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**RAPID REPORT**

*Extracellular Vesicles in Lung Health, Disease, and Therapy*

# Microvesicles in bronchoalveolar lavage as a potential biomarker of COPD

**Erica Bazzan,[1]* Claudia Maria Radu,[2,3]* Mariaenrica Tinè,[1] Tommaso Neri,[4] Davide Biondini,[1] Umberto Semenzato,[1] Alvise Casara,[1] Elisabetta Balestro,[1] Paolo Simioni,[3] Alessandro Celi,[4] Manuel G. Cosio,[1,5]* and Marina Saetta[1]***

[1]*Department of Cardiac, Thoracic, Vascular Sciences, and Public Health, University of Padova, Padua, Italy;* [2]*Department of Women's and Children's Health, University of Padova, Padua, Italy;* [3]*Department of Medicine, University of Padova, Padua, Italy;* [4]*Respiratory Pathophysiology Unit, Department of Surgery, Medicine, Molecular Biology, and Critical Care, University of Pisa, Pisa, Italy; and* [5]*Meakins-Christie Laboratories, Respiratory Division, McGill University, Montreal, Quebec, Canada*

## Abstract

Microvesicles (MVs) released from almost all cells are recognized as cell communication tools. MVs have been investigated in several inflammatory diseases but poorly in biological fluids like bronchoalveolar lavage (BAL) of smokers. The purpose of this study was to investigate the presence and source of MVs in BAL of smokers with and without chronic obstructive pulmonary disease (COPD) compared with nonsmoking controls. Using flow cytometry in BAL, we detected endothelial and alveolar macrophage (AM)-derived MVs and found a higher number of AM-MVs in the BAL of smokers with COPD than in smokers without COPD and nonsmokers, which correlated with the pack-years ($r$ = 0.46; $P$ = 0.05) and with the degree of airway obstruction measured by the forced expiratory volume in 1 s percent predicted ($r$ = −0.56; $P$ = 0.01). Endothelial and alveolar macrophage-derived MVs are present and measurable in human BAL fluid. In response to smoking and to the development of COPD, inflammatory signals in AM-derived MVs can be quantified, and their numbers are related to the pack-years and the decrease in lung function. These results open the opportunity for future investigation of these microvesicles as biomarkers and possible mechanistic guides in COPD.

*cigarette smoking; chronic obstructive pulmonary disease; cytofluorimetric analysis; extracellular vesicles*

## INTRODUCTION

Cell-to-cell communication is an essential component for mammalian development and preservation of homeostasis and for efficient responses to threats within the surrounding environment. Beyond signaling through cell-cell contact, extracellular vesicles (EVs) have recently emerged as important information shuttles that can disseminate homeostatic and disease signals (1). It has become increasingly clear that these vesicles, originally considered cell debris, are in fact actively involved in a variety of physiologically relevant processes and participate in cell-cell communication. Vesicles released by healthy cells are found in circulation and contain cell-derived biomolecules such as miRNA, cytokines, and inflammatory mediators (1). Recognized as "extracellular vesicles" (EVs), they are stratified by size and include exosomes (<100 nm), microvesicles (MVs), also called microparticles or ectosomes (=100–1, 000 nm), and apoptotic bodies (≥1,000 nm).

Most cells in blood are thought to release EVs (1); however, studies in blood have practical difficulties both in assaying the low EV concentrations in circulation and identifying their tissue of origin (2). Nevertheless, studies of EVs in cancer, cardiovascular, metabolic, neurological, and infectious diseases among others have started to illuminate a role for EVs as promising candidate biomarkers for diagnosis and prognosis in lung diseases (3).

