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Increased Circulating CD62E^{+} Endothelial Extracellular Vesicles Predict Severity and in- Hospital Mortality of COVID-19 Patients

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Abstract

COVID-19 and infectious diseases have been included in strategic development goals (SDG) of United Nations (UN). Severe form of COVID-19 has been described as an endothelial disease. In order to better evaluate Covid-19 endotheliopathy, we characterized several subsets of circulating endothelial extracellular vesicles (EVs) at hospital admission among a cohort of 60 patients whose severity of COVID-19 was classified at the time of inclusion. Degree of COVID-19 severity was determined upon inclusion and categorized as moderate to severe in 40 patients and critical in 20 patients. We measured citrated plasma EVs expressing endothelial membrane markers. Endothelial EVs were defined as harboring VE-cadherin (CD144+), PECAM-1 (CD31 + CD41-) or E-selectin (CD62E+). An increase in CD62E + EV levels on admission to the hospital was significantly associated with critical disease. Moreover, Kaplan-Meier survival curves for CD62E + EV level showed that level ≥ 88,053 EVs/µL at admission was a significant predictor of in hospital mortality (p = 0.004). Moreover**,** CD62E + EV level ≥ 88,053 EV/µL was significantly associated with higher in-hospital mortality (OR 6.98, 95% CI 2.1–26.4, p = 0.002) in a univariate logistic regression model, while after adjustment to BMI CD62E + EV level ≥ 88,053 EV/µL was always significantly associated with higher in-hospital mortality (OR 5.1, 95% CI 1.4–20.0, p = 0.01). The present findings highlight the potential interest of detecting EVs expressing E-selectin (CD62) to discriminate Covid-19 patients at the time of hospital admission and identify individuals with higher risk of fatal outcome.

Keywords COVID-19 · Extracellular vesicles · Endothelial · E-selectin · UN SDG3

Introduction

Infection with SARS-CoV-2 leads in some patients to severe forms described as an endothelial disease [1]. This endotheliopathy is probably at the origin, at least in part, of COVID-19 prothrombotic state [2] giving rise to microthrombosis. Extent of microthrombosis participate to severity of the disease and is a unique characteristic of COVID-19 and in particular of acute respiratory distress syndrome (ARDS) [3]. Despite preventive anticoagulation proposed as soon the COVID-19 outbreak started, D-Dimer levels are associated with severity at admission, with worsening during hospital stay and with mortality independently from venous thrombotic events [4–6]. The

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SARS-CoV-2 infection of endothelial cells is still a matter of debate but modification of cell morphology, endothelial cells apoptosis or abnormal endothelial organization like intussusceptive angiogenesis is clearly demonstrated [7, 8]. In terms of circulating biomarkers, increase of circulating endothelial cells, angiopoietin-2 or von Willebrand factor have been described as good predictive marker of severity and in-hospital mortality [9–12]. Mechanistically, endothelial activation is probably occurring after combination of hypoxemia and/or inflammatory burst response [2].

Extracellular vesicles (EVs) are a heterogeneous group of small-sized vesicles released by most cell types in response to different stimuli [13, 14]. They are composed of a lipid bilayer that encloses a wide range of bioactive material; in particular, they are largely involved in hemostasis since they carry protein involved in thrombosis and fibrinolysis. For that, EVs quantification and characterization has become of great interest in all thromboembolic disease. Thus, in COVID-19 several studies have reported a higher numbers in plasma of EVs derived from platelets, endothelial cells, leukocytes, neutrophils, alveolar-macrophages or alveolar-epithelial cells [15]**.** Moreover, increased levels of EVs bearing tissue factor (TF) in severe COVID-19 has been found and could directly contribute to thrombosis [16].

The study aimed to better evaluate circulating endothelial EVs with different patterns in confirmed COVID-19 patients at hospital admission. These snapshot indexes of endothelial injury or activation could help to better contribute in COVID-19 severity characterization at entrance in hospital.

