Respiratory Medicine 223 (2024) 107563

Contents lists available at ScienceDirect

Respiratory Medicine

journal homepage: www.elsevier.com/locate/rmed

Original Research

Enhanced prothrombotic and proinflammatory activity of circulating extracellular vesicles in acute exacerbations of chronic obstructive pulmonary disease

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ARTICLE INFO

Keywords:
Extracellular vesicles
Thrombosis
Inflammation
Chronic obstructive pulmonary disease
Cardiovascular risk

ABSTRACT

Background: Acute exacerbations of chronic obstructive pulmonary disease (AE-COPD) are associated with a high rate of cardiovascular events. Thromboinflammation (the interplay between coagulation and inflammation) is probably involved in these events. Extracellular vesicles (EV) increase during AE-COPD, but their role in thromboinflammation in COPD is still unknown. We investigated EV-associated prothrombotic and proinflammatory activity in COPD.

Methods: Patients with AE-COPD, stable COPD (sCOPD) and age- and sex-matched subjects (controls) were enrolled. AE-COPD patients were evaluated at hospital admission and 8 weeks after discharge (recovery; longitudinal arm). In a cross-sectional arm, AE-COPD were compared with sCOPD and controls.

EV-mediated prothrombotic activity was tested by measuring the concentration of EV-associated phosphatidylserine, as assessed by a prothrombinase assay, and tissue factor, as assessed by a modified one-stage clotting assay (EV-PS and EV-TF, respectively). Synthesis of interleukin-8 (IL-8) and C–C motif chemokine ligand-2 (CCL-2) by cells of the human bronchial epithelial cell line 16HBE incubated with patients' EV was used to measure EV-mediated proinflammatory activity.

Results: Twenty-five AE-COPD (median age [interquartile range] 74.0 [14.0] years), 31 sCOPD (75.0 [9.5] years) and 12 control (67.0 [3.5] years) subjects were enrolled.

In the longitudinal arm, EV-PS, EV-TF, IL-8 and CCL-2 levels were all significantly higher at hospital admission than at recovery. Similarly, in the cross-sectional arm, EV-PS, EV-TF and cytokines synthesis were significantly higher in AE-COPD than in sCOPD and controls.

Conclusions: EV exert prothrombotic and proinflammatory activities during AE-COPD and may therefore be effectors of thromboinflammation, thus contributing to the higher cardiovascular risk in AE-COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a heterogeneous condition characterized by chronic respiratory symptoms and airflow limitation which usually progress over time. Its natural history is punctuated by acute exacerbations (AE), defined as worsening of symptoms and increased sputum production within <14 days [1]. COPD is associated with both pulmonary and systemic inflammation that

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https://doi.org/10.1016/j.rmed.2024.107563
Received 30 December 2023; Received in revised form 7 February 2024; Accepted 9 February 2024
Available online 9 February 2024

increase steeply during AE [2]. Systemic inflammation is a key feature of COPD and is currently believed to represent the biological link between pulmonary and extrapulmonary manifestations [3], the latter responsible for a relevant part of the burden of the disease.

Cardiovascular diseases are probably the most important extrapulmonary manifestations of COPD, and are responsible for a relevant number of deaths in these patients [4], especially during AE (AE-COPD). Indeed, AE-COPD are associated with a particularly elevated cardiovascular risk which tends to return to normal within 31–90 days [5]. Although the mechanisms underlying such increased risk have not been fully elucidated, the clinical association between systemic inflammation and cardiovascular diseases, epitomized by atherothrombosis, is well known [6]. The biological bases of the association between inflammation and coagulation in a process currently referred to as thromboinflammation are also partially known [7].

Extracellular vesicles (EV) are emerging as key players in thromboinflammation, and their role has been extensively investigated in cardiovascular diseases [8]. They have also been studied in the field of respiratory diseases, where they have been essentially investigated as potential biomarkers [9]. EV are submicron structures fully enclosed by a lipid bilayer, shed by cells either constitutively or upon activation. They express on their outer membrane, and contain within their cytoplasm, molecules present in the parental cell, and participate in intercellular communication. Medium-large EV (formerly referred to as microparticles or microvesicles) can express on their surface the negatively charged phospholipid, phosphatidylserine (PS), an essential component of the multimolecular complexes involved in blood coagulation [9]. Furthermore, a subset of EV express tissue factor (TF), a 46-Kd integral membrane protein that functions as an essential cofactor for coagulation factor (F)VII(a) in the initiation of the coagulation cascade [10]. PS- and TF-bearing EV are therefore procoagulant and prothrombotic, and play a role, for example, in cancer associated thrombosis [11]. Along with their prothrombotic role, EV can also exert a proinflammatory activity [12,13]. *In vitro* studies have shown that subsets of EV induce the synthesis of proinflammatory mediators by bronchial epithelial cells, consistent with a potential pathogenic role for EV in COPD [14,15].