Only few studies have described EVs in blood in chronic obstructive pulmonary disease (COPD) secondary to smoking, in which innate and adaptive immune inflammation (4, 5) play an important mechanistic role. In fact, epithelial and endothelial cell-derived-EVs are known to be increased in the blood of COPD patients, whereas in bronchoalveolar lavage (BAL) only the smallest EVs, the exosomes, but not MVs, have been investigated in the context of COPD in humans (6). The potential presence of MVs in tissue fluids, like BAL from the lung, offers the possibility of focusing specifically on the

* E. Bazzan and C. M. Radu contributed equally to this work. M. G. Cosio and M. Saetta contributed equally to this work as senior authors.
Correspondence: M. Saetta (marina.saetta@unipd.it).
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**Table 1.** *Clinical characteristics of the subjects in the study*

| | Smokers with COPD | Smokers w/o COPD | Nonsmokers |
|---|---|---|---|
| Subjects, *n* | 19 | 16 | 9 |
| Age, yr | 69 ± 5* | 62 ± 13 | 55 ± 12 |
| Smoking history, pack-years | 46 ± 23† | 27 ± 20 | |
| Current/ex-smokers, *n*/*n* | 4/15 | 6/10 | |
| $FEV_1$% predicted | 61 ± 20* | 102 ± 8 | 96 ± 18 |
| $FEV_1$/FVC % | 59 ± 11* | 86 ± 11 | 94 ± 13 |

Values are expressed as means ± SD. COPD, chronic obstructive pulmonary disease; $FEV_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; w/o. without. *Significantly different from smokers without COPD and nonsmokers ($P < 0.05$). †Significantly different from smokers without COPD ($P < 0.05$).

presence, origin, and roles of these vesicles in human lung pathology.

The purpose of this study was to investigate the presence and source of MVs in BAL of smokers with and without COPD compared with nonsmoking controls.

## MATERIALS AND METHODS

### Clinical Characteristics

BAL samples were obtained according to standard protocols (7) in 9 nonsmokers [forced expiratory volume in 1 s ($FEV_1$): 96 ± 18% predicted], 16 smokers without COPD ($FEV_1$: 102 ± 8% predicted), and 19 smokers with COPD ($FEV_1$: 61 ± 20% predicted) undergoing clinically indicated bronchoscopy. The study was approved by Ethics Committee (Ref. No. 0006045), and all subjects gave written informed consent before their enrollment.

### MV Isolation and Characterization

BAL fluid was obtained (7) and immediately processed. BAL samples were filtered by a gauze filter (50-μm-size pore) to remove any mucus and centrifuged at 350 *g* for 10 min at room temperature, to separate supernatant from BAL cells. The BAL supernatants were centrifuged at 10,000 *g* 30 min at 4°C to isolate EV pellets. Finally, EVs were resuspended in ultrafiltered PBS and stored at 80°C (8–10).

For the characterization and analysis of the MVs, 20 μL (μl) of MV suspension were incubated in the dark for 30 min at room temperature with 2 μl of fluorescent-conjugated monoclonal antibodies against cell-type specific antigens and 2 μl of Annexin V-FITC (Bender MedSystems), which binds exposed phosphatidylserine on MV surface and can signal apoptotic but also immune-stimulated cells/MVs (11–15).

The new generation of flow cytometer (CytoFLEX Beckman Coulter), which can detect EVs to a size as low as 100 nm, was used to identify and separate the EV population according to dimension, as suggested by minimal information for studies of extracellular vesicles (MISEV) guidelines (9, 10). For the calibration of the flow cytometer for EV size, fluorescent polystyrene beads in sizes of 0.1, 0.16, 0.2, 0.24, 0.3, 0.5, and 0.9 μm (Megamix FSC & SSC Plus, BioCytex, France) covering the range of EV size were used. EVs ranging from 150 to 900 nm were recognized as microvesicles (MVs) and analyzed.

Macrophage-derived MVs were identified using CD14-APC (allophycocyanin, eBioscience). Nonactivated and activated endothelial-derived MVs were identified using CD146-PC5.5 (cyanine 5.5, Beckman Coulter) and CD62E-PE (phycoerythin, Beckman Coulter) respectively. Annexin V-FITC was used to investigate parental cell derived MV characteristics (apoptosis, immune activation). The incubation of sample with the appropriate isotype controls was subtracted from the positive antibody sample to avoid nonspecific signals. To confirm the presence and integrity of MVs, calcein-AM (Sigma-Aldrich) was used (16, 17).

MV absolute count was expressed as events per microliters of the volume measured by the CytoFLEX. Files were exported, and data were evaluated by CytExpert Software (Version 2.3, Beckman Coulter).

