

RESEARCH ARTICLE

Extracellular Vesicles in Lung Health, Disease, and Therapy

Intra-alveolar neutrophil-derived microvesicles are associated with disease severity in COPD

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Abstract

Despite advances in the pathophysiology of chronic obstructive pulmonary disease (COPD), there is a distinct lack of biochemical markers to aid clinical management. Microvesicles (MVs) have been implicated in the pathophysiology of inflammatory diseases including COPD, but their association to COPD disease severity remains unknown. We analyzed different MV populations in plasma and bronchoalveolar lavage fluid (BALF) taken from 62 patients with mild to very severe COPD (51% male; mean age: 65.9 yr). These patients underwent comprehensive clinical evaluation (symptom scores, lung function, and exercise testing), and the capacity of MVs to be clinical markers of disease severity was assessed. We successfully identified various MV subtype populations within BALF [leukocyte, polymorphonuclear leukocyte (PMN; i.e., neutrophil), monocyte, epithelial, and platelet MVs] and plasma (leukocyte, PMN, monocyte, and endothelial MVs) and compared each MV population to disease severity. BALF neutrophil MVs were the only population to significantly correlate with the clinical evaluation scores including forced expiratory volume in 1 s, modified Medical Research Council dyspnea score, 6-min walk test, hyperinflation, and gas transfer. BALF neutrophil MVs, but not neutrophil cell numbers, also strongly correlated with BODE index. We have undertaken, for the first time, a comprehensive evaluation of MV profiles within BALF/plasma of COPD patients. We demonstrate that BALF levels of neutrophil-derived MVs are unique in correlating with a number of key functional and clinically relevant disease severity indexes. Our results show the potential of BALF neutrophil MVs for a COPD biomarker that tightly links a key pathophysiological mechanism of COPD (intra-alveolar neutrophil activation) with clinical severity/outcome.

biomarker; chronic obstructive pulmonary disease; extracellular vesicles; microvesicles; neutrophil

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a heterogeneous chronic inflammatory disease, defined by persistent airflow limitation that is not fully reversible and is characterized by a mixture of small airways disease and parenchymal destruction (1). COPD is a significant health-care burden and is the third most prominent cause of death worldwide (2). Until recently, the only disease-modifying treatments available to patients were smoking cessation, pulmonary rehabilitation, and domiciliary oxygen therapy (for hypoxic patients). However, advances such as lung volume reduction surgery and endobronchial valve implantation

have improved lung function and survival in selected patients (3, 4). While this progress has accompanied increased understanding in the disease pathology, there remains a distinct lack of laboratory tests to guide therapeutic management in COPD. This has been highlighted in a recent statement published by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) board of directors, calling for urgent research to improve the diagnosis and management of COPD (5).

There has been considerable interest investigating the role of extracellular vesicles and in particular microvesicles (MVs) in inflammatory disease. MVs are membrane-circumscribed extracellular particles of 100–1,000 nm in size and



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are derived from eukaryotic cells following direct cell activation or injury. They are increased in numerous pathophysiological states and thus have been proposed as potential biomarkers in many inflammatory diseases (6, 7). Several studies have reported elevated levels of circulating endothelial-derived MVs in patients with COPD (8, 9), while a recent publication demonstrated that extracellular vesicles originating from neutrophils play a vital role in the pathophysiology of COPD (10). Despite these studies, the biomarker potential of MVs, particularly those originating in the intra-alveolar space, remains largely unknown. MVs have several potential advantages as diagnostic tools in lung inflammatory disease such as COPD because 1) they are very stable in their environment; 2) the cells that have produced each MV population can be easily determined by their surface marker analysis; and 3) they carry a number of inflammatory cargoes including cytokines, chemokines, and miRNA that possibly play pathogenic roles in these diseases (6, 11, 12). If readily identifiable within the bronchoalveolar lavage fluid (BALF), MVs may represent an inflammatory blueprint of the activation status of various pulmonary cells, producing a detailed account of local inflammatory states and underlying pathological processes.

In this study, we undertook a systematic evaluation of the profiles of intra-alveolar (within BALF) and circulating (within plasma) MVs in COPD patients, characterizing a variety of MV subtype populations by flow cytometry. We demonstrated that polymorphonuclear leukocyte (PMN; i.e., neutrophil)-derived MVs are actively released within BALF in these patients and importantly their levels highly correlate with a number of vital physiological markers of COPD severity. Our results show the exciting potential of BALF neutrophil MVs for a COPD biomarker that tightly links, for the first time, a key pathophysiological mechanism of COPD (intra-alveolar neutrophil activation) with clinical severity/outcome.

MATERIALS AND METHODS

The COPD-Microenvironment study (NCT03010592) evaluated the airway inflammatory milieu before and after interventional bronchoscopic and surgical treatments for individuals with mild to very severe COPD. This trial was approved by the London-Camden & King's Cross (REC No. 17/LO/0136) Ethics Committee and Health Research Authority and all participating subjects provided written informed consent. This substudy focuses on the baseline data and the primary aim at this initial stage was to evaluate the presence of different MV populations within baseline BALF/blood samples and assess their correlation with COPD severity (before any intervention or lung volume reduction procedure). Sixty-two consecutive patients were enrolled between the 6th February 2017 and 3rd September 2018. Written informed consent was obtained from all patients. All participants were patients with a confirmed diagnosis of COPD and were over the age of 18.

Inclusion and Exclusion Criteria

Study inclusion criteria were as follows: postbronchodilator forced expiratory volume in 1 s/forced vital capacity

(FEV₁/FVC) \leq 70%; PaCO₂ \leq 7.3 kPa and PaO₂ \geq 6.0 kPa on room air; right ventricular systolic pressure $<$ 45 mmHg on transthoracic echocardiogram; a minimum exercise tolerance of 140 m in 6 min; a smoking history \geq 10 pack-years but no smoking for $>$ 6 mo; have had a recent influenza vaccine; and are under optimal medical treatment (including steroid inhalers) according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (13).

Patients with the following conditions were excluded: exacerbation of COPD within 6 wk of bronchoscopy; asthma; clinically significant bronchiectasis; giant bullae; previous lung volume reduction surgery; immunomodulatory therapy to treat moderate to severe chronic inflammatory autoimmune disorder; antiplatelet or anticoagulant therapy that could not be stopped before the procedure; Known sensitivities to drugs required to perform bronchoscopy; and any other disease or condition that would increase risk of bronchoscopy or assessments of the patient.

Importantly, we ensured that each patient was free of exacerbations for at least 6 wk, did not have any signs of current infection or a systemic inflammatory response (using symptom scores, examination, and routine inflammatory marker measurement) and had stopped smoking for at least 6 mo. This ensured that any relationship between disease severity and MVs was due to disease pathogenesis and progression rather than infection, systemic inflammation, or cigarette smoke.

