Online Supplementary File

Circulating desmosine levels do not predict emphysema progression but are associated with cardiovascular risk and mortality in COPD

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APPENDIX 1

METHODS

Study Population and Ethics

Nine hundred and ninety one patients with COPD enrolled in the ECLIPSE study (GSK Study No. SCO104960, NCT00292552) [1], and 186 patients with COPD and 110 age and gender matched controls with a history of smoking from a second independent cohort were studied.

ECLIPSE is an international multicentre observational longitudinal cohort study of subjects with COPD, smokers with normal spirometry and non-smokers.

The primary selection criteria were based on lung function (decliners vs. non-decliners). Secondly the 500 patients with most loss of lung function and the 500 patients with no loss of lung function were selected. Within each group patients were stratified by emphysema severity (100 patients with no or minimal emphysema, 200 with moderate emphysema and 200 with severe emphysema). 991 samples were available for analysis.

ECLIPSE participants were aged 40–75 years and the presence of significant lung disease was determined by history, physical examination and screening investigations. Patients with COPD were current or ex-smokers (≥10 pack years), with baseline post-bronchodilator FEV_1<80% of the reference value and FEV_1/FVC<0.7. Smokers with normal spirometry were free of significant lung disease, were current or ex-smokers (≥10 pack years), and had baseline post-bronchodilator FEV_1>85% and FEV_1/ FVC>0.7. Non-smokers were free from

significant lung disease, had smoking history of <1 pack year, and had baseline post-bronchodilator FEV_1>85% and FEV1/FVC>0.7. Key exclusion criteria were the presence of respiratory disorders other than COPD and a reported COPD exacerbation within 4 weeks of enrolment. Comorbidities were not excluded, but these had to be clinically stable at the time of assessment.

The American Thoracic Society (ATS) respiratory questionnaire, the modified Medical Research Council (mMRC) questionnaire and the COPD-specific version of the St. George's Respiratory Questionnaire (SGRQ-C) were used to record data related to symptoms, disease history and quality of life. Exacerbations requiring treatment with antibiotics, oral corticosteroids or hospitalization (moderate or severe exacerbations respectively) in the year prior to the study were also recorded. Co-morbidities were self-reported using the ATS-DLD-78 questionnaire. Nutritional status was assessed by body mass index (BMI) and fat-free mass index (FFMI) measured by bioelectrical impedance. Spirometry and the 6-minute walking distance (6MWD) were performed according to international guidelines [2]. Spirometric reference values were obtained from the European Community for Coal and Steel (ECCS) tables [3]. The BODE index was calculated according to Celli *et al*. [4].

The second independent cohort consisted of 186 patients with COPD and 110 age and gender matched controls recruited from "The association of lung function and cardiovascular risk" study [5]. Patients were recruited during 2011 – 2013 from department research database, out-patient clinics and by posters.

All subjects were over 40 years of age, of European descent, had a significant smoking history of ≥10 pack years and were studied at clinical stability.

No subject recruited had active or suspected malignancy, terminal disease or known alpha 1 antitrypsin deficiency. Controls had no respiratory diagnosis and no

respiratory symptoms.

The second independent cohort (The Association of Lung Function and Cardiovascular Risk – Nottingham) consisted of 186 patients with COPD and 110 age and gender matched controls recruited during 2011 – 2013 from department research database, out-patient clinics and by posters [5]. All subjects were over 40 years of age, of European descent, had a smoking history of ≥10 pack years and were studied at clinical stability.

Ethics committees of all participating institutions approved the study and written informed consent was obtained from all subjects.