Although initial evidence of thromboinflammatory mechanisms involving platelets, monocytes and endothelium in COPD patients has been provided [16–18], studies specifically investigating the potential role of EV in thromboinflammation in these patients are currently lacking. EV of different cell origin increase in COPD patients according to disease severity [19], and in AE-COPD compared to stable patients [20], but their possible pathogenic role has not been investigated in AE-COPD.

Based on the above considerations, we investigated the prothrombotic and proinflammatory activity of EV derived from the peripheral blood of COPD patients in different disease states. Our hypothesis is that the prothrombotic and proinflammatory activity of EV is higher in AE-COPD than in stable disease.

2. Methods

2.1. Subjects and study design

We conducted an observational study enrolling 3 groups of subjects: a) patients within 48 h of admission to the Emergency Department with symptoms compatible with severe AE-COPD (i.e.: AE-COPD requiring hospitalization) and in whom this diagnosis was confirmed at the follow-up visit (see below) (AE-COPD); b) COPD patients that had been free of AE for at least 12 months (stable COPD, sCOPD); c) sex- and age-matched control subjects, recruited among volunteers (controls).

The study had a double design: a) in a cross-sectional arm, EV prothrombotic and proinflammatory activity was compared across the aforementioned groups (AE-COPD, sCOPD and controls); b) in a longitudinal arm, only patients with AE-COPD were re-evaluated 8 weeks after hospital discharge (recovery). At each visit, in both the cross-sectional and the longitudinal arm, complete medical history collection and physical examination were performed. Moreover, all subjects underwent pulmonary function tests (PFTs) according to current recommendations [21]. In AE-COPD group, PFTs were performed at recovery to confirm the diagnosis of COPD, as recommended [1].

Main exclusion criteria were: recent (<3 months) acute cardiovascular events (myocardial infarction, stroke, pulmonary embolism), New York Heart Association stage III-IV chronic heart failure, severe renal failure (estimated glomerular filtration rate <30 mL/min/1.73 m^2), history of asthma, and active cancer.

The study was approved by the Ethic Committee of the Area Vasta Nord-Ovest Toscana, Italy (protocol number 18722); it was conducted according to the Helsinki Declaration, and each participant signed an informed written consent.

2.2. EV isolation from patients blood

At each visit, a sample of 8 mL peripheral blood was withdrawn into sodium citrate (0.9% w/v) and immediately submitted to low-speed centrifugation (1500×*g* for 15 min at 4 °C) to remove whole cells and cell debris. Platelets were then cleared by high-speed centrifugation (16000×*g* for 2 min at 4 °C) to obtain platelet-poor plasma (PPP). Medium-large EV were finally sedimented by a further high-speed centrifugation step (16000×*g* for 45 min at 4 °C). The pellet was resuspended in 100 μL normal saline and stored at −80 °C until analysis. For transmission electron microscopy analysis the pellet was resuspended in 30 μL PBS and stored at 4 °C until analysis, performed within 3–4 days.

2.3. Electron microscopy

Transmission electron microscopy was performed as previously described [22] except that a Zeiss Gemini SEM 500 microscope, equipped with a scanning Transmission Electron Microscopy detector (Zeiss, Oberkochen, Germany) was used.

2.4. EV prothrombotic activity

The prothrombotic activity of EV was evaluated both in terms of EV-associated PS and TF concentration (EV-PS and EV-TF, respectively).

EV-PS was detected in PPP using the Zymuphen MP-activity kit (Hyphen Bio-Med, Neuville Sur Oise, France) according to the manufacturer's instructions. Briefly, the assay is based on the property of annexin-V, immobilized onto plastic wells, to bind PS. PPP is added to the wells and, after extensive washing, captured EV are detected by the addition of FVa, FXa, Ca^{2+} and prothrombin. Under the conditions used, the rate of thrombin formation is limited by PS availability. A chromogenic substrate is finally added to quantify thrombin concentration with a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad, Milan, Italy). Known amounts of PS are used to obtain a standard curve.

