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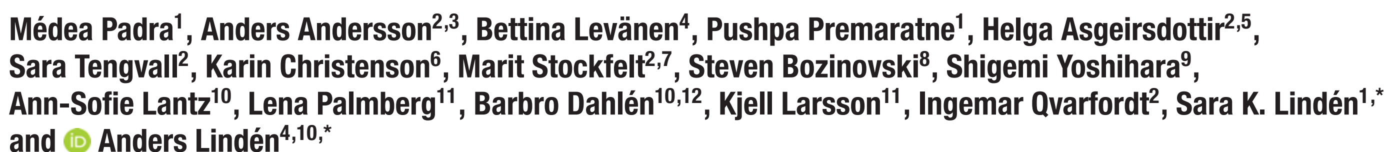
Research Article

Increased MUC1 plus a larger quantity and complex size for MUC5AC in the peripheral airway lumen of long-term tobacco smokers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There is little information on mucins *versus* potential regulatory factors in the peripheral airway lumen of long-term smokers with (LTS+) and without (LTS−) chronic obstructive pulmonary disease (COPD). We explored these matters in bronchoalveolar lavage (BAL) samples from two study materials, both including LTS+ and LTS− with a very similar historic exposure to tobacco smoke, and healthy non-smokers (HNSs; n=4–20/group). Utilizing slot blot and immunodetection of processed (filtered and centrifuged), as well as unprocessed BAL samples from one of the materials, we compared the quantity and fraction of large complexes of mucins. All LTS displayed an enhanced (median) level of MUC5AC compared with HNS. LTS− displayed a higher level of large MUC5AC complexes than HNS while LTS+ displayed a similar trend. In all LTS, total MUC5AC correlated with blood leukocytes, BAL neutrophil elastase and net gelatinase activity. Large mucin complexes accounted for most MUC5B, without clear group differences. In all LTS, total MUC5B correlated with total MUC5AC and local bacteria. In the same groups, large MUC5B complexes correlated with serum cotinine. MUC1 was increased and correlated with BAL leukocytes in all LTS whereas MUC2 was very low and without clear group differences. Thus, the main part of MUC5AC and MUC5B is present as large complexes in the peripheral airway lumen and historic as well as current exposure to tobacco smoke emerge as potential regulatory factors, regardless of COPD *per se*. Bacteria, leukocytes and proteinases also constitute potential regulatory factors, of interest for future therapeutic strategies.

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Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a chronic airway disorder caused by inhalation of noxious particles or gases, including tobacco smoke; a disorder that is characterized by persistent respiratory symptoms and airflow limitation, as well as variable inflammatory patterns and tissue remodeling throughout the airways [1–4]. At the global level, COPD is predicted to become the third most

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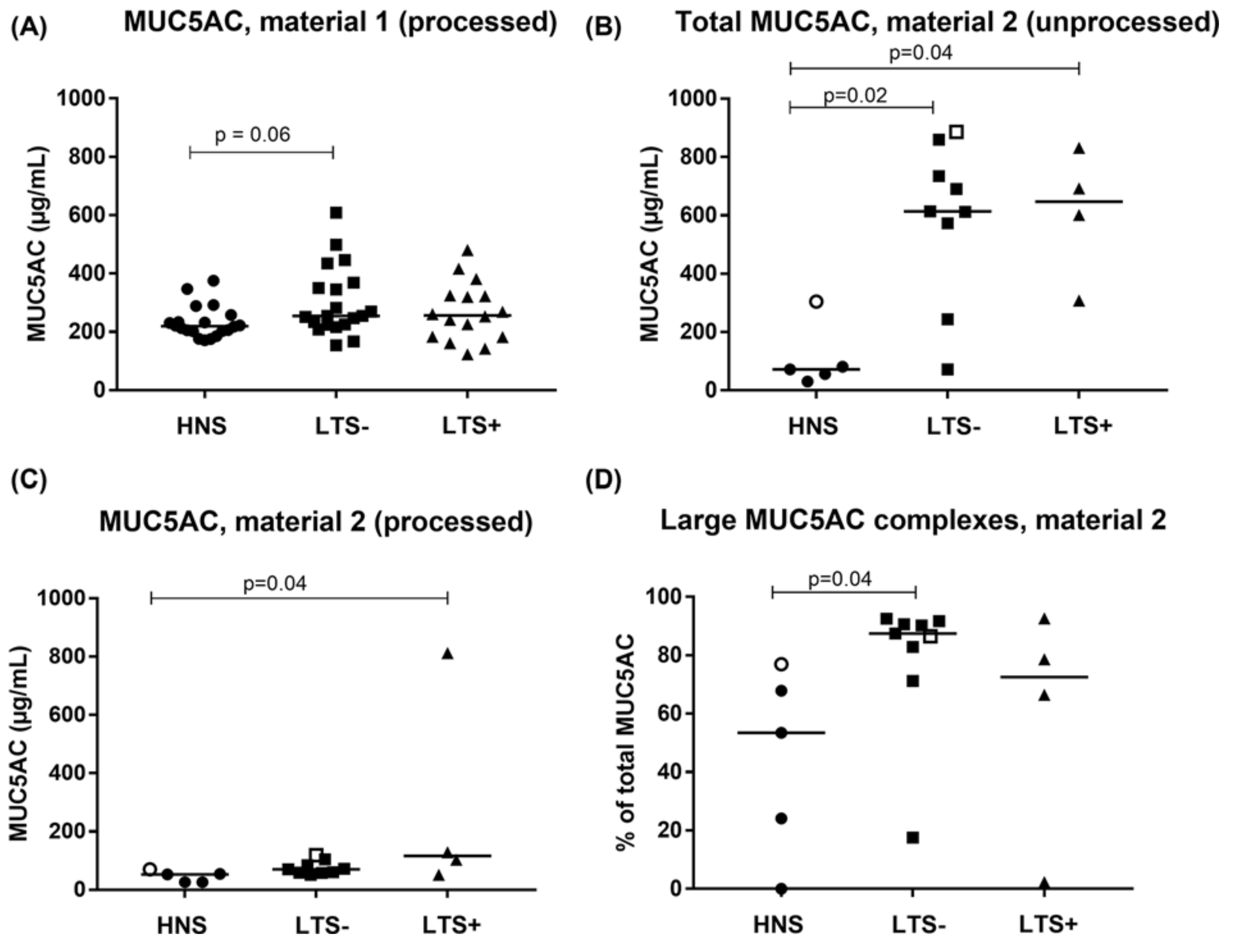

Figure 1. Level and proportion of MUC5AC in large complexes in the airway lumen of smokers with and without COPD in comparison with HNSs

Datasets including LTSs with (LTS+; triangles) and without (LTS−; squares) COPD compared with HNSs (circles) are shown as follows: (**A**) level of MUC5AC in processed (filtration and centrifugation) BAL fluid samples from Material 1; (**B**) level of MUC5AC in unprocessed (no filtration and no centrifugation) BAL fluid samples from Material 2; (**C**) level of MUC5AC in processed (filtration and centrifugation) BAL fluid samples from Material 2 and (**D**) proportion (%) of MUC5AC present in large complexes from Material 2. Each data point represents the mucin concentration in one subject. Solid (black) symbols denote subjects without colonization by bacterial pathogens whereas open (white) symbols denote the subjects with this colonization in Material 2. Horizontal bold lines depict the median value for each study group. Analytical statistics are shown according to Kruskal–Wallis one-way ANOVA on ranks.

common cause of disease-related mortality within a few years [1,5]. Still, COPD is largely a preventable disease, since long-term tobacco smoking remains the most common known cause [1].

There is a gradual decline in ventilatory capacity of the lungs in COPD, with more rapid loss of forced expiratory volume during 1 s (FEV_1) than vital capacity (VC), often associated with progressively impaired gas diffusion capacity (DLCO) over time [1,3]. The decline in FEV_1 correlates with the frequency of exacerbations, in particular among patients who also suffer from chronic productive cough caused by the comorbidity chronic bronchitis [1,3].

Among active smokers with COPD, smoking cessation remains the most effective treatment [1]. In addition, pharmacological treatments provide relief of symptoms and immunization against Influenza virus and *Streptococcus pneumoniae* reduces the risk for exacerbations [1]. However, there is an unmet medical need for more efficacious and tolerable therapy to improve the long-term prognosis of COPD. To develop such therapy, the understanding of pathogenic target mechanisms must be improved.

The increased accumulation of total mucus in the small airways correlates with enhanced mortality among human subjects in a statistically significant manner [6,7], suggesting that excess mucus *per se* is an important pathogenic

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factor. In population studies, it has been demonstrated that the decline in FEV_1 over time is enhanced in individuals with productive cough [8]. In addition, the risk of developing COPD is increased in smokers who early in life have increased airway phlegm and experience productive cough [9]. It is also known that the soluble mucins MUC5B and MUC5AC constitute the main components of mucus in humans under normal conditions [10]. Down to the tenth generation of bronchi, surface goblet cells account for most of the production of MUC5AC and submucosal glands account for most of the production of MUC5B, with some additional production in club cells [10,11]. However, from the tenth generation of bronchi and beyond where glands are lacking, surface goblet cells and club cells jointly account for the production of MUC5B [10,11] .

