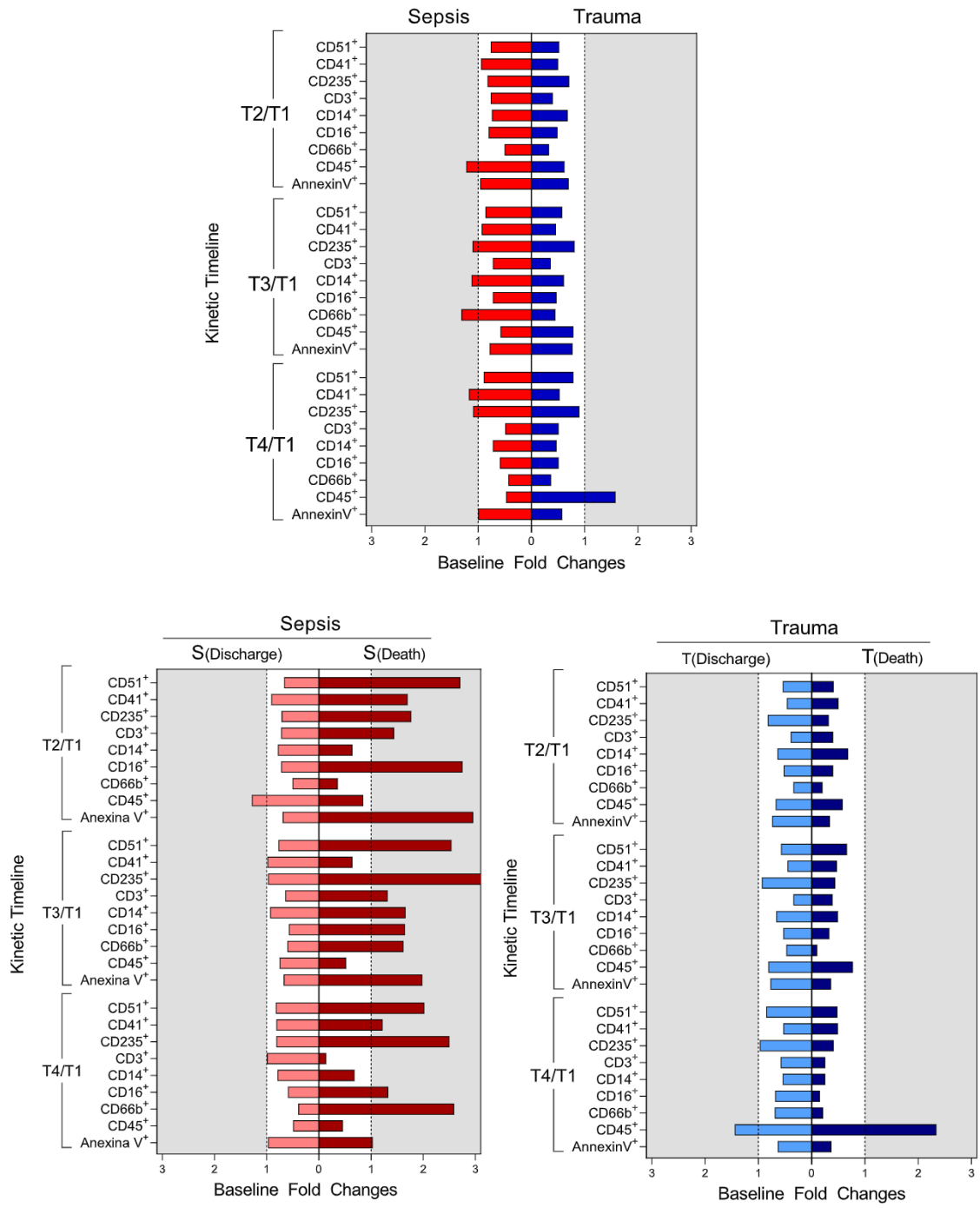

Fig. 6. *Kinetic of baseline fold changes in plasma microvesicles in critically ill patients according to clinical outcome.* MV levels were quantified by flow cytometry in plasma samples from sepsis/septic shock patients (Sepsis, n = 10) and trauma victims (Trauma, n = 19). Critically ill patients were subsequently categorized according to the outcome into subgroups named: sepsis/septic shock evolving to discharge [S(Discharge), n = 7], sepsis/septic shock progressing to death [S(Death), n = 3]; trauma victims evolving to discharge [T(Discharge), n = 16], trauma victims progressing to death [T(Death), n = 3], as described in materials and methods. The results are presented in bar charts showing the baseline folds calculated as the ratio of MV levels observed at 24 h (time 2-T2), 72 h (time 3-T3) and at outcome (time 4-T4) by those observed at admission/baseline (time 1-T1) represented as: T2/T1; T3/T1; T4/T1. Baseline fold changes data were analysed considering the following criteria: unaltered (=1), decreased (<1) or increased (>1) values throughout the kinetic timeline.