To test EV-TF a one-stage clotting assay using a semi-automated coagulation analyzer (Start Max, DiagnosticaStago, Milan, Italy) was performed, as described [23]. Briefly, the test is based on the time to clot formation upon recalcification of citrated normal plasma with 25 mM $CaCl_2$; TF availability is the rate-limiting factor for this reaction. For each experimental session, calibration curves were generated using recombinant human relipidated TF (pg/mL) (BioMedica Diagnostics-Windsor-NS Canada). In our experimental conditions, clotting times of 19 ± 2 s and 627 ± 84 s were obtained with 100 pg/mL and 0.001 pg/mL TF, respectively.

2.5. Cell culture

SV-40 immortalized human bronchial epithelial cells (16HBE) were kindly provided by Dr. M. Profita (National Research Council, Palermo,

Italy). Cells were maintained in minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum, 0.2 mg/mL L-glutamine and 2.5 mM HEPES buffer. 16HBE cells were maintained in a humidified 95% air/5% CO_2 atmosphere at 37 °C.

2.6. EV proinflammatory activity

To test EV proinflammatory activity, we measured EV-induced production of interleukin-8 (IL-8) and C–C motif chemokine ligand-2 (CCL-2) from target cells. Specifically, total EV isolated from study subjects were incubated with 16HBE cells, grown to confluence in 96-well plates for 24 h. Following an 18-h incubation, the conditioned medium was harvested and cleared by centrifugation for 5 min at 16,000×*g*. IL-8 and CCL-2 concentration were measured in the conditioned medium from 16HBE cells by a sandwich ELISA kit (IL-8 and CCL-2 Kit-Elisa-Ready-SET-Go! Affimetrix, USA) with a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad, Milan, Italy) according to the manufacturer's instructions.

2.7. Statistical analysis

Data distribution was assessed for normality by the Shapiro-Wilk test. Data are shown as mean ± standard deviation or median [interquartile range], as appropriate. Comparisons among independent groups were performed by analysis of variance or the Kruskal-Wallis test, and by Mann Whitney or *t*-test for two independent groups, as appropriate. Comparison between non-normally distributed paired data was made by the Wilcoxon matched pairs signed rank test; Fisher exact test was used for categorical variables. A p value < 0.05 was assumed for statistical significance.

Prism software (v. 9.4.1 for MacOS; GraphPad, San Diego, CA, USA) was used for statistical analysis and graph preparation.

3. Results

3.1. Study population

The confirmed AE-COPD group includes 25 patients. Thirty-one and 12 subjects were enrolled in sCOPD and control group, respectively. Table 1 shows the clinical and functional characteristics of the study subjects. The three groups are homogeneous in terms of age and sex distribution, while smoking history is more relevant in COPD patients, as expected. Pulmonary function was obviously worse in COPD patients (both AE-COPD and sCOPD) than in control subjects, with slightly, though not significantly, lower forced expiratory volume in the 1st second (FEV1) values in AE-COPD than in sCOPD. Moreover, AE-COPD patients had higher dyspnea scores and lower blood eosinophils count than sCOPD. There were no significant differences in the distribution of the main comorbidities.

While all study subjects were tested for EV-PS and EV-TF, IL-8 and CCL-2 values are not available for 4 AE-COPD and 3 sCOPD patients for technical reasons unrelated to the patients' characteristics.

Fig. 1 shows a representative electron micrograph image of EV isolated as described in the methods section.

3.2. EV prothrombotic activity

We first analyzed EV-associated prothrombotic activity in the longitudinal arm of the study, and we found that both EV-PS and EV-TF decreased significantly from AE-COPD to recovery (Figs. 2A and 3A, respectively). Moreover, EV-PS and EV-TF were significantly higher in AE-COPD at hospital admission than in sCOPD patients and in control subjects; last, EV-PS and EV-TF were still significantly higher in AE-COPD at recovery than in sCOPD (cross-sectional arm of the study, Figs. 2B and 3B, respectively). There was no difference between sCOPD and controls in both EV-PS and EV-TF levels in the cross-sectional arm of the study (Figs. 2B and 3B).

Table 1
Clinical and functional characteristics of the study subjects.

