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Cigarette smoking prior to blood sampling acutely affects serum levels of the chronic obstructive pulmonary disease biomarker surfactant protein D

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1 **Cigarette smoking prior to blood sampling acutely**
2 **affects serum levels of the Chronic Obstructive**
3 **Pulmonary Disease biomarker Surfactant Protein D**

4
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Keywords (4/5):

- biomarker
- chronic obstructive pulmonary disease
- cigarette smoking
- pre-analytical variability

Abbreviations:

- COPD = chronic obstructive pulmonary disease
- LC-MS = liquid chromatography-mass spectrometry
- SPD = surfactant protein D
- sRAGE = soluble receptor for advanced glycation end-products

Short title:

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To the Editor,

Biomarker tests in pulmonary medicine show great promise with regard to improving patient care, yet their translation into widely-used clinical tests is a slow and rather ineffective process. Very few biomarkers pass the crucial stages of the biomarker development pipeline (*e.g.* analytical validation, clinical validation, establishing broad clinical utility), and many efforts are thus needed to secure a good return on biomarker development investments by eventually providing health care professionals with clinically useful tests [1, 2].

Reliable analytical methods are a cornerstone of biomarker testing, and the clinical usefulness of such methods depends on whether or not pre-analytical variables, which may potentially affect the validity of tests results, can be controlled. In chronic obstructive pulmonary disease (COPD), there has been a distinct focus on ensuring such analytical validity [1]. For example, cigarette smoking was recently identified as critical pre-analytical variable for serum measurements of the soluble receptor for advanced glycation end-products (sRAGE), a promising and (predominantly) lung-derived biomarker candidate for emphysema severity assessment in COPD [3]. Corresponding findings put previously reported associations between sRAGE and specific COPD characteristics into a different perspective given that smoking status prior to blood sampling is typically not reported to be controlled in clinical biomarker studies.

In this study, we examined the acute effects of cigarette smoking on serum levels of surfactant protein D (SPD), which represents another promising and (predominantly) lung-derived COPD biomarker candidate. This protein is present in pulmonary surfactant and is involved in the innate immune defense against various pathogens [1]. Higher SPD levels were reported for COPD patients compared to control subjects, and SPD levels were found to be

associated with exacerbations, emphysema progression, and mortality [1, 2]. Previous publications furthermore revealed associations between questionnaire-based smoking status (non-smoker *versus* current smoker) and circulating SPD levels [4, 5], and we hereby aimed to explore these findings by studying the effects of cigarette smoking on SPD levels experimentally.

To this end, biobanked serum samples (stored at -80 °C for approx. 5 years) were obtained from an acute smoking study (NCT00807469) which included COPD patients, young and old individuals that have a low familial risk to develop COPD, and young individuals that have a high familial risk to develop COPD (see Table 1). In the corresponding study, serum samples were taken at baseline and two hours after smoking three cigarettes within one hour. Prior to cigarette smoking, subjects did not smoke for two days, which was checked by means of exhaled carbon monoxide (CO) measurements using the Micro+ Smokerlyzer (CO levels needed to be below 5 ppm before smoking and needed to be increased after smoking) [6]. Blood samples were collected as described previously [7], the study was approved by the medical ethical review board of the University Medical Center Groningen (UMCG; METc 2008/136), and the study adhered to the Declaration of Helsinki. In all samples, serum SPD was quantified using a validated liquid chromatography-mass spectrometry (LC-MS) method targeting the SPD protein by means of the SPD-specific peptides NEAAFLSMTDSK and SAAENAALQQLVVAK [8].

[INSERT TABLE 1]

In all four study groups, similar patterns of cigarette smoke-induced SPD level changes were observed (Mann-Whitney U test; p-values ≥ 0.28), thereby disqualifying SPD as susceptibility marker (based on how susceptibility was defined in the respective clinical study [6]). Data from all study groups were thus combined revealing a statistically significant

increase of serum SPD levels after cigarette smoking (one sample t-test; p-values < 0.0001; see Figure 1A), irrespective of the initial SPD level (see Figure 1B). Moreover, potential associations based on linear regression between the combined relative SPD level changes, as dependent variable, and the individual variables listed in Table 1, as independent variable, did not reveal any other significant association (linear regression; p-values ≥ 0.21).