### Statistics

Data are shown as means ± SE or median (range). Shapiro-Wilk normality test was applied to evaluate normal distribution of the data. Once it was verified that the data were not normally distributed, nonparametric statistic tests were performed. Group differences were evaluated by Kruskall-Wallis test and Mann-Whitney *U* test. Correlation coefficients were calculated by the Spearman Rank method. All data were analyzed by R statistical software (version 3.5.2). $P < 0.05$ was considered statistically significant.

## RESULTS

The subjects' clinical characteristics are shown in Table 1. Smokers with COPD were older than smokers without COPD and nonsmokers ($P = 0.013$) and smoked more than those without COPD ($P = 0.021$).

The total number of MVs (events/μl) was similar in nonsmokers (4,297.84 ± 1,969.21 MVs/μl), smokers without COPD (3,786.05 ± 913.33 MVs/μl) and smokers with COPD (4,728.57 ±

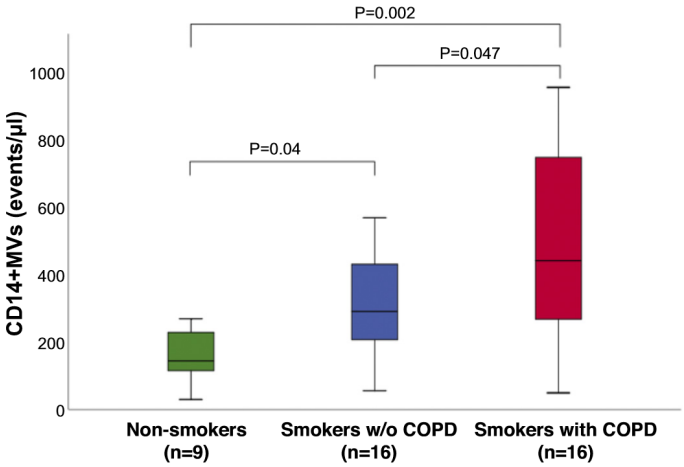


**Figure 1.** Macrophage-derived microvesicles (MVs) ($CD14^+$ events/μl) in nonsmokers ($n = 9$), smokers without (w/o) chronic obstructive pulmonary disease (COPD; $n = 16$), and smokers with COPD ($n = 19$). *Bottom* and *top* of each box plot, 25th and 75th percentiles; solid line, median; brackets, minimum and maximum. *P* values in the figure represent the results of Mann-Whitney *U* tests. Kruskal-Wallis test: $P < 0.001$.

**Table 2.** *Number of MVs in the BAL*

| | Smokers with COPD, events/μL ($n$ = 19) | Smokers w/o COPD, events/μL ($n$ = 16) | Nonsmokers, events/μL ($n$ = 9) |
|---|---|---|---|
| **CD14$^+$** | **440 (49–952)***† | **291 (56–567)*** | **143 (29–583)** |
| CD14$^+$/Annexin V$^-$ | 349 (32–894)* | 233 (19–506)* | 126 (11–526) |
| CD14$^+$/Annexin V$^+$ | 91 (16–321)* | 58(23–116) | 41 (2–131) |
| **CD146$^+$** | **23 (9–110)** | **14 (5–28)** | **18 (2–41)** |
| CD146$^+$/Annexin V$^-$ | 14 (0–80) | 6 (2–18) | 9 (0–22) |
| CD146$^+$/Annexin V$^+$ | 9 (2–30) | 7 (2–10) | 7 (2–27) |
| **CD62E$^+$** | **641 (241–1905)** | **543 (236–1290)** | **536 (344–737)** |
| CD62E$^+$/Annexin V$^-$ | 373 (128–1325) | 417 (139–1117) | 417 (218–551) |
| CD62E$^+$/Annexin V$^+$ | 140 (11–707) | 129 (53–268) | 119 (98–201) |

Values are expressed as median (range). Annexin V has been traditionally used as a marker of apoptosis. Recently, many other functions of Annexin V have been described, including immune modulation and coagulation, suggesting a significance that goes beyond marking MVs derived from apoptotic cells. Bold characters identify the total number of microvesicles per parental cell type, regardless Annexin V staining. BAL, bronchoalveolar lavage; MVs, microvesicles; COPD, chronic obstructive pulmonary disease; w/o, without. *Significantly different from nonsmokers ($P < 0.05$). †Significantly different from smokers w/o COPD ($P < 0.05$).