Clinical Evaluation

Baseline demographic data included age, sex, and body mass index (BMI). Symptom scores, pulmonary function, exercise testing, blood leukocyte and neutrophil levels, fibrinogen, and C-reactive protein were recorded in all patients.

Symptom Scores

Dyspnea and health-related quality of life scores were measured using standardized questionnaires, the modified Medical Research Council (mMRC) dyspnea score (14) and St. George's Respiratory Questionnaire (SGRQ) (15), respectively. The MRC dyspnea scale assesses the effect of breathlessness on daily activities and classifies patients on a scale of 0 (not troubled by breathless except on strenuous exercise) to 5 (too breathless to leave the house or breathless when dressing undressing) (14). The SGRQ is a health status questionnaire and designed to measure the impact of COPD on health-related quality of life and wellbeing. Patients complete responses to 50 items based upon symptoms, activity and impact of disease, to generate an aggregate score (16).

Lung Function and Exercise Capacity

Postbronchodilator spirometry (17), lung volumes (assessed by body plethysmography) (18), and gas transfer (19) were measured using the MasterScreen PFT system (Jaeger, Hoechberg, Germany) in accordance with European Respiratory Society (ERS) and American Thoracic Society (ATS) guidance. At least three measurements were obtained and the highest (spirometry) and mean (lung volumes and gas transfer) values compared with predicted values of the European Steel and Coal Community (20, 21). Earlobe capillary samples were analyzed for pH, PCO₂, PO₂, and

HCO₃⁻ using the RapidLab Siemens system. Six-minute walk testing was conducted using a 30-m course and in line with ERS/ATS guidance (22).

Blood Sampling

Blood was obtained as previously described (23). In brief, patient had a venous cannula inserted using a mild tourniquet. The first 10 ml were discarded, and the remaining blood was taken for full blood count (1 × 4 mL Purple BD Vacutainer K2 EDTA tube), coagulation (1 × 4.5 mL Blue BD Vacutainer Sodium Citrate tube), biochemistry (1 × 5 mL Gold BD Vacutainer Serum Separator Tube II Advance), and MV enumeration. For MV analysis, 2 × 4 mL green BD Vacutainer Lithium Heparin tubes were used, which is optimal for MV preservation (24). Full blood count, coagulation, and biochemistry profiles were analyzed routinely in the Royal Brompton Hospital's Clinical Laboratory. The heparin tubes were transferred on wet ice to the Chelsea and Westminster Research Laboratory within 3 h of sample acquisition. Samples were handled with care to minimize any artifactual production of MVs. The blood was centrifuged at 200g for 10 min to obtain platelet-rich plasma (PRP), which was then aspirated (without interfering with the packed cells interface), numbered and then stored anonymously within cryotubes at -80°C for analysis at a later date [freeze thawing has no measurable effect on MV counts (23, 24)].

MV numbers were measured in PRP rather than platelet poor plasma (PPP) since a dramatic drop in MV yield occurs during the centrifugation steps in preparation of PPP (23, 25). Therefore, we measured MVs in PRP [low-speed centrifugation (200g for 10 min) to remove only the red and white blood cells]. This strategy prevented analysis of platelet-derived MVs [as the size of platelet MVs overlaps that of platelets (1–3 μm) and flow cytometry would not allow us to reliably differentiate between them], but we considered that more accurate quantification of other MV subtypes (leukocyte-derived neutrophil MVs, monocyte MVs, and endothelial MVs) was a priority for this pilot study.

Bronchoalveolar Lavage Sampling

Bronchoscopy was performed under deep sedation or general anesthesia in accordance with BTS guidelines (26). A bronchoalveolar lavage (BAL) was obtained by instilling 50 ml of normal saline into the target lobe (the primary treatment site) and aspirated manually using a 50-mL syringe followed by entrained suction. The BAL was divided into two aliquots: several milliliters were sent for standard microbiology microscopy and culture; the remainder was transferred on wet ice to the research laboratory within 3 h of acquisition. This was centrifuged at 200g for 5 min at 4°C, and the pellet was analyzed for cell counts. The remaining cell free supernatant was passed through a 100-μl strainer to remove any debris and centrifuged again at 200g for 5 min at 4°C to remove residual debris or larger particles. The refined MV-rich sample was numbered and stored anonymously in cryotubes at -80°C.

BALF Cell Analysis

BALF was analyzed for neutrophil and alveolar macrophage counts. Cell pellets were isolated by centrifugation as

described above and incubated for 30 min at 4°C with antibody cocktail containing fluorescence-conjugated antibodies of 0.5 μg/ml of CD45 (clone 30-F11; Biolegend), 0.5 μg/ml of CD11b (M1/70; BD Biosciences, San Jose, CA) and 0.5 μg/ml CD66b (G10F5; Biolegend) for neutrophils and CD45, 0.5 μg/ml of CD11c (N418; Biolegend), and 0.5 μg/ml of F4/80 (BM8; Biolegend) to identify alveolar macrophages.

Microvesicle Analysis

The stored numbered BALF and plasma samples underwent blind analysis. As described previously (11, 12), we identified MVs by flow cytometry as “plasma membrane-derived” particles that were as follows: 1) size <1 μm; 2) positive for specific surface markers of their precursor cells; and 3) sensitivity to 0.1% Triton X-100 detergent. In brief, samples were thawed and combined with fluorophore-conjugated monoclonal antibodies (as stated in Supplemental Table S1; see also Supplemental Methods) in pairs or alone to identify MV phenotype. Samples were incubated in the dark at 4°C for 30 min before dilution in 1 mL of filtered PBS. Accucheck counting beads (Invitrogen, Paisley, UK) were added to determine MV counts and acquired with CyAn ADP flow cytometer. Forward scatter and side scatter (trigger threshold 0.01) were used to elucidate a 1-μm gate that was delineated using sizing beads (upper size: 1.3 μm, Polysciences, Inc., Hamburg, Germany) as previously shown (11, 12) (Supplemental Fig. S1, A and B). Data were analyzed using FlowJo software. All stained MV samples were also treated with 0.1% Triton detergent (Supplemental Fig. S1C) to correctly differentiate MVs from nonvesicular antibody-bound events (27). Any detergent-insensitive events were subtracted from total MV counts to eliminate any false-positive events. Unstained samples were also analyzed to exclude the phenomenon of auto-fluorescence and accurately delineate positive events (Supplemental Fig. S1D) while samples were also stained with isotype controls (Supplemental Fig. S1E). Furthermore, BALF samples were also harvested from healthy volunteers and assessed for MV populations as an additional control group (Supplemental Fig. S2, A and B). The centrifugation and flow cytometry methods to isolate and characterize MVs in this study have been previously validated both by high-resolution imaging and electron microscopy (11).