Materials and Methods

Study Design and Participants

This observational cohort study was conducted in Georges Pompidou European hospital in Paris, France (2020-A01048–31, March–June 2020 - NCT04624997) and included 60 consecutive hospitalized COVID-19 patients classified according to World Health Organization guidance as moderate/severe (non-critical; n = 40; median oxygen requirement 3 L/min; score 4–7) or critical (n = 20; requiring mechanical ventilation, score 8–9) as previously described [12]. In-hospital mortality was followed up for 90 days for all patients. The two groups were comparable at admission for most clinical and biological characteristics, but critical patients had higher body mass index (BMI = 29.50 kg/m^2 [IQR, 25.8–33.6] vs. 26.15 kg/m^2 [IQR, 23.6–27.9], $p < 0.019$), higher white cell and granulocyte counts (respectively p = 0.009 and 0.005) and higher D-dimer, troponin and CRP circulating levels ($p < 0.001$ for these 3 biomarkers) than non-critical patients.

Plasma Preparation and Extracellular Vesicles (EVs) Isolation

Blood was collected from 60 individuals after admission via venipuncture or pre-existing arterial lines in 3,5 ml 0.109 M citrated plastic tubes (Tube Vacuette, Greiner). Poor platelet plasma (PPP) was prepared from whole blood within 1 hour of sampling by centrifugation twice at 2500 *g* for 15 min, at 21 °C with the lowest deceleration settings and then stored at −80 °C. Large EVs (lEVs) were then isolated from 500 µl of PFP by one step centrifugation at 20500 *x g* for 2 h, at 4 °C. lEVs rich-pellet was resuspended in 0.1 µm filtrated PBS 1X and aliquots of 50 µl ×4 have been stored at −80 °C [17, 18].

Large Extracellular Vesicles (lEVs) Characterization by Flow Cytometry

Circulating lEVs were analyzed using Cytoflex flow cytometer (Beckman Coulter, USA) equipped with 3 lasers (405 nm, 488 nm, 638 nm) and 13 band pass filters: 450/45, 525/40 (2), 585/42, 610/20 (2), 660/10 (2), 690/50, 712/25, 780/60 (3). FSC and SSC were resulting from 488 nm laser line excitation while vSSC (violet SSC) was resulting from 405 nm laser line excitation. Before analyzing lEVs, gating strategies were required to define events with a diameter of 0.1 µm to 0.9 µm according to the application note set-up by Spittler, 2015. lEV gating was determined by using a combination of FITC labelled fluorescent Megamix-Plus SSC (Cat# 7803, Biocytex, France) and Megamix-Plus FSC beads (Cat# 7802, Biocytex, France), hereby termed as Gigamix. The Gigamix contains beads of sizes 0.1 µm, 0.16 µm, 0.2 µm, 0.24 µm, 0.3 µm, 0.5 µm and 0.9 µm (Fig. 1). Acquisition settings for lEVs were adjusted as FSC gain at 106, SSC gain 61, vSSC gain at 61 and threshold set on vSSC at 6500 in height. For experiments using fluorescence-conjugated antibodies to stain lEV surface markers, antibodies were directly added to EV-containing sample. All antibodies were centrifuged for 5 min at 13,000×*g* at 4 °C before they were applied to EV samples. Endothelial EVs were defined as harboring either VE-cadherin (CD144$^+$), PECAM-1 (CD31$^+$CD41$^-$) or E-selectin (CD62E$^+$). All antibodies were from Beckman-Coulter, Villepinte, France) except CD62E, obtained from Becton-Dickinson, Rungis, France. Following MISEV guidelines, prior to EVs staining, the antibodies and their corresponding isotypes were titrated and the appropriate dilution have been determined as summarized in Table 1. All samples were diluted in 0,1 µm filtrated NaCl 0,9% to appropriate

