ON-LINE SUPPLEMENTARY INFORMATION

Clinical validity of plasma and urinary desmosine as biomarkers for chronic obstructive pulmonary disease

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METHODS

Study subjects

Potentially eligible patients were identified by scrutiny of hospital records, from general practice asthma clinic registers, by advertisements in local papers and from computerised general practice records.

Stable asthma: Objective confirmation by methacholine airway hyper responsiveness (airway hyperactivity determined by a ≥ 20% drop in FEV_1 at a methacholine dose of ≤ 8 mg/mL) or where this was not safe (FEV_1 < 60%) by evidence of airflow variability and a ≥ 12% and 200 mL increase in FEV_1 following nebulised salbulamol 2.5 mg.

Sample collection

The majority of individuals (Cohort 1) had spot urine, blood and sputum samples collected during two visits in order to establish the stability of uDES and bDES levels in each group. The period between the two visits was between 6-8 weeks and samples were collected when the subjects was stable. The remaining participants (Cohort 2) were hospitalised because of the exacerbation of COPD. Spirometry was performed in the majority of participants in both cohorts whilst only subjects in Cohort 1 had gas transfer factor for carbon monoxide (TLCO%) measured. Urine was collected in a polystyrene urine collection bottle (ThermoFisher), aliquoted and frozen at -80ºC until analysis. Plasma samples were collected in K_2-EDTA tubes (BD Biosciences). Serum samples were collected in plain tubes (BD Biosciences). Sputum samples were collected and processed using the previous published methods (1).

uDES, bDES and sputum MMP-9 activity assays

A summary of assay performance for uDES, bDES and sputum MMP-9 activity assays is shown in On-line Supplementary **Table E1.** For uDES and bDES assays, the selection of deuterium-5 isotope as an internal standard in the uDES and bDES assays was based on minimal interference signals of D_5-DES transitions from the matrix.

RESULTS

Clinical characteristics of study cohorts

FEV1 (% predicted) in patients with stable COPD were lowest (62 ± 20%) compared to other groups in Group 1 (Table 1). Asthmatic non-smokers and smokers also had a significant lower FEV_1 than healthy non-smokers and smokers ($p<0.05$, Table 1). There was no difference in FEV_1 between healthy non-smokers and smokers, or between asthmatic smokers and asthmatic non-smokers in Group 1. In Group 2, "during an exacerbation" COPD patients had significantly lower FEV_1 than healthy volunteers ($p<0.001$). In terms of TLCO%COHb, stable COPD patients had the lowest values followed by asthmatic smokers and asthmatic non-smokers (Table 1).

uDES and bDES levels in patients with stable asthma, stable and "during an exacerbation" COPD and healthy volunteers.

Similar uDES and bDES results were found in the samples collected from a subsequent visit (On-line Supplementary **Table E2**). We did not observe a significant difference between healthy non-smokers, smokers and ex-smokers (On-line Supplementary **Table E2**). There was a significant negative correlation between uDES levels and FEV_1 in healthy non-smokers but not in healthy smokers (On-line Supplementary **Figure E2)**. The uDES levels were not associated with disease severity (i.e. FEV_1% predicted) in these two COPD groups although an increased variability in very severe COPD patients (FEV_1 < 30% predicted) was noticeable (On-line Supplementary **Figure E2A**). We did not observe a correlation between FEV_1 and bDES levels in each group ((On-line Supplementary **Figure E2B**).

REFERENCE

1. Pavord ID, Pizzichini MM, Pizzichini E, Hargreave FE. The use of induced sputum to investigate airway inflammation. Thorax1997 Jun;52(6):498-501.

SUPPLEMENTARY TABLES

Table E1. Summary of performance for uDES, bDES and sputum MMP9 (sMMP9) activity assays

Performance Items	bDES assay	uDES assay	sMMP9 activity
Low limit of quantification	0.12 ng/mL	1 ng/mL	0.25 ng/mL
High limit of quantification	160.0 ng/mL	480 ng/mL	16 ng/mL
Intraday assay imprecision for QCs(%CV)	0 to 12 %	5 to 16 %	6-10%
Interday assay imprecision for QCs (%CV)	3 to 7%	2 to 5%	5-9%
Intraday assay accuracy for QCs (%Bias)	-7 to 8%	-11 to 0%	-4 to 5%
Interday assay accuracy for QCs (%Bias)	-4 to 3%	-1 to 0%	-4 to 5%
Dilution linearity	Up to 4 folds	Up to 8 folds	Up to 4 folds
SPE recovery	130% to 133%	110% to 112%	101% to 110%
Sample stability	RT or 4ºC for 20 h; up to 3 freeze/thaw cycles	RT or 4ºC for 20 h; up to 3 freeze/thaw cycles	4ºC for 24h, RT for 2 h, recommend assay of sputum samples after a single F/T cycle
Reference range (healthy volunteers, n=10-18)*	0.14-0.24 ng/mL	2-19 ng/mg creatinine	13-751 ng/mL
Reference range (COPD patients, n=10-18)*	0.15-0.75 ng/mL	12-77 ng/mg creatinine	20-1553 ng/mL