	AE-COPD (n = 25)	sCOPD (n = 31)	Controls (n = 12)	p
Age, years	74.0 [14.0]	75.0 [9.5]	67.0 [3.5]	n.s
Sex, female:male	14:11	10:21	6:6	n.s.
Smoking history				
● current	12	8	1	p < 0.01
● former	9	22	5	
● never	4	1	6	
Pack-years, n	48.0 [15.0]*	40.0 [20.5]§	0.5 [12.8]*§	*p < 0.01 §p < 0.01
BMI, Kg/m^2	27.3 [6.4]	29.0 [7.5]*	23.9 [3.6]*	*p < 0.05
FEV1, L[a]	1.31 (±0.76)§	1.60 (±0.54)#	3.03 (±0.42)§#	§p < 0.01
% predicted[a]	53.4 (±19.3)§	64.7 (±17.1)#	113.0 (±15.7)§#	#p < 0.01
FEV1/FVC[a]	0.50 (±0.15)*	0.56 (±0.12)§	0.79 (±0.08)*§	*p < 0.01 §p < 0.01
GOLD severity of airflow obstruction[a], n				
● FEV1≥80% pred.	4	8	n.a.	n.s.
● 50≤FEV1 < 80% pred.	6	16		
● 30≤FEV1 < 50% pred.	9	7		
● FEV1 < 30% pred.	1	0		
mMRC	3 [1]*	1 [1]*§	0 [0]*§	*p < 0.01 §p < 0.05
CAT®	15.8 (±7.2)*	11.9 (±7.0)*	n.a.	*p < 0.01
Exacerbations in the previous 12 months, n	2 [1]	0 [0]	n.a.	n.a.
Non-invasive mechanical ventilation in ED, n	6	n.a.	n.a.	n.a.
Eosinophils, μL^{-1}	30 [161]*	150 [173]*	n.a.	*p < 0.05
%	0.2 [2.9]*	2.4 [2.7]*		
Inhaled treatment				
● LAMA/LABA/ICS	18	11	n.a.	p < 0.05
● LAMA/LABA	3	10		
● LAMA	1	7		
● LABA/ICS	3	2		
● none	0	1		
Associated conditions				
● hypertension	19	22	5	n.s.
● heart and arterial disease	10	11	1	n.s.
● diabetes	4	4	3	n.s.
● obesity	6	10	1	n.s.
● psychiatric disorders	8	1	2	n.s.

AE-COPD: acute exacerbations of COPD; sCOPD: stable COPD; BMI: body mass index; FEV1: Forced Expiratory Volume in the 1stsecond; FEV1/FVC: Forced Expiratory Volume in the 1st second to Forced Vital Capacity ratio; mMRC: modified Medical Research Council scale for dyspnoea; CAT®: COPD Assessment Test; ED: Emergency Department; LAMA: long-acting anti-muscarinic drugs; LABA: long-acting β_2-agonist drugs; ICS: inhaled corticosteroids; n.a.: not applicable; n.s.: not significant.
Data are presented as mean (±standard deviation) or median [interquartile range], as appropriate.
[a] Spirometric data for 5 patients belonging to AE-COPD group with a previous reliable diagnosis of COPD available in our records were not recorded at recovery because of clinical conditions..

3.3. EV proinflammatory activity

When analyzing the proinflammatory activity of EV, we found that the EV-induced IL-8 and CCL-2 synthesis by 16HBE cells decreased significantly from hospital admission to recovery in AE-COPD

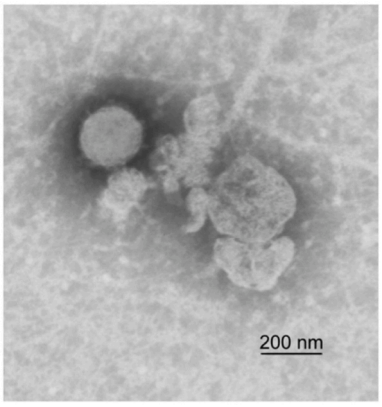

Fig. 1. Representative electron micrograph image of circulating extracellular vesicles isolated as described in the Methods section.

(Figs. 4A–5A, longitudinal arm). Furthermore, cytokines levels were significantly higher in AE-COPD at hospital admission than in sCOPD and controls, and they remained significantly higher in AE-COPD at recovery compared to sCOPD (cross-sectional arm, Figs. 4B–5B). While we found no difference in CCL-2 levels between sCOPD and controls (Fig. 5B), IL-8 synthesis was significantly higher in sCOPD than in controls (Fig. 4B).