MEASUREMENTS

Circulating inflammatory biomarkers

Peripheral venous blood was collected in the morning after an overnight fast on all study visits. White blood cell (WBC) count was measured in a central clinical laboratory. Serum was prepared by centrifugation of whole blood at 1500 *g* for 10-15 min and plasma (anticoagulated with ethylene-diamine tetra-acetate) was obtained by centrifugation at 2000 *g* for 10-15 min. Samples were stored at -80ºC until analyzed centrally. Serum interleukin (IL)-6, IL-8 and tumour necrosis factor alpha (TNF-α) concentrations were determined by validated enzyme-linked immunosorbent assays (ELISAs; SearchLight Array Technology, Thermo Fisher Scientific, Rockford, IL, USA). Serum surfactant protein D (SP-D) was measured using a colorimetric sandwich immunoassay method (BioVendor, Heidelberg, Germany). Chemokine ligand 18 (CCL-18) concentrations were measured using a human CCL18/PARC DuoSet ELISA kit (R&D systems). Soluble receptor for advanced glycation endproducts (sRAGE) was measured using the Quantikine human RAGE ELISA kit

(R&D). High-sensitivity C-reactive protein (hs-CRP) (Roche Diagnostics, Mannheim, Germany) and fibrinogen (K-ASSAY fibrinogen test, Kamiya Biomedical Co., Seattle, WA, USA) concentrations were measured using immunoturbidometric assays validated for use with EDTA plasma.

Plasma desmosine (pDES) measurements

One thousand patients were selected from the ECLIPSE cohort to assess pDES. Patients were selected to cover all ranges of FEV_1 decline and emphysema progression at both ends of the spectrum. 981 patients had available samples collected at year 1 for analysis. No samples collected at baseline were available. Total pDES concentration was measured in EDTA plasma using a modified assay of a validated isotope dilution LC-MS/MS method [6-8]. All samples underwent less than 3 freeze-thaw cycles. pDES levels were measured at year 1 and year 2 visit in the ECLIPSE cohort and at baseline in the Nottingham cohort.

Computed tomography

In the ECLIPSE study, subjects underwent a low-dose CT of the chest using multi-detector scanners with a minimum of four rows (GE Healthcare or Siemens Healthcare) on the initial visit of the study. Imaging was performed in the supine position, at suspended full inspiration, without administration of intravenous contrast. Exposure settings were 120 kVp and 40 mAs and images were reconstructed using 1.0 mm (Siemens) or 1.25 mm (GE) contiguous slices and a low spatial frequency reconstruction algorithm (GE: Standard; Siemens: b35f).

All CT scans were analysed at a central laboratory using Pulmonary Workstation 2.0 software (VIDA Diagnostics, Coralville, IA. USA) [9]. Quantitative assessment of the percentage of low attenuation areas (%LAA), representing the presence of emphysema, was performed using a threshold of −950 Hounsfield units. The total lung volume was calculated by summing the CT voxels that contained lung. CT density values were calculated from the apparent x-ray attenuation values measured in Hounsfield units (HU) by adding 1000 to the HU (eg, −950 HU equals 50 g/L) [10]. Patients were considered to have emphysema if the low attenuation area was greater than 10% of the total lung volume (appendix). Ct Scans were repeated after 3 years.

The percentile density method was also used to quantify emphysema and the lowest 15th percentile point of the entire frequency distribution of lung density within patients was determined (PD15) [11]; a higher PD15 value therefore indicates less emphysema. The annual change of lung density was calculated as the change in PD15 per year, corrected for total lung volume [12].

Coronary artery calcium score

CACS was assessed on the standard CT lung images with a low spatial frequency algorithm as previously described [13]. Images were analysed on a workstation using dedicated analysis software (VScore, Vitrea Fx, V.3.1.0, Vital Images, Minnetonka, Minnesota, USA).

CACS was assessed using the Agatston scoring method [14]. Coronary calcification was defined as an area in the course of a coronary artery that had an attenuation threshold of ≥130 HU and was ≥1 mm ^{2}. On each axial slice the area of calcification that met these requirements was measured and then multiplied by a weighting factor

dependent on the peak attenuation within the region. These weighted areas were summed to produce the total Agatston CACS. For the interpretation of Agatston score the absolute value can be used or it can be considered as low (<100 Agatston units (AU)), intermediate (101–400 AU), high (401– 1000 AU) or very high (>1000 AU). There are many ways to interpret the Agatston score in both clinical and research context. One of the most common, and useful, is the used in our study. This is largely based on work from the MESA study (Multi-ethnic study of atherosclerosis), which assessed calcium score in asymptomatic patients and correlated with future CHD events [15]. This has been supplemented by multiple other studies including those assessing the prognostic implications [16, 17] and correlation with myocardial ischaemia [18]. Normal ranges for Agatston scores in healthy asymptomatic individuals stratified by age, gender and ethnicity have been published [19].