outcome, therefore it is important to know if there is a change in status over time (T2, T3 and T4) as compared to admission (T1). Baseline folds were determined with this purpose, calculating the change in the MV response pattern in the sepsis/septic shock and trauma groups over time concerning baseline values. This analysis reaffirmed that septic patients who progressed to death presented a progressive increase in MV when compared to the group who were discharged, revealing the pathophysiological basis of the uncontrolled inflammatory reaction of sepsis again. This finding is consistent with what was described by Janota J. et al., [22], who carried out a review study on the origin of MV derived from cell membranes. Conversely, this was not observed in the subgroups of trauma victims, who showed only an increase in MV derived from total leukocytes ($CD45^+$) at time T4/T1, regardless of the outcome.

Our results also demonstrate that there is a pattern of MV in sepsis/septic shock patients and trauma victims, which may be related to the pathophysiology. However, it is necessary to question whether the

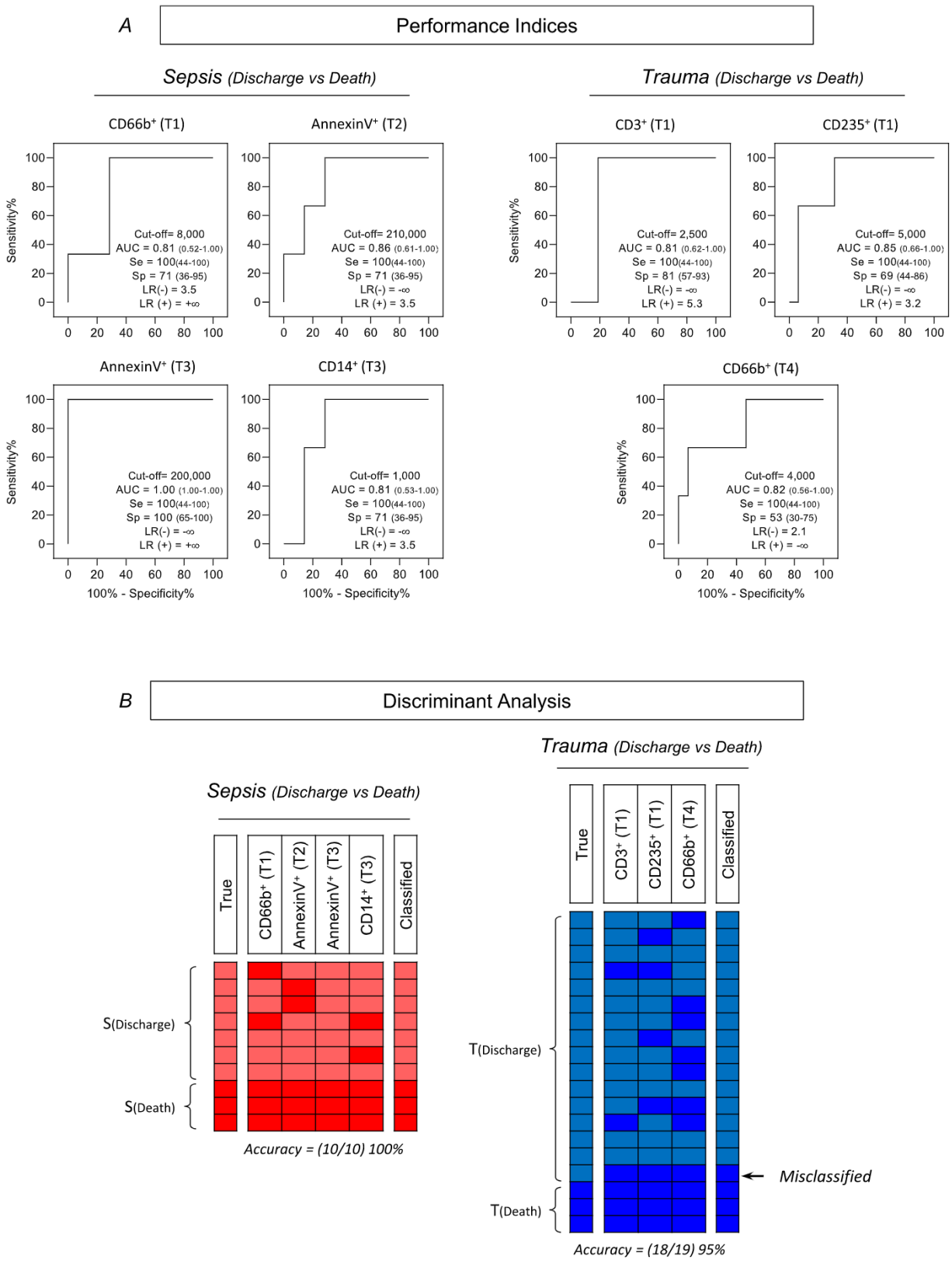

Fig. 7. *Performance of plasma microvesicles as a prognostic biomarker in critically ill patients according to the clinical outcome.* MV levels were quantified by flow cytometry in plasma samples from critically ill patients classified according to the outcome in subgroups named: sepsis/septic shock evolving to discharge [S (Discharge) ▭, n = 7], sepsis/septic shock progressing to death [S(Death) ▬, n = 3]; trauma victims evolving to discharge [T(Discharge) ▭, n = 16], and trauma victims progressing to death [T(Death) ▬, n = 3], as described in materials and methods. (A) ROC curves were assembled to define the cut-off points for each MV and calculate the performance indices (sensitivity, specificity and likelihood ratio - LR), as well as the area under the curve (AUC) as an indicator of global accuracy. (B) Discriminant analysis of "true" (clinical outcome) and "classified" (based on the proposed biomarkers) categories showing the accuracy for the set of biomarkers proposed for the classification of Sepsis and Trauma clinical outcome.

pattern shown so far could point to a possible pattern of MV as a laboratory predictor of sepsis/septic shock or trauma outcome. To answer this question, the performance of these markers through the ROC curve was evaluated, analysing the potential of a given laboratory biomarker to segregate groups by evaluating accuracy. The ROC curve illustrates a panoramic assessment, which exposes the performance of all biomarkers evaluated to discriminate the sepsis/septic shock and trauma groups according to the outcome and compare them with the control group. The markers relevant to segregate groups are those that presented an accuracy above 0.8, which is considered a moderate to high accuracy. Based on the understanding of the pathophysiology of sepsis when compared to the control, the importance of neutrophilic participation throughout the evolution of sepsis in the four stages is evident.