2,837.57 MVs/μl). In 85% of the cases, the dimension of the MVs ranged between 200 and 500 nm and there was no difference between the three groups regarding MV size ($P = 0.341$).

The number of MVs/μl derived from macrophages (CD14$^+$) was significantly higher in smokers with ($p = 0.002$) and without COPD ($p = 0.04$) than in nonsmokers and also significantly higher in smokers with COPD than in smokers without COPD ($P = 0.047$, Fig. 1 and Table 2). When CD14$^+$ MVs were separated by the Annexin V expression, the number of CD14$^+$/Annexin V$^-$ MVs was significantly higher in smokers with and without COPD than in nonsmokers ($P < 0.05$, Table 2), while the number of MVs derived from CD14$^+$/Annexin V$^+$ macrophages were significantly higher in smokers with COPD than in nonsmokers ($P = 0.02$, Table 2). Both the Annexin − and Annexin V$^+$ CD146$^+$ MVs/μl, an endothelial cell marker, had a low and similar expression in all groups, while the Annexin V$^-$ and Annexin V$^+$ CD62E$^+$ microvesicles per microliter, a marker of activated endothelium expressing E-Selectin, was similar but highly expressed in the three groups (Table 2).

In smokers with COPD, the CD14$^+$ MVs correlated positively with the pack-years of smoking ($r = 0.46$; $P = 0.05$) and inversely with the lung function expressed as $FEV_1$% predicted ($r = -0.56$; $P = 0.01$, Fig. 2). This last correlation remained significant after adjustment for smoking history ($P = 0.004$). When CD14$^+$ MVs were separated by the Annexin V expression, only the CD14$^+$/Annexin V$^-$ were inversely correlated with $FEV_1$% predicted ($r = -0.6$; $P = 0.007$). When all smokers were analyzed together, CD14$^+$ MVs were correlated with the pack-years of smoking ($r = 0.34$; $P = 0.05$) and with $FEV_1$% predicted ($r = -0.46$; $P = 0.005$), while CD14$^+$/Annexin V$^-$ MVs correlated only with $FEV_1$% predicted ($r = -0.41$; $P = 0.01$).

No correlation was found between MVs derived from endothelial cells and clinical (age, sex, and pack-years) or functional parameters ($FEV_1$% predicted and $FEV_1$/FVC%).

## DISCUSSION

Microvesicles (MVs) in specific biological fluids represent a sample of accessible MV-forming parent cells (3). As such blood contains a mixture of MVs from lungs, extrapulmonary organs, and circulating blood cells, while biological fluids specific to the lung such as bronchoalveolar lavage (BAL) ought to contain lung-specific sources of MVs. For these reasons, we studied the MVs in the BAL of human lung and the possible effects smoking and COPD would have on MV load and characteristics.

We found that MVs expressing CD14, a key molecule in the activation of innate immune cells expressed on cells of the myelomonocyte lineage including monocytes, macrophages, and some granulocytes (18), were increased in smokers with and without COPD. Although expressed in all smokers, CD14 was significantly overexpressed in COPD, indicating a high level of alveolar macrophage (AM) activation in smokers developing the disease. CD14 expression not only correlated with the decline in lung function but also with smoking pack-years, highlighting the potential use of lung-derived MVs as a prognostic biomarker for COPD.

BAL also showed that the presence of MVs expressing CD146, a marker for endothelial cells involved in cell-cell cohesion and permeability (19), was similar in all groups studied. CD62E, a marker of activated endothelial cells expressing E-Selectin, which is crucial for inflammatory cells recruitment and accumulation in the sites of inflammation (20), was highly expressed in smokers with and

