Detection of cells in EBC holds potential for pathophysiological insights in pulmonary diseases

Letizia Palomba, Ph.D.[1†]; Debora Paris, Ph.D.[2†]; Annabella Tramice, Ph.D.[2]; Pasquale Ambrosino, M.D., Ph.D.[3]; Mauro Maniscalco, M.D., Ph.D.[4,5*]; Andrea Motta, Ph.D.[2*]

[1]Department of Biomolecular Sciences, "Carlo Bo" University; 61029 Urbino, Italy.

[2]Institute of Biomolecular Chemistry, National Research Council; 80078 Pozzuoli (Naples), Italy.

[3]Istituti Clinici Scientifici Maugeri IRCCS, Cardiac Rehabilitation Unit of the Telese Terme Institute; 82037 Telese Terme (Benevento), Italy.

[4]Istituti Clinici Scientifici Maugeri IRCCS, Pulmonary Rehabilitation Unit of the Telese Terme Institute; 82037 Telese Terme (Benevento), Italy.

[5]Department of Clinical Medicine and Surgery, Section of Respiratory Disease, University of Naples Federico II; 80131 Naples, Italy.

[†]These authors contributed equally to this work.

*Corresponding authors: Andrea Motta (e-mail: **andrea.motta@icb.cnr.it**) and Mauro Maniscalco (e-mail: **mauro.maniscalco@icsmaugeri.it**).

Funding This work was partially supported by the "*Ricerca corrente*" funding scheme of the Ministry of Health, Italy.

Keywords: biomarkers, bronchiectasis, COPD, epithelial cells, liquid biopsy, macrophages, outcomes, rehabilitation.

To the Editor:

Exhaled breath condensate (EBC), the liquid form of expired air, is non-invasively collected and can be considered a lung-specific liquid biopsy (1, 2). We hypothesized that EBC could also be a carrier of viable cells from the airway mucosa, and if present, they could be used to investigate pulmonary pathologies as done for the bronchoalveolar lavage (BAL) cells (3–5). Due to noninvasiveness of EBC, cell analysis could also be easily extended to evaluation of pediatric lung diseases. To test our hypothesis we examined samples from healthy subjects and COPD as disease model. COPD was chosen as it is currently the third leading cause of death worldwide, and detection of noninvasive biomarkers could possibly address the unmet need of identifying early-stage disease, monitor therapeutic response, and evaluate new therapies (6).

After approval by the local Ethic Committee, we collected EBC from healthy nonsmokers (n=10), patients with stable COPD [n=10, confirmed negative for bronchiectasis by high-resolution computed tomography (HRCT)] and with COPD positive for bronchiectasis [n=10, diagnosed by HRCT], the last two classes being ex-smokers for 1 year. All patients presented no exacerbations in the prior month and were under ICS/LABA/LAMA treatment (Table 1).

EBC was collected with a TURBO-DECCS condenser (Medivac, Pilastrello, Italy) as reported (7), obtaining 5.0 ± 0.8 ml from each subject. No salivary contamination was detected by the α-amylase test (Infinity Amylase Reagent, Sigma, Milan, Italy), and NMR spectra (8). EBCs were centrifuged at 800*g* for 10 min, the pellets re-suspended in 0.05 mL DMEM/F12 supplemented with 10% fetal bovine serum (Life Technologies), penicillin/streptomycin (50 units/ml), hydrocortisone (0.2 μM) and insulin (50 μg/ml), counted in a Bürker chamber and lastly plated on polylysine-coated coverslips. Viability was determined by Trypan blue exclusion assay. After a 24-hour incubation at 37°C in 5% CO_2 in 35-mm culture dishes (Life Technologies), the attached cells were analyzed by immunofluorescence assay. Mouse anti-pan-cytokeratin (anti-P-CK Abcam, Cambridge, UK; 1:100) and rabbit anti-CD14 (GeneTex, Irvine, CA, 1:100) were used as primary antibodies for lung

epithelial cells (ECs) and monocytes/macrophages identification, respectively. Appropriate Alexa-488, or -546 donkey anti IgGs (Invitrogen Life Technologies) secondary antibodies were used before counterstained with nuclear dye Hoechst 33342. Stained cells were examined with confocal Nikon Eclipse Ti2 microscope equipped with the DS-Qi2 digital camera, and the images analyzed with NIS-Elements C software (Nikon, Florence, Italy). The average of total cell number was obtained by counting Hoechst positive cells in at least four random fields (magnification 40 μm). For negative control, we substituted the primary antibodies with nonspecific mouse IgG. Statistical analysis was performed using ANOVA test with Bonferroni post-hoc p adjustment after evaluation of normal data distribution with Shapiro-Wilk test.

Hoechst positive cells were detected for both healthy and COPD subjects (Figures 1A-1B, panels 1), with the total number increasing from healthy ($17.40\pm5.56\times10^3$ cells/ml of EBC) to COPD ($38.93\pm3.61\times10^3$ cells/ml), and to COPD/bronchiectasis ($64.80\pm1.13\times10^3$ cells/ml) (Figure 1C). Interestingly, COPD/bronchiectasis presented the highest number of the total cells (Figure 1C), suggesting that pathological differences are reflected in the total cell number.

We then investigated the cell type and their distribution. Since EBC contains lung inflammatory biomarkers, we looked for macrophages using CD14 that is strongly positive in monocytes/macrophages. CD14-positive cells were detected in all groups [Figures 1A-1B (panels 2), 1D] thus confirming the presence of macrophages. Their number significantly increased from healthy subjects ($4.40\pm3.03\times10^3$ macrophages/ml of EBC; mean-percentage value 23.75±8.88%) to COPD ($27.70\pm4.03\times10^3$ macrophages/ml; mean-percentage value 70.67±3.79%, $p=2.40\times10^{-4}$), and to COPD/bronchiectasis ($52.40\pm1.70\times10^3$ macrophages/ml; mean-percentage value 81.00±1.41%, $p=1.66\times10^{-4}$; red boxes in 1D). Since BAL also contains ECs (9), we looked for them in EBC using an anti-cytokeratin antibody. ECs were clearly seen [Figures 1A-1B (panels 3), 1D], presenting a significant decrease, as percent of total cells, from healthy subjects ($13.00\pm2.87\times10^3$ ECs/ml of EBC; mean-percentage value 76.25±8.80%) to COPD ($9.60\pm1.60\times10^3$ ECs/ml; mean-percentage value

25.00±6.56%, p=2.42×10^{-4}), and to COPD/bronchiectasis (12.40±0.56×10^{3} ECs/ml; mean-percentage value 19.00±1.41%, p=2.73×10^{-4}; blu boxes in Figure 1D). Interestingly, the EC-macrophages ratio was remarkably different between healthy donors and COPD: ECs were predominant for healthy subjects (2.95) while the CD14+ cells were dominant for COPD patients [(0.35) for COPD and (0.24) for COPD/bronchiectasis] (Figure 1D).

The cells observed in EBC could also originate from buccal/tracheal cells' contamination because they are also CD14 and cytokeratin positive. However, the presence of a saliva trap in the condenser, the α-amylase test and the absence in the NMR spectra of carbohydrates' signals could safely exclude detectable salivary contamination. The fact that buccal ECs presents flat pancake-liked profile, centerline located nucleus, and large cytoplasm-to-nucleus ratio (10), features not observed in Figures 1A-1B (panels 3), lends further support to the absence of salivary contamination. Currently, we cannot rule out a possible tracheal contamination as tracheal ECs also stain positive for cytokeratin. However, since they are widely used as *in vitro* models of lung diseases (11–13), the possible detection in EBC may provide a rapid means to investigate respiratory diseases.

We reported for the first time that EBC carries viable respiratory cells and that their composition varies with the presence of a pathological state. Clearly, the study presents limitations: first and foremost, the cellular district should be clearly identified, as this dictates to what process they participate. Furthermore, no statistically significant correlation was observed between cell data and lung functions. As promising aspects, we found significant differences between healthy and COPD subjects, and, based on cell distribution, also between COPD subsets (COPD/bronchiectasis *vs.* COPD, 19.00±1.41%, *vs.* 25.00±6.56%, p=0.019 for ECs, and 81.00±1.41% *vs.* 70.67±3.79%, p=0.020 for macrophages), therefore suggesting a possible phenotype/endotype characterization in airways diseases probing the EBC cellular component. Lastly, EBC cells could be investigated by single-cell analysis with excellent resolution even in the presence of a limited number of cells (14).

We are currently investigating the origin and distribution of cells, and their correlation with lung functions in an increased number of patients with the aim of extending these results to liquid biopsy of lung pathologies for clinical applications.

Acknowledgments: We thank the participants, their families and caregivers. We also acknowledge the help of L. Cristino and B. Marfella (ICB-CNR) for help with cell images.

Authors contributions: A. Motta, L. Palomba and M. Maniscalco conceptualized the study. A. Motta, L. Palomba and M. Maniscalco contributed to the study design. M. Maniscalco and P. Ambrosino enrolled and evaluated the patients. L. Palomba, D. Paris and A. Tramice carried out experiments and data acquisition. A. Motta, L. Palomba, D. Paris and A. Tramice contributed to data interpretation and analysis. A. Motta, L. Palomba, D. Paris and M. Maniscalco wrote the paper. All authors revised the manuscript and approved the final version prior to submission.

Conflict of interest: All the authors declare no conflict of interest.

References

1. Koc A, Goksel T, Pelit L, Korba K, Dizdas TN, Baysal E, *et al.* cfDNA in exhaled breath condensate (EBC) and contamination by ambient air: toward volatile biopsies. *J Breath Res* 2019;13(3):036006.

2. Verzè M, Minari R, Gnetti L, Bordi P, Leonetti A, Cosenza A, *et al.* Monitoring cfDNA in plasma and in other liquid biopsies of advanced EGFR mutated NSCLC patients: A pilot study and a review of the literature. *Cancers (Basel)* 2021;13(21):5403.

3. Davidson KR, Ha DM, Schwarz MI, Chan ED. Bronchoalveolar lavage as a diagnostic procedure: a review of known cellular and molecular findings in various lung diseases. *J Thorac Dis* 2020;12(9):4991–5019.

4. Shanthikumar S, Colenutt S, Cole T, Conyers R, Rozen T, Harrison J, *et al.* Clinical utility of bronchoalveolar lavage in pediatric oncology patients. *Pediatr Infect Dis J* 2022;41(11):899–903.

5. Bergantini L, d'Alessandro M, Cameli P, Perrone A, Cekorja B, Boncompagni B, *et al.* Integrated approach to bronchoalveolar lavage cytology to distinguish interstitial lung diseases. *Eur J Intern Med* 2021;89:76–80.

6. Martinez FJ, Agusti A, Celli BR, Han MK, Allinson JP, Bhatt SP, *et al.* Treatment trials in young patients with chronic obstructive pulmonary disease and pre-chronic obstructive pulmonary disease patients: Time to move forward. *Am J Respir Crit Care Med* 2022;205(3):275–287.

7. Maniscalco M, Paris D, Cuomo P, Fuschillo S, Ambrosino P, Tramice A, *et al.* Metabolomics of COPD pulmonary rehabilitation outcomes via exhaled breath condensate. *Cells* 2022;11(3):344.

8. de Laurentiis G, Paris D, Melck D, Maniscalco M, Marsico S, Corso G, *et al.* Metabonomic analysis of exhaled breath condensate in adults by nuclear magnetic resonance spectroscopy. *Eur Respir J* 2008;32(5):1175–1183.

9. Heron M, Grutters JC, ten Dam-Molenkamp KM, Hijdra D, van Heugten-Roeling A, Claessen AM, *et al.* Bronchoalveolar lavage cell pattern from healthy human lung. *Clin Exp Immunol* 2012;167(3):523–531.

10. Theda C, Hwang SH, Czajko A, Loke YJ, Leong P, Craig JM. Quantitation of the cellular content of saliva and buccal swab samples. *Sci Rep* 2018;8(1):6944.

11. Aghapour M, Raee P, Moghaddam SJ, Hiemstra PS, Heijink IH. Airway epithelial barrier dysfunction in chronic obstructive pulmonary disease: Role of cigarette smoke exposure. *Am J Respir Cell Mol Biol* 2018;58(2):157–169.

12. Gon Y, Hashimoto S. Role of airway epithelial barrier dysfunction in pathogenesis of asthma. *Allergol Int* 2018;67(1):12–17.

13. Liu WK, Xu D, Xu Y, Qiu SY, Zhang L, Wu HK, *et al.* Protein profile of well-differentiated versus un-differentiated human bronchial/tracheal epithelial cells. *Heliyon* 2020;6(6):e04243.

14. Schoof EM, Furtwängler B, Üresin N, Rapin N, Savickas S, Gentil C, *et al.* Quantitative single-cell proteomics as a tool to characterize cellular hierarchies. *Nat Commun* 2021;12(1):3341.

Table 1. Characteristics and clinical parameters of the subjects enrolled in the study[a]

Clinical data (unit)	*Healthy controls*	*COPD*	*COPD/Bronchiectasis*
N	10	10	10
Sex (F/M)	5/5	5/5	5/5
Age (y)	60.75 ± 10.21	69.40 ± 4.20	71. 80 ± 5.22
BMI (kg/m^2)	24.68 ± 2.01	27.30 ± 1.72	26.50 ± 2.12
GOLD	-	D	D
FEV_1 (L)	3.20 ± 1.20	2.20 ± 0.14	1.43 ± 0.57
FEV_1 (% predicted)	101.27 ± 16.70	46.34 ± 3.93	44.10 ± 4.21
FVC (L)	4.17 ± 0.68	3.79 ± 0.55	2.15 ± 0.46
FVC (% predicted)	111.36 ± 14.20	70.58 ± 5.42	68.33 ± 7.88
FEV_1/FVC	75.83 ± 10.42	57.77 ± 9.04	66.61 ± 11.21
6MWD (m)	-	170.30 ± 8.43	129.10 ± 39.28
Former tobacco exp. (pk/yr)	-	28.4 ± 2.1	27.2 ± 1.9
ICS/LABA/LAMA	-	10	10

[a]COPD and COPD/Bronchiectasis were diagnosed by high-resolution computed tomography (HRCT). FEV_1, forced expiratory volume during the first second of a forced breath; FVC, forced vital capacity; FEV_1/FVC, (Tiffeneau-Pinelli index) ratio between the forced expiratory volume in the first second (FEV_1) and the forced vital capacity (FVC) of the lungs; ICS, inhaled corticosteroid; LABA, Long-acting beta agonist; LAMA, Long-acting muscarinic antagonists; 6MWD, six-minute walking distance.

Figure caption

Figure 1. Expression of lung monocytes/macrophages and alveolar epithelial cells in EBC samples from healthy, COPD and COPD with bronchiectasis subjects. *(A, B)* Representative micrographs of immunocytochemical staining of CD14 (panels 2, green signal) and P-CK (panels 3, red signal) positive cells. Hoechst 33342 (panels 1, blu signal) was used for nuclear staining. Scale bar: 40 μm. *(C)* Box and whiskers plot of the total average cell number × 10^3/ml of EBC obtained by counting Hoechst 33342 positive cells (magnification 40 μm). *(D)* Box and whiskers plot reporting the percentage of CD14 (red boxes) and P-CK (blu boxes) positive cells with respect to the total cells. Results are expressed as mean ± standard deviation. *, $p<0.05$; **, $p<10^{-3}$; and ***, $p<10^{-4}$.

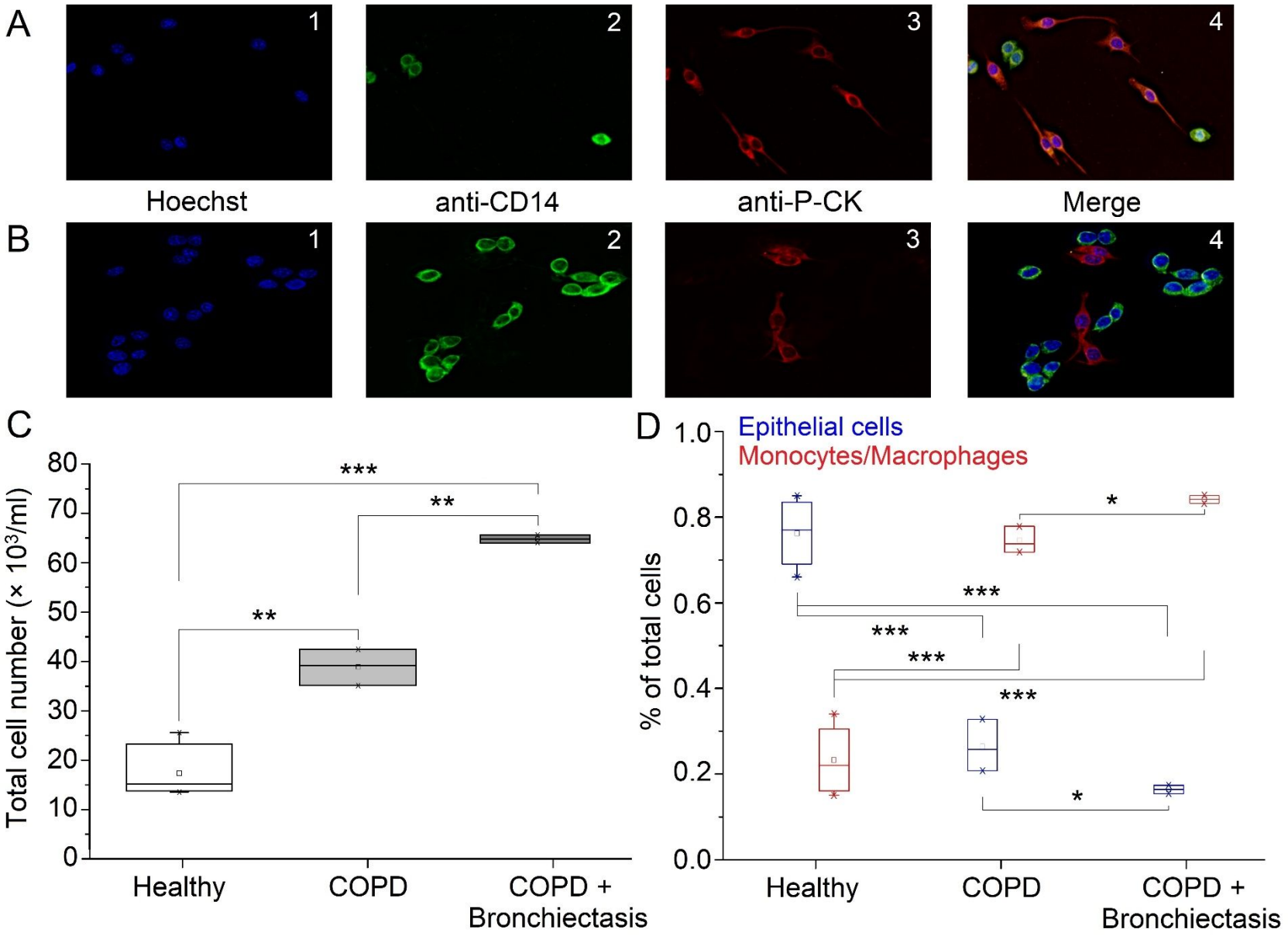
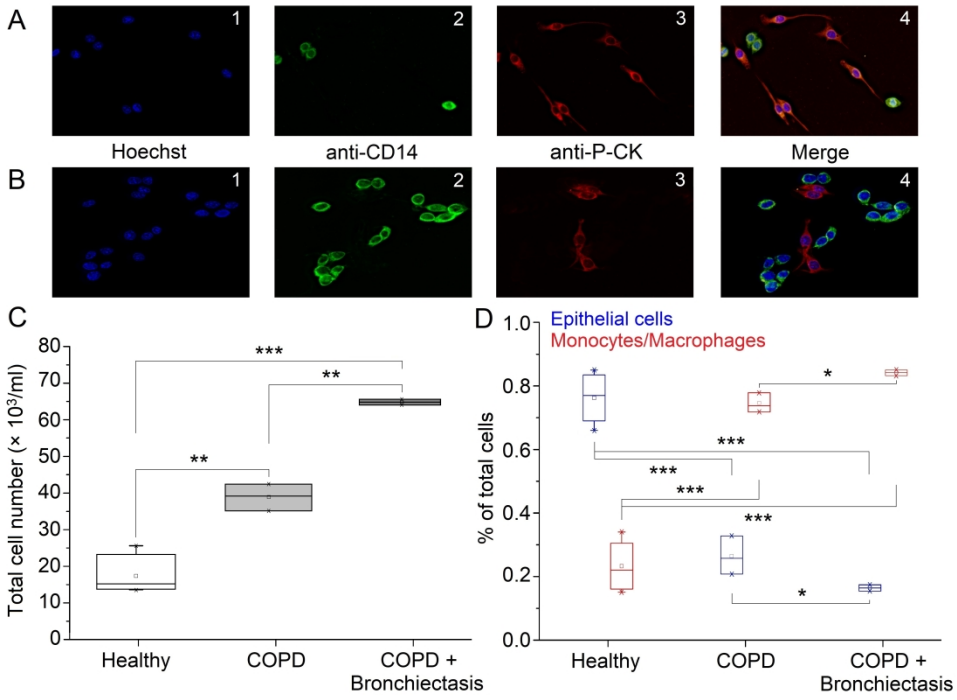

A
1
2
3
4
Hoechst
anti-CD14
anti-P-CK
Merge
B
1
2
3
4
C
Total cell number (× 10³/ml)
80
70
60
50
40
30
20
10
0
***
**
**
Healthy
COPD
COPD +
Bronchiectasis
D
Epithelial cells
Monocytes/Macrophages
% of total cells
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
***
***
*
Healthy
COPD
COPD +
Bronchiectasis

Figure 1



.

1646x1219mm (96 x 96 DPI)