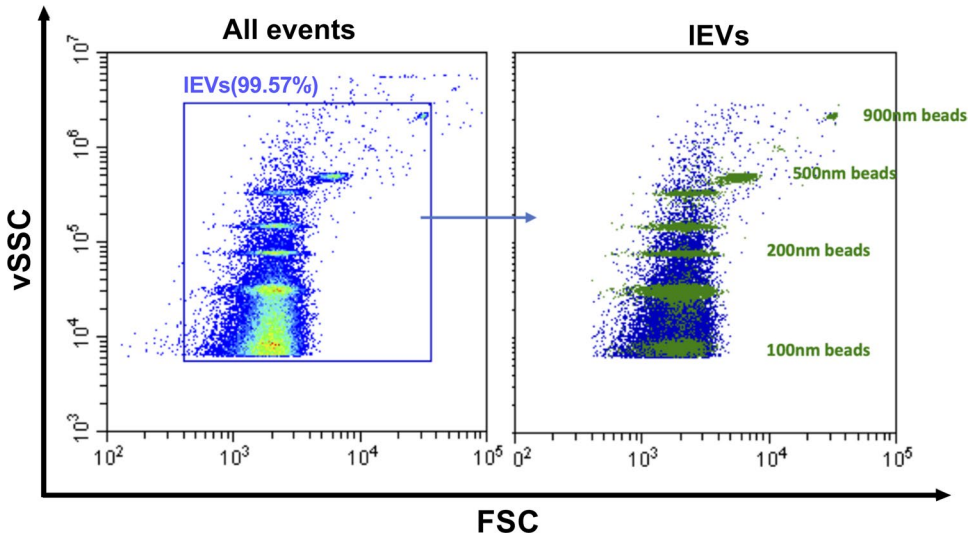

Fig. 1 IEVs gating strategy: gating strategy have been performed using a combination of Megamix-Plus SSC Megamix-Plus FSC fluorescent beads, containing beads of sizes 0.1 µm, 0.16 µm, 0.2 µm, 0.24 µm, 0.3 µm, 0.5 µm and 0.9 µm

dilutions in order to avoid swarm detection. Controls include for all analyses of lEVs negative control with isotype, detergent lysis, buffer-only, buffer with reagent (without EVs) and unstained samples. For the buffer-only control, 0.1 µm-filtered NaCl 0,9% was recorded at the same acquisition settings as all other samples and had a count of less than 100 events s^{-1}. For detergent lysis controls, stained samples were evaluated immediately after addition of Triton 0,05% as described by Gyorgy [17]. Triton detergent exposure decreased over 90% of the positive labeling (Fig. 2). Samples concentrations were measured using the instrument flow rate sensors, resulting in a flow rate of 10 µL min^{-1}. Prior to analysis, a calibration has been performed using weighed volumes of deionized water. After acquisition, data were analyzed

Table 1 Summary of reagents used for flow cytometry experiments

Characteristic being measured	Analyte	Analyte detector	Reporter	Isotype	Clone	Dilution	Manufacturer	Cat. Number	Lot Number
Cell surface protein	Human PECAM	Anti-human CD31antibody	PE	Mouse IgG1	1F11	1/50	Beckman coulter	IM2409	200042
Non-specific binding of antibody	NA	Mouse IgG1	PE	NA	679.1Mc7	1/50	Beckman coulter	A07796	200053
Cell surface protein	Human GPIIb	Anti-human CD41antibody	APC	Mouse IgG1	P2	1/33	Beckman coulter	B16894	200026
Non-specific binding of antibody	NA	Mouse IgG1	APC	NA	679.1Mc7	1/67	Beckman coulter	IM2475	200064
Cell surface protein	Human VE-Cadherin	Anti-human CD144 antibody	PE	Mouse IgG1	TEA 1/31	1/20	Beckman coulter	A07481	200031
Non-specific binding of antibody	NA	Mouse IgG1	PE	NA	679.1Mc7	1/50	Beckman coulter	A07796	200056
Cell surface protein	Human E-Selectin	Anti-human CD62E antibody	BV421	Mouse IgG1, κ	68-5H11	1/33	BD	563360	5337980
Non-specific binding of antibody	NA	Mouse IgG1, κ	BV421	NA	X40	1/67	BD	562438	9301751

using CytExpert software and EVs concentrations were normalized to plasma volume.