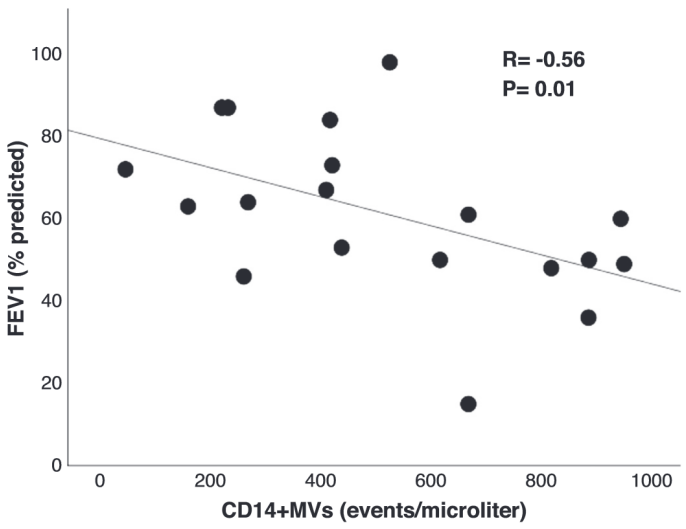


**Figure 2.** Relationship between macrophage-derived microvesicles (MVs) [CD14$^+$ events/μl) and forced expiratory volume in 1 s ($FEV_1$); %predicted] in smokers with chronic obstructive pulmonary disease (COPD). Spearman rank correlation $r = -0.56$ and $P = 0.01$. The correlation remained significant after adjustment for smoking history ($P = 0.004$).

without COPD and nonsmokers. Of interest $CD62E^+$, which is promptly expressed after activation by inflammatory stimuli (21–24), might have been induced by the irritation produced by the bronchoscopy itself, which would indicate that $CD62E^+$ MVs are likely derived from pulmonary endothelium.

MVs were also marked for Annexin V, which is commonly used for the detection of MVs derived from apoptotic cells (11–15). The expression of Annexin V was higher in macrophage-derived MVs from smokers with COPD compared with nonsmokers. The biological importance of Annexin V is complex and goes well beyond the marking for apoptosis. The main mechanism of action of Annexin V is related to its binding to phosphatidylserine expressed in outer cell membranes and in MVs, which is associated with immune modulation and anticoagulation (13, 15). In view of this observation, the high expression of Annexin V in MVs from smokers with COPD could represent a sign of immune activation rather than a signal of apoptosis as it is often believed.

While the presence of microvescicles has been demonstrated in BAL fluid of patients with different chronic diseases, including interstitial lung diseases (25) and cystic fibrosis (26), to our knowledge this is the first study to characterize MVs in BAL of smokers with and without COPD. The study of BAL has the advantage of identifying the cell origin of MVs beyond the cargo, an important distinction that might increase the prognostic and possible mechanistic value of MVs in the study of lung events in chronic lung diseases.

The main limitation of our study is that a limited number of markers were used to investigate the origin of MVs. In particular, the presence of epithelial-derived MVs was not evaluated. However, since the presence and the possible significance of EVs in BAL had never been investigated before in COPD, we approached our study as a feasibility study, starting with a limited number of markers (macrophages and endothelial cells). Of interest the analysis of our data showed that smokers with COPD had 37% of MVs in BAL that were not identified by our staining, while nonsmokers had 70% of MVs nonidentified. These data are important and call for further studies to identify the missing MVs and their significance not only in COPD but also in other lung diseases.

### Conclusions

MVs obtained directly from the lung BAL show that, in response to smoking and to the development of COPD, measurable inflammatory signals in alveolar macrophages can be quantified and that their numbers are related to the pack-years and the decrease in lung function. These findings support the known inflammatory role of alveolar macrophages in COPD and the potential contribution of the lung capillary endothelial cells in enhancing this inflammation. These results open the opportunity for future investigation of these microvesicles as biomarkers and possible mechanistic guides in COPD.

## ETHICAL APPROVAL

The study was approved by Padova Hospital Ethics Committee (Ref. No. 0006045) and all subjects gave written informed consent before their enrollment.

## GRANTS

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

E. Bazzan, C.M.R., M.G.C., and M.S. conceived and designed research; E. Bazzan, C.M.R., and M.T. performed experiments; E. Bazzan, A. Casara, M.T., D.B., U.S., and E. Balestro analyzed data; E. Bazzan, M.T., T.N., A. Casara, A. Celi, and M.G.C. interpreted results of experiments; E. Bazzan prepared figures; E. Bazzan, M.T. M.G.C., and M.S. drafted manuscript; P.S. A. Celi, M. G.C., and M.S. edited and revised manuscript; E. Bazzan, C.M.R., T.N., D.B., A. Casara, E. Balestro, P.S., A. Celi, M.G.C., and M.S. approved final version of manuscript.

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