[INSERT FIGURE 1]

Our study thus revealed an acute effect of cigarette smoking on serum SPD levels and substantiates the previously reported associations between questionnaire-based smoking status and circulating SPD levels [4, 5]. Reported findings should be explored in different and larger populations, and further research on the mechanistic nature of this effect is warranted. Nonetheless, it is recommended to put a tight control of cigarette smoking as source of pre-analytical variability into practice for future studies on this promising and (predominantly) lung-derived protein biomarker. This recommendation thereby supports the previously reported recommendation to standardize blood sampling conditions for SPD, which emanated from the observations that SPD exhibits some degree of circadian variation and that SPD levels are influenced by physical activity prior to blood sampling [9].

An important consideration with regard to the reported findings is the fact that we measured SPD levels using a validated LC-MS method, which detects SPD by means of two protein-specific peptides in the C-type lectin, ligand binding domain of the protein [8]. This method showed adequately low bias (accuracy; within $\pm 15\%$) and coefficient of variation (precision; $\leq 15\%$) values during method validation (see [8]). Matching data for in-study quality control (Supplemental Figure 3) and incurred sample reanalysis samples (see [8]) were observed during clinical sample analysis, which were all in agreement with prevailing regulatory guidelines [10] thereby supporting the relevance of the observed changes. This

method furthermore showed a very good correlation ($R^2 = 0.9$; average (Bland-Altman) bias = +37%; $N = 32$) with a commercial ELISA (BioVendor, Cat. No. RD194059101), which holds an '*in vitro* diagnostic (IVD)' status for the European Union (see [8]). A similar correlation ($R^2 = 0.9$; average (Bland-Altman) bias = -3%; $N = 14$) was also found when comparing both methods based on the change in SPD levels due to cigarette smoking (see [8]), which argues against a method-specific artefact underlying the observed effect. At last, extensive sample stability parameters (*e.g.* 5× freeze-thaw stability, 27-day benchtop stability) were addressed during method validation (see [8]) to ascertain that SPD in serum is not susceptible to storage-related interferences. Extrapolating such data to the specific conditions that applied to the study samples should admittedly be done prudently, as holds true for most studies targeting biobanked samples. Nonetheless, these stability data indicate that serum SPD is a rather stable marker, at least when measured with the validated LC-MS method, thereby further supporting the plausibility of a true biological effect underlying the cigarette smoke-induced changes observed in this study.

In conclusion, cigarette smoking prior to blood sampling was found to induce acute changes in serum levels of SPD and should thus be considered as a critical pre-analytical variable for this (predominantly) lung-derived protein. Based on these findings and similar findings for sRAGE, as reported previously, but also due to the apparent ineffectiveness of biomarker development in pulmonary medicine, we believe that we should consider controlling, or at least monitoring, a person's smoking status prior to (blood) sampling for basically any lung-derived biomarker. To this regard, it may be useful to measure exhaled breath carbon monoxide (CO) levels before sampling to detect recent exposure to CO, which is often present in large quantities in cigarette smoke.

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146 collection, analysis, and interpretation of data; in the writing of the report; or in the decision to
147 submit the report for publication.

148 **Data availability:** All mass spectrometry data presented in this manuscript are available in the
149 PASSEL repository under accession code ‘PASS01363’.

150

Tables and figure legends

Tables:

Table 1: Baseline characteristics of the study subjects.

Variable ^{a,b,c}	Young subjects		Old subjects	
	Non-susceptible	Susceptible	Non-susceptible	COPD
n	28	21	27	13
Age, years	21 (19-39)	31 (18-42)*	51 (39-71)	66 (50-74)**
Gender, male	17/28 (61%)	11/21 (52%)	23/27 (85%)	13/13 (100%)
Current smokers, yes	28/28 (100%)	13/21 (62%)	26/27 (96%)	10/13 (77%)
FEV ₁ , % predicted	106 (90-122)	110 (97-132)	106 (87-136)	65 (41-80)**
FEV ₁ /FVC, %	85 (74-98)	81 (76-97)*	78 (71-91)	50 (32-65)**
RV/TLC, %	22 (11-53)	25 (18-32)*	32 (24-42)	39 (33-55)**
MEF ₅₀ , % predicted	96 (72-150)	94 (74-145)	90 (59-162)	23 (10-41)**
hsCRP, mg/L	0.7 (0.2-12.5)	1.0 (0.4-3.0)	1.9 (0.3-12.7)	2.9 (0.8-6.2)
Blood neutrophils, ×10 ⁹ /L	3.4 (1.0-8.4)	3.8 (1.2-5.0)	3.5 (1.5-6.1)	3.8 (2.9-5.2)
Blood eosinophils, ×10 ⁹ /L	0.19 (0.05-0.68)	0.12 (0.07-0.50)	0.17 (0.06-0.63)	0.21 (0.08-0.50)

^aContinuous data are presented as median (range), and categorical data are presented as fractions (percentages).

^bContinuous variables were tested using the Mann Whitney U test, and p-values below 0.05 for young non-susceptible *versus* young susceptible are indicated with single asterisks (*) whereas p-values below 0.05 for old non-susceptible *versus* old susceptible (COPD) are indicated with double asterisks (**).

^cCOPD = chronic obstructive pulmonary disease; FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; hsCRP = high-sensitivity C-reactive protein; MEF₅₀ = maximal expiratory flow at 50% of vital capacity; TLC = total lung capacity; RV = residual volume.

Figure legends

Figure 1: Relative changes between SPD levels measured in serum samples that were taken two hours after smoking three cigarettes within one hour and samples that were taken at baseline (N = 89) presented as (A) histogram and (B) Bland-Altman plot. For preparation of these figures, data from all four study groups were combined due to the absence of statistically significant group differences (Mann-Whitney U test; p-values ≥ 0.28). Figures containing data for the separate groups is included as Supplemental Figure 1 and 2.

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195

SUPPLEMENTAL MATERIAL

Cigarette smoking prior to blood sampling acutely affects serum levels of the Chronic Obstructive Pulmonary Disease biomarker Surfactant Protein D

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216 **Table of Contents**

217		<u>Page:</u>
218	Supplemental Figure 1: Relative changes in serum SPD levels per study group	3
219	Supplemental Figure 2: Absolute changes in serum SPD levels per study group	4
220	Supplemental Figure 3: Overview of in-study quality control data	5
221

Supplemental Figure 1: Relative changes in serum SPD levels due to cigarette smoking in young (<40 years) and old (>40 years) individuals that are non-susceptible ('NS') for developing COPD, in young individuals that are susceptible ('S') for developing COPD, and in COPD patients ('COPD'). Presented differences are different from zero ($p < 0.05$, one sample t-test) for every group, and no differences ($p > 0.05$, Mann-Whitney U test) for the average changes were found between the groups.

Supplemental Figure 2: Absolute differences between serum SPD levels measured at baseline ('baseline') and levels measured two hours after smoking three cigarettes within one hour ('after') in young and old individuals that are non-susceptible ('NS') for developing COPD, in young individuals that are susceptible ('S') for developing COPD, and in COPD patients ('COPD'). For all groups, presented differences are statistically significant ($p < 0.05$, two-tailed Wilcoxon signed rank test) with the exception of the young susceptible subjects ($p_{\text{peptide 1}} = 0.08$; $p_{\text{peptide 2}} = 0.09$).

Supplemental Figure 3: Overview of the in-study quality control (QC) data obtained during the three analytical runs carried out for quantification of SPD in the clinical samples. QC samples with SPD levels around 2-3 times the lower limit of quantification (QC-low), with midrange SPD levels (QC-medium), and with high SPD levels (QC-high) were processed in duplicate during each analytical run. As is shown in the figure, biases within $\pm 15\%$ were observed for a sufficient number of QC samples in order to meet the regulatory requirements [1] specifying that at least 4 out of 6 of the QC samples per run (and at least one of the two samples at the same QC level) should be within $\pm 15\%$ of their respective nominal value.

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