Results and Discussion

Levels of $CD62E^+$ EVs at admission were significantly higher in critical patients ($p = 0.03$, Fig. 3A) while other endothelial EVs subtypes ($CD31^+CD41^-$: $p = 0.26$; $CD144^+$: $p = 0.10$) did not differ between the two groups. We next evaluated the predictive value of $CD62E^+$ EVs on in-hospital mortality. This mortality reached 65% (n = 13) in critical patients, as compared to 10% (n = 4) in the non-critical group ($p < 0.01$). Time from admission to outcome (death/discharge) was also greater ($p < 0.01$) in critical patients (20.00 CI 8.00–40.50), when compared to non-critical subjects (10.00 CI 4.00–22.00). The optimum cut-off value for $CD62E^+$ EVs level at admission to predict in-hospital mortality was 88,053 EVs/µL according to the receiver operating characteristic (ROC) curve with a sensitivity of 74.4% (95% CI 44.0–88.0), a specificity of 70.5% (95% CI 58.0–86.0), a positive predictive value of 52.1% (95% CI 31.0–72.0) and a negative predictive value of 86.5% (95% CI 70.0–95.0). Area Under Curve (AUC) for in-hospital mortality was 70.0% (95% CI 54.0–85.0). Therefore, $CD62E^+$ EVs levels ≥88,053 EVs/µL at admission were associated with higher in-hospital mortality (OR 7.0, 95% CI 2.1–26.4, $p = 0.002$) in a univariate logistic regression model, and remained so after adjustment for BMI (OR 5.1, 95% CI 1.4–20.0, $p = 0.01$). Kaplan-Meier survival curves confirmed this result ($p = 0.004$, Fig. 3B), including after adjustment to BMI using a Cox proportional hazard analysis (HR 4.0 95% CI 1.4–11.5, $p = 0.009$). Plasma levels of endothelial EVs expressing intercellular junctional proteins PECAM-1