The membrane-bound mucins in the airways include MUC1 [12]; a mucin that may act as a 'decoy' when it is released, thereby limiting the exposure of epithelial cells to pathogens [12]. In experimental models *in vivo*, murine *Muc1* may attenuate epithelial inflammation and injury in response to the opportunistic pathogen *Pseudonomas aeruginosa* [13], whereas murine *Muc5b* plays an important role in pulmonary host defense [14]. In patients with COPD, there is immunoreactivity for MUC5AC in lining epithelial cells and for MUC5B in submucosal gland cells [10]. However, it is still uncertain whether immunoreactivity for MUC5B is more prominent in the epithelium of patients with COPD than it is in clinically healthy smokers [10]. Moreover, there is evidence that the total area of MUC5AC immunoreactivity is increased in bronchial submucosal glands for patients with COPD, in comparison with smokers without COPD and healthy non-smokers (HNSs), respectively [15]. In terms of soluble mucins from the airway lumen, there is evidence for increased levels of MUC5AC and MUC5B in induced sputum from ever smokers with severe COPD, in comparison with non-smoking controls [16]. However, the underlying study did not specifically address historic (pack-years) or current (cotinine) exposure to tobacco smoke. In fact, this has been the case for several previous studies on mucins in the airways, including those claiming that the MUC5B/MUC5AC ratio is enhanced in sputum or bronchoalveolar lavage (BAL) samples from COPD patients, compared with smokers without COPD [10,17]. This means that it is difficult to judge the pathogenic significance of exposure to tobacco smoke *per se* in relation to qualitative and quantitative alterations in mucins from smokers with and without COPD. Moreover, relatively few studies have addressed extracellular mucins in BAL samples; samples that are likely to reflect the truly peripheral airway lumen.

It is evident that there is still an incomplete understanding of the quantitative and qualitative alterations among mucins in the peripheral airway lumen of smokers with and without COPD and even less known about potential regulatory factors, such as current and historic exposure to tobacco smoke or local and systemic signs of inflammation. Here, we addressed these matters using blood and BAL samples from current smokers with and without COPD, ascertaining that these particular study groups had a very similar historic exposure to tobacco smoke, and by comparing them with HNSs.

Materials and methods

Human study materials

Ethical review

The current study was based upon the analyses of samples from HNSs and long-term tobacco smokers (LTSs) with (LTS+) and without (LTS−) COPD, obtained from two study materials – from Stockholm (*Material 1*) and from Gothenburg (*Material 2*). The study populations were recruited and characterized after careful review and approval by the regional committee for ethical review in Stockholm (*Material 1:* Diary No. 2005/733-31/1-41) and Gothenburg (*Material 2:* Diary No. 968-11), respectively. After receiving oral and written information, all participating subjects gave oral and written informed consent, in accordance with the recommendations of the code of ethics of the World Medical Association (Declaration of Helsinki).

Protocols

More extensive information on details of the protocols, including the clinical characterization of *Material 1* and *Material 2*, is available online (Supplementary Material).

Clinical protocols

We recruited study subjects at the specialized Outpatient Clinic for Respiratory Medicine of respective University Hospital. Each subject attended a screening visit for recording of clinical history, clinical chemistry, medical examination, ventilatory capacity before and after bronchodilation (reversibility test), DLCO, radiology of the chest (X-ray) and electrocardiography (ECG); always performed prior to bronchoscopy. A negative history of atopy and lung disease other than COPD or chronic bronchitis or emphysema was required for all study subjects. For inclusion, we required

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Table 1 Yield from bronchoalveolar lavage (BAL)

Parameter (median (range))	Material 1		
	HNS	**LTS−**	**LTS+**
(A)			
Number of subjects	**20**	**20**	**16**
BAL volume (ml)	250	250	250
BAL return volume (ml)	155.5 (88–186)	148.5 (75–185)	112.5 (55–170)[3]
BAL recovery (%)	62.5 (35–74)	59.5 (49–74)	46.5 (22–68)[3]
BAL leukocyte count (million cell/l)	118.7 (53–171.5)	314.3 (117.6–1196)[3]	283 (100–1283)[3]
BAL macrophage (%)	91 (70–172)	93.15 (84.6–98.3)	89.65 (79.6–98)
BAL lymphocyte (%)	5.3 (0–23.6)	4.15 (0.6–10.3)	4.95 (0.1–6.3)
BAL neutrophil (%)	2 (0–17.3)	2.15 (0–6.6)	2.3 (0–9.6)
BAL eosinophil (%)	0 (0–2.3)	0.3 (0–1.6)	1 (0–4.6)[2]
BAL basophil (%)	0 (0–0)	0 (0–0.3)	0 (0–0.6)
	Material 2		
	HNS	**LTS−**	**LTS+**
(B)			
Number of subjects	**5**	**9**	**4**
BAL volume (ml)	150	150	150
BAL return volume (ml)	85 (38–111)	78 (48–103)	55.5 (33–91)
BAL recovery (%)	57 (25–74)	53 (32–68)	36.8 (22–60)
BAL leukocyte count (million cell/l)	99 (44–221)	223 (157–528)[1]	292.5 (216–433)
BAL macrophage (%)	93 (70–97)	98 (95–99)[1]	86 (69–96)
BAL lymphocyte (%)	3 (2–29)	1 (0–3)[1]	1.5 (0–14)
BAL neutrophil (%)	1 (1–4)	1 (0–2)	8.5 (2–18)
BAL eosinophil (%)	0 (0–0)	0 (0–0)	2 (0–5)[1]
BAL basophil (%)	NA	NA	NA

[1] $P<0.05$.
[2] $P<0.01$.
[3] $P<0.001$ compared with HNS (Kruskal–Wallis test).

a negative history of atopy and, for *Material 2* only, we also required a negative test for specific IgE against airborne allergens in blood. For all LTS, we required a tobacco load of at least 5 pack-years in *Material 1* and at least 20 pack-years in *Material 2*. For all LTS, a history of chronic bronchitis was merely recorded. For HNSs and LTS−, the inclusion criterion was a post-bronchodilatory FEV_1/FVC ratio of ≥70% and an FEV_1 of >80% of predicted whereas DLCO was merely recorded for all study groups. For LTS+, the inclusion criterion was a post-bronchodilatory FEV_1/FVC ratio of <70% [1]. The X-ray had to be normal for HNS. For all LTS, it had to be without significant pathology beyond hyperinflation, direct signs of bronchitis and indirect signs of emphysema. To ascertain a high level of clinical safety during bronchoscopy, we required a post-bronchodilatory FEV_1 > 40% predicted. Clinical or laboratory signs of infection within 4 weeks prior to bronchoscopy resulted in a rescheduled investigation. The clinical characteristics for the included subjects in *Material 1* and *Material 2* is summarized separately (Supplementary Table S1A,B).

Bronchoscopy

For both *Material 1* and *Material 2*, bronchoscopy was performed by an experienced pulmonologist during stable clinical conditions, in accordance with clinical routine of the interventional unit at respective university hospital (Supplement Material). Briefly, after premedication and local anesthesia, the bronchoscope was positioned and wedged in a subsegmental bronchus (middle lobe or lingula). The BAL was then performed by repeatedly instilling aliquots of sterile and body-tempered phosphate-buffered saline (PBS). The BAL samples were aspirated (yield presented in Table 1) in a sterile plastic bottle and kept on ice until transportation to the laboratory for processing (see below). During bronchoscopy, medication for bronchodilation and anesthesia was administered as needed.

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Table 2 Bacteria in lower airway samples from Material 2

Patient group	Bacterial species	Quantity (CFU/ml)
HNS	No bacteria	
HNS	No bacteria	
HNS	No bacteria	
HNS	Normal oral microflora	4000
HNS	*S. pneumoniae/H. influenzae/α-Streptococcus*	10000/10000/10000
LTS−	Normal oral microflora	2000
LTS−	Normal oral microflora	121000
LTS−	Normal oral microflora	12000
LTS−	Normal oral microflora	<100
LTS−	Normal oral microflora	300
LTS−	Normal oral microflora	3000
LTS−	No bacteria	
LTS−	Normal oral microflora/*S. pneumoniae*	2000/1000
LTS-	No bacteria	
LTS+	No bacteria	
LTS+	No bacteria	
LTS+	No bacteria	
LTS+	Normal oral microflora	100000

Growth of bacteria (colony-forming units, CFU) in BAL fluid or bronchial.
Brush samples from the study subjects of Material 2 (see methods section), including HNSs, LTSs without COPD (LTS−) and LTSs with COPD (LTS+).

Growth of bacteria

For *Material 2* only, a lower airway sample (Supplementary Material) was obtained from each subject and sent to the Department of Bacteriology at Sahlgrenska University Hospital, for a qualitative and quantitative analysis of the growth of aerobic bacteria. The principal bacteriological results are summarized separately (Table 2).

Processing of BAL samples

The processing of BAL samples was performed according to clinical routine at the respective study site, as follows: after arrival to the laboratory, the entire BAL sample from *Material 1* was filtered under sterile conditions and the yield was then centrifuged at low temperature to divert extracellular fluid (i.e., cell-free BAL fluid) from cells and debris. The cell pellet (i.e., BAL cells) was separated from the obtained supernatant and the latter was frozen. For *Material 2* only, unprocessed BAL samples were also collected and, once retrieved, the cell pellet was resuspended in PBS and the cells were counted to determine total leukocyte concentrations. Smears for differential cell counts were prepared using cytocentrifugation and the obtained preparations were stained for cell differential counts. The principal findings in the BAL samples are presented separately (Table 1).

Processing of blood samples

For *Material 2*, venous blood samples were collected as plasma and as serum. Blood cell total (see Results below) and leukocyte differential (Supplementary Table S2) counts were determined utilizing an automated flow cytometer according to clinical routine. The obtained plasma and serum samples were frozen until further analysis.

Characterization and quantification of mucins

In brief, mucin analyses were performed as follows: a protease inhibitor cocktail was added to the BAL samples during thawing. The BAL samples were diluted in reduction buffer. To expose the MUC5B and MUC2 epitopes, the BAL samples were reduced and then alkylated for these specific analyses. Each sample was loaded on to a polyvinylidene difluoride membrane using a slot blot apparatus. In addition, serial dilutions of the mucin standards were also loaded. Vacuum was applied to attach the mucins to the membrane and the membranes were then air-dried, pre-wetted in methanol, rinsed with water and incubated in PBS. The membranes to be analyzed for MUC1 were analyzed in duplicates, whereof one of the membranes was subject to periodate oxidation. The unspecific binding was attenuated by incubating with a blocking buffer. The membranes were then incubated with specific rabbit serum against MUC5B or MUC2 or with mouse monoclonal antibodies against MUC5AC or MUC1 in blocking buffer with gentle shaking. The membranes were then washed and were thereafter incubated with infrared-labeled secondary antibody diluted

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in blocking buffer. The membranes were washed again and the blots were subsequently visualized and quantified for determination of the type and level (i.e., concentration) of respective mucin.