Arterial stiffness

Patients were asked to refrain from short acting bronchodilators for a minimum of four hours and long acting bronchodilators for >12 hours prior to the study. Further, they were asked to refrain from caffeine products for >6 hours. Tests were performed after a period of resting supine for at least 10 minutes. Heart rate (HR) and peripheral blood pressure (BP) were performed in the seated position and the mean of two technically acceptable results recorded (Omron 705IT, UK).

Arterial stiffness was measured as the carotid - femoral pulse wave velocity (aortic pulse wave velocity, PWV) using Vicorder (Skidmore Medical, UK) in triplicate and the average recorded [5, 20].

Statistical analysis

Data are expressed as mean±SD. Each variable was tested for normal distribution using the Kolmogorov-Smirnov test and statistical test selected accordingly.

pDES levels between paired samples (e.g. year 1 vs year 2; death vs survivors) was assessed using Wilcoxon test. Comparison between groups were conducted using analysis of variance (ANOVA) with Student-Newman-Keuls as a post-hoc test for comparison between the different groups or the Kruskall Wallis equivalent with Dunn's test as a post-hoc test for comparison between the different groups for non-normally distributed variables. We used analysis of covariance (ANCOVA) to control for potential relevant confounders (%LAA, age, gender, cumulative smoking history (pack/year history), years smoked and inflammation. Chi square tests were used to compare frequencies (i.e. gender).

Correlations were calculated as Pearson's correlation coefficient or Spearman's correlation coefficient for non-normally distributed variables. Logistic regression was conducted with death as the dependent variable and pDES, age, gender, smoking history, mMRC, hospitalisations, inflammation, CACS and cardiovascular comorbidities as independent relevant variables. The selection of covariates was based on the significant of univariate correlation and biological relevance. Cox proportional hazards models were constructed to compare mortality between pDES quartiles adjusted for age, gender, smoking history, mMRC, hospitalisations, inflammation, CACS and cardiovascular comorbidities. Benjamini and Hochberg False Discovery Rate (FDR) method was used to adjust the multiple hypothesis tests. Analyses were conducted using the SAS Version 9.3 (SAS Institute Inc, Cary, NC, USA). A p value <0.05 was considered statistically significant.

APPENDIX 2

PRINCIPAL INVESTIGATORS AND CENTERS PARTICIPATING IN ECLIPSE (NCT00292552, SC0104960)

Bulgaria: Y Ivanov, Pleven; K Kostov, Sofia. Canada: J Bourbeau, Montreal; M Fitzgerald, Vancouver; P Hernández, Halifax; K Killian, Hamilton; R Levy, Vancouver; F Maltais, Montreal; D O´Donnell, Kingston. Czech Republic: J Krepelka, Praha. Denmark: J Vestbo, Hvidovre. The Netherlands: E Wouters, Horn. New Zealand: D Quinn, Wellington. Norway: P Bakke, Bergen, Slovenia: M Kosnik, Golnik. Spain: A Agusti, Jaume Sauleda, Palma de Mallorca. Ukraine: Y Feschenko, Kiev; V Gavrisyuk, Kiev; L Yashina, W MacNee, Edinburgh; D Singh, Manchester; J Wedzicha, London. USA: A Anzueto, San Antonio, TX; S Braman, Providence. RI; R Casaburi, Torrance CA; B Celli, Boston, MA; G Giessel, Richmond, VA; M Gotfried, Phoenix, AZ; G Greenwald, Rancho Mirage, CA; N Hanania, Houston, TX; D Mahler, Lebanon, NH; B Make, Denver, CO; S Rennard, Omaha, NE; C Rochester, New Haven, CT; P Scanlon, Rochester, MN; D Schuller, Omaha, NE; F Sciurba, Pittsburg, PA; A Sharafkhaneh, Houston, TX; T Siler, St Charles, MO; E Silverman, Boston, MA; A Wanner, Miami, FL; R Wise, Baltimore, MD; R ZuWallack, Hartford, CT.