4. Discussion

We conducted this study to explore the potential prothrombotic activity (evaluated as EV-associated PS and EV-associated TF) and proinflammatory activity (evaluated as EV-induced IL-8 and CCL-2 synthesis by 16HBE) of circulating EV isolated from the peripheral blood of COPD patients in different states of the disease. Our data show that EV-PS levels, EV-TF levels, and EV-mediated cytokines production are significantly higher at the onset of AE-COPD than 8 weeks after hospital discharge (recovery). Moreover, EV-associated prothrombotic and proinflammatory activity is higher in AE-COPD patients, both at hospital admission and at recovery, than in sCOPD and control subjects.

Previous studies have investigated the presence of a COPD-related prothrombotic status. Vaidyula et al. found a higher level of "circulating" TF in 11 moderate-to-severe COPD patients compared to 45 healthy controls [24]. However, the authors did not differentiate among cell- and EV-associated TF, while we specifically looked for EV-associated TF. Moreover, they did not investigate patients during AE. Another study demonstrated detectable TF activity in the blood of a small percentage of 60 stable COPD patients. Again, the Authors did not investigate EV-associated TF activity, nor did they evaluate a possible change in TF levels during AE, specifically stating that it would have been an interesting topic for future studies [25]. A more recent work [26] described a "prothrombotic state" in 103 stable COPD patients, characterized by increased levels of FII, FV, FVIII and FX; TF was not tested. Finally, to the best of our knowledge, EV-PS has not been previously investigated in this context. Taken together, the aforementioned results confirm that COPD is associated with an increase in a number of molecules involved in blood coagulation, but no studies have specifically evaluated prothrombotic EV in the context of COPD, especially during AE. Our finding that total EV-PS and EV-TF are significantly higher at the onset of AE than 8 weeks after hospital discharge therefore provides a theoretical basis for the increased risk for acute cardiovascular events that has been described in these patients. In particular, since our AE patients were recruited after an event that required hospitalization, our findings are consistent with the observation that the relative risk for myocardial infarction, sudden cardiac death or stroke is particularly elevated during, or immediately after, a severe AE-COPD [27]. Moreover, the observation that EV-PS and EV-TF measured several weeks after a severe AE remain higher compared to stable patients (Figs. 2B and 3B) are consistent with their involvement in the long-lasting cardiovascular risk after an AE [5].

Since COPD is characterized by chronic inflammation with acute bouts during AE, we also hypothesized a proinflammatory action for EV isolated from AE-COPD patients. The role of IL-8 and CCL-2 as main attractants for neutrophils and monocytes in the pathogenesis of COPD has been extensively described [28]; interestingly, these cytokines have also been studied in cardiovascular diseases. Indeed, both IL-8 and CCL-2 are involved in the biological events that eventually lead to the formation of the atherosclerotic plaque [29]. Moreover, IL-8 has been proposed both as a marker of systemic inflammation in COPD [30] and as a biomarker in cardiovascular diseases [29,31]. We have previously demonstrated a proinflammatory activity for EV using *in vitro* models

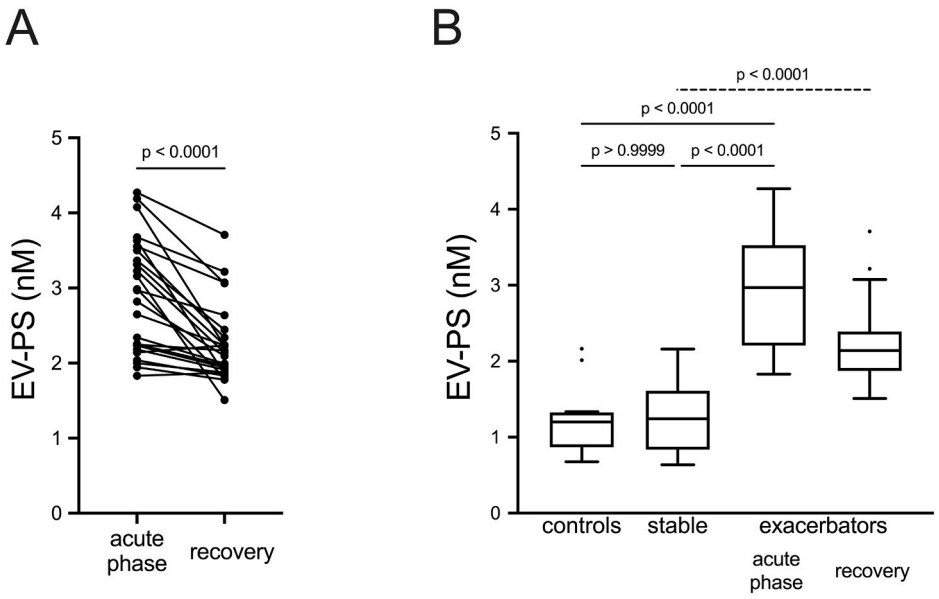

Fig. 2. Extracellular vesicle-associated phosphatidylserine concentration (EV-PS). Panel A: comparison between acute phase and recovery in exacerbators (longitudinal arm of the study; Wilcoxon matched pairs signed rank test). Panel B: comparisons among control subjects, stable COPD patients and exacerbators in the acute phase (Kruskall Wallis test; solid lines); comparison between exacerbators after recovery and stable patients (Mann Whitney test; dashed line).

