ORIGINAL RESEARCH

Microparticles in sputum of COPD patients: a potential biomarker of the disease?

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Background: Microparticles (MPs) are small membrane vesicles of 0.1–1 µm which are released by cells following chemical, physical, and apoptotic stimuli. MPs represent more than a miniature version of the cell. Their composition and function depend not only on cellular origin, but also on stimuli. Chronic obstructive pulmonary disease (COPD) is a lung disease characterized by nearly irreversible lung destruction which results in airway limitation.

Purpose: We investigated the presence and source of MPs in sputum of COPD patients to evaluate if changes in MP number and origin may reflect the pathophysiological conditions of disease and may serve as potential biomarkers for diagnostic and prognostic use.

Methods: Induced sputum samples were collected from 18 male subjects and liquefied with Sputasol. MPs obtained were immunolabeled for leukocyte (CD11a), granulocyte (CD66b), monocyte-macrophage (CD11b), platelets and megakaryocytic cells (CD41), endothelial cells (CD31), and red blood cells (CD235ab) and analyzed by cytofluorimetry.

Results: There was a negative correlation between CD31-MPs and forced expiratory volume in 1 second ($R=-53$, $P<0.05$) and CD66b-MP level was correlated with worse performance index of COPD such as the Body mass index airflow Obstruction, Dyspnea, and Exercise capacity (BODE); they were negatively correlated with 6-minute walking test: 0.65 and −0.64, respectively ($P<0.05$). CD235ab-MPs showed a negative correlation with body mass index ($R=-0.86$, $P<0.05$), while there was a positive correlation with dyspnea index ($R=0.91$, $P<0.05$).

Conclusion: The main finding of this study was that MPs were detected in the sputum of patients affected by COPD. The phenotype of some of them was related to the main COPD parameters. These results suggest that MPs could be implicated in the pathogenesis of COPD.

Keywords: COPD, microparticles, sputum, lung

Background

Extracellular vesicles have received a great deal of attention during the last decade as a novel approach to detect diseases as messengers or mediators of disease pathophysiology. The main classes of extracellular vesicles generally include exosomes, microvesicles/microparticles (MPs), and apoptotic bodies, which are differentiated by their biogenesis and secretion mechanisms. Exosomes are 50–150 nm in diameter and are characterized by their endosomal origin. Exosomes are released by endocytosis following intracellular assembly in multivesicular bodies that contain intraluminal vesicles.

MPs are shed from the plasma membrane through direct outward budding, and they are larger than exosomes (100–2,000 nm). MPs are enriched in phosphatidylserine and contain a membrane component that is similar to that of the parent cell membrane.[1] Apoptotic bodies are 1–4 µm in diameter and are released from the plasma membrane as blebs of cells undergo apoptosis. Apoptotic bodies may contain DNA

fragments, noncoding RNAs, and cell organelles.[2] Several cell types (such as macrophages, platelets, endothelial cells, granulocytes, monocytes, lymphocytes) release MPs following chemical (cytokines, thrombin, and endotoxin), physical (shear stress and hypoxia), and apoptotic stimuli. MPs play an active role in the initiation and amplification of the coagulation cascade, and a pivotal role has been proposed for them in thrombosis, propagating inflammation, modulating vascular tone, angiogenesis, stem cell engraftment, and tumor metastasis.[3] MPs have been isolated from different biological fluids including plasma,[4] serum,[5] cerebrospinal fluid,[6] bronchoalveolar lavage,[7] and synovial fluid.[8]

The phenotype of circulating MPs and, consequently, their origin are different in various pathological conditions, and detection of their cellular origin may serve as a predictor or marker of diseases.[9]

MPs are more than just a miniature version of the specific cell of origin, although the antigens found on the surface of MPs and their cargo resemble those of their parental cells (eg, lineage markers), as certain MP components are selectively enriched compared to their parental cell.

Recent reports have underlined their role as signaling elements in cell–cell communication. Interest has been attracted to MPs because of their positive correlations with various vascular diseases, but now investigation is also being made of MPs in pulmonary diseases in view of their amplified numbers, procoagulant properties, and participation in inflammatory events.