Quantification of C-reactive protein

Protein concentrations of C-reactive protein (CRP) in serum were determined with a high-sensitivity commercial assay (Supplementary Material), utilizing the agglutination of CRP to latex particles. These particles were subsequently exposed to anti-CRP antibodies and then detected by a turbidimetric analysis instrument.

Quantification of cotinine

Protein concentrations of the nicotine metabolite cotinine were determined in serum samples utilizing a competitive immunochemical method with detection by a chemiluminescence instrument.

Quantification of neutrophil elastase

Levels (i.e., concentrations) of the neutrophil elastase-α-1 proteinase inhibitor complex in cell-free BAL fluid samples were determined utilizing ELISA.

Quantification of net gelatinase and serine proteinase activity

Fluorometric substrates were used to measure two types of net proteinase activity (Supplementary Material) in cell-free BAL fluid samples, where the increase in fluorescence is proportional to proteolytic activity.

Statistical analyses

Analytical statistics for key outcomes were addressed separately for *Material 1* and *Material 2* with a non-parametric approach, using GraphPad Prism 7.0 (GraphPad Software Inc. San Diego, U.S.A.) software package. Median (range) values are presented unless otherwise stated. Kruskal–Walllis one-way ANOVA on ranks with Dunn's multiple comparisons test was utilized for comparisons between groups. The Spearman's rank order correlation test was utilized to analyze associations between mucin levels and other parameters among LTS+ and LTS−. The HNS group was excluded from the analysis to avoid false positive correlations due to the mucin changes occurred with smoking *per se*. The level of statistical significance was set at $P<0.05$.

Results

Clinical characteristics

In *Material 1*, the number (n) of included subjects ranged from 16 to 20 per study group and in *Material 2*, it ranged from 4 to 9 per study group (Supplementary Table S1A,B). Both genders were represented, the body mass index (BMI) was normal and similar among all study groups and the range of age was similar for all study groups. Importantly, the historic exposure to tobacco smoke (i.e., tobacco load) was very similar for all LTS (LTS− and LTS+) in both *Material 1* and *Material 2*. For *Material 2* only, we also monitored chronic bronchitis but only two subjects had chronic bronchitis. One such subject was included in the LTS− group and one in the LTS+ group. The results for cotinine and CRP in *Material 2* are presented below.

In terms of ventilatory capacity, the FEV_1 (% predicted) suggested mild to moderate chronic airflow obstruction in the LTS+ group of both study materials, with normal FEV_1 for the LTS− and HNS groups in these materials (Supplementary Table S1A,B). In terms of gas diffusion capacity, there was a modest reduction in DLCO (% predicted) for the LTS− group and a substantial reduction in the LTS+ group, with normal DLCO (% predicted) for the HNS group, in both study materials (Supplementary Table S1A,B).

Growth of bacteria

The lower airway samples harvested from *Material 2* displayed growth of any bacteria in two subjects from the HNS group, in seven subjects from the LTS− group and in one subject from the LTS+ group (Table 2). Two subjects only displayed cultures positive for pathogenic species and these subjects belonged to the HNS and LTS− group, respectively.

Yield and viability for BAL samples

We obtained BAL samples from all included study subjects. The median volume yield was of a similar order of magnitude for the HNS, LTS− and LTS+ groups, respectively, in *Material 1* (Table 1A) and *Material 2* (Table 1B), with

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a gradual decline in BAL yield for these groups, as expected. There was a high cellular viability (>96%) among BAL cells in both study materials (Supplementary Material). In both study materials, the LTS groups tended to display a higher total leukocyte count in BAL samples than did the HNS group (Table 1A,B). However, this enhancement of leukocytes was not statistically significant for the LTS+ group in *Material 2*. Among the subpopulations of leukocytes in BAL samples, the percentage of neutrophils tended to be higher than that for eosinophils in both study materials, particularly so in the LTS+ groups. Moreover, in the BAL samples from *Material 2*, the percentage of neutrophils tended to be more increased in the LTS+ group compared with the HNS group in *Material 2* whereas this increase was less evident in *Material 1* (Table 1A,B). Finally, a similar pattern was observed for the percentage of eosinophils in BAL samples. However, in this case, it was for eosinophils only that the difference in percentage proved statistically significant.

Group-related differences for level and proportion of MUC5AC in large complexes

We quantified the level of MUC5AC in cell-free BAL fluid samples from both *Material 1* and *Material 2*. The processed (filtered with a pore size of 70 μm) samples from *Material 1* displayed a higher ($P=0.046$) level of MUC5AC in the pooled group of all LTS (254 [72–886] μg/ml; $n=36$) than in the HNS group (219 [72–305] μg/ml; $n=20$, Figure 1A). There was a trend in the same direction for MUC5AC in the LTS− group. Since a 2.5-fold increase in the level of MUC5AC in sputum has previously been observed in long-term smokers (LTSs) [18], we found the corresponding difference that we detected in BAL samples smaller than expected. We therefore hypothesized that a larger proportion of the secreted mucins forms large complexes in LTSs, with and without COPD, and therefore utilized the access to unprocessed material, that was available for the subjects in *Material 2* only. In the unprocessed BAL samples from *Material 2*, the MUC5AC content was 8.5-fold higher in the pooled group of all LTS compared with the HNS group (Figure 1B). There was a similar level for the LTS+ and LTS− groups. However, along the lines of the findings in *Material 1*, the level of MUC5AC in filtered BAL fluid from the pooled group of all LTS in *Material 2* was only ∼35% higher than that in the HNS group (Figure 1C). The proportions of mucins that were present as large complexes (i.e., those removed by processing including filtration) were indeed larger in the LTS− and LTS+ group, respectively, than in the HNS group from *Material 2* (Figure 1D).

Potential regulatory factors

Potential regulatory factors of mucins were analyzed in *Material 2* only.

Cotinine

The level of cotinine in serum was markedly higher ($P=0.00021$; $n=13$) in the pooled group of all LTS (336 [19–500] pg/ml) than in the HNS group (10 [10–10] pg/ml) (Figure 2A). This was also true for the LTS− group whereas the LTS+ group displayed a similar trend only.

Leukocytes

The leukocyte count in blood samples was higher in LTS+ than in HNS and tended to display an intermediate level in the LTS− group (Figure 2B). The percentage of neutrophils tended to be higher in both LTS groups than in the HNS group. Moreover, this percentage of neutrophils tended to be higher than that for eosinophils in all study groups (Supplementary Table S2).

CRP

The level of CRP in serum tended to be higher in the LTS+ group than in the HNS group, with the LTS− group displaying an intermediate level (Figure 2C).

Neutrophil elastase

The level of neutrophil elastase protein in cell-free BAL fluid samples did not markedly differ for either the LTS+ or the LTS− group, in comparison with the HNS group (Figure 2D).

Net gelatinase activity

The level of net gelatinase activity in cell-free BAL fluid samples did not markedly differ for either the LTS+ or the LTS− group, compared with the HNS group (Figure 2E).

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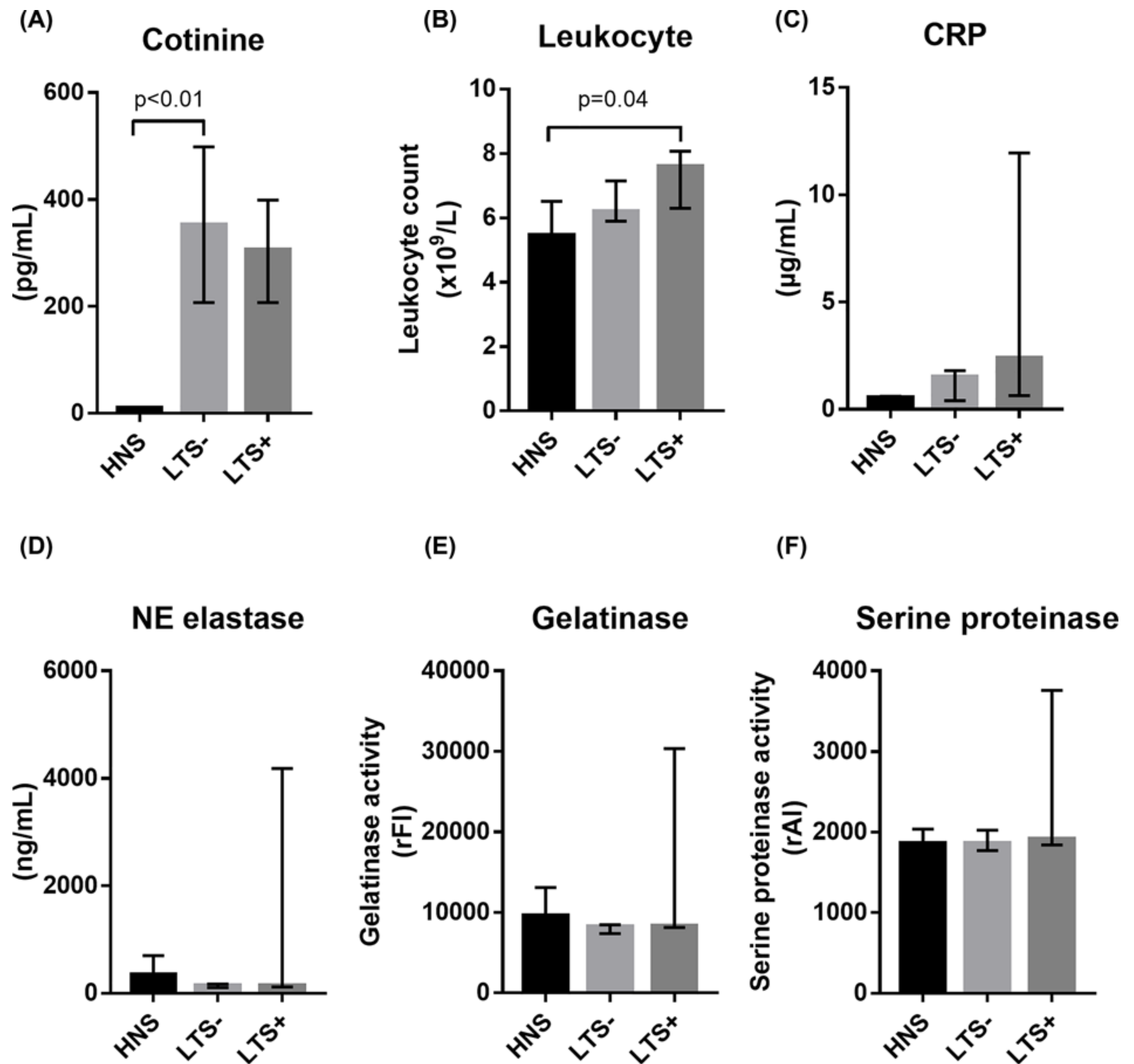