Cytokine Analysis

Sandwich ELISAs were conducted to measure TNF-α/IL-1β/IL-6/CXCL8 (R&D Systems, Abingdon, UK) as per manufacturer recommendations. Detectable ranges were TNF: 15.6–1,000 pg/ml (DY210); IL-1β: 3.9–250 pg/ml (DY201); IL-6: 9.4–600 pg/ml (DY206); and CXCL8: 31.2–2,000 pg/ml (DY208).

Statistical Analysis

No statistical power calculation was conducted at the time of study design because of the lack of data available regarding lung neutrophil MV production in bronchoalveolar lavage fluid (BALF) from patients with COPD. Flow cytometric data were analyzed using FlowJo software. IBM SPSS was used to conduct normality tests for all acquired data, where

a normal distribution of data was defined with a Shapiro-Wilk value of $P > 0.05$. Parametric data are presented as means \pm SD while nonparametric data are presented as median and interquartile range. The Pearson's correlation coefficient was used to assess the relationship between MVs and the various clinical characteristics for parametric data whereas Spearman's rank correlation coefficient (r) was used for nonparametric data. All data were analyzed on GraphPad Prism, and $P < 0.05$ was defined as the minimum threshold for statistical significance.

RESULTS

Microvesicle Characterization in BALF and Plasma

The patient demographics for our study population are described in Table 1. Sixty-two patients with mild to very severe COPD (FEV₁ %predicted range: 16.40% to 84.60%) who were free from disease exacerbation and respiratory infection for at least 6 wk before bronchoscopy were recruited. All patients were under optimal medical treatment (including steroid inhalers) according to GOLD (13). As our clinical characteristics demonstrate, while all patients had significant airways disease, the population was heterogeneous with varying degrees of symptom severity, lung hyperinflation and airflow obstruction.

We investigated a number of different MV subtype populations in patient BALF samples (Fig. 1) and demonstrated the presence of leukocyte derived-MVs (CD45+), PMN (i.e., neutrophil) MVs (CD66b+/CD11b+), and monocyte MVs (CD45+/CD14+), as previously described (23, 28, 29). Alveolar macrophage-derived MVs were identified by expression of mannose (CD206) and transferrin (CD71). Alveolar macrophage MVs have not been reported in human samples previously, and our detection strategy was based on analysis of human lung myeloid cells where alveolar macrophages are clearly distinguished as CD206-positive and CD14 low-expressing cells (30, 31). Epithelial-derived MVs (T1 alpha+ or EpCAM+) and platelet MVs (CD42b+/CD31) were also readily identifiable in BALF. When considering all these different MV populations within BALF taken from our patients, leukocyte-derived MVs, specifically of PMN (i.e., neutrophil) origin were those in the greatest numbers (Fig. 1 and Supplemental Table S2A). Interestingly, BALF PMN (i.e., neutrophil) MVs were much higher (by greater than one order of magnitude) in patients with COPD compared with our healthy volunteer controls (Supplemental Fig. S2).

In platelet-rich plasma, we measured total leukocyte-derived MVs (CD45+), PMN (i.e., neutrophil) MVs (CD66b+/CD11b+), and monocyte MVs (CD45+/CD14+). Endothelial markers may not be coexpressed in individual MVs (8) and so single endothelial markers were chosen to identify endothelial MVs (CD144+, CD146+, or CD62E+) (Fig. 2). The largest population of MVs detected were derived from leukocytes although we also detected significant numbers of endothelial MVs (Fig. 2 and Supplemental Table 2B).

BALF Neutrophil-Derived Microvesicles Correlate with COPD Severity

Having identified various MV populations within BALF, we compared BALF MV numbers to the clinical variables

Table 1. Demographics and clinical characteristics of study population

| Variable | Group total (n = 62) |
|---|----------------------|
| Age, yr | 65.90 \pm 7.68 |
| Sex | |
| Male | 51% |
| Female | 49% |
| BMI, kg/m ² | 24.37 \pm 3.80 |
| Active comorbidities | 2 (1–3) |
| Pack year smoking history | 44 (33–54) |
| Exacerbation frequency (last 12 mo) | 1 (0–3) |
| mMRC score (grades 1–5) | 2 (2–3) |
| SGRQ score, % | 56.59 \pm 16.98 |
| GOLD Stage | |
| Stage 1 | 2% (1/62) |
| Stage 2 | 10% (6/62) |
| Stage 3 | 45% (28/62) |
| Stage 4 | 43% (27/62) |
| FEV ₁ , %predicted | 34.76 \pm 12.49 |
| RV, %predicted | 212.38 \pm 44.57 |
| TLC, %predicted | 136.50 \pm 13.42 |
| RV/TLC, % | 60.02 \pm 9.73 |
| IC, liter | 2.13 \pm 0.74 |
| IC/TLC, % | 26.42 \pm 7.53 |
| TLCoc, %predicted | 37.35 \pm 13.48 |
| KCOc, %predicted | 44.24 \pm 13.34 |
| 6MWD, m | 348.31 \pm 118.92 |
| BODE survival index | 5 (4–7) |
| pH | 7.44 \pm 0.03 |
| PCO ₂ | 4.93 \pm 0.67 |
| PO ₂ | 9.43 \pm 1.30 |
| HCO ₃ ⁻ | 24.72 \pm 2.74 |
| White cell count, cells \times 10 ⁹ /L | 7.58 \pm 2.06 |
| Neutrophil count, cells \times 10 ⁹ /L | 4.59 \pm 1.79 |
| Fibrinogen, g/L | 3.46 \pm 0.61 |
| CRP, mg/L | 4.47 \pm 4.17 |

Data are presented as means \pm SD or median with interquartile range (IQR). Baseline demographics and clinical characteristics of patient cohort are given. Study participants had significant burden of symptoms, severe airways obstruction, hyperinflation, impairment of gas transfer, and a predicted 4-yr survival of 57%. Patients did not have any signs of active infection or a systemic inflammatory response as evidenced by normal white cell counts and C-reactive protein (CRP) levels. BMI, body mass index; mMRC, modified medical research council dyspnea scale; SGRQ, St. George's respiratory questionnaire; GOLD, global initiative for obstructive lung disease; FEV₁, forced expiratory volume in 1 s; RV, residual volume; TLC, total lung capacity; IC, inspiratory capacity; TLCoc, transfer factor of the lungs for carbon monoxide corrected for hemoglobin; KCOc, transfer coefficient of the lungs for carbon monoxide corrected for hemoglobin; 6MWD, 6-min. walk distance; PCO₂, partial pressure of carbon dioxide; PO₂, partial pressure of oxygen; HCO₃⁻, bicarbonate; BODE index for COPD survival, composite score comprising body mass index, airflow obstruction, dyspnea score on mMRC, and exercise capacity on 6-min walk test.