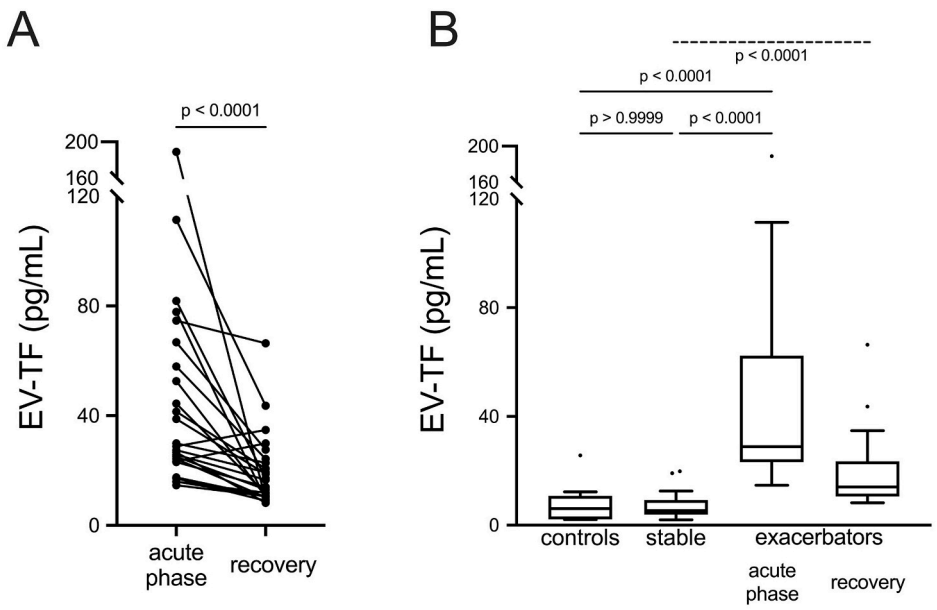

Fig. 3. Extracellular vesicle-associated tissue factor concentration (EV-TF). Panel A: comparison between acute phase and recovery in exacerbators (longitudinal arm of the study; Wilcoxon matched pairs signed rank test). Panel B: comparisons among control subjects, stable COPD patients and exacerbators in the acute phase (Kruskall Wallis test; solid lines); comparison between exacerbators after recovery and stable patients (Mann Whitney test; dashed line).