Many studies have been conducted in order to characterize circulating MPs in pulmonary diseases. Mutschler et al showed, for the first time ever, the presence of MPs, derived from platelets, in pulmonary air–liquid interfaces in sedated pigs.[8] Recent investigations conducted in bronchoalveolar lavage fluid characterized intra-alveolar procoagulant MPs in patients with acute respiratory distress syndrome and hydrostatic pulmonary edema. In acute respiratory distress syndrome patients, intra-alveolar MPs contain high levels of tissue factor, show a highly procoagulant activity, and likely contribute to intra-alveolar fibrin formation, a critical pathogenic feature of acute lung injury.[10,11]

Chronic obstructive pulmonary disease (COPD) is a lung disease characterized by irreversible lung destruction which results in airflow limitation. The severity of the disease depends largely on the degree of airflow limitation, which is measured by forced expiratory volume in 1 second (FEV_1).[12]

In 2010, Porro et al[13] provided evidence of the presence of MPs in sputum obtained from cystic fibrosis patients and also found that MPs obtained from cystic fibrosis sputum are proinflammatory when injected into the lungs of mice.[14]

The goal of the present study is to investigate the presence and source of sputum MPs in COPD patients and to correlate the number and source of MPs to the clinical picture. Changes in MP number and composition may reflect the disease pathophysiological conditions and, therefore, could have potential prognostic value for diagnostic use. Understanding MP involvement in COPD may provide insight into disease mechanisms and also aid in the development of novel therapeutic strategies.

Methods
Study patients
The study was approved and performed according to the ethical standards of CE Azienda Ospedaliero-Universitaria Ospedali Riuniti di Foggia on human experimentation. Written informed consent was obtained from each subject.

Induced sputum samples were collected from 18 male subjects with mild to severe COPD (I–IV stages according to Global initiative for chronic Obstructive Lung Disease guidelines) enrolled at the Institute of Respiratory diseases (Ospedali Riuniti of Foggia). All subjects were former smokers, and stopped smoking for at least 1 year. Presence of main comorbidities was also evaluated and it was found that the majority of patients had been affected by cardiovascular diseases (Table 1). All subjects completed the study questionnaires on smoking status, COPD Assessment Test score, and general data, and performed standardized spirometry. The equipment was calibrated daily using a 3 L syringe. In accordance with the Global initiative for chronic Obstructive Lung Disease guidelines, the subjects were defined as COPD when FEV_1/forced vital capacity was <70% post-bronchodilation. All patients with COPD had stable disease with no exacerbation or respiratory tract infection 2 months before the study. Drugs for COPD (inhaled corticosteroid/long-acting β2-agonist or long-acting muscarinic antagonist) were interrupted at least 7 days before the collection of sputum. The data about the 6-minute walking test (6MWT), and body mass index (BMI), and dyspnea by Borg scale[15] were also collected, and the BODE index was then calculated according to international guidelines were also calculated according to international guidelines.[16]

Collection of sputum samples
Sputum induction was achieved by making the patients inhale hypertonic saline solution via UltraNeb DevilBiss (Sunrise Medical, Wollaston, UK) treated with Sputasol (Oxoid,

Table 1 General characteristics of COPD patients at enrollment in the study

	Mean	SD
Age (years)	70.33	8.98
BMI (kg/m²)	28.39	5.10
Pack/years	39.13	20.34
FEV_1/FVC	50.83	15.21
FEV_1 (%)	52.39	22.19
FVC (%)	81.28	23.44
GOLD stage I (N)	3	
GOLD stage II (N)	7	
GOLD stage III (N)	4	
GOLD stage IV (N)	4	
mMRC	2.46	1.81
Borg	11.00	3.51
Number of exacerbations	1.69	0.63
BODE index	2.64	1.96
6MWT (m)	326.08	103.40
pH	7.41	0.03
$paCO_2$ (mmHg)	45.49	8.33
paO_2 (mmHg)	64.43	11.61
HCO_3 (mEq)	28.34	5.84
SaO_2 (%)	92.46	6.11
Hypertension (%)	22	
Heart diseases (%)	44	
Diabetes (%)	11	
Others diseases (%)	28	

Abbreviations: 6MWT, 6-minute walking test; BMI, body mass index; BODE, Body mass index airflow Obstruction, Dyspnea, and Exercise capacity; COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; GOLD, Global initiative for chronic Obstructive Lung Disease; mMRC, modified Medical Research Council scale for dyspnea; SD, standard deviation.

Hampshire, UK) according to European Respiratory Society guidelines.[17] The whole expectorate was collected directly into a clear plastic Petri dish where the selection process was performed. We used forceps to pull the sputum out of the surrounding saliva. The selected sputum (plugs) was dissolved in Sputasol. After homogenization, the solution was filtered through a nylon mesh filter (53 µm nylon mesh).

The filtered cell suspension was centrifuged at $600\times g$ for 10 minutes at 4°C–8°C, and the supernatant was aspirated and stored at −80°C for the analysis of MPs, performed later. The total cell count and viability of sputum cells were obtained simultaneously in a Bürker counting chamber. Cytospins were prepared, stained with Diff Quick Stain (Medion Diagnostics, Düdingen, Switzerland), and two researchers with training in reading induced sputum slides independently counted 400 nonsquamous cells on the stained slides.