measured in our patient cohort (P and r values for each variable are illustrated in Supplemental Table S2). PMN (i.e., neutrophil-derived) MVs alone were found to strongly correlate the BODE index score (i.e., increasing levels were associated with worsening BODE index, $P = 0.0034$), which is a robust predictive marker of COPD severity and mortality (32–34) (Fig. 3A). This correlation persists after excluding patients with microbiology-positive isolates using routine laboratory culture techniques ($P = 0.0034$), confirming the relationship is not a consequence of an infective process driving a neutrophilic infiltration (Fig. 3B).

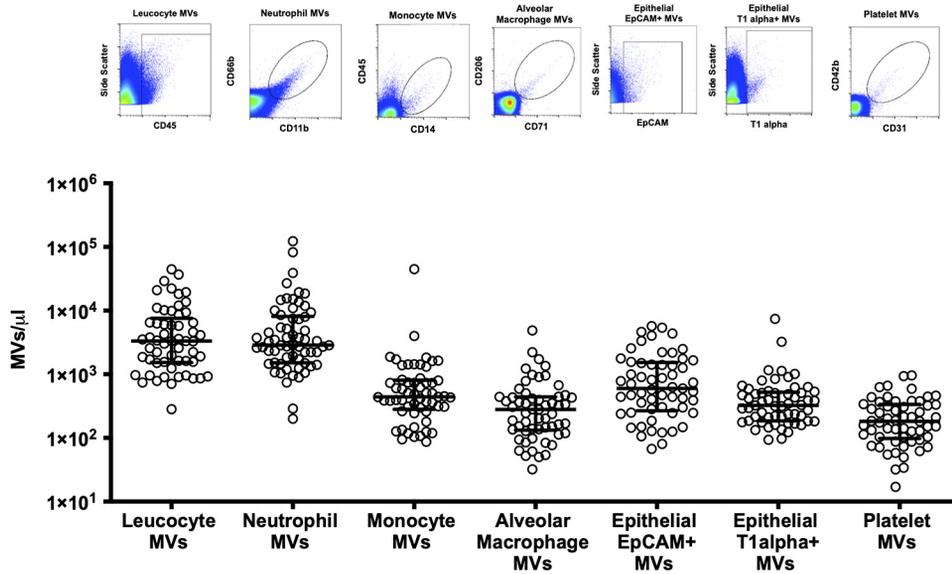


Fig. 1. Flow cytometry plots and corresponding scatter dot graphs illustrating the relative expressions of microvesicle (MV) subpopulations in bronchoalveolar fluid (BALF) characterized as follows: leukocyte (CD45 +); neutrophil (CD66b + / CD11b +); monocyte (CD45 + / CD14 +); alveolar macrophage (CD206 + / CD71 +); epithelial (as either EpCAM + or T1alpha +); and platelet-derived MVs (Cd42b + / CD31 +). All events were under 1 μm in diameter and detergent sensitive, validating the respective MV subpopulation. Leukocyte-derived MVs, specifically of neutrophil origin, are the predominant population. Scatter dot graphs display log-transformed data with median and interquartile range; *n* = 62.

BALF neutrophil MVs also correlated significantly with many individual indicators of COPD disease severity including: airways obstruction (FEV₁: higher neutrophil MV numbers were associated with decreased FEV₁, *P* = 0.0405); exercise tolerance (6-min walking distance: higher neutrophil MV numbers were related to decreased exercise capacity, *P* = 0.0382); symptom scores (mMRC dyspnea score: higher neutrophil MV numbers were associated with increased disability from breathlessness, *P* = 0.0102); hyperinflation [inspiratory capacity and total lung capacity (IC/TLC) and residual volume to total lung capacity (RV/TLC): higher neutrophil MV numbers were associated with severe gas entrapment: *P* = 0.0280 and *P* = 0.0253, respectively]; and lung parenchymal damage [transfer factor of the lungs for carbon monoxide corrected for hemoglobin. (TLCOC%):

higher MV numbers were related to worse gas transfer, *P* = 0.0369] (Fig. 4, A-F). MVs originating from other cell populations did not correlate with any of these clinical parameters including the BODE score (Supplemental Table S3). Interestingly, however, BALF EpCAM + ve epithelial cell-derived MVs correlated with the number of exacerbations patients had in the preceding 12 mo before sampling (i.e., increasing levels of epithelial MVs were associated with higher frequency of exacerbations, *P* = 0.0494).

Circulating Microvesicles and COPD Severity

We also investigated the relationship of circulating MVs and disease severity. Contrasting with BALF data, no correlations existed between circulating PMN (i.e., neutrophil-derived) MVs and any of the recorded clinical parameters

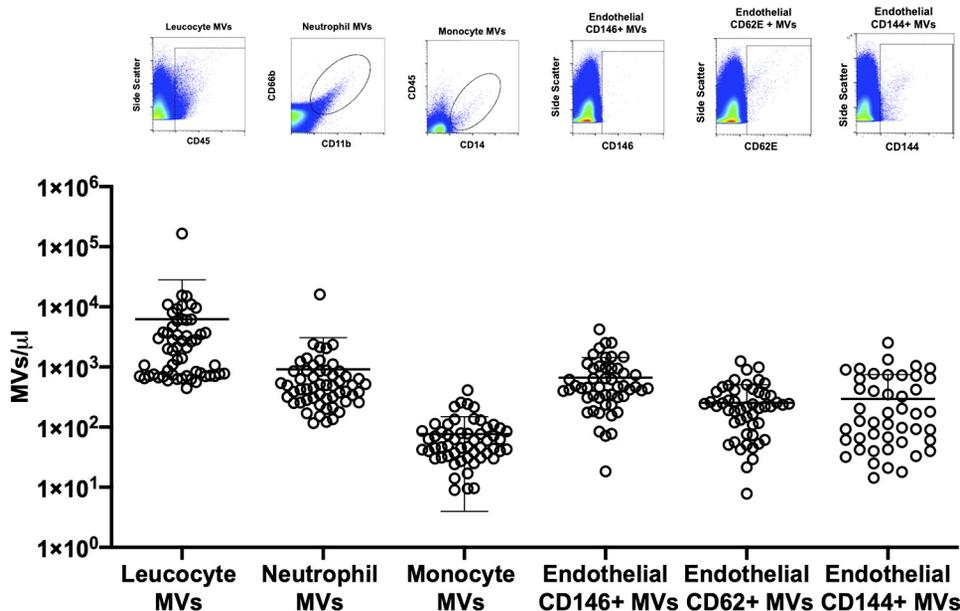


Fig. 2. Flow cytometry plots and corresponding scatter dot graphs illustrating the relative expressions of microvesicle (MV) subpopulations in platelet rich plasma. Endothelial MVs were identified as either CD146 +, CD62E + or CD144 +. All events were under 1 μm in diameter and detergent sensitive, validating the respective MV subpopulation. Leukocyte-derived MVs are the largest population. Scatter dot graphs display log-transformed data with median and interquartile range; *n* = 62.

