Detection and characterisation of extracellular vesicles in exhaled breath condensate and sputum of COPD and severe asthma patients

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To the Editor:

Extracellular vesicles, nano-sized (20–1000 nm) membranous structures released from cells, play critical roles in both physiological and pathological processes [1]. Extracellular vesicles can be classified based on their size into small (sEVs; <200 nm, also known as exosomes) and medium (mEVs; >200 nm, also known as microvesicles) [1]. Extracellular vesicles might be a target for personalised medicine, given their content and biological origin [2]. Indeed, extracellular vesicles detected in various biological fluids, including sputum supernatants, mucus, epithelial lining fluid, the pulmonary circulation and bronchoalveolar lavage fluid represent a unique tool for both investigating the pathophysiology of respiratory disease and for biomarker discovery [2, 3].

Micro (mi)RNAs, mostly in a membrane-encapsulated form, have previously been detected in exhaled breath condensate (EBC) [4], a noninvasive technique for collecting aerosol particles generated in the airways [5]. However, no studies have tried to detect, isolate and quantify EBC sEVs and mEVs. Respiratory tract lining fluid proteins could be detected in endogenous particles in exhaled breath [6]. We aimed to investigate whether extracellular vesicles are present and detectable in EBC and to perform a preliminary comparison of their concentrations in severe asthma, COPD and healthy control subjects. We also compared the nature and quantity of extracellular vesicles isolated from EBC with those isolated from induced sputum supernatants [7]. Our results strongly suggest that extracellular vesicles can be isolated from both fluids.

We isolated and characterised sEVs and mEVs following international guidelines [1]. Briefly, 1 mL EBC or sputum supernatant samples were washed with PBS and centrifuged at low speed (750×*g* for 15 min and 1500×*g* for 5 min). Next, samples were centrifuged at 17 000×*g* for 30 min and at 120 000×*g* for 1.5 h to isolate mEVs and sEVs, respectively. The isolated fractions were resuspended in 100 µL PBS and were characterised using dynamic light scattering for size (figure 1a) and by scanning electron microscope for morphology (figure 1b). In addition, we performed a Western blot on proteins extracted from sEVs (figure 1c) using an anti-CD63 antibody (dilution 1:500, sc-5275; Santa Cruz Biotechnology, Dallas, TX, USA). CD63 is a tetraspanin mostly expressed on the surface of nano-sized (20–120 nm) extracellular vesicles of endocytic origin [8]. The results confirmed that we had indeed isolated sEVs and mEVs from both EBC and sputum supernatant. Next, both extracellular vesicle fractions were bound to 4 µm aldehyde sulfate latex beads and examined by cytofluorometry (figure 1d). They were then stained with calcein, a dye commonly used to label extracellular vesicles because it is converted to green-fluorescent calcein by intracellular esterases which are not present in damaged vesicles and debris [9]. Both extracellular vesicle subpopulations demonstrated enzymatic activity and were stained by calcein (figure 1d). Moreover, flow cytometry confirmed the expression of exosomal markers (CD63, CD9) in the sEV fraction (dilution 1:20, Brilliant Violet 421 anti-human CD63 antibody; BioLegend, San Diego, CA, USA; dilution, 1:20, PE anti-human CD9 antibody, BioLegend). sEVs displayed greater expression of the tetraspanins CD9 and CD63 compared to mEVs. Previous *in vitro* studies showed that extracellular vesicles from mast cells induce mesenchymal transition in pulmonary adenocarcinoma A549 cells [10] and that airway epithelium-derived exosome-like vesicles play a functional role in lung cell-to-cell communication by

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Small and medium extracellular vesicles, identified and characterised in exhaled breath condensate and induced sputum supernatants, might provide pathophysiological insights and novel biomarkers in severe asthma and COPD patients https://bit.ly/3fiTnXY