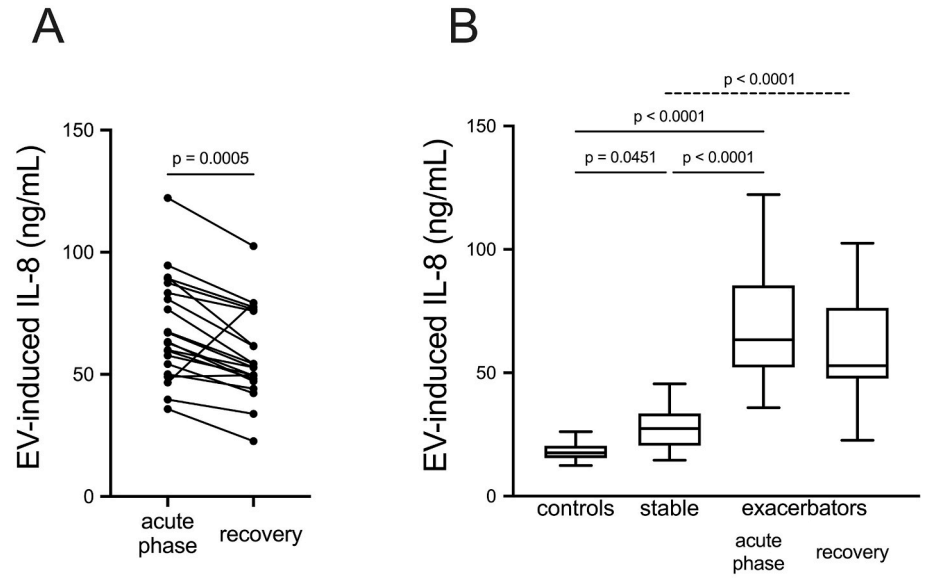

Fig. 4. Extracellular vesicle-induced interleukin-8 (IL-8) synthesis by 16HBE cells. Panel A: comparison between acute phase and recovery in exacerbators (longitudinal arm of the study; Wilcoxon matched pairs signed rank test). Panel B: comparisons among control subjects, stable COPD patients and exacerbators in the acute phase (Kruskall Wallis test; solid lines); comparison between exacerbators after recovery and stable patients (Mann Whitney test; dashed line).

involving bronchial epithelial cell lines [14,32], while recent elegant studies have shown that small EV (commonly referred to as exosomes) isolated from the bronchoalveolar lavage fluid (BALF) of both stable COPD patients [33] and current smokers without COPD [34] are involved in the pathogenesis of emphysema. In particular, these exosomes were proven to carry neutrophil elastase and matrix metalloproteinase 12 on their outer surface; once transferred to murine models, exosomes caused the pathologic damages characteristic of pulmonary emphysema [33,34]. While these studies show a pivotal role for airway-derived EV in the pulmonary inflammatory milieu, our results have the potential advantage of reflecting the systemic inflammation characterizing COPD, since it focuses on circulating EV. We demonstrate that circulating EV exert a proinflammatory role after incubation with target bronchial epithelial cells (16HBE), thus inducing the production of cytokines involved in both COPD and cardiovascular diseases. It is conceivable that this mechanism, involving EV, can be part of the so-called "spillover" of pulmonary inflammation, which is thought to be responsible for COPD extrapulmonary manifestations [3,35]: actually, once secreted by bronchial cells, IL-8 and CCL-2 could pass into the general blood circulation and provoke systemic effects in distant organs like blood vessels. In this light, our data may be regarded as complementary to those found in the aforementioned studies [33,34].

The proinflammatory activity, in terms of IL-8 or CCL-2 production, of airways-derived EV has also been investigated in respiratory diseases other than COPD. In asthma, exosomes derived from BALF of asthmatic subjects, but not from normal controls, induced a significant increase in

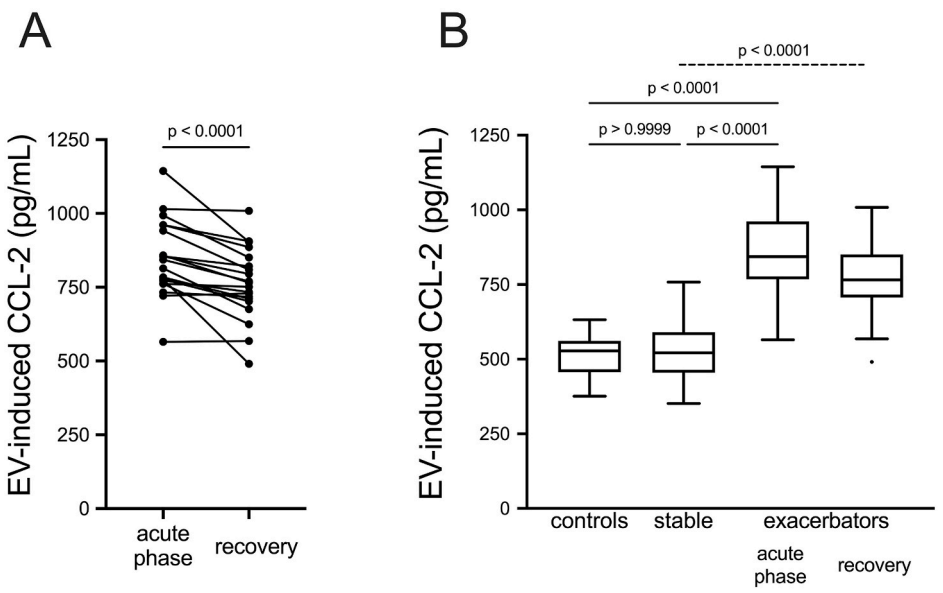

Fig. 5. Extracellular vesicle-induced C–C motif chemokine ligand-2 (CCL-2) synthesis from 16HBE cells. Panel A: comparison between acute phase and recovery in exacerbators (longitudinal arm of the study; Wilcoxon matched pairs signed rank test). Panel B: comparisons among control subjects, stable COPD patients and exacerbators in the acute phase (Kruskall Wallis test; solid lines); comparison between exacerbators after recovery and stable patients (Mann Whitney test; dashed line).

IL-8 production by cells of the bronchial line, 16HBE [36]. Furthermore, a significant production of IL-8 and CCL-2 was demonstrated after incubating exosomes isolated from BALF of patients affected by sarcoidosis with 16HBE cells and monocytes, respectively [37,38]. While all the aforementioned studies investigated EV isolated from respiratory secretions, we chose to investigated EV isolated from blood because they are quite easier and minimally invasive to obtain and could reflect systemic inflammation better than bronchial and alveolar inflammation in COPD [39]. On the other hand, the cell biology model we used is conceptually similar to those provided by some of those studies [36–38], thus showing its repeatability across different laboratory settings. Since some previous studies showed that IL-8-induced neutrophilic inflammation can worsen the atherosclerotic process and that CCL-2 can be involved in both atherogenesis and plaque instability [40,41], it is conceivable that the proinflammatory role of EV we found can also be involved in the higher cardiovascular risk of COPD patients, especially after severe AE.

The application of a cell biology-based *ex vivo* model to study EV isolated from COPD patients, and the demonstration of simultaneous EV-associated prothrombotic and proinflammatory activity represent in our opinion the main strengths and the novelty of our study.

This study also has some limitations. First, the small sample size, while comparable with those of the aforementioned studies on TF in COPD [24,25] and EV-induced cytokine synthesis in other respiratory diseases [36–38], does not allow for firm conclusions. Second, the nature of the study does not allow us to establish a definite causal relationship between the higher levels of prothrombotic and proinflammatory EV and cardiovascular risk. Third, we decided to focus only on medium-large EV-associated TF, since TF has historically been associated only with these vesicles. However, a recent paper published when our study was well underway showed a significant association of TF activity also with small EV (defined as vesicles isolated through centrifugation at 100000×*g*), thus suggesting that measuring both large and small EV-associated TF activity would provide more complete information [42]. Moreover, measuring total plasmatic TF along with EV-associated TF activity would have provided useful information to further clarify the individual contribution of EV-TF to prothrombotic activity in COPD. Fourth, we do not know if the higher EV-associated prothrombotic and proinflammatory activity that we observed at the onset of AE-COPD was due to an increased number of EV or to the generation of a similar number of more potent EV. Of note, a previous study found higher levels of some subtypes of circulating EV at the onset of AE-COPD than at recovery [20]. However, in our opinion, this does not invalidate the results, since they can still be used to explain the mechanisms underlying the increased prothrombotic and proinflammatory activity of EV in these patients. Finally, we did not investigate the cell source of the EV we tested, since this was not the main focus of this study, and we cannot know whether different EV populations are present in different stages of disease (AE-COPD onset versus recovery, stable COPD).

5. Conclusions

In conclusion, our data shed light on the role of EV as effectors of thromboinflammation, a possible pathogenetic drive of AE-COPD and, more specifically, of the increased risk of cardiovascular events that characterizes these events. Moreover, the observation that EV-PS, EV-TF, and EV-induced synthesis of IL-8 and CCL-2 measured several weeks after an AE remain higher compared to stable disease is consistent with the involvement of EV in the long-lasting thromboinflammatory state following an AE-COPD. In fact, one might hypothesize the existence of a specific endo-phenotype characterized by persistent increased concentrations of EV-PS, EV-TF and proinflammatory EV. Prospective studies are needed to confirm these findings and to further investigate whether the risk for further episodes of AE and of cardiovascular events can be predicted at the individual level based on the analysis of EV-associated prothrombotic and/or proinflammatory activity.

CRediT authorship contribution statement

Dario Nieri: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Camilla Morani:** Writing – review & editing, Investigation, Data curation. **Miriam De Francesco:** Writing – review & editing, Investigation, Data curation. **Roberta Gaeta:** Writing – review & editing, Investigation, Data curation. **Mariapia Niceforo:** Writing – review & editing, Investigation, Data curation. **Mariella De Santis:** Writing – review & editing, Investigation, Data curation. **Ilaria Giusti:**

Methodology, Investigation. **Vincenza Dolo:** Methodology, Investigation. **Marta Daniele:** Writing – review & editing, Investigation, Data curation. **Alberto Papi:** Writing – review & editing, Investigation, Data curation. **Alessandro Celi:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Tommaso Neri:** Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None of the authors has relevant competing interests to declare.

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