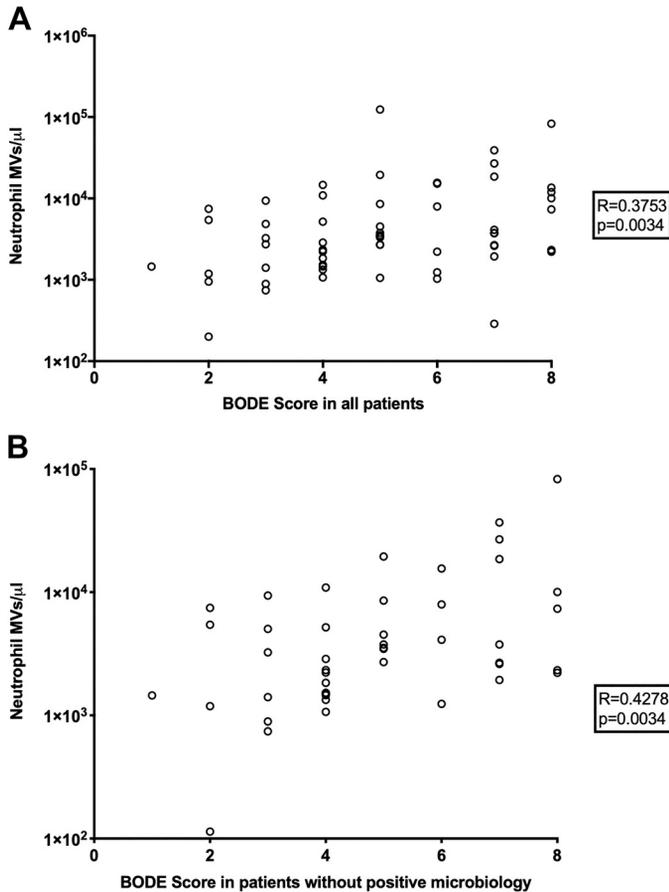


Fig. 3. Correlation of neutrophil-derived microvesicles (MVs) and BODE index. **A:** higher numbers of bronchoalveolar lavage fluid (BALF) neutrophil-derived MV numbers were associated with worsening BODE index score ($n = 62$). **B:** this correlation persisted after excluding those patients with a microbiology isolate found in the BALF on routine laboratory analysis ($n = 59$). Data are log transformed. *R*, Spearman's rank correlation coefficient.

(Supplemental Table S4). Monocyte MVs did correlate with gas transfer (TLC_{CO}: increasing monocyte MV numbers were related to worsening gas transfer; $P = 0.0099$), and with the BODE index but only when patients with isolates positive for microbiology were excluded ($P = 0.0273$) (Supplemental Table S4), but did not correlate with any of the other markers of disease severity.

Cytokines and COPD Severity

For assessment of alternative inflammation markers and comparisons with the MV-COPD severity relationship, we undertook a comprehensive evaluation of cytokines in BALF obtained from our patient cohort, which have been previously shown to relate to COPD pathology, including TNF, IL-1 β , IL-6, and CXCL8 (1, 24). However, we found no correlation between the cytokines measured and the clinical parameters or severity markers described above (Fig. 5 and Supplemental Table S5). We did find that these BALF cytokines correlated with various MV populations (Supplemental Table S5).

Origin of Neutrophil-Derived Microvesicles

BALF neutrophil MV numbers correlated, albeit weakly, with BALF neutrophil cell numbers ($P = 0.0386$) (Fig. 6A) but

did not correlate with circulating neutrophil MV numbers ($P = 0.0957$), suggesting that these MVs were released locally rather than translocating from the circulation (Fig. 6B). However, despite this apparent relationship between intra-alveolar polymorphonuclear cells and MVs, there was no relationship between BALF neutrophils cell numbers and disease severity (i.e., BODE index; $P = 0.4740$) (Fig. 6C). Therefore, MVs appear to be a more accurate indicator of COPD severity than either cytokines or parent cells.

DISCUSSION

This study comprehensively identifies and characterizes the presence of different MV subtype populations within the BALF and plasma of patients with mild to very severe COPD. We demonstrated that PMN (i.e., neutrophil)-derived MVs are substantially increased within BALF in these COPD patients and that these BALF neutrophil MV levels strongly correlate with clinical indexes reflecting COPD disease severity. Importantly, we observed increasing levels of neutrophil MVs were related to worsening pulmonary function (i.e., airflow limitation), exercise tolerance, quality of life measures (i.e., disability from breathlessness), and a higher BODE index, a composite COPD severity score that closely reflects risk of mortality (32, 33) and subsequent COPD exacerbations (34). To the best of our knowledge, this is the first such study to propose a potential COPD clinical marker that correlates with such a broad range of indexes of disease severity (35, 36).

Neutrophils MVs in BALF are likely produced locally within the alveoli from infiltrating neutrophils rather than translocating from the circulation, and therefore, the strong correlation observed between their levels and COPD disease severity is consistent with the prominent role of neutrophils in COPD, not only in the stable state but also in progression of disease. Excessive numbers of neutrophils are found within the airways of COPD patients (37), associated with increased air trapping (38). BALF neutrophils infiltrate into the lungs following the release of chemokines and cytokines by resident lung cells in response to cigarette smoke (39). This composite inflammatory milieu within the alveoli, which is full of various inducers of MV production, would contribute to significant production of MVs from neutrophils (11, 40).

Intriguingly, we did not find a clear relationship between absolute BALF neutrophil numbers and COPD disease severity, which merits some consideration and discussion. First, this may simply reflect practical difficulties in harvesting “cells” by the bronchoalveolar lavage procedures, rather than MVs of much smaller sizes, as activated or injured cells tend to aggregate and become more resistant to be efficiently recovered by lavage (41). Second, neutrophils would produce MVs when they are activated, but not under the resting state, and hence levels of neutrophil-derived MVs may reflect neutrophil “activation,” not just their numbers, within the air space (42). Finally, and most interestingly, this may suggest a potential role of MVs in mediating neutrophil-induced inflammatory injuries within the lungs during the progression of COPD. Once in the airways, neutrophils release proteases that illicit several of the pathogenic hallmarks of COPD: epithelial cell injury, disruption of the epithelial

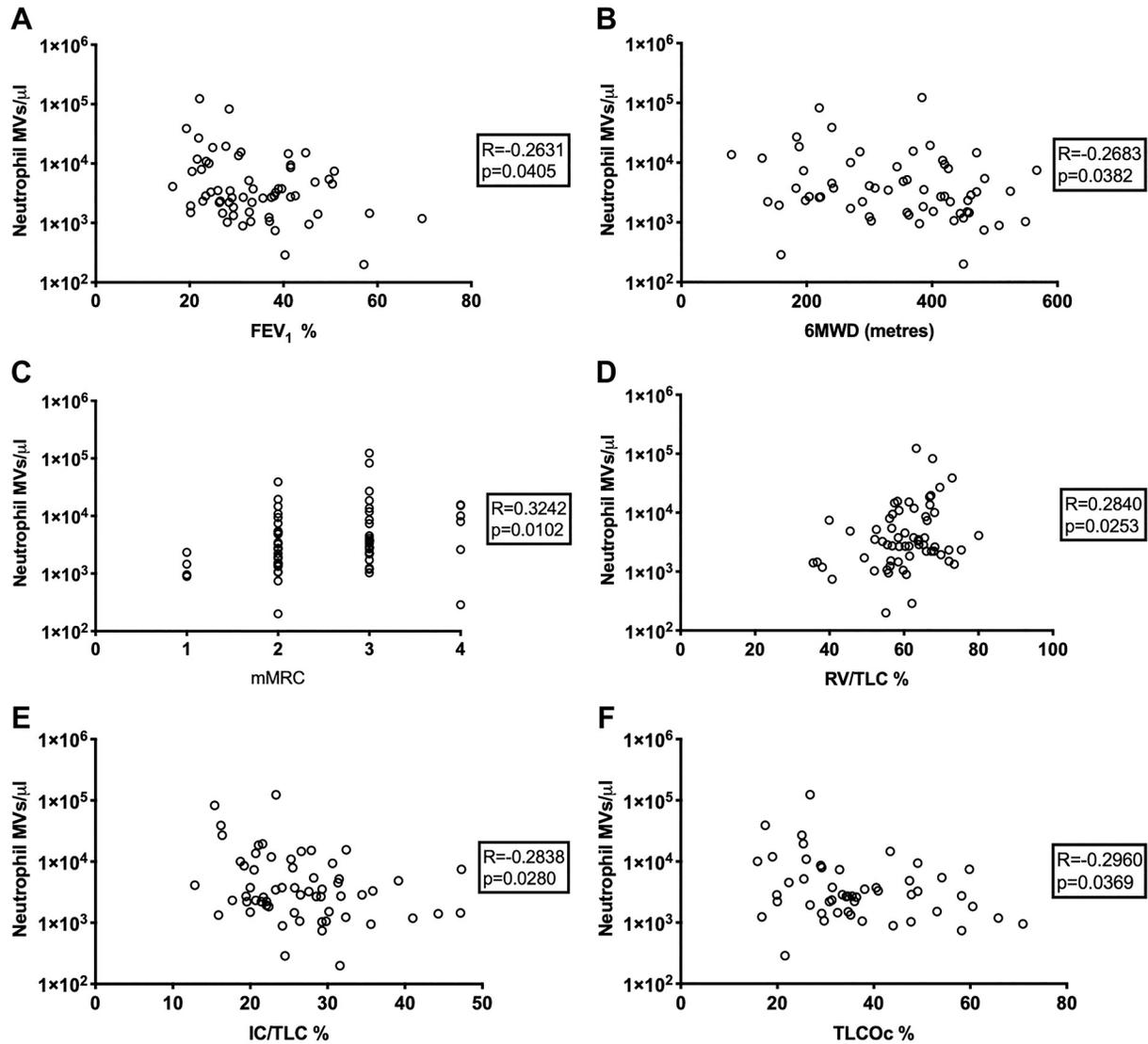


Fig. 4. Correlation of bronchoalveolar lavage fluid (BALF) neutrophil-derived microvesicles (MVs) with discrete physiological measures of chronic obstructive pulmonary disease (COPD) severity: airways obstruction, hyperinflation, gas transfer, and breathlessness. Higher BALF neutrophil-derived MV numbers were significantly associated with deteriorating airways obstruction (FEV_1) (A); decreased 6-min walk distance (6MWD; in meters) (B); declining modified Medical Research Council (mMRC) dyspnea score (C); and increased hyperinflation, as quantified using the percentage ratio of residual volume to total lung capacity (RV/TLC) (D) and of inspiratory capacity and total lung capacity (IC/TLC) (E); and of impaired gas transfer (TLCoc) (F); $n = 62$. R , Spearman's rank correlation coefficient.

barrier, cilia beat frequency reduction, increased mucus secretion, and increased permeability of the bronchial mucosa (43, 44). Indeed, neutrophil MVs have been shown to transport a variety of these proteases including matrix metalloproteinases, cathepsin G, and elastase (45, 46) and to possess a variety of proinflammatory capabilities including disruption of epithelial barrier function (45). This has been highlighted in a recent publication that demonstrated that neutrophil-derived extracellular vesicles harboring neutrophil elastase play a vital role in COPD, causing emphysema when instilled into the lungs of mice (10).

We also undertook a detailed evaluation of “circulating” MVs since previous investigations of circulating MVs in COPD have focused on endothelial MVs. These plasma MVs were predominantly composed of those of neutrophil origin, but did not correlate with the disease severity markers

measured. Interestingly, circulating monocyte MVs showed some correlation with an index of lung parenchymal damage (gas transfer) and the BODE index when microbiology-positive isolates had been excluded. Monocytes have been implicated in the pathogenesis of COPD, and monocyte recruitment into the lungs is increased (47), potentially explaining this finding. However, monocyte MVs do not correlate with other indicators of COPD severity so are less informative than BALF neutrophil MVs as a potential diagnostic tool. Notably, we could not replicate previous published data, which have shown associations between circulating endothelial MVs and hyperinflation (9) or history of frequent exacerbations (8). These studies were performed in a much more homogenous patient group (mild COPD patients only) compared with our patient group and therefore less translatability to the general population. Consequently, we

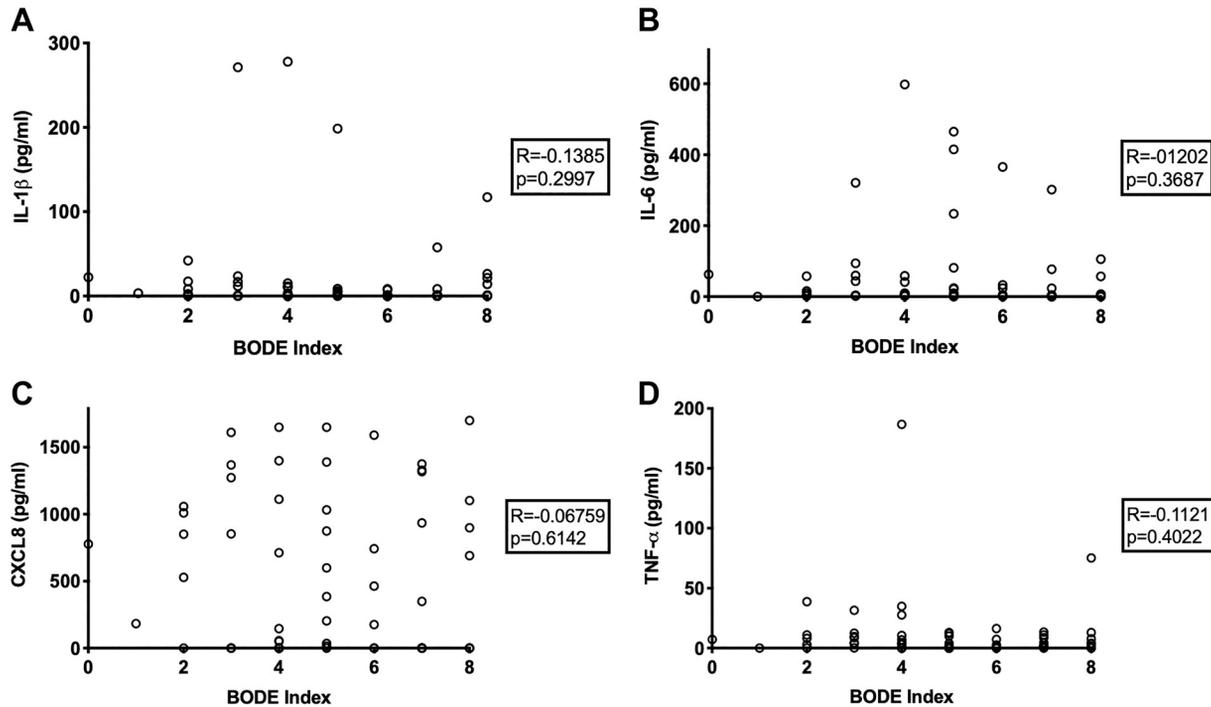


Fig. 5. Correlation of bronchoalveolar lavage fluid (BALF) cytokines and BODE index. IL-1 β (A), IL-6 (B), CXCL8 (C), and TNF (D) levels in BALF were not correlated to any of the measures of COPD disease severity including BODE index; $n = 59$. R , Spearman's rank correlation coefficient.

cannot support a previously proposed hypothesis that circulating endothelial MVs represent a potential biomarker for exacerbation risk (48).

Sampling of the intra-alveolar space via bronchoscopy is the ideal modality to study markers of airway inflammation involved in the initiation and progression of the emphysematous process, but the procedure is invasive and there may be some difficulty in obtaining a true alveolar sample. Consequently, in contrast to peripheral blood and sputum, there are few studies investigating prognostic markers in BALF. To the best of our knowledge, this is the first paper to examine multiple soluble cytokines in BALF and their relation to COPD severity, particularly those that have been implicated in the pathophysiology of this disease. Despite previous reports indicating increased cytokines such as CXCL8 (24) or IL-6 (49) in BALF of COPD patients compared with non-COPD controls, we could not find a correlation between TNF, IL-1 β , IL-6, CXCL8, and COPD disease severity. Many of such reports have relied solely on indexes such as FEV₁ (50) alone or have been limited by low sample numbers (50, 51), rather than comprehensively phenotyping patients in a large sample population as we have performed in this study. Furthermore, while soluble mediators/cytokines are upregulated in accordance with the activation of pulmonary cells, they may actually be rapidly decreased by neutralization or degradation within the alveolar space (e.g., TNF) where many inhibitors and enzymes are present. This highlights the potential problem with soluble mediators/cytokines as unreliable markers of disease severity (52–54). Interestingly though, we found that TNF, IL-1 β , IL-6, and CXCL8 correlate with various MV populations and specifically IL-1 β , IL-6, and CXCL8 correlate with neutrophil MVs. Cytokines do stimulate MV release, which may account for

this finding (55), although this deserves further mechanistic investigation in future studies. Another potential explanation is that these cytokines are enclosed and transported within MVs, as we have previously shown (12), which are then inadvertently measured in our ELISA assays.

At present there are still no ideal clinical diagnostic tests for COPD to assess the disease severity, predict the clinical outcome, and guide treatments. Spirometry is conventionally used in drug trials, but while FEV₁ is easy to obtain and reproducible, it is often unresponsive to medical therapies that improve survival, e.g., domiciliary oxygen, and does not correlate with other clinical outcomes in COPD (56). Composite clinical scores such as the BODE index provide better representation of the whole clinical picture of COPD, thus better predicting disease severity and outcome (32–34). However, a similar criticism to these other clinical indexes (i.e., not directly reflecting the pathogenesis of disease) would be applicable, and hence, they may not change timely or dynamically in response to effective treatments. Our results show the exciting potential of BALF neutrophil MVs as a clinical marker in COPD that tightly links, for the first time, a key pathophysiological mechanism of COPD (intra-alveolar neutrophil activation) with clinical severity/outcome. Because of this mechanistic link, neutrophil MVs can effectively represent an inflammatory blueprint of the alveolar/lung microenvironment during COPD progression and thus have potential advantages compared with other soluble markers or clinical indexes.