Figure 2. Level of potential regulatory factors among smokers with and without COPD in comparison with HNSs

Datasets from *Material 2* including LTSs with (LTS+) and without (LTS−) COPD compared with HNSs are shown as level of: (**A**) cotinine in serum; (**B**) leukocyte count in blood samples; (**C**) CRP in serum; (**D**) neutrophil elastase protein in BAL samples; (**E**) net gelatinase activity (relative fluorescence intensity, rFI) in BAL samples and (**F**) net serine proteinase activity (relative absorbance intensity, rAI) in BAL samples. Bars depict the median and interquartile range for each study group. Analytical statistics are shown according to Kruskal–Wallis one-way ANOVA on ranks (n=4–8).

Net serine proteinase activity

The level of net serine proteinase activity in cell-free BAL fluid samples did not markedly differ for either the LTS+ or the LTS− group, compared with the HNS group (Figure 2F).

Associations between MUC5AC and potential regulatory factors

To identify likely causes of the increased level of MUC5AC among smokers, we performed statistical correlations between factors that may induce or be associated with the level of MUC5AC among LTSs with and without COPD in *Material 2*. We found that the leukocyte count in peripheral blood and the level of MUC5AC in BAL samples from the pooled group of all LTS displayed a positive correlation (Figure 3A). In the same group, the level of the mucin secretagogue neutrophil elastase [19] and the level of MUC5AC in BAL samples displayed a positive correlation

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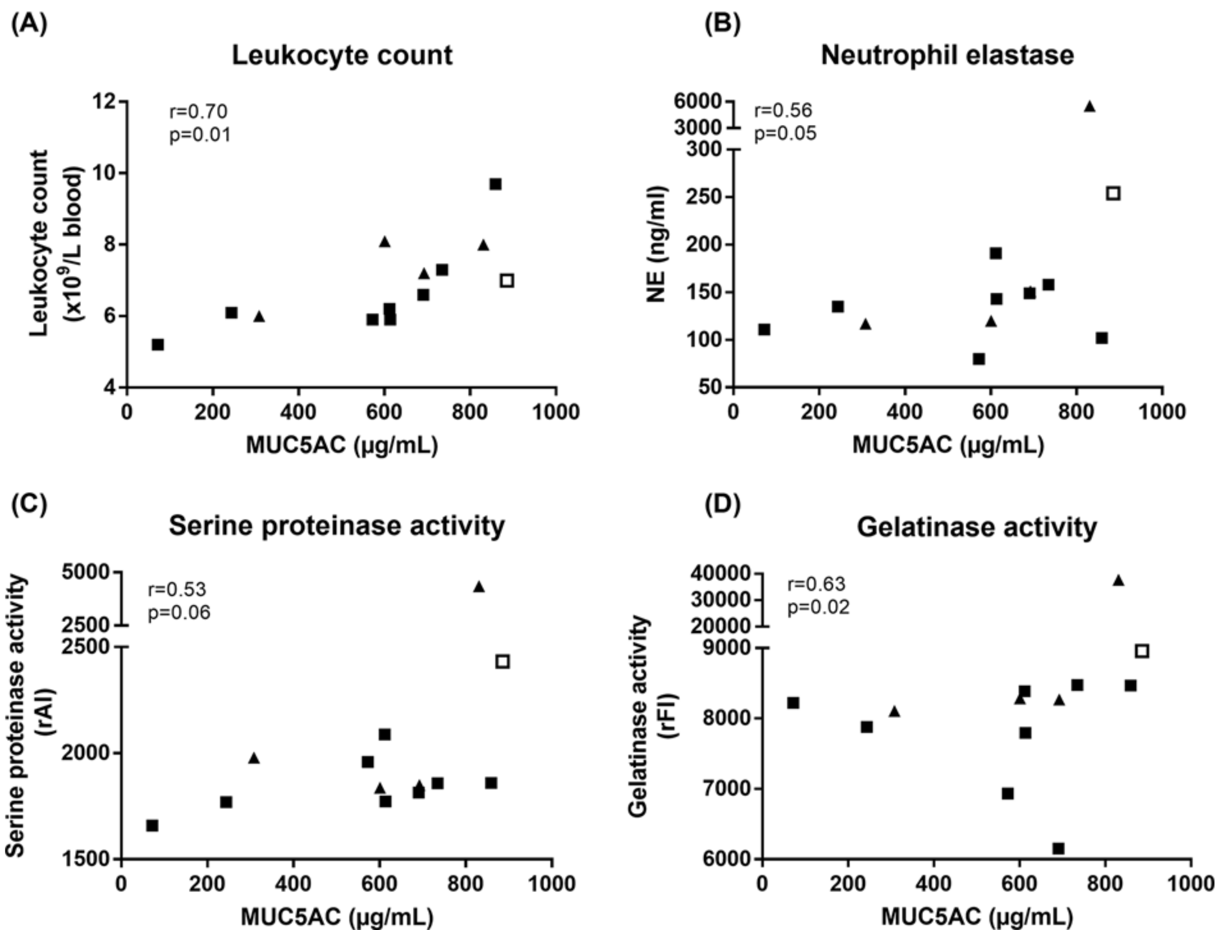

Figure 3. Correlations between potential regulatory factors and MUC5AC among LTSs with and without COPD
Datasets from BAL samples from *Material 2* including LTSs with (LTS+; n=4; triangles) and without (LTS−; n=9; squares) COPD are shown as follows: level of MUC5AC in relation to level of (**A**) blood leukocyte count; (**B**) concentration of neutrophil elastase protein; (**C**) net serine proteinase activity (relative absorbance intensity, rAI); (**D**) net gelatinase activity (relative fluorescence intensity, rFI). Solid (black) symbols denote subjects without bacterial colonization whereas open (white) symbols denote subjects with bacterial colonization. Analytical statistics are shown according to Spearman's rank order correlation.

(Figure 3B). A similar trend was present for the association between the net serine proteinase activity and the level of MUC5AC in BAL samples (Figure 3C). Furthermore, the gelatinase metalloproteinase-9 has been experimentally implicated in the production of MUC5AC and accounts for net gelatinase activity in human airways [20]. In line with this, the level of net gelatinase activity and the level of MUC5AC displayed a positive correlation in BAL samples from all LTS (Figure 3D). However, we detected no statistically significant correlations between these potentially regulatory factors and the proportion of MUC5AC present as large complexes in BAL samples from the pooled group of all LTS (data not shown).

Group-related differences for level and proportion of MUC5B in large complexes

We detected no statistically significant differences between the group of LTS+ and LTS− in terms of the total level of MUC5B in BAL samples from *Material 2* (Figure 4A). However, both LTS groups displayed a modest trend toward higher levels of MUC5B in comparison with HNS. Despite the lack of statistical significance, half of the subjects in the pooled group of all LTS displayed a 2- to 15-fold higher level of MUC5B in BAL samples than did the subject with the highest level of MUC5B in the HNS group, when comparing subjects without any detected colonization by pathogenic bacteria. Notably, the two subjects that had pathogenic colonization had very similar levels of MUC5B in BAL samples, although one belonged to the HNS group and one belonged to the LTS- group (Figure 4A). Approximately 70% of

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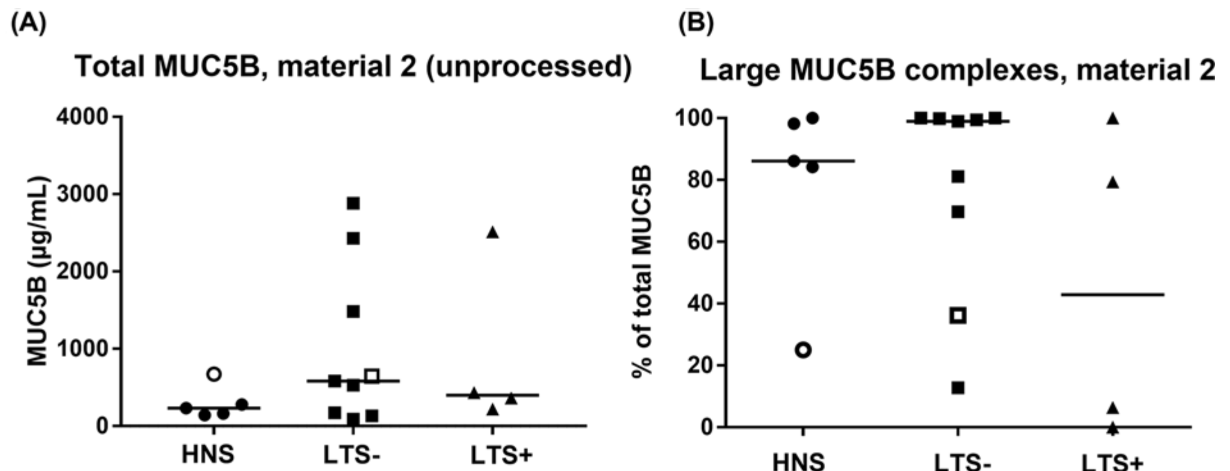

Figure 4. Level of MUC5B and proportion of large complexes in the airway lumen of LTSs with and without COPD compared with HNSs

Datasets from LTSs with (LTS+; triangles) and without COPD (LTS−; squares) compared with non-smokers (HNS; circles) showing (**A**) concentration of MUC5B in unprocessed (no filtration and no centrifugation) BAL samples and (**B**) proportion (%) of MUC5B present in large complexes in BAL samples, all from *Material 2*. Solid (black) symbols denote subjects without bacterial colonization whereas open (white) symbols denote subjects with bacterial colonization. The horizontal bars depict the median for each study group. Analytical statistics according to Kruskal–Wallis one-way ANOVA on ranks did not verify any significant difference between groups.

all subjects in the study had 80% or more of their MUC5B present as large complexes in BAL samples (Figure 4B). Therefore, we quantified MUC5B only in BAL samples from *Material 2*.