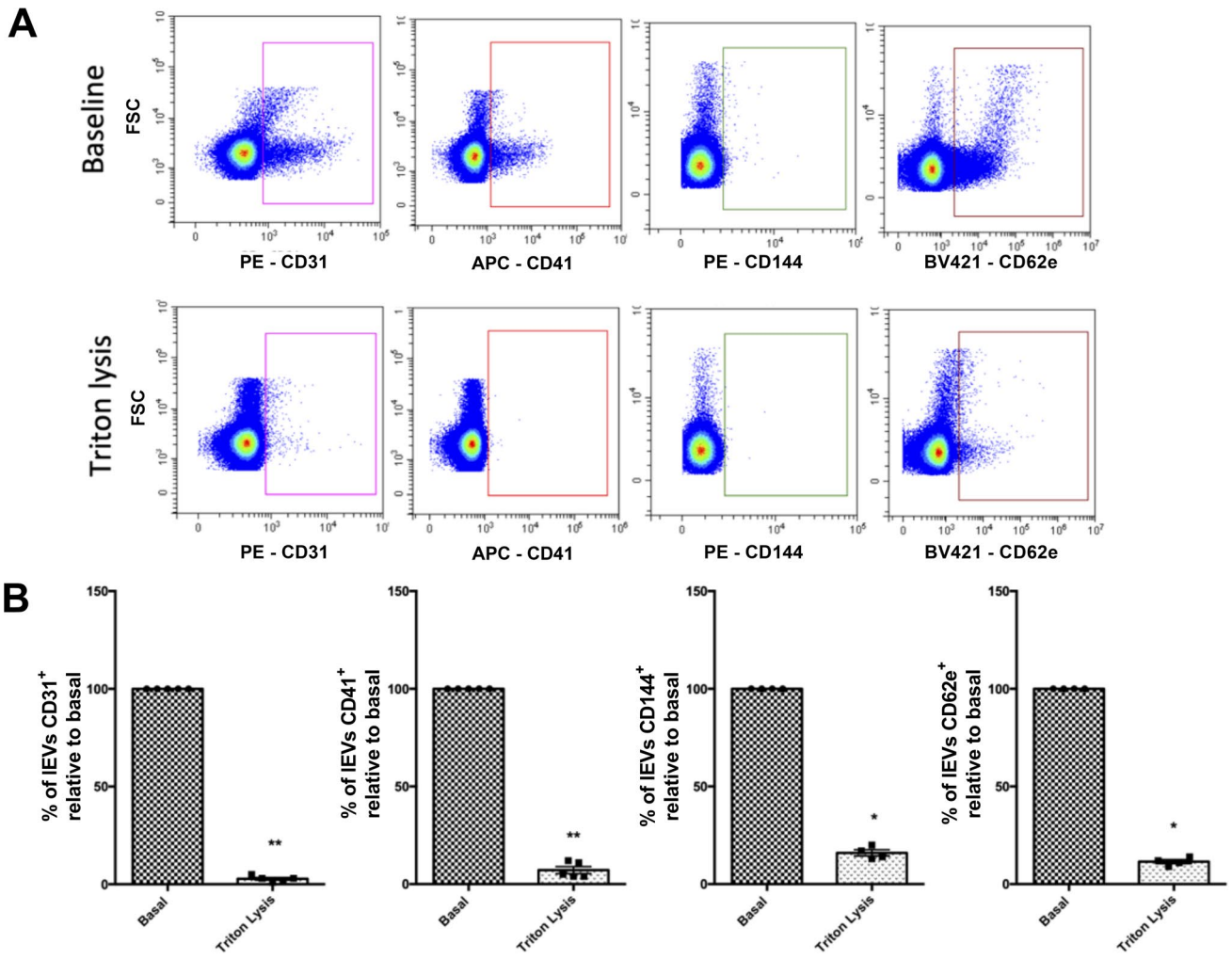

Fig. 2 Effect of Triton lysis on lEVs flow cytometry analysis. (A) Representative experiment assessing levels of endothelial-derived lEVs either in absence (top panels; baseline) or presence of Triton (bottom panels). lEVs number dramatically decreases after Triton lysis. **(B)** Plasma lEVs levels (n = 4) before and after Triton lysis. Data are expressed as mean ± SEM. (*$P < 0.05$; Mann Withney test)