MP isolation

The processed sputum was centrifuged at $37\times g$ for 3 minutes. The supernatant was then centrifuged at $253\times g$ for 10 minutes and recentrifuged at $253\times g$ for 20 minutes to remove the cells and large debris, respectively. Two hundred microliters of each MP-containing supernatant was frozen and stored at −80°C until characterization by flow cytometry.

MP characterization

The MP population was characterized in the sputum supernatant according to the expression of membrane-specific antigens. Antihuman CD11a labeling was used to count leukocyte MPs, while counting of granulocyte MPs was performed using antihuman CD66b. Platelets and megakaryocytic MPs were counted using antihuman CD41, platelet–endothelial MPs (EMPs) using antihuman CD31, and MPs from red blood cells were counted using antihuman CD235ab. Human Immunoglobulin M was used as isotype-matched negative control for CD66b, IgG1 was used as isotype-matched negative control for CD11a, CD41, and CD31, while IgG2 was the negative control for CD235ab. For these studies, 10 µL of supernatant MPs was incubated with 10 µL of specific antibody (1 µg/mL; fluorescein isothiocyanate conjugated; BioLegend, San Diego, CA, USA). After 15 minutes of incubation at +4°C, the samples were diluted in 500 µL of 0.9% saline solution. Then, 10 µL of flow count beads were added to each sample and analyzed in a flow cytometer (Beckman Coulter Epics XL-MCL, Miami, FL, USA). Sample analysis was stopped after counting 10,000 events.

Statistical analysis

All data are reported as mean ± standard deviation and analyzed by Statistica Software (StatSoft, Inc, Tulsa, OK, USA). Continuous data are presented as mean ± standard deviation in the tables. The relationships between variables were determined by measuring the Pearson's correlation coefficient or Spearman's correlation for the variables which were not normally distributed. A P-value of <0.05 was considered statistically significant.

Results

Patients

Patient characteristics are summarized in Table 1. Study participants were aged between 51 and 85 years and had a mean FEV_1 of 52.39%±22.19%. Induced sputum was characterized by a high level of neutrophils (86.33%±13.98%) and although in two patients we found a higher number of eosinophils, in both cases, the level was lower than 10%, while the prevalence of neutrophils was higher than 85% (Table 2).

Table 2 Cellularity of induced sputum in COPD patients

	Mean	SD
Eosinophils (%)	5.00	4.18
Neutrophils (%)	86.33	13.98
Macrophages (%)	10.33	13.54
Lymphocytes (%)	0.67	0.58

Abbreviations: COPD, chronic obstructive pulmonary disease; SD, standard deviation.

MP phenotype

MPs were found in induced sputum of all 18 subjects enrolled. The MP phenotype was analyzed by evaluating the presence of different antigens representing all cell types. The expression of CD66b-MPs (granulocytes) was higher than that of other MPs, CD235ab (erythrocytes), and CD31-MPs (platelets/endothelial cell adhesion molecules 1) were also frequently found, instead the levels of CD41-MPs and CD11a-MPs were generally low (Table 3).

Correlation between main COPD parameters and MP phenotype

Table 4 summarizes the correlations between the MP phenotype and the main COPD parameters.

There was a negative correlation between CD31-MPs and FEV_1 ($R=-0.53$, $P<0.05$; Figure 1). CD66b-MPs were correlated with a worse COPD performance index, being positively correlated with the BODE index and negatively correlated with 6MWT: 0.65 and -0.64, respectively ($P<0.05$). CD235ab-MPs showed a negative correlation with BMI ($R=-0.86$, $P<0.05$) and a positive correlation with dyspnea index ($R=0.91$, $P<0.05$). CD41-MPs and CD11a-MPs did not show correlations with the other parameters analyzed (data not shown).

Finally, no correlation was found between the number of MPs and induced sputum cellularity, or with the number of disease exacerbations.

Discussion

The main result of the present study is the demonstration that in the sputum of patients affected by COPD, it is also

Table 3 Characterization of MPs in COPD sputum

	Mean	SD
MPs ($\times10^3$)/μL	20.02	27.09
Cells/mL in sputum ($\times10^6$)	1.18	0.21
CD66b-MPs (%)	37.70	28.89
CD11a-MPs (%)	4.42	13.14
CD41-MPs (%)	7.71	7.82
CD31-MPs (%)	14.52	12.95
CD235ab-MPs (%)	19.86	21.86

Abbreviations: COPD, chronic obstructive pulmonary disease; MPs, microparticles; SD, standard deviation.