Associations between MUC5B and potential regulatory factors in all LTS

In *Material 2*, the level of MUC5B displayed a positive correlation with the level of MUC5AC in BAL samples from the pooled group of all LTS (Figure 5A). In this material, the level of large complexes of these two mucins also correlated in a positive manner (Figure 5B). It is known that MUC5B can be released from airway epithelial cells after stimulation by neutrophil elastase [21] and, along these lines, we detected a trend toward a positive correlation between the level of neutrophil elastase and the level of MUC5B in BAL samples from the pooled group of all LTS in *Material 2* (Figure 5C). Given that MUC5B is involved in antibacterial host defense in the airways and that bacterial products increase the mRNA for MUC5B [22], we also examined the association between MUC5B and the quantity of any bacteria in lower airway samples from the pooled group of all LTS in *Material 2*. Here, we detected a statistically significant positive correlation between the bacterial count (i.e., CFU) and the level of MUC5B (Figure 5D). Moreover, we detected a correlation between the level of cotinine in serum and the proportion of MUC5B present as large complexes in BAL samples from the pooled group of all LTS from *Material 2* (Figure 5E).

Group-related levels and proportion of MUC2 in large complexes

Although the presence of significant amounts of MUC2 in the airways has been questioned [23], a recent study suggested the presence of large amounts of MUC2 in the airways of patients with COPD, including mainly former but also some current smokers [24]. Since MUC2 is a large oligomerizing mucin that is likely to be lost during sample filtration, we quantified MUC2 in *Material 2* only, from which we harvested the unprocessed samples. Notably, the level of MUC2 in our BAL samples was very low and close to the detection limit of our assay, despite of the loading of a three-fold larger volume of BAL sample for its detection here, compared with the analyses of other mucins. Using this approach, the integrated density of MUC2 that we obtained (Figure 6A) was nine- and seven-fold lower than that of MUC5AC and MUC5B, respectively. Moreover, the level of MUC2 in the collected BAL samples was close to the detection limit in the three study groups, making quantification uncertain in processed as well as unprocessed BAL samples. Therefore, we here present the data on MUC2 as integrated density only (Figure 6A) and we did not detect any clear differences between the study groups for this mucin. Nor did we detect any corresponding difference in the proportion of large MUC2 complexes (Figure 6B).

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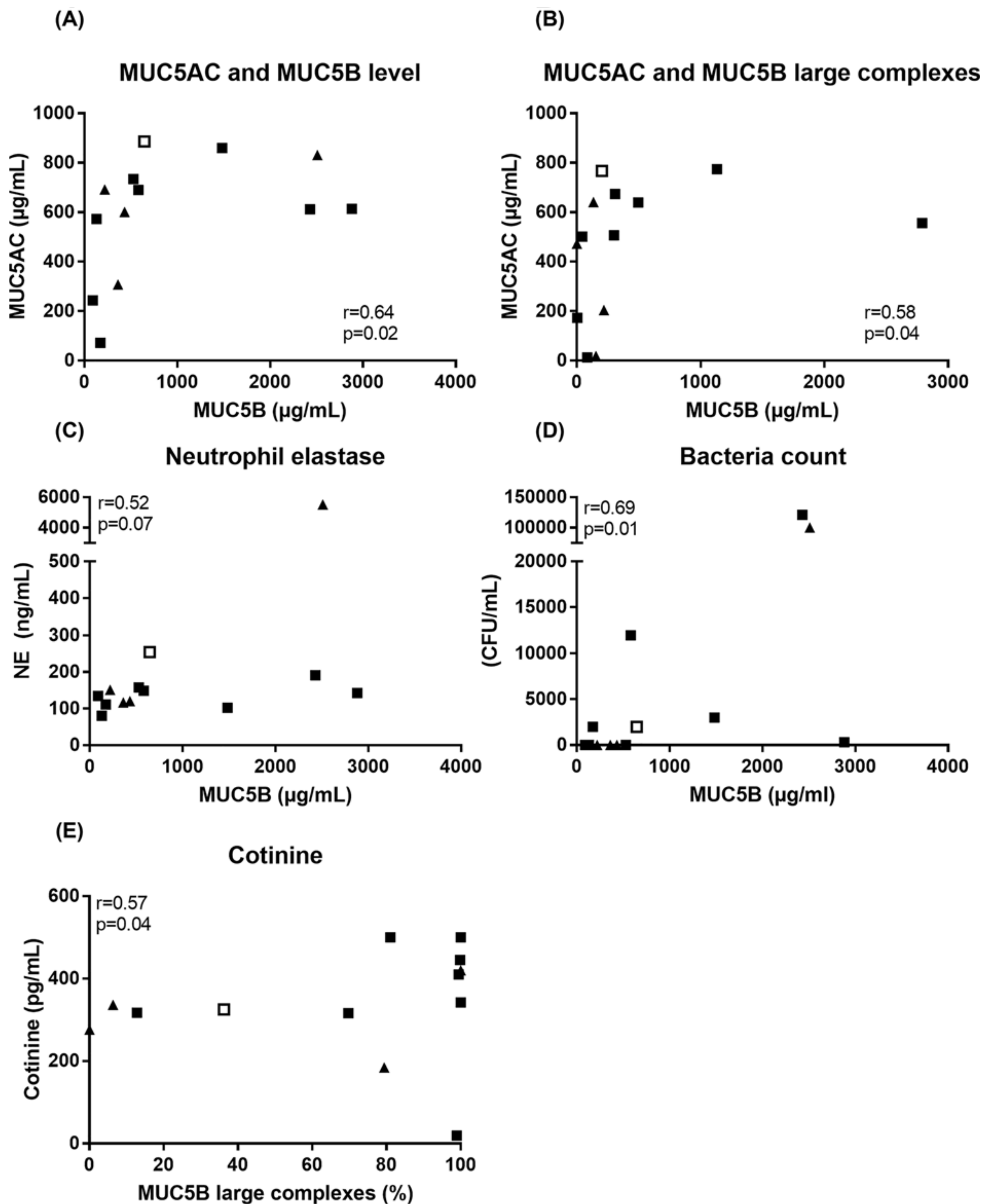

Figure 5. Correlations between potential regulatory factors and MUC5B among LTSs with and without COPD

Datasets from LTSs with (LTS+; *n*−=4; triangles) and without COPD (LTS−; *n*=9; squares) in *Material 2* showing correlations between (**A**) level of MUC5AC and MUC5B in BAL samples; (**B**) level of large complexes of MUC5AC and MUC5B in BAL samples; (**C**) level of neutrophil elastase and MUC5B in BAL samples; (**D**) bacterial count (i.e., CFU for normal flora or pathogens) in lower airway samples and level of MUC5B in BAL samples; (**E**) level of serum cotinine and the proportion (%) of MUC5B present in large complexes in BAL samples. Solid (black) symbols denote subjects without bacterial colonization whereas open (white) symbols denote subjects with bacterial colonization. Analytical statistics are shown according to Spearman's rank order correlation.

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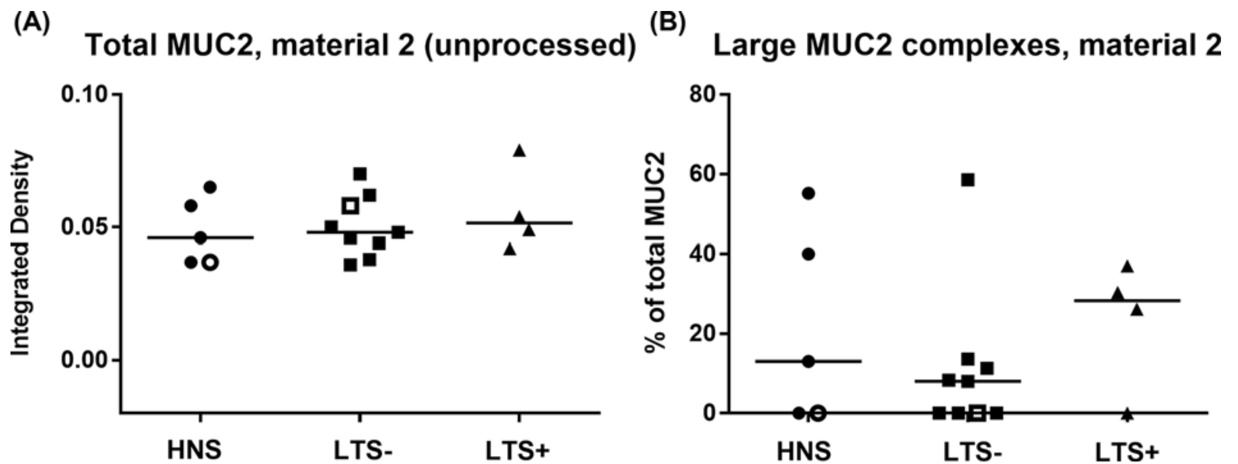

Figure 6. Level of MUC2 and its proportion in large complexes in the airway lumen of LTSs with and without COPD compared with HNSs

Datasets from LTSs with (LTS+) and without COPD (LTS−) compared with HNSs showing (**A**) level of MUC2 in unfiltered BAL samples and (**B**) proportion (%) of large MUC2 complexes in BAL samples from *Material 2*. Open (white) symbols denote subjects with colonization by bacterial pathogens whereas solid (black) symbols denote subjects without this type of colonization. The horizontal bars depict the median value for each study group. Analytical statistics are shown according to Kruskal–Wallis one-way ANOVA on ranks.