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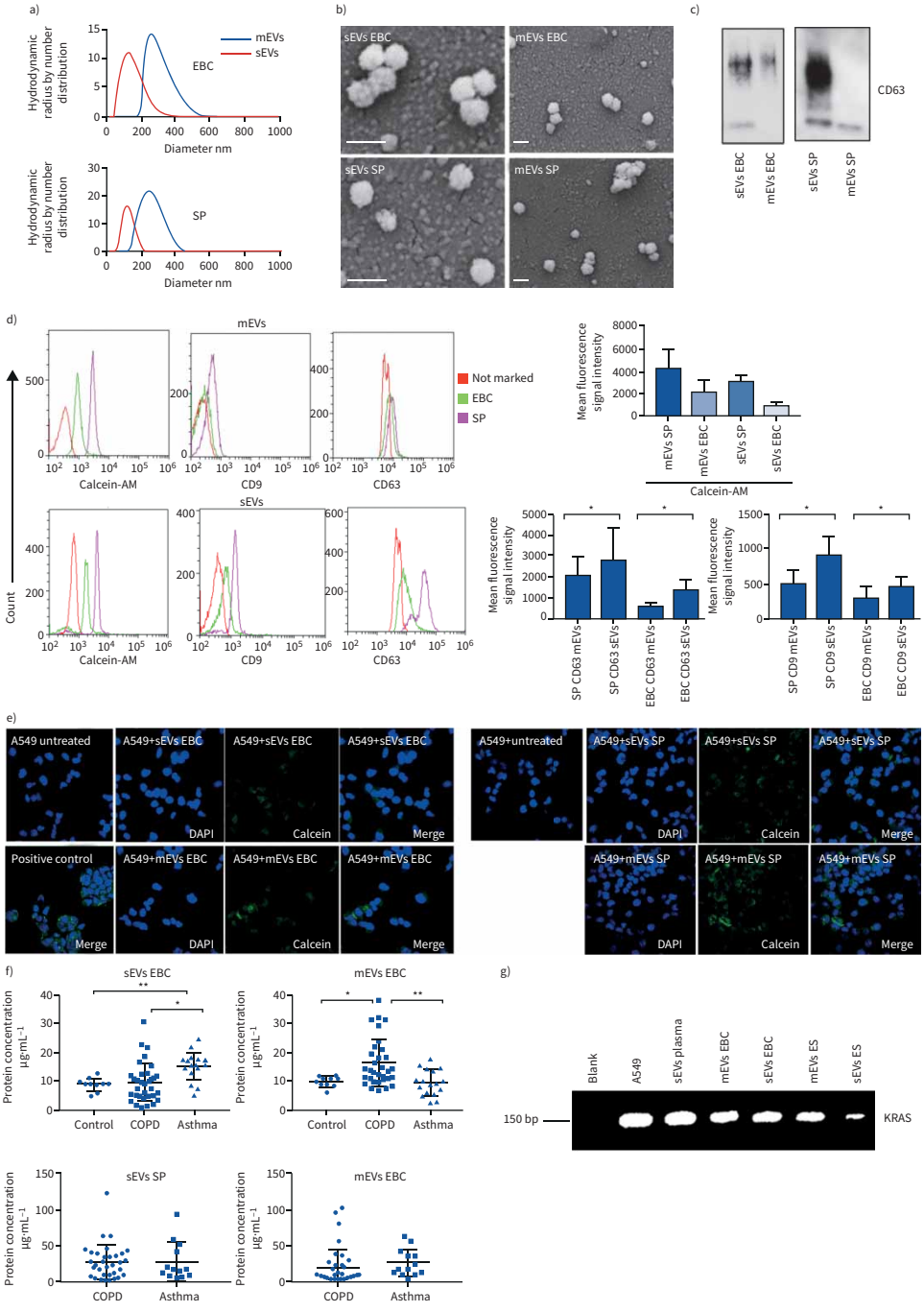

FIGURE 1 Characterisation of small (sEVs; <200 nm) and medium (mEVs; >200 nm) extracellular vesicles in exhaled breath condensate (EBC) and sputum supernatants (SP). a) Size measurement of sEVs and mEVs by dynamic light scattering. b) Scanning electron microscope for morphology characterisation of sEVs and mEVs isolated from EBC and SP. Scale bars sEVs=100 nm; mEVs=500 nm. c) Western blot with the anti-CD63 antibody, used as a marker of sEVs (exosomes). CD63 core protein is 26 kDa protein which can be glycosylated. The antibody detects the various glycosylated forms ranging from 30 to 60 kDa. d) Flow cytometry analysis of sEV (exosomal) markers: sEVs showed higher expression of tetraspanins CD9 (lower right histograms) and CD63 (lower left histograms) compared to mEVs (p=0.0001 for all comparisons: SP mEVs CD63 *versus* SP sEVs CD63; EBC mEVs CD63 *versus* SP sEVs CD63; SP mEVs CD9 *versus* SP sEVs CD9; EBC mEVs CD9 *versus* CD9 sEVs EBC). SP-derived sEVs and mEVs showed greater enzymatic activity compared with their respective counterparts in EBC (upper histograms). Data are presented as mean±SEM. AM: acetoxymethyl. e) Confocal microscopy of sEVs and mEVs internalisation in A549 cells. Representative images of A549 staining after 1 h of incubation with extracellular vesicles (either mEVs or sEVs) isolated from EBC or SP. Blue: nuclei (4′,6-diamidino-2-phenylindole