There are some limitations to our work. Cells release other species of extracellular vesicles including exosomes and apoptotic bodies. Their role in COPD was beyond the scope of this project, but we appreciate that it would be prudent to investigate these in future studies. Secondly, we only used 50 ml,

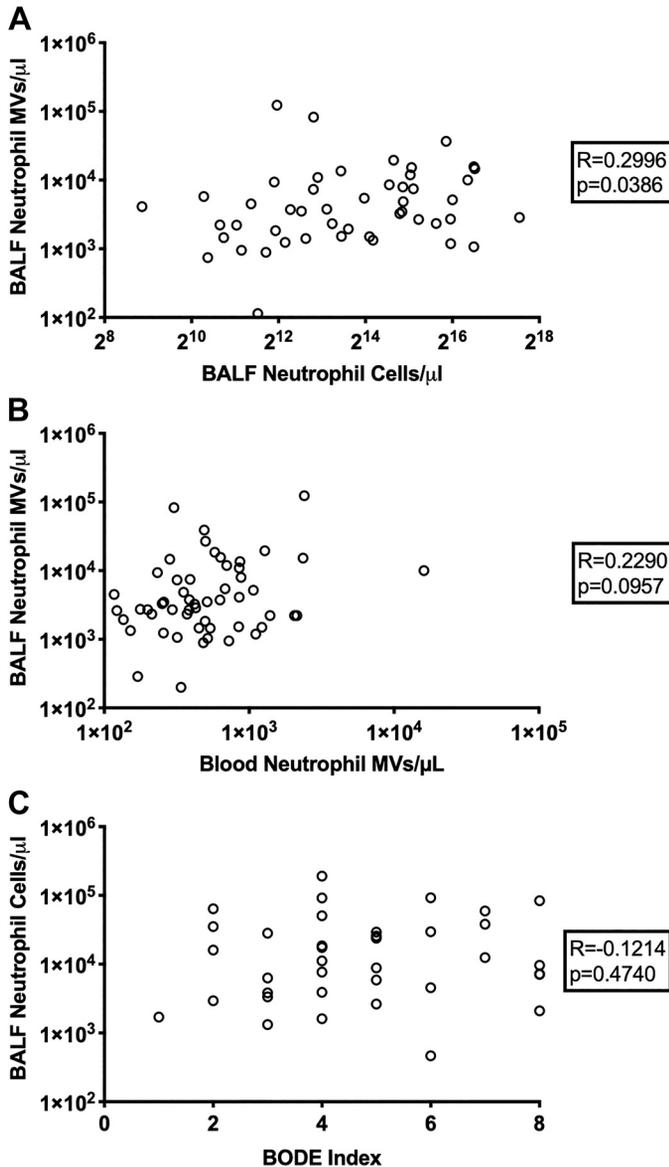


Fig. 6. Origin of bronchoalveolar lavage fluid (BALF) neutrophil-derived microvesicles (MVs). BALF neutrophil-derived MV numbers did correlate with BALF neutrophil cells, albeit weekly (A). However, BALF neutrophil-derived MVs did not correlate with circulating neutrophil-derived MVs suggesting local release rather than translocation from the circulation (B). Having shown increased BALF neutrophil-derived MVs correlate with worsening BODE index, we next examined if numbers of BALF neutrophils, their parent cells, also correlated with disease severity (i.e. BODE index) and found no relationship (C); *n* = 62. *R*, Spearman's rank correlation coefficient.

rather than larger volumes, to perform BAL during bronchoscopy to reduce complications in our patients (some with considerable disease burden), which carries the risk of a more “bronchial” washing as opposed to pure alveolar samples. Thirdly, to assess blood MV profiles we used platelet-rich plasma since there is a dramatic drop in MV yield during the centrifugation steps in preparation of platelet-poor plasma (23). This strategy prevented analysis of circulating platelet-derived MVs (as the size of platelet MVs overlaps that of platelets (1–3 μm) in flow cytometry), but we considered that more accurate quantification of other MV subtypes was a priority for this study. Fourthly, we used a CyAn ADP flow cytometer,

which has previously been used widely in the literature to characterize MVs (11, 57) but is designed primarily for cell analysis. However, we used our established stringent approach (12, 23) to detect relevant MVs using particle size, surface marker expression and detergent sensitivity. We believe that this methodology, while missing some MVs of smaller sizes (and exosomes), is appropriate for the analyses in this study, for which “reproducibility” of MV identification/detection is essential. Finally, the present study does not directly investigate the mechanistic relationship between neutrophil-derived MVs and COPD, as it is not possible to clearly demonstrate such cause-effect relationships in human patient studies. However, there is already a large body of pre-clinical evidence indicating the crucial importance of neutrophils in COPD, and more recently, the potential pathogenic role of neutrophil-derived EVs is suggested in the progression of COPD. The strength of our study is that it has been able to translate such pathophysiological mechanisms into patients and for the first time provide strong evidence linking the key neutrophil-related pathophysiological mechanism with clinical severity/outcome in COPD patients.

In conclusion, we have identified a variety of MV subtype populations within the BALF and plasma of COPD patients with a spectrum of disease severity. In this heterogeneous patient cohort ranging from mild to very severe COPD, BALF PMN (i.e., neutrophil) MVs strongly correlate with the BODE index as well as multiple other markers of COPD severity: worsening dyspnea score, degree of airway obstruction and hyperinflation, lung parenchymal damage, and exercise tolerance. Our data suggest that these BALF neutrophil MVs are a novel, clinically relevant marker for COPD that has a strong biological plausibility in terms of its role in disease pathogenesis as well as a high-degree association with clinical severity and thus can be readily integrated into clinical practice as diagnostic and/or therapeutic targets.

SUPPLEMENTAL DATA

Supplemental Tables S1, S2, S3, S4, S5: <https://doi.org/10.6084/m9.figshare.11980584>.

Supplemental Figs. S1 and S2: <https://doi.org/10.6084/m9.figshare.11980584>.

Supplemental Methods: <https://doi.org/10.6084/m9.figshare.11980764>.

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

S.Soni, J.L.G., S.Singh, M.R.W., S.V.K., O.S.U., P.L.S., and M.T. conceived and designed research; S.Soni, J.L.G., K.P.O., M.K., L.F., N.T., K.S., E.D.T., A.M.A., S.Singh, M.R.W., J.A.W., S.V.K., O.S.U., and P.L.S. performed experiments; S.Soni, J.L.G., K.P.O., M.K., J.A.W., S.V.K., O.S.U., P.L.S., and M.T. analyzed

data; S.Soni, J.L.G., K.P.O., S.V.K., O.S.U., P.L.S., and M.T. interpreted results of experiments; S.Soni, S.V.K., O.S.U., P.L.S., and M.T. prepared figures; S.Soni, J.L.G., K.P.O., A.M.A., S.Singh, M.R.W., S.V.K., O.S.U., P.L.S., and M.T. drafted manuscript; S.Soni, J.L.G., K.P.O., E.D.T., A.M.A., S.Singh, M.R.W., S.V.K., O.S.U., P.L.S., and M.T. edited and revised manuscript; S.Soni, A.M.A., S.Singh, M.R.W., S.V.K., O.S.U., and P.L.S. approved final version of manuscript.

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