Group-related differences for level and proportion of MUC1 in large complexes

In *Material 2*, the level of MUC1 in BAL samples from the LTS– group was 20% higher than that of HNS (Figure 7A). However, MUC1 is a relatively small mucin and does not form the same type of large oligomeric structures as the other gel forming mucins analyzed here [12]. To verify this, we compared the levels of MUC1 in unprocessed (Figure 7A) and processed (Figure 7B) BAL samples from *Material 2*, and indeed, less than 3% of MUC1 in the HNS group, and less than 8% in the LTS+ and LTS— groups, was lost due to sample processing (Figure 7C). Since a relatively low proportion of MUC1 was lost in the mesh spacer and centrifugation steps here, we analyzed MUC1 in *Material 1* as well. We found an increased level of MUC1 being present in the BAL samples from all LTS in this larger material as well (Figure 7D).

It seems likely that the small loss of MUC1 due to sample processing was due to a portion of MUC1 interacting with the other and larger mucin complexes, as in *Material 2*, the quantity of MUC1 in large complexes tended to correlate with the quantity of MUC5AC large complexes (Figure 8A) in a positive manner and clearly correlated in this manner with MUC5B in large complexes (Figure 8B). The level of MUC1 did not correlate ($r = -0.13$; $P=0.60$; $n=18$) with the level of net gelatinase activity for all study subjects in *Material 2*, in spite of previous studies indicating that certain gelatinases, such as MMP-14, are involved in the shedding of the MUC1 in response to bacteria [12]. However, the level of MUC1 did correlate in a positive manner with the total leukocyte count per subject in BAL samples from the pooled group of all LTS from *Material 2* (Figure 8C). Together, this suggests that some of the MUC1 detected may originate from leukocytes, especially in the case of unprocessed BAL samples.

Association between MUC1 and DLCO

We detected a moderate and negative correlation between the level of MUC1 and DLCO among all study subjects in *Material 1* only (Supplementary Figure S1). We were unable to detect such a correlation in the substantially smaller dataset from *Material 2* (data not shown).

Discussion

The results from several previous studies on mammalian airways are suggestive of a pathological up-regulation of MUC5AC in a variety of airway disorders, including COPD, but the role of exposure to tobacco smoke *per se* has not been thoroughly addressed in these studies [17,25–27]. Given this, we think that the most important aspect of the current study is the novel evidence that historic and current exposure to tobacco smoke constitute two potentially

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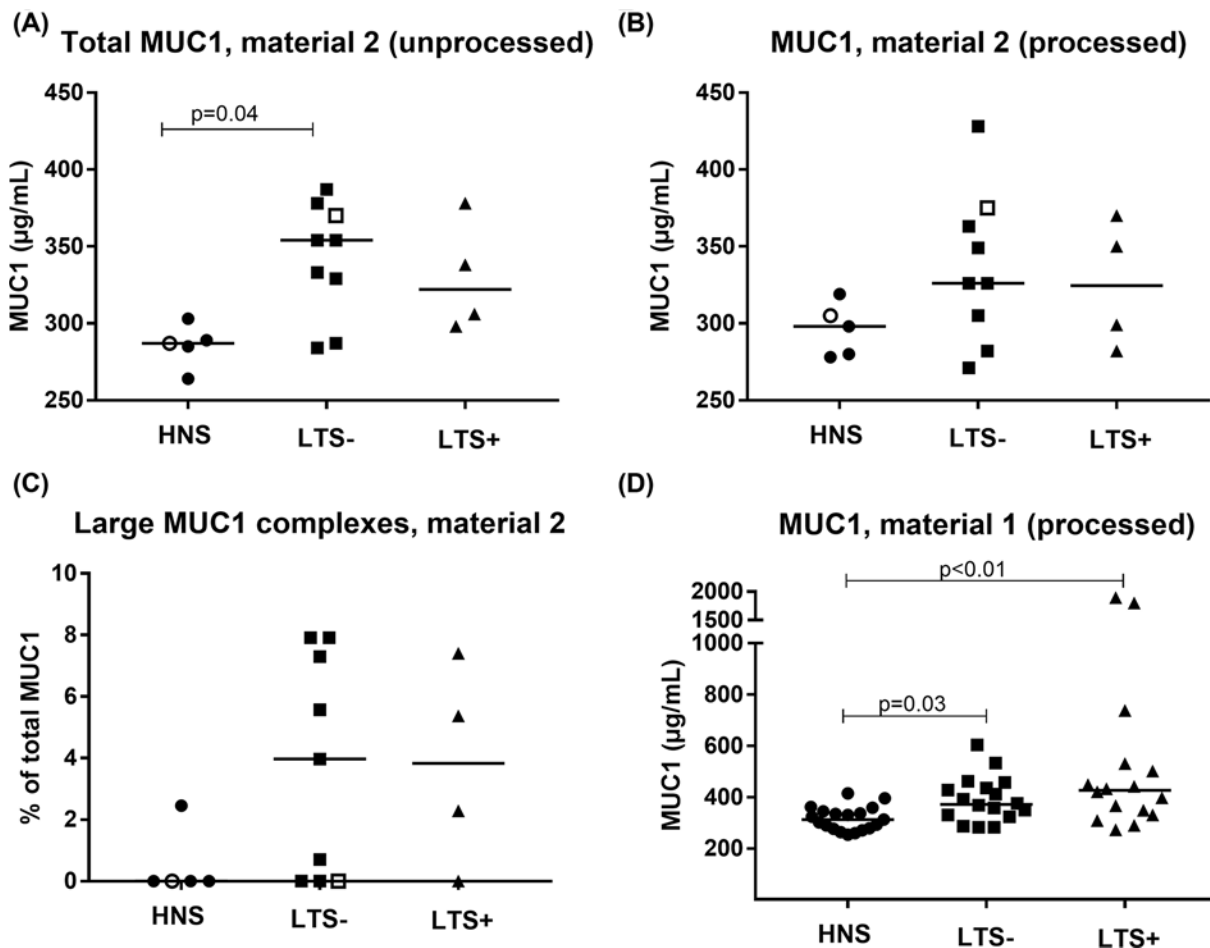

Figure 7. Level of MUC1 in the airway lumen of LTSs with and without COPD compared with HNSs

Datasets from LTSs with (LTS+; triangles) and without (LTS−; squares) COPD compared with HNSs (circles) showing (**A**) total level of MUC1 in unprocessed BAL samples from *Material 2*; (**B**) level of MUC1 in processed BAL samples from *Material 2*; (**C**) proportion (%) of MUC1 present in large complexes from *Material 2*; (**D**) level of MUC1 in processed BAL samples from *Material 1*. Open (white) symbols denote subjects with bacterial colonization whereas solid (black) symbols denote subjects without bacterial colonization according to culture of lower airway samples from *Material 2*. Analytical statistics are shown according to Kruskall–Wallis one-way ANOVA on ranks.

important regulatory factors, explaining both qualitative and/or quantitative alterations of mucins in the peripheral airway lumen of humans. As judged from the current data, the exposure to tobacco smoke emerges as more important than COPD *per se* and we think that this principal finding was facilitated by three key aspects of our study design. First, we investigated LTS+ and LTS− with a very similar tobacco load and HNS without any substantial tobacco load. Second, we compared mucins in unprocessed with those in processed BAL samples. Third, we explored BAL samples originating from the peripheral airways and not induced sputum sample originating from the more proximal airways. Importantly, this allowed us to characterize alterations of mucins among smokers in the most critical compartment in terms of typical pathology.

Despite limitations in statistical power due to a modest sample size for our materials, we detected quantitative differences in terms of enhanced levels in all LTS for MUC5AC and MUC1 in comparison with HNS. In contrast, we failed to do this for MUC5B and MUC2. Moreover, we detected an enhanced proportion of large MUC5AC complexes among all LTS. When we addressed associations with potential regulatory factors, we detected positive correlations between blood leukocytes and MUC5AC as well as between BAL leukocytes and MUC1 in BAL samples from all LTS. This is compatible with a mechanistic link to systemic inflammation as well as with a causative relationship, since neutrophils can produce neutrophil elastase and other proteinases that may act as secretagogues [19–21]. Along these lines, we detected positive correlations between two different types of net proteinase activity and MUC5AC in