(DAPI)); green: sEVs or mEVs (calcein-AM); untreated: A549 cells not treated with either EBC or SP-derived extracellular vesicles (sEVs or mEVs); positive control: HCT116 cells treated with HT29 cell-derived sEVs; merge: DAPI+calcein-AM. f) Quantification of vesicle total proteins in patients with severe asthma or COPD and healthy control subjects: EBC sEVs protein concentrations were higher in severe asthma patients (n=17) compared to control subjects (n=10) and COPD patients (n=35); EBC sEVs protein concentrations in control and COPD subjects were similar. In three out of 20 severe asthma patients, EBC was not collected because of technical failure. EBC mEVs protein concentrations were elevated in COPD patients compared with severe asthma and control subjects. In the latter groups, EBC mEVs protein levels were similar. In two out of 35 COPD patients, mEVs protein concentrations in EBC were undetectable. sEVs and mEVs protein concentrations isolated from SP were not significantly different in asthma (n=13) and COPD patients (n=35). Seven out of 20 severe asthma patients did not provide a valid sputum sample; in one patient with COPD, sEVs protein concentrations in sputum supernatant were undetectable. g) sEVs and mEVs contain double-stranded DNA. Representative example of amplified DNA-KRAS isolated from A549 (positive control), plasma sEVs, EBC sEVs, EBC mEVs, SP sEVs and SP mEVs. DNA extraction and amplification were performed in pooled biological samples. Outliers have not been considered in statistical analysis. One-way ANOVA followed by Bonferroni's *post hoc* comparison tests were performed in all statistical analyses. *: p<0.05; **: p<0.01.

transferring cellular cargo, including nucleic acids (mRNA and miRNA), proteins and lipids, which may be relevant to airway biology and remodelling [11]. We investigated the internalisation of calcein-labelled sEVs and mEVs in A549 cells by confocal microscopy to functionally characterise the extracellular vesicle fractions. HCT116 cells treated with sEVs isolated from HT29 cell line and untreated A549 cells were used as positive and negative controls, respectively [12]. Extracellular vesicles from both EBC and sputum supernatant were taken up by pulmonary epithelial cells (figure 1e), suggesting a possible functional role which warrants further investigation.

In a preliminary quantitative study, we measured the EBC and sputum extracellular vesicle total protein content, an indirect measure of extracellular vesicle number [1]. We recruited an opportunistic sample of 20 patients with severe asthma (females/males 12/8; mean±SEM age 59.0±3.0 years; ex-/current/nonsmokers 3/3/14; 14 atopic; pre-bronchodilator forced expiratory volume in 1 s (FEV_1) 1.4±0.1 L, 54.9±3.0% predicted; post-bronchodilator FEV_1 1.7±0.2 L, 66.2±2.9% pred; pre-bronchodilator forced vital capacity (FVC) 2.4±0.2 L, 78.2±3.9% pred; post-bronchodilator FVC 2.7±0.2 L, 89.3±3.4% pred; Asthma Control Questionnaire (ACQ) 2.4±0.2; median (interquartile range (IQR)) fractional exhaled nitric oxide (F_{ENO}) 27 (13.4–33) ppb; inhaled corticosteroids (ICS)/long-acting β_2-agonists (LABA)/long-acting muscarinic receptor antagonists (LAMA) 19/20/3; four prescribed oral corticosteroids); 35 COPD patients (females/males 12/23; age 71.6±1.1 years; ex-/current/nonsmokers 26/7/2; Global Initiative for Chronic Obstructive Lung Disease A/B/C/D category 15/6/8/6; seven atopic; pre-bronchodilator FEV_1 1.7±0.1 L, 67.9±3.5% pred; post-bronchodilator FEV_1 1.8±0.1 L, 71.8±3.5% pred; pre-bronchodilator FVC 2.6±0.1 L, 81±3.0% pred; post-bronchodilator FVC 2.8±0.2 L, 86.3±2.8% pred; St George's Respiratory Questionnaire (SGRQ) total score 36.3±3.5; F_{ENO} 18 (13–26) ppb; ICS/LABA/LAMA 14/29/28; none prescribed oral corticosteroids); and 10 healthy control subjects (10 males, age 43.4±1.6 years; ex-/current/nonsmokers 3/0/7; pre-bronchodilator FEV_1 4.1±0.2 L, 104.1±4.1% pred; pre-bronchodilator FVC 5.1±0.3 L, 105.9±4.8% pred; F_{ENO} 17.4 (10.1–48.6) ppb; no pharmacological treatment). Continuous data were expressed as mean±SEM or median (IQR (25th and 75th percentiles)), depending on data distribution. ANOVA or Kruskal–Wallis and Chi-squared tests were used for between-group comparisons, as appropriate. Categorical data were expressed numerically. Significance was defined as a value of p<0.05. Diagnosis of severe asthma was based on European Respiratory Society/American Thoracic Society guidelines [13]. Participants attended the clinical pharmacology laboratory, Catholic University of the Sacred Heart, University Hospital Agostino Gemelli Foundation IRCCS (Rome, Italy), on one occasion for spirometry, F_{ENO} measurement, 15-min EBC collection, sputum induction and to complete the ACQ (severe asthma patients) and SGRQ (COPD patients). Spirometry [14], F_{ENO} measurement [15], EBC collection [5] using an EcoScreen condenser (Jaeger, Hoechberg, Germany) and sputum induction and analysis [16] were performed according to international standardised procedures. This study was approved by the ethics committee of the University Hospital Agostino Gemelli Foundation (approval numbers 0013996/16 and P/1034/CE2012) and written consent was obtained from participants. We acknowledge that the groups were not matched for age (p<0.0001), smoking history (p<0.0001), atopic status (p=0.0002) and prescribed treatment (p<0.03), so interpretation of the findings must be cautious. EBC sEV protein concentrations were higher in severe asthma patients (15.7±1.2 μg mL^{-1}) compared to COPD (9.9±1.1 μg mL^{-1}, p<0.05) and healthy controls (9.2±1.1 μg mL^{-1}, p<0.01), the results of which were similar (figure 1f). By contrast, EBC mEV protein concentrations were elevated in COPD (16.3±1.5 μg·mL^{-1}) compared with severe

asthma (9.5±1.1 $\mu g \cdot mL^{-1}$, $p<0.01$) and healthy controls (9.9±0.5 $\mu g \cdot mL^{-1}$, $p<0.05$) (figure 1f). Protein concentrations in sEVs and mEVs isolated from sputum supernatant were similar in severe asthma (sEVs 26.3±4.8 $\mu g \cdot mL^{-1}$; mEVs 28.7± 6.9 $\mu g \cdot mL^{-1}$) and COPD (sEVs 20.0±4.4 $\mu g \cdot mL^{-1}$; mEVs 28.2±4.2 $\mu g \cdot mL^{-1}$). Although there was no control group, this finding suggests that EBC might be a better biological fluid to study quantitative differences in extracellular vesicles between severe asthma and COPD patients. Finally, we were able to isolate DNA from both EBC sEVs and mEVs, thus demonstrating that they might be a valuable source of nucleic acid suitable for genetic analysis in lung diseases including lung cancer (figure 1g). DNA was isolated from pooled EBC and sputum supernatant samples obtained from three individuals using QIAamp MinElute Virus Spin Kit (Qiagen, Venlo, the Netherlands). Genotyping for KRAS was performed using nested-PCR.

Overall, these preliminary and exploratory data show that extracellular vesicles are detectable in EBC and sputum and that EBC sEVs might be more useful in severe asthma, whereas measurement of EBC mEV concentrations might be more informative in COPD patients. The next step is to use specific quantitative techniques to measure extracellular vesicles in biological fluids. Our *ex vivo* approach cannot identify the cellular source(s) of extracellular vesicles for which *in vitro* studies are likely to be required. Further studies are needed to better characterise extracellular vesicles in EBC, quantify their content, including proteins, define variability over time and potential confounding factors and clarify the utility of these measurements in respiratory diseases.

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Author contributions: D. Lucchetti provided substantial contributions to the acquisition and analysis of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. G. Santini provided substantial contributions to the acquisition and analysis of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; L. Perelli provided substantial contributions to the acquisition and analysis of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. C. Ricciardi-Tenore provided substantial contributions to the acquisition and analysis of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. F. Colella provided substantial contributions to the acquisition and analysis of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. N. Mores provided substantial contributions to the interpretation of data for the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. G. Macis provided substantial contribution to the design of the work; revised the manuscript critically for important intellectual content; approved the final version of the manuscript to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. A. Bush provided

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