Table 4 Main correlations between the MP phenotype and some COPD characteristics

%	CD66b-MPs	CD31-MPs	CD235ab-MPs
BMI	0.01	−0.34	−0.86*
FEV$_1$%	0.28	−0.53*	−0.45
Borg	0.24	−0.46	0.91*
BODE	0.65*	−0.08	−0.09
WT (m)	−0.64*	0.25	0.39
Neutrophils	0.43	0.19	0.21
Macrophages	−0.54	−0.05	−0.08
Eosinophils	0.43	−0.42	0.27

Note: *$P<0.05$.
Abbreviations: BMI, body mass index; BODE, Body mass index airflow Obstruction, Dyspnea, and Exercise capacity; COPD, chronic obstructive pulmonary disease; FEV$_1$, forced expiratory volume in 1 second; MPs, microparticles; WT, walking test.

possible to detect the presence of MPs. The MPs were obtained with the same protocol used in a previous study[13] and they were identified through measures of cytofluorimetric analysis.

The phenotype of some MPs is related with the main COPD parameters such as FEV_1, BODE index, or 6MWT. These results, together with other data, suggest that MPs are likely implicated in the pathogenesis of COPD.

There are various subtypes of MPs that are defined according to specific membrane antigens, such as endothelial/platelet cell adhesion molecule 1 (CD31), leukocytes (CD11a), megakaryocytic (CD41), granulocytes (CD66b), monocyte-macrophages (CD11b), and red blood cells (CD235ab), which have recently been described in a number of diseases including pulmonary hypertension and acute coronary syndrome.[18]

FEV$_1$% vs CD31
$r=-0.5347$; $P=0.0222$; $r^2=0.2859$
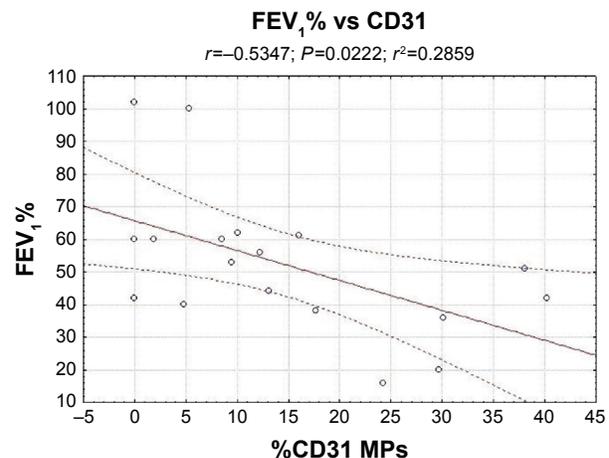
Figure 1 Correlation between FEV$_1$% and CD31-microparticles.
Note: COPD patients with a worse lung function have higher levels of CD31-MPs in sputum.
Abbreviations: COPD, chronic obstructive pulmonary disease; FEV$_1$, forced expiratory volume in 1 second; MPs, microparticles.

Different MPs were found in induced sputum of all patients enrolled; the levels of CD41-MPs and CD11a-MPs were low, CD235ab-MPs and CD31-MPs were frequently found, and CD66b-MPs were the most abundant among all other MPs.

No correlation was found between the number of MPs and induced sputum cellularity, as well as the number of exacerbations. This could mean that MPs were not strictly derived from sputum cells or influenced by exacerbations.

Platelet/endothelial cell adhesion molecule 1 (CD31) is a signaling molecule that plays various roles in vascular biology, in particular, in the regulation of platelet function, angiogenesis, T- and B-cell activation, endothelial cell permeability, and transmigration across the endothelium.[19–24] PECAM-1 is concentrated at endothelial junctions and is also expressed on the surface of platelets, neutrophils, and subsets of lymphocytes. Unlike vascular endothelial-cadherin, PECAM-1 is located outside the adherence junctions on endothelial cells.[25]

Takahashi et al[26] reported that CD31-EMPs are released from pulmonary microvascular endothelial cells mainly in response to apoptosis induced by stimulation by H_2O_2 or cigarette smoke extract.

Thus, the released EMPs likely reflect the apoptosis of injured endothelial cells.[6] In a recent paper, Thomashow et al[27] demonstrated that circulating levels of CD31-MPs were higher in COPD patients compared to control subjects and, moreover, that there was a negative correlation with FEV_1 and with the percentage of emphysema. Liu et al[28] also found a relationship between CD31+ and the severity of obstruction in animal models.

In our study, we found high levels of CD31-MPs also, in the sputum of COPD patients, the numbers being negatively correlated with the severity of disease. Patients with a worse lung function have highest levels of CD31-MPs (Figure 1).

Thus, we can hypothesize that CD31-MPs could be directly correlated with lung damage; in fact, these data indicate that the high levels of circulating and local MPs could reflect the decline of small airway function in COPD patients. Moreover, the presence of CD31-MPs in sputum could hypothesize lung epithelium and vascular endothelium damage in COPD patients.

On the other hand, CD66b-MPs and CD235ab-MPs were more strongly correlated with a worsening of the main COPD indexes such as BODE and 6MWT. Moreover, CD235ab-MPs showed a negative correlation with BMI and a positive correlation with dyspnea index. In this case, it is more difficult to explain the relationship with MPs because they are multiparametric indexes and, therefore, different

components could be involved in their decline. We can only suppose that during the progression of disease, endothelial activations are increased, and this mechanism could upregulate the expression of a pool of MPs, including CD66b and CD235ab. However, clinical relevance of these correlations should be evaluated in future larger studies.

Recent studies, in fact, demonstrated that the main causes of death in COPD patients are not respiratory events, but cardiovascular events such as ischemic heart disease and stroke.[29] Vascular abnormalities in the endothelium have been reported in both pulmonary[30] and systemic vasculatures[31] in COPD patients. Impaired endothelial function, as assessed by flow-mediated dilation of the brachial artery, is associated with a low FEV_1 in COPD patients.[6,32] Endothelial injury in the pulmonary capillary vasculature leads to lung destruction, and since cardiovascular diseases are the main cause of death among individuals with COPD, EMPs are now receiving attention as potential biomarkers for COPD.

The number of circulating EMPs is increased in patients with vascular disorders, such as acute coronary disease,[33,34] renal failure,[35] and metabolic diseases,[36] and reflects the endothelial damage occurring in these patients. Moreover, the number of EMPs is a sensitive marker of pulmonary capillary endothelial damage induced by smoking in healthy active smokers.[37]

The number of apoptotic epithelial and endothelial cells is increased in emphysematous lung as compared to normal lung.[38] The senescence of alveolar epithelial and endothelial cells is accelerated in patients with emphysema.[39] Greater numbers of apoptotic lung cells are observed in lung tissues from COPD patients than in those from smokers without COPD.[40–42] Furthermore, morphological and biochemical markers of autophagy are increased in the lungs of patients with COPD compared with normal lung tissue. These results indicate the importance of injured cells in the pathophysiology of lung destruction and COPD.[6]

MPs are not passive agents induced from activated or injured cells, but rather active modulators that promote both proinflammatory and anti-inflammatory signals.[43] MPs contain proteins and microRNAs and can deliver those components to distant endothelial cells.[44] Therefore, increased EMPs may influence vascular function and systemic inflammation under COPD exacerbation.[17]

Increased DNA fragmentation in the pulmonary capillaries and arteriolar endothelium of individuals with COPD was shown by Segura-Valdez et al.[45] In addition, Kasahara et al[38,46,47] reported increased septal cell death (endothelial and epithelial cells) in human emphysematous lungs compared with lungs of nonsmokers or smokers without emphysema.[37]

Conclusion

The main result of our study is not only the presence of MPs in COPD patients' sputum, but also the relation between the number of EMPs and FEV_1. This indicates that endothelial injury is closely connected to the pathophysiology of COPD.

Since COPD is a heterogeneous disease characterized by various combinations with small airway disease and emphysema, the relationships between the severity of the emphysema and the EMP count are of great interest. Moreover, as quicker responses can be seen in circulating EMP levels compared with an annual FEV_1 decline, monitoring EMP levels is valuable as a means of estimating COPD progression.

Simple and noninvasive biomarkers in COPD are needed to monitor disease progression, identify exacerbations, and evaluate the efficacy of novel therapies. Sputum is a rich, noninvasive source of biomarkers of inflammation and infection, and has been used extensively to assess inflammation in lung airways pathologies. The presence of CD31-MPs in COPD sputum could be a new noninvasive method to monitor the disease course.

Main limitations of this study are that only a limited number of subjects with lung diseases were enrolled, so it was not possible to evaluate different expression of MPs according to severity of the disease, as well as the obvious absence of a control group with healthy subjects in whom it would be difficult to obtain sputum even if induced. However, our preliminary data suggest that high levels of MPs reflect the presence of endothelial inflammation. CD31-MPs, CD66b-MPs, and CD235ab-MPs could be good new candidates for the study of pulmonary endothelial injury and COPD progression.

Future studies could aim to evaluate if different stages of diseases can influence the phenotype of MPs and define the possible role of them in monitoring the effectiveness of medication.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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