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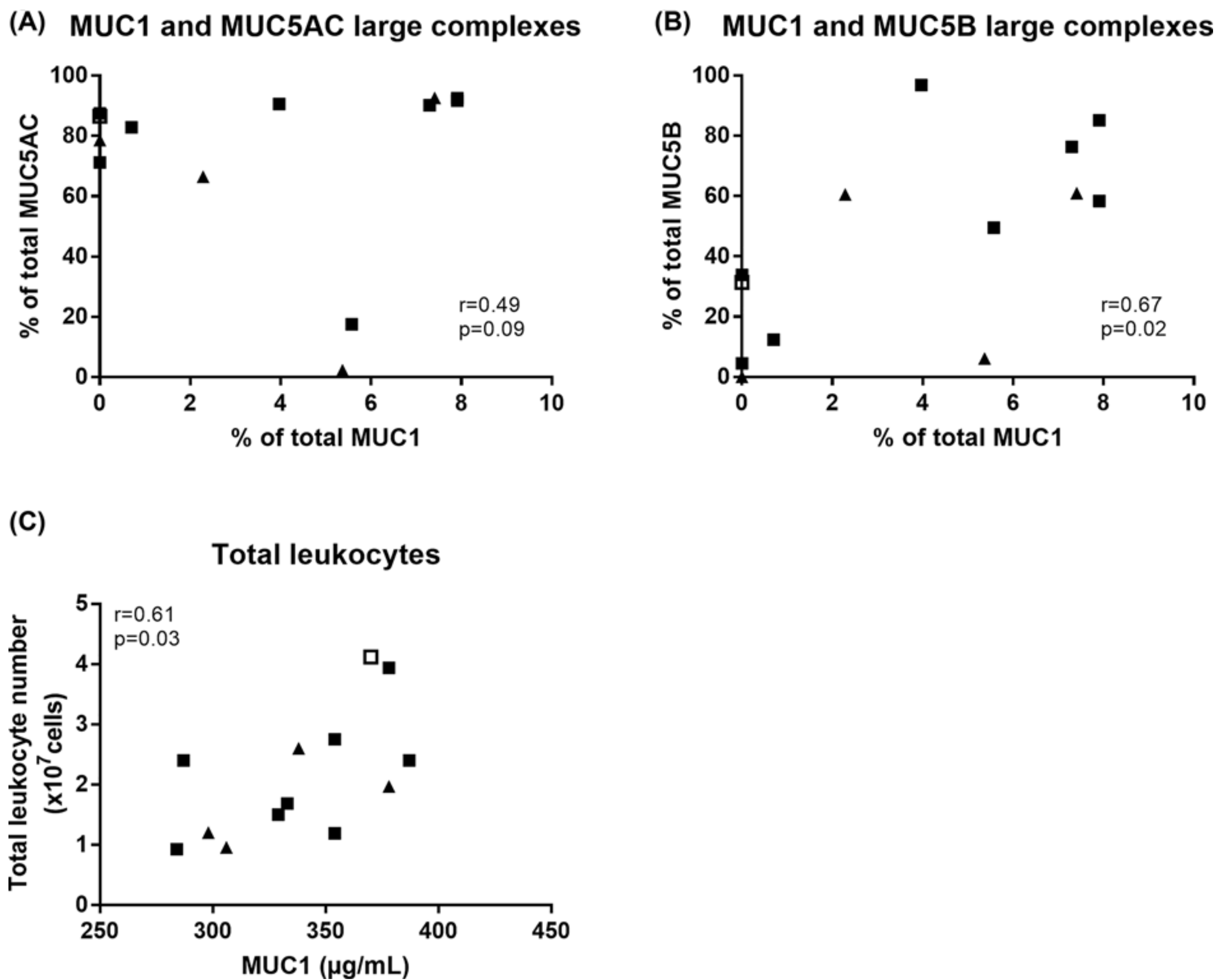

Figure 8. Correlations between large complexes of MUC1, other mucins and leukocytes from the airways of LTSs with and without COPD

Datasets from long-term smokers with (LTS+; n=4; triangles) and without COPD (LTS−; n=9; squares) in *Material 2* showing correlations between (**A**) large complexes of MUC1 and MUC5AC; (**B**) large complexes of MUC1 and MUC5B; (**C**) total leukocyte count per subject and MUC1 (**C**) in BAL samples. Open (white) symbols denote subjects with bacterial colonization whereas solid (black) symbols denote subjects without bacterial colonization. Analytical statistics are shown according to Spearman's rank order correlation.

BAL samples from all LTS. Taken together, these observations are in line with the previously suggested association of MUC5AC with acute lung injury, neutrophil trafficking and inflammation [28].

From previous studies, it is known that the mucins MUC5AC and MUC5B constitute the major secretory mucins in the airways. It is also known that both these mucins form large oligomeric complexes and although the complexes are normally large, they can become even larger in chronic inflammatory airway disease, such as asthma [29]. We therefore quantified the mucins in processed (filtration and centrifugation) and unprocessed (no filtration and no centrifugation) BAL samples for each of the three study groups in the one material where this was possible (*Material 2)*. From a methodological point-of-view, our results clearly suggest that the combination of centrifugation and filtration with a large mesh does affect the content of mucins in a detrimental manner. To us, the most obvious explanation for this is that the large mucin complexes are blocked by the filter membrane and/or lost due to centrifugation; a mild centrifugation step that was originally designed to precipitate large objects such as cells. Notably, in some samples, the processing of BAL samples removed as much as 95% of the mucins, by average 77% of MUC5AC, 47% of MUC5B and 5 % of MUC1. These observations highlight the importance of awareness of the impact of this type of processing of BAL samples in future studies of the airways. Given the outcome of our comparison between processed and unprocessed samples, we suspect that the 'methodological confounders' filtering and centrifugation may account for

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the discrepancy between some of our current findings and previous findings [18]. Furthermore, our methodological approach allowed the identification of the proportion of mucins present in large complexes, a factor of potential mechanistic importance for the clearance of mucus. In addition, the qualitative similarities in group-related differences for the level of MUC5AC in *Material 1* and *Material 2* do support that the results obtained in *Material 2* are representative, despite of the limited sample size of this material.

Interestingly, the level of MUC5B tended to follow a similar trend to that of MUC5AC, albeit the group-related differences were not statistically significant in our study, presumably due to limitations in statistical power. In a previous publication, it was reported that the total amount of mucin and goblet cells of the bronchiolar epithelium in peripheral airways were similar in HNSs, smokers and patients with COPD [15]. However, the investigators concluded that the presence of intraluminal mucin was more frequent among the patients with COPD and that the predominant intraluminal mucin was MUC5B, which was also more frequent among the patients with COPD. Given that there was more historic smoking (i.e., higher tobacco load) in the group of patients with COPD than in the group of smokers (without COPD), it seems feasible that tobacco load *per se* was a confounder that contributed to the results in the referred study. Moreover, tethering of MUC5AC to epithelial mucous cells can lead to the accumulation of luminal mucins in the airways of patients with the chronic inflammatory airway disorder asthma and this impairs mucociliary clearance [30]. Collectively, these results suggest that it may not solely be the quantity of mucin produced but also the quality and clearance of mucus from the lumen that constitute keys to an improved understanding pathology in chronic inflammatory disorders of the lungs.

When addressing associations with potential regulatory factors in our current study, we detected a positive correlation between the level of MUC5B large complexes in BAL samples and the level of serum cotinine in samples from all LTS. Given that cotinine is a relatively short-lived metabolite of nicotine, this forward current smoking as a potential regulatory factor; in line with clinical data suggesting that current smoking drives chronic bronchitis [31]. Given our observation, it is interesting to note that nicotine bears the mechanistic potential to hinder post-exocytotic swelling and hydration, leading to higher viscosity and enhanced self-aggregation of the mucin polymer [32].

Currently, a common perception is that the main role of both MUC5AC and MUC5B is to facilitate mucociliary clearance and that MUC5B is required to maintain immune homeostasis and antibacterial host defense [33]. For example, the trachea is cleaned by MUC5B mucin bundles coated with MUC5AC and mice lacking Muc5b have inflammatory infiltrates and bacteria, such as *Stapylococcus aureus* in the lower airways [14,34]. The results from our current study support the role of antimicrobial host defense given the positive correlation between the level of MUC5B and the quantity of any bacteria present in lower airway samples from the pooled group of all LTS displaying growth of bacteria. This is in line with previous observations, showing that microbial products and inflammatory cytokines can cause a massive discharge of mucins [35]. In addition, published datasets indicate that microbes affect goblet cell dynamics and the mucus layer directly via local release of bioactive factors, in addition to an indirect effect via the activation of leukocytes [19,35,36]. In this context, it is interesting that a previous publication on BAL fluid from COPD patients, with and without colonization by bacterial pathogens, indicated no difference in the levels of MUC5AC and MUC5B [24]. However, in that study, current and former smokers were mixed, so current smoking was not specifically addressed, and there was no matching for anti-inflammatory treatment with steroids either. Moreover, similar reservations can be forwarded against the claims that there is a high level of MUC2 in the sputum and BAL samples of COPD patients, with a lower amount of MUC2 in the colonized group. In addition, the referred results regarding MUC2 should be considered with caution due to concerns with potential cross-reactivity of antibodies in the quantification assay with other mucins [23]. In fact, most recent studies suggest that there is very little or no MUC2 in human airways [10,26]. The latter is in line with our current finding of a very low level of MUC2 in the BAL samples, with no clear difference between study groups or in relation to the presence of local bacteria.

It is widely accepted that cell-surface mucins play an important role in mucosal host defense since they constitute both a barrier and a messenger. Notably, these intriguing filamentous molecules extend further than most other cell surface structures [35]. Moreover, several membrane-bound mucins are produced in mammalian airways, including MUC1, a mucin that has been shown to act as a releasable decoy that limits the pathogen's access to epithelial cells in the stomach, as indicated in human cells *in vitro* and knockout mice *in vivo* [12]. In addition, it has been reported that there is decreased mRNA for MUC1 in lung tissue, bronchial epithelial cells and neutrophil samples from smokers and clinically stable patients with COPD compared with HNSs [37]. In contrast, in other studies, enhanced MUC1 levels have been detected in airway epithelial cells and sputum samples from patients with clinically stable COPD compared with the corresponding samples from HNSs [38]. This has also been the case for sputum samples from patients with exacerbations of COPD [39]. These differences may be explained by the high degree of post-translational regulation of mucins since they mainly comprise carbohydrate, and as a consequence, the levels of mucin mRNA levels do not reflect mucin glycoprotein levels [36,40]. In our current study, we detected an increased level of MUC1 in the

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LTS- group and a trend toward a similar increase in the LTS+ group, compared with the HNS group, in the samples from *Material 2*. Of note here, compared with MUC5AC and MUC5B, MUC1 is a smaller, non-oligomerizing mucin [12,35]. Given this, the quantification of MUC1 is much less likely to be affected by filtering of BAL samples, which presumably made it possible to detect increased MUC1 levels in both LTS groups in the filtered samples from *Material 1*.

Several studies provide evidence of the anti-inflammatory role of MUC1 in the airways [41,42]. Thus, murine *Muc1* can attenuate epithelial inflammation and injury in response to *Pseudomonas aeruginosa* [13]. Moreover, there is both clinical and experimental evidence that the level of MUC1 correlates with the severity of airway inflammation [39] as well as with the levels of TNF-α [43] and neutrophil elastase [44]. In our study, the level of MUC1 correlated in a positive manner with total leukocyte count per subject in BAL samples. This is a finding compatible with luminal MUC1 originating from local leukocytes responding to inflammatory stimuli or inflammatory mediators from leukocytes increasing the epithelial shedding of MUC1 [12].