When assessing the sensitivity, specificity and likelihood ratio in both groups, it was possible to perform a discriminant analysis and select four screening parameters capable of separating the outcomes: 1) MV derived from neutrophils ($CD66b^+$) in T1, 2) total MV (Annexin V^+) at T2, 3) total MV (Annexin V^+) at T3 and T4) monocyte-derived MV ($CD14^+$) at T3. Three screening parameters were selected to segregate the outcome in the trauma patients' group: 1) MV derived from T-cells ($CD3^+$) at T1, 2) MV derived from red blood cells ($CD235^+$) at T1 and 3) MV derived from neutrophils ($CD66b^+$) at T4.

An individual detailing of the patients was made to assess the values within the established cut-off points by using cut-off points in mm^3. Later, a new classification was performed, segregating the outcome. For these cases the variables selected in both groups (sepsis/septic shock and trauma) were evaluated at the laboratory level, using the cut-off points established by the ROC curve with the best performance to segregate the outcome.

This comparative analysis by categories demonstrated 100% accuracy (10/10) for the performance of the proposed set of biomarkers in septic patients and 95% accuracy (18/19) in trauma patients.

The use of MV in clinical practice at the bedside is still a challenging task that needs further clinical studies. This is a pilot study that evaluated patients with specific inclusion criteria, in the acute phase of the disease and with exclusion criteria strictly defined to maintain the pathophysiological substrate under evaluation, which explains the small number of patients. Furthermore, there are few studies in the literature on trauma patients. Another limitation refers to the non-segregation of MV according to their nanometric dimensions. According to some authors, there are different functions performed by MV depending on their dimensions [23], and this study only performed immunophenotyping. Hence, it seems clear that studies similar to ours that investigate microvesicles in sepsis namely in different dimensions must be validated in further studies with a larger number of participants.

6. Conclusions

Our findings suggest that MV might be useful as a potential role in discriminating outcome in patients with sepsis/septic shock and trauma with high accuracy. However, further studies with a larger number of participants will be necessary to validate our findings.

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CRediT authorship contribution statement

Marcelo Lourencini Puga: Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Mayra Gonçalves Menegueti**: Data curation, Writing - original draft, Writing – review, editing and statistical analysis. **Marina Malheiros Araújo Silvestrini:** Investigation, Data curation. **Lorena Júnia de Souza Santos:** Investigation, Data curation, **Raquel Ferreira-Nogueira:** investigation. **Anibal Basile-Filho:** Investigation. **Andréa Teixeira-Carvalho:** Conceptualization, Funding aquisiton, Data curation, Formal analysis,Project administration, Writing - original draft, Writing - review & editing. **Olindo Assis Martins-Filho:** Conceptualization, Funding aquisiton, Data curation, Formal analysis,Project administration, Writing - original draft, Writing - review & editing. **Maria Auxiliadora-Martins:** Conceptualization, Funding aquisiton, Data curation, Formal analysis,Project administration, Writing - original draft, Writing - review & editing.

Conflict of interest statement

The authors declare that there are no conflict of interest.

Data Availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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