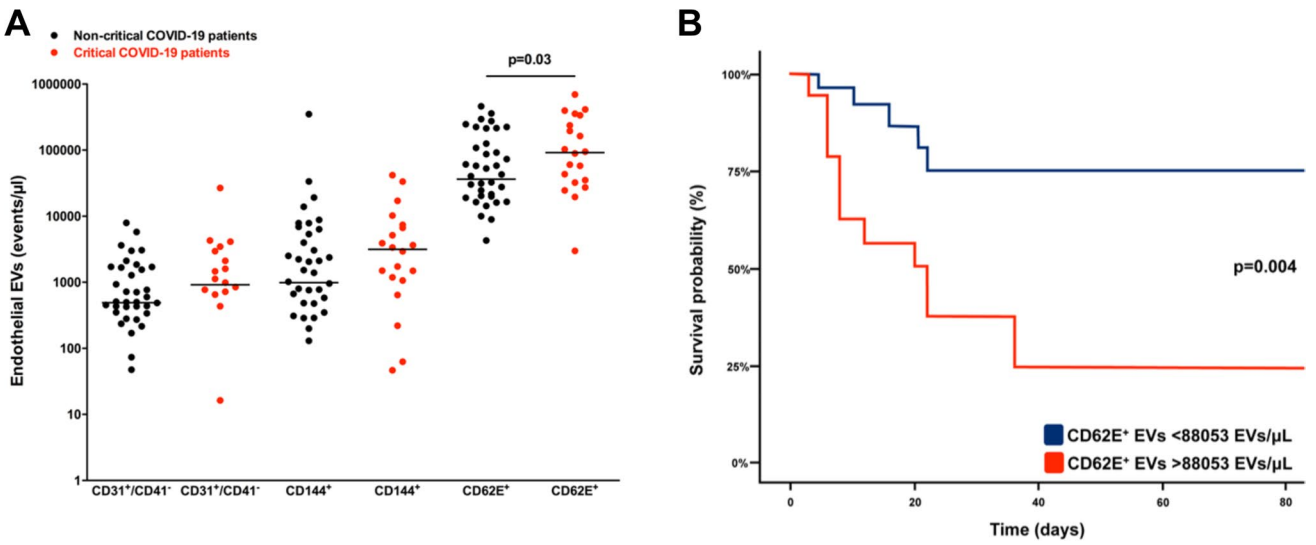

Fig. 3 Endothelial circulating EVs in COVID-19 patients. 3A: CD31 + CD41-, CD144+ and $CD62E^+$ EVs levels at admission (Mann-Whitney and Fisher's tests) **3B**: Kaplan–Meier survival curves, illustrating prognostic impact of $CD62E^+$ EVs

(CD31) or VE-Cadherin (CD144) did not discriminate between critical and non-critical COVID-19 patients.

In this study, we demonstrated that lEVs expressing CD62 (E-selectin) in COVID-19 patients were related to severity of patients at admission and to in-hospital mortality. To our knowledge, this study is the first one to examine different panel of endothelial circulating EVs in different range of COVID-19 severities, from symptomatic to critically ill patients. On the contrary, plasma levels of endothelial EVs expressing the intercellular junctional proteins PECAM-1 (CD31) or VE-Cadherin (CD144) do not discriminate patients with COVID-19. Endothelial EVs, more than just a biomarker, are active partners in coagulation, fibrinolysis, inflammation, cell survival, endothelial regeneration and angiogenesis and have been described to actively contributing to vascular diseases progression [19]. EVs have been described to regulate cellular components of innate immunity, including macrophages, monocytes, granulocytes, NK cells, and dendritic cells as well as soluble components of the innate immunity system, including the ComC [20]. In COVID-19, several stimuli leading to endothelial cell activation could explain EVs increase including hypoxic conditions [21], and inflammation [22]. Interestingly, increased interleukin-6 levels in late complicated COVID-19 stages have been shown to stimulate $CD62E^+$ EVs release in vitro [23]. Moreover, in COVID-19, increased EVs could clearly be active partners in cross talk between cells by participating to complications of inflammation and coagulopathy but also could have beneficial effect as cardioprotection or reducing thromboinflammatory process by decreasing endothelial ICAM-1 expression on endothelial cells [24]**.** According to current COVID-19 outbreak, there are several limitations in our study. First, we did not performed an iterative biomarker measurement over time to provide a more accurate picture of endothelial activation during COVID-19 evolution. Second limitation is the absence of comparison of this increased EVs levels in different wave of COVID-19 considering new variants, new treatments and vaccination effect on COVID-19 severity. Indeed, dexamethasone has now been incorporated into the standard of care for COVID-19 hospital patients [25]. Lower mortality after dexamethasone incorporation into standard of care have been observed: relationship between endothelial EVs regarding anti-inflammatory drugs should be of interest.

All in all, our results highlight the potential for EVs expressing E-selectin (CD62E) to discriminate COVID-19 patients at admission and identify higher risk of in-hospital mortality. Understanding contribution of endothelial EVs biological effects on thrombosis, thromboinflammation or tissular regeneration is still to determine. Plasma levels of $CD62E^+$ EVs at admission may help to identify patients for anti-inflammatory therapies.

Data availability Raw Data are available upon request to corresponding author.

Authors Contributions All the undersigning authors have substantially contributed to the paper. DMS and CMB designed the present study and wrote the manuscript. RC performed statistical analyses. FM, CLG and AP performed and/or analyzed the data, NG, LS, OS, TM and JLD included patients and edited the manuscript. All authors declare that the submitted work is original and has not been published before (neither in English nor in any other language) and that the work is not under consideration for publication elsewhere.

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Declarations

Ethical Approval for SARCODO Study Authorization number 2020–04-048/ 2020-A01048–31/ 20.04.21.49318) - NCT: NCT04624997.

Consent to Participate The study SARCODO was performed in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrollment.

Consent to Publish All patients provided written informed consent to have their data published.

Competing Interests All authors declare no conflict.

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