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(GlaxoSmithKline, USA), E Wouters (The Netherlands), J Yates (GlaxoSmithKline, USA).

SUPPLEMENTARY TABLES

Table 1S. Demographic of the study population and remaining ECLIPSE cohort.

	STUDY COHORT	REMAINING ECLIPSE COHORT	p-value
n	991	1175	
Men	635 (64%)	775 (66%)	NS
Hypertension (%)	41.2	41.7	NS
Angina (%)	11.4	10.7	NS
Heart Attack (%)	9.6	8.5	NS
Heart Failure (%)	5.7	8.4	<0.05
Stroke (%)	4.2	3.4	NS
Age (Years)	63.1 ± 7.2	63.5 ± 7.1	NS
BMI ($kg.m^{-2}$)	26.8 ± 5.8	26.2 ± 5.5	<0.01
Pack/Year	47.4 ± 26.0	49.2 ± 27.5	NS
mMRC	2.6 ± 1.0	1.7 ± 1.1	<0.001
FEV_1 (L)	1.41 ± 0.5	1.30 ± 0.5	<0.0001
FEV_1 (% pred)	50.5 ± 15.2	46.5 ± 15.9	<0.0001
FVC (L)	3.1 ± 0.9	3.0 ± 0.9	<0.0005
FVC (% pred)	89.2 ± 19.7	84.8 ± 20.2	<0.0001
FEV_1/FVC	0.46 ± 0.1	0.44 ± 0.1	<0.0001
SpO_2 (%)	94.7 ± 2.8	94.4 ± 3.2	<0.05
6MWD (m)	384 ± 118.9	356.9 ± 122.9	<0.0001
6MWD (%pred)	59.5 ± 18.0	57.2 ± 18.4	<0.0001
BODE	2.9 ± 2.0	3.3 ± 2.2	<0.0001

Definition of abbreviations: COPD = patients with Chronic Obstructive Pulmonary Disease; BMI = Body mass index; Pack/Year: cumulative history of smoking; mMRC = modified medical research council dyspnoea score; FEV_1 = forced expiratory volume in the first second; FVC = forced vital capacity; SpO_2 = oxygen saturation; 6MWD = six minute walking distance; BODE= BODE index. Benjamini Hochberg correction was applied to prevent α-error accumulation.

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TABLE 2S. DESMOSINE QUARTILES.

	Q1	**Q2**	**Q3**	**Q4**	p-value
	<= 0.28 ng/mL	0.29 - 0.35 ng/mL	0.36 - 0.44 ng/mL	>= 0.45 ng/mL	
Current smokers (n/%)	108/41	83/36	92/37	79/32	NS
Age (Years)	59.1 ± 7.1	62.7 ± 7.1	64.3 ± 6.4	66.5 ± 5.9	<0.0005
FEV_1 (L)	1.5 ± 0.58	1.4 ± 0.5	1.3 ± 0.4	1.4 ± 0.5	<0.05
SpO_2 (%)	94.9 ± 2.8	94.8 ± 3.2	94.3 ± 2.8	94.5 ± 2.7	<0.05
mMRC	1.4 ± 0.9	1.6 ± 1.1	1.6 ± 1.1	1.8 ± 1.1	<0.0005
Pack/Year	44.1 ± 24.1	47.9 ± 26.1	48.9 ± 24.5	48.6 ± 25.9	<0.05
Years Smoked	36.5 ± 8.6	40.1 ± 8.6	40.3 ± 10.2	40.6 ± 10.8	<0.0005
Hospitalisations	0.61 ± 1.6	0.54 ± 1.3	0.70 ± 1.5	0.81 ± 1.7	<0.05
6MWD (m)	2.6 ± 1.9	2.7 ± 1.9	3.2 ± 2.0	3.0 ± 2.1	<0.0005
BODE index	2.6 ± 2.0	2.8 ± 1.9	3.3 ± 1.9	3.1 ± 2.1	<0.05
%LAA	15.4 ± 11.3	16.1 ± 11.1	18.3 ± 12.2	16.9 ± 11.4	NS
Fibrinogen (mg/dL)	435.4 ± 94.4	459.8 ± 95.8	464.2 ± 94.9	466.7 ± 109.3	<0.005
IL-6 (pg/mL)	3.1 ± 12.2	3.5 ± 10.9	3.9 ± 9.7	8.0 ± 47.7	<0.001
IL-8 (pg/mL)	11.7 ± 23.9	17.1 ± 17.1	11.4 ± 11.4	15.8 ± 15.8	<0.05
CCL-18 (pg/mL)	105.9 ± 40.2	108.4 ± 38.9	114.3 ± 48.8	119.9 ± 44.9	<0.05
Lymphocytes	2.0 ± 0.7	2.0 ± 0.6	12.0 ± 0.7	1.8 ± 0.7	<0.05
Lymphocytes (%)	26.6 ± 7.6	26.7 ± 7.5	26.3 ± 8.0	24.1 ± 7.5	<0.01
Neutrophils	5.1 ± 1.6	5.1 ± 2.0	5.0 ± 1.8	5.3 ± 2.0	NS
Neutrophils (%)	64.1 ± 8.2	63.6 ± 8.6	64.2 ± 8.6	66.3 ± 8.5	<0.05
SP-D (pg/mL)	127.9 ± 69.6	141.1 ± 84.9	136.7 ± 61.5	148.7 ± 88.6	<0.05
CACS	312.7 ± 633.9	386.0 ± 666.9	466.7 ± 677.9	629.4 ± 995.3	<0.0001
Cardiovascular hist.	55 (23.0%)	46 (19.2%)	62 (29.9%)	76 (31.8%)	<0.05
Hypertension	78 (20.0%)	94 (24.1%)	101 (25.9%)	117 (30.0%)	<0.001
Angina	28 (26.4%)	19 (17.9%)	21 (19.8%)	38 (35.8%)	<0.05
Heart Attack	21 (22.6%)	14 (15.1%)	24 (25.8%)	34 (36.6%)	<0.05
Heart Failure	9 (16.7%)	9 (16.7%)	18 (33.3%)	18 (33.3%)	NS

Definition of abbreviations: Q1-Q4: desmosine quartiles 1 & 4. mMRC = modified medical research council dyspnoea score; 6MWD = six minute walking distance; ; Hospitalisations = number of hospitalisations recorded in the first three years of the study; IL-6: Interleukin six; IL-8: Interleukin eight; CCL-18: Chemokine (C-C motif) ligand eighteen; SP-S: Surfactant protein D; CACS: coronary artery calcification score. Comparisons among groups were done using Wilcoxon test and Dunn test as a post-hoc test. Benjamini Hochberg correction was applied to prevent α-error accumulation.

SUPPLEMENTARY FIGURES

Figure 1S. Bland and Altman plot with pDES assessed at year 1 and year 2.

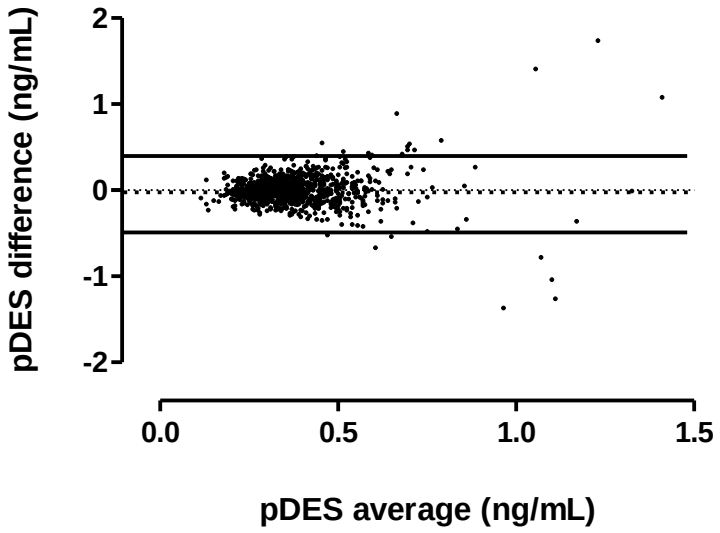

Figure 1S: Bland and Altman plot with pDES assessed at year 1 and year 2. pDES assessed at year 1 and year 2. Dashed line represents the bias while full lines represent limit of agreement.

Figure 2S. pDES levels and cardiovascular comorbidities.

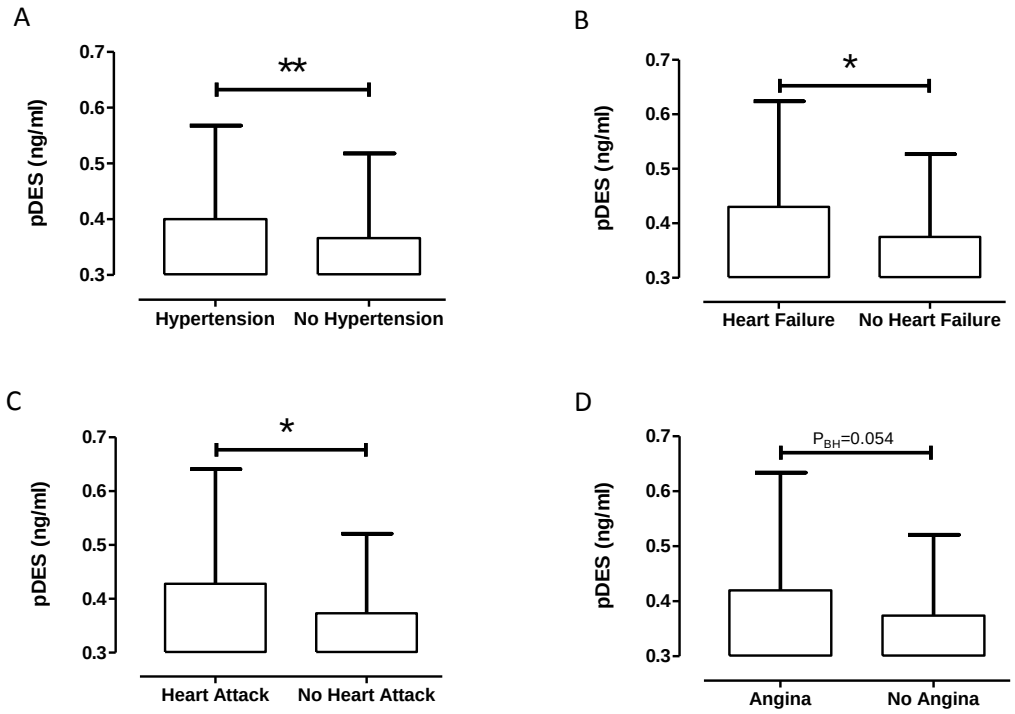

Figure 2S: pDES and cardiovascular comorbidities. pDES in patients with history of hypertension (Panel A), heart failure (Panel B), heart attack (Panel C), and angina (Panel D). (*p<0.05, ** p<0.0005).

Figure 3S. pDES levels and cardiovascular comorbidities.

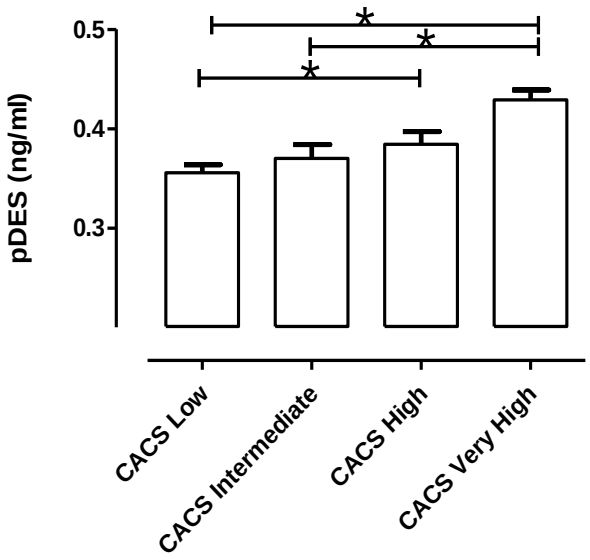

Figure 3S: Relationship between pDES and CACS. This figure shows pDES levels in the different CACS categories (*p<0.0001).

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