*The reference ranges were performed in small number of subjects randomly selected from Cohort 2 as a part of assay validation process. The results related to uDES assay have been published in a technical report (18).

Table E2. uDES and bDES levels in patients with stable asthma and COPD as well as healthy volunteers on a subsequent visit

	Healthy non-smokers	Healthy smokers	Asthma non-smokers	Asthma smokers	Stable COPD patients
uDES (ng/mg creatinine	8 (7-10)	11 (7-15)	7 (5-11)	8 (7-12)	11* (7-17)
bDES (ng/mL)	0.21 (0.19-0.25)	0.23 (0.19-0.27)	0.21 (0.19-0.24)	0.23 (0.20-0.25)	0.29 (0.25-0.34)[$]

* $p<0.01$, vs. asthma non-smokers

[$] $p<0.01$ vs. healthy smokers; $p<0.001$, vs. the other 3 groups

Kruskal-Wallis ANOVA with Dunn's method for pairwise multiple comparisons.

Data are shown as median (IQR)

Note: See **Table 1** for the demographics.

Table E3. uDES and bDES levels for healthy non-smokers, current smokers and ex-smokers in Group 2.

Sample type	Urine			Blood	
Sub-group	**Healthy volunteer-non-smokers**	**Healthy volunteer-current smokers**	**Healthy volunteer-ex-smokers**	**Healthy volunteer-non-smokers**	**Healthy volunteer-current smokers**
Age (yrs)	22 (20-35)	24 (20-47)	25 (23-57)	66 (65-69)	69 (67-73)
N	41	13	8	9	10
Gender	15/16	3/10	6/2	9/0	9/1
BMI	25 ± 4	26 ± 5	24 ± 3	n.a.	n.a.
FEV_1[% predicted)	105 (95-111)	91 (87-100)	104 (101-110)	n.a.	n.a.
uDES (ng/mg creatinine) or bDES (ng/mL)	7 (5-10)	9 (7-12)	7 (5-10)	0.18 ± 0.08	0.19 ± 0.08

n.a.: Not available

Note that data are expressed as means ± SD where data are normally distributed and as median (IQR) otherwise.