Given that we performed our current pilot study on two clinical materials of modest sample size, it could be argued that variations in these limited materials due to mere chance would prevent us from making compatible findings. However, even if there was some variation in the quantitative outcomes of *Material 1* and *Material 2*, such as the BAL leukocyte counts, these outcomes displayed quite similar patterns for group-related differences from a qualitative point-of-view, including the trends toward group-related differences in BAL neutrophil and eosinophil counts. Clearly, these variations were within what could be expected given the previous literature [45–47].

In conclusion, the present study forwards evidence that both historic and current exposure to tobacco smoke constitute regulatory factors determining the quality and/or quantity of mucins in the peripheral airway lumen of humans. In addition, the study forwards evidence that bacteria, leukocytes and proteinases may also constitute regulatory factors. Thus, these factors may represent targets for future therapy of pathological alterations in airway mucins, possibly regardless of COPD. The present study also indicates that a correct understanding of the pathological implications for mucins in long-term tobacco smokers requires the analysis of mucins in unprocessed BAL samples, allowing large gel forming mucins to be taken into account. We think that our novel findings motivate verification in larger study materials and a further elucidation of the underlying pathogenic mechanisms in translational models.

Clinical perspectives

- Mucins have been attributed a pathogenic role in COPD but there is an incomplete understanding of how mucins relate to potential regulatory factors in current tobacco smokers with and without COPD but yet a similar exposure history.
- The current results show that not only the quantity of specific mucins but also the proportion of large mucin complexes in the peripheral airways relate to tobacco smoking *per se* and is suggestive that local bacteria and proteinases, as well as systemic leukocytes, exert an impact too. Moreover, critical information on these mucins can be obtained by comparing processed and unprocessed samples.
- The most important clinical implication is that smoking *per se,* rather than COPD alone, accounts for important alterations in mucins located in the peripheral airway lumen.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

Authors A.L., I.Q., M.P. and S.K.L. outlined the clinical and experimental study concepts. The study subjects were identified and examined by authors A.A., A.-S.L., B.D., H.A., I.Q., K.L., L.P. and S.T. The clinical samples were collected and investigated by A.S.L., L.P., M.P., P.P., K.C., M.S., S.T. and B.L. Author M.P. performed the main bulk of mucin analyses with supervision by S.K.L. The proteinase analyses were conducted by S.B. The analysis of neutrophil elastase was organized and supervised by S.Y. Authors M.P., P.P., M.S. and B.L. performed the additional laboratory analyses and summarized all datasets. All authors reviewed the completed datasets. The manuscript was outlined, completed and revised by M.P., S.K.L. and A.L. All authors critically reviewed and approved the manuscript prior to its final submission.

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Abbreviations

BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DLCO, gas diffusion capacity; FEV_1, forced expiratory volume during 1 s; FVC, forced vital capacity; HNS, healthy non-smoker; LTS, long-term smoker; MMP-14, matrix metalloproteinase 14; PBS, phosphate-buffered saline.

References

1 https://goldcopd.org/ Global initiative for chronic obstructive lung disease. Accessed online: 14 April 2020
2 Backman, H., Eriksson, B., Ronmark, E., Hedman, L., Stridsman, C., Jansson, S.A. et al. (2016) Decreased prevalence of moderate to severe COPD over 15 years in northern Sweden. *Respir. Med.* **114**, 103–110, https://doi.org/10.1016/j.rmed.2016.03.013
3 Pesci, A., Balbi, B., Majori, M., Cacciani, G., Bertacco, S., Alciato, P. et al. (1998) Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* **12**, 380–386, https://doi.org/10.1183/09031936.98.12020380
4 Celli, B.R., MacNee, W. and Force, A.E.T. (2004) Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur. Respir. J.* **23**, 932–946, https://doi.org/10.1183/09031936.04.00014304
5 Murray, C.J. and Lopez, A.D. (1997) Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet* **349**, 1436–1442, https://doi.org/10.1016/S0140-6736(96)07495-8
6 Lange, P., Nyboe, J., Appleyard, M., Jensen, G. and Schnohr, P. (1990) Relation of ventilatory impairment and of chronic mucus hypersecretion to mortality from obstructive lung disease and from all causes. *Thorax* **45**, 579–585, https://doi.org/10.1136/thx.45.8.579
7 Annesi, I. and Kauffmann, F. (1986) Is respiratory mucus hypersecretion really an innocent disorder? A 22-year mortality survey of 1,061 working men. *Am. Rev. Respir. Dis.* **134**, 688–693
8 Vestbo, J., Prescott, E. and Lange, P. (1996) Association of chronic mucus hypersecretion with FEV_1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group. *Am. J. Respir. Crit. Care Med.* **153**, 1530–1535
9 Guerra, S., Sherrill, D.L., Venker, C., Ceccato, C.M., Halonen, M. and Martinez, F.D. (2009) Chronic bronchitis before age 50 years predicts incident airflow limitation and mortality risk. *Thorax* **64**, 894–900, https://doi.org/10.1136/thx.2008.110619
10 Kirkham, S., Kolsum, U., Rousseau, K., Singh, D., Vestbo, J. and Thornton, D.J. (2008) MUC5B is the major mucin in the gel phase of sputum in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **178**, 1033–1039, https://doi.org/10.1164/rccm.200803-391OC
11 Okuda, K., Chen, G., Subramani, D.B., Wolf, M., Gilmore, R.C., Kato, T. et al. (2019) Localization of secretory mucins MUC5AC and MUC5B in normal/healthy human airways. *Am. J. Respir. Crit. Care Med.* **199**, 715–727, https://doi.org/10.1164/rccm.201804-0734OC
12 Linden, S.K., Sheng, Y.H., Every, A.L., Miles, K.M., Skoog, E.C., Florin, T.H. et al. (2009) MUC1 limits *Helicobacter pylori* infection both by steric hindrance and by acting as a releasable decoy. *PLoS Pathog.* **5**, e1000617, https://doi.org/10.1371/journal.ppat.1000617
13 Umehara, T., Kato, K., Park, Y.S., Lillehoj, E.P., Kawauchi, H. and Kim, K.C. (2012) Prevention of lung injury by Muc1 mucin in a mouse model of repetitive *Pseudomonas aeruginosa* infection. *Inflamm. Res.* **61**, 1013–1020, https://doi.org/10.1007/s00011-012-0494-y
14 Roy, M.G., Livraghi-Butrico, A., Fletcher, A.A., McElwee, M.M., Evans, S.E., Boerner, R.M. et al. (2014) Muc5b is required for airway defence. *Nature* **505**, 412–416, https://doi.org/10.1038/nature12807
15 Caramori, G., Di Gregorio, C., Carlstedt, I., Casolari, P., Guzzinati, I., Adcock, I.M. et al. (2004) Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. *Histopathology* **45**, 477–484, https://doi.org/10.1111/j.1365-2559.2004.01952.x
16 Kesimer, M., Ford, A.A., Ceppe, A., Radicioni, G., Cao, R., Davis, C.W. et al. (2017) Airway mucin concentration as a marker of chronic bronchitis. *N. Engl. J. Med.* **377**, 911–922, https://doi.org/10.1056/NEJMoa1701632

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17 Fernandez-Blanco, J.A., Fakih, D., Arike, L., Rodriguez-Pineiro, A.M., Martinez- Abad, B., Skansebo, E. et al. (2018) Attached stratified mucus separates bacteria from the epithelial cells in COPD lungs. *JCI Insight* **3**, https://doi.org/10.1172/jci.insight.120994
18 Baraniuk, J.N., Casado, B., Pannell, L.K., McGarvey, P.B., Boschetto, P., Luisetti, M. et al. (2015) Protein networks in induced sputum from smokers and COPD patients. *Int. J. Chron. Obstruct. Pulmon. Dis.* **10**, 1957–1975, https://doi.org/10.2147/COPD.S75978
19 Quintana-Hayashi, M.P., Navabi, N., Mahu, M., Venkatakrishnan, V., Fernandez, H.R., Haesebrouck, F. et al. (2017) Neutrophil elastase and IL17 expressed in the pig colon during Brachyspira hyodysenteriae infection synergistically with the pathogen induce increased mucus transport speed and production via MAPK3. *Infect. Immun.* **85** (8)), https://doi.org/10.1128/IAI.00262-17
20 Deshmukh, H.S., Shaver, C., Case, L.M., Dietsch, M., Wesselkamper, S.C., Hardie, W.D. et al. (2008) Acrolein-activated matrix metalloproteinase 9 contributes to persistent mucin production. *Am. J. Respir. Cell Mol. Biol.* **38**, 446–454, https://doi.org/10.1165/rcmb.2006-0339OC
21 Nadel, J.A. (1991) Role of enzymes from inflammatory cells on airway submucosal gland secretion. *Respiration* **58**, 3–5, https://doi.org/10.1159/000195961
22 Kim, Y.D., Bae, C.H., Song, S.Y. and Choi, Y.S. (2015) Effect of beta-glucan on MUC4 and MUC5B expression in human airway epithelial cells. *Int. Forum Allergy Rhinol.* **5**, 708–715
23 Dickey, B.F., Fahy, J.V., Kesimer, M., Boucher, R.C., Evans, C.M. and Thornton, D. (2016) Measuring airway Mucin 2 in patients with severe chronic obstructive pulmonary disease with bacterial colonization. *Ann. Am. Thorac. Soc.* **13**, 2103–2104, https://doi.org/10.1513/AnnalsATS.201607-532LE
24 Sibila, O., Garcia-Bellmunt, L., Giner, J., Rodrigo-Troyano, A., Suarez-Cuartin, G., Torrego, A. et al. (2016) Airway Mucin 2 is decreased in patients with severe chronic obstructive pulmonary disease with bacterial colonization. *Ann. Am. Thorac. Soc.* **13**, 636–642, https://doi.org/10.1513/AnnