-Online supplementary data-

Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility.

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METHODS

Blood Sampling

Peripheral blood (20 mL) from COPD patients and healthy non-COPD volunteers was collected in heparinised tubes from a peripheral vein using a 21-gauge needle and processed for assays within 2 hours. Peripheral blood from patients with exacerbated COPD was collected 1, 8 and 28 days after the onset of exacerbation. The samples were centrifuged for 10 min at 170*g*, and plasma was then harvested and centrifuged for 20 min at 1,500*g* to obtain platelet-free plasma (PFP)[1].

Characterisation of MP phenotype

Plasma samples were measured using a BD FACS Canto II flow cytometer and BD FACS DIVA software version 1.2.6 (BD Biosciences, Erembodegem Belgium) as previously described[1-3]. Microparticles (MPs) were defined as particles whose diameter was less than 1 um. Four EMP phenotypes were defined: CD144+ (FITC) MPs (VE-cadherin EMPs), CD146+ (PE) MPs (MCAM EMPs), CD31+ (FITC)/CD41- (PE) MPs (PECAM EMPs) and CD62E+ (PE) MPs (E-selectin EMPs). Alveolar capillaries

are negative for von Willebrand factor (vWF), whereas arterioles and venules in the lungs and endothelial cells in other organs are positive for vWF[4, 5]. We defined EMPs derived from pulmonary capillary endothelial cells as von Willebrand factor-negative (vWF: APC)- EMPs. All antibodies used for flow cytometry were obtained from BD Biosciences, except that anti-CD41-PE was obtained from Beckman Coulter and anti-vWF-APC was obtained from R and D systems (Table E1). Ten microlitres of PFP was incubated with each specific antibody. After 30 min of incubation at room temperature, samples were diluted in 300 μl of a 0.9% saline salt solution. Equal volumes of sample and Flowcount beads (Beckman-Coulter) were then added and analysed by FACS using settings with a threshold of 200. MP sizes were determined using 1 μm beads (Fluka, Sigma), and FSC and SSC gates were drawn to include events smaller than 1 μm (Figure E1). EMP measurements were performed twice to ensure repeatability. Appropriate isotype control antibodies or fluorescence minus one (FMO) controls were used to discriminate true events from noise and to increase the specificity of MP detection.

Pulmonary function tests

Spirometry measurements were conducted by a well-trained technician following the ATS/ERS guidelines[6] after inhalation of a bronchodilator before sample collection and one year after sample collection. Acceptable manoeuvres for spirometry measurements were defined as those with a sufficient peak expiratory flow, a rapid start, an absence of major flow fluctuations and an adequate duration of expiration. Pulmonary function testing was performed in duplicate. The best FEV1 and FVC values were recorded from acceptable manoeuvres[7].

Visual assessment of low attenuation areas (LAAs) by chest CT images

We visually assessed the severity of lung alveolar destruction according to the Goddard classification[8]. We used a high-resolution CT to quantify LAA. Scans were performed in the supine position using the same CT scanner (XVigor; Toshiba, Tokyo, Japan). LAA was scored for the right and left sides of the upper, middle and lower lung fields. Zero represented no abnormality, while 1 was given for up to 25%, 2 for up to 50%, 3 for up to 75%, and 4 for total involvement or almost the total absence of normal

lung tissue. The total possible scores ranged from 0 to 24. The assessment was independently performed by two thoracic surgeons in a blinded fashion, and the final score was calculated with the means of the scores assigned by the two readers.

PECAM-1 and vWF immunohistochemical staining of peripheral lung tissues

Human lung tissue samples from COPD patients were taken at non-emphysematous, non-fibrotic areas distant from isolated solitary nodules in lobectomy specimens. Paraffin slides were stained with anti-human PECAM-1 (Dako) or vWF (Dako) according to the manufacturer's protocols.