SUPPLEMENTARY FIGURES

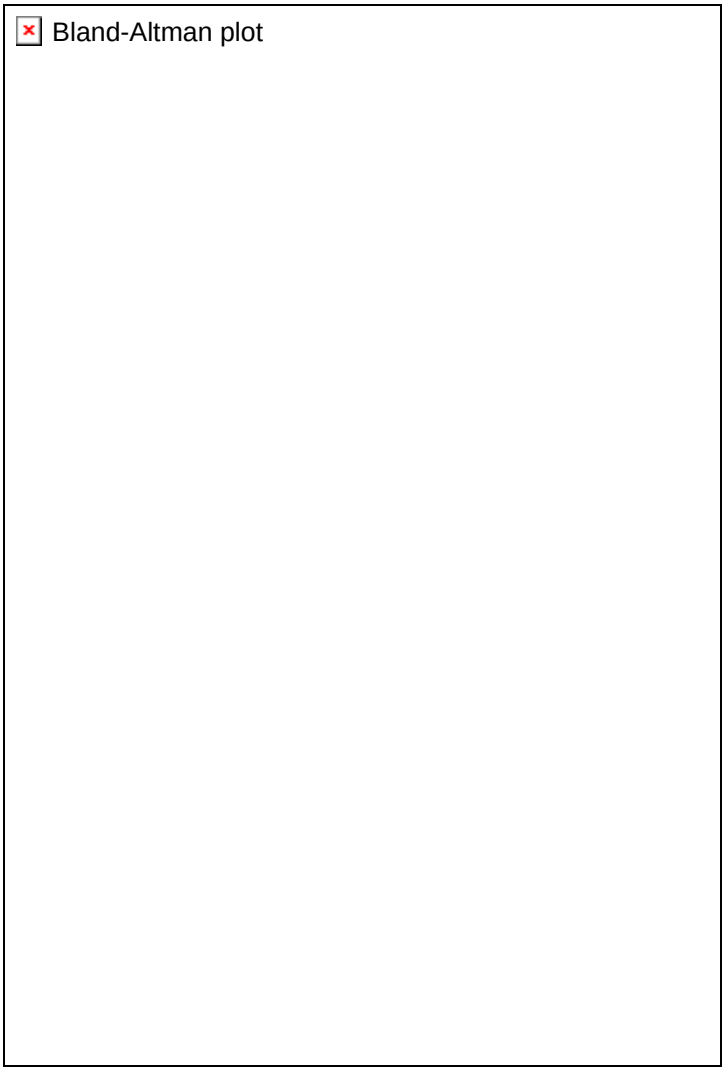

Figure E1. Assessing intra-subject variability of uDES and bDES levels in healthy non-smokers, healthy smokers and stable COPD patients.

A. Bland-Altman plots of uDES levels between two visits in healthy non-smokers, healthy smokers and stable COPD patients. The intra-subject variability (expressed as coefficient of variation(CV)) of uDES levels was 47 ± 35% (range = 1-108%), 41 ± 27% (range = 0-84%) and 50 ± 34% (range = 0-117%) for healthy non-smokers, healthy smokers and COPD patients.

B. Bland-Altman plots of bDES levels between two visits in healthy non-smokers, healthy smokers and stable COPD patients. The intra-subject variability of bDES levels was 6 ± 4% (range = 1-16%), 12 ± 11% (range = 0-43%) and 10 ± 11% (range=0-53%) for healthy non-smokers, healthy smokers and COPD patients. Data were expressed as mean ± S.D.

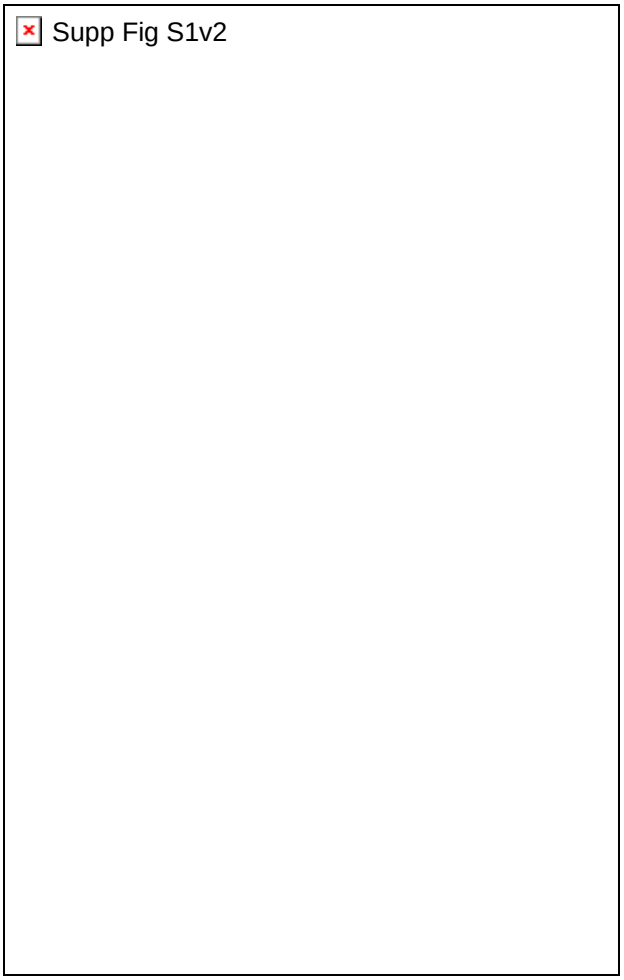

Figure E2. Correlation of uDES or bDES levels to FEV_1 in patients with stable and "during an exacerbation" COPD and healthy volunteers.

A. The correlation coefficients and p values for FEV_1(%predicted) vs. uDES expressed as (r, p) were (-0.48, 0.003), (-0.01, 0.94), (-0.14, 0.19) and (-0.27, 0.10) for healthy non-smokers, healthy smokers, stable and "during an exacerbation" COPD patients,

respectively (Spearman correlation). The only significance was found in healthy non-smokers.

B. The correlation coefficient and p values for FEV_1(%predicted) vs. bDES were (0.14, 0.36), (-0.12, 0.48), (-0.10, 0.32) and (0.16, 0.13) for healthy non-smokers, healthy smokers, stable and "during an exacerbation" COPD patients, respectively (Spearman correlation).