Statistical Analysis

The Kruskal-Wallis test was used to compare age, body mass index, PaO_2, pack-years smoking index, leukocyte counts, CRP, SAA, D-dimer or EMP numbers among the 4 GOLD stages. The analysis of repeatability is based on the method proposed by Bland and Altman[9] for comparing twice EMP measurements (TIME 1 and TIME 2) of one sample. The differences in the EMP numbers between TIME1 and TIME2 were

plotted against the average of the twice EMP measurements. The limits of agreement were defined as the mean difference ± 1.96 SD. The regression line was used to evaluate significant decreases or increases between the average and difference of the twice EMP measurements. *p* values less than 0.05 were considered significant. All analyses were performed using the SAS system (Version 8.2, SAS Institute, Cary, NC).

RESULTS

Repeatability of EMP measurements

Supplementary Figure 4 shows the Bland-Altman plot representations between the twice (Test 1 and Test 2) measurements of four EMP phenotypes. In all these plots, dots were randomly dispersed above and below the mean of the error (Test1 –Test2) line over a wide range of the EMP numbers, and the regression lines showed no significant decreases or increases. These data suggested good repeatability of the measures.

Table E 1: Antigens used for EMP identification.

Differentiation cluster	Antigen	Fluorescence	Clone	Manufacture
CD144	VE-cadherin	FITC	55-7H1	BD Biosciences
CD31	PECAM-1	FITC	WM59	BD Biosciences
CD62E	E-selectin	PE	68-5H11	BD Biosciences
CD41	GPIIb	PE	P2	Beckman Coulter
Not applicable	Von Willebrand factor	APC	210905	R&D Systems

Table E 2: Comparison of the characteristics of 80 COPD patients in stable condition.

GOLD stage	I (n = 25)	II (n = 26)	III (n = 19)	IV (n= 10)	*p* value
Age	73.0 ± 12.5	70.0 ± 9.00	75.0 ± 6.50	71.0 ± 13.0	0.4685
Body mass index	23.0 ± 3.40	23.0 ± 5.00	21.3 ± 3.24	21.1 ± 6.20	0.0648
PaO_2, Torr (At sample correction)	83.0 ± 7.50	80.0 ± 13.0	78.0 ± 11.5	82.5 ± 22.1[1)]	0.1956
Pack-years smoking index	30 (0-90)	37.5 (0-80)	40 (0-80)	45 (5-80)	0.3503
Leukocyte count	5700 ± 1850	6600 ± 2400	6800 ± 2000	6000 ± 1900	0.2697
CRP	0.100 ± 0.200	0.200 ± 0.400	0.100 ± 0.300	0.150 ± 0.300	0.3112
SAA	4.60 ± 3.00	5.95 ± 8.60	5.00 ± 7.70	7.40 ± 21.4	0.7890
D-dimer	0.940 ± 0.670	0.880 ± 1.47	1.08 ± 0.448	1.46 ± 1.74	0.7895

1) Including 6 patients with long-term oxygen therapy

FIGURE LEGENDS

Supplementary Figure 1: Expression of PECAM-1 and vWF on pulmonary capillary endothelial cells from non-emphysematous regions in moderate COPD patients. PECAM was clearly expressed by endothelial cells in pulmonary venules, arteries and capillary vasculature. In contrast, vWF was rarely expressed by endothelial cells in pulmonary capillary vasculature, although it was expressed in pulmonary venules and arterioles. Scale bars, 50 μm.

Supplementary Figure 2: Gating strategy for EMPs. (A) 1.0 μm bead calibration. (B) Isotype control. (C) Platelet-free plasma stained by CD31 & CD41.

Supplementary Figure 3: Correlations between predicted FEV1% and (A) VE-cadherin EMPs, (B) PECAM EMPs, (C) E-selectin EMPs. r_s: Spearman correlation coefficient.

Supplementary Figure 4: Bland-Altman plot representation between two measurements of (A) VE-cadherin EMPs, (B) PECAM EMPs, (C) E-selectin EMPs and MCAM EMPs. Each dot stands for one subject and the dashed lines represent the mean ± 1.96 SD interval. The regression line showed no significant decreases or increases in all four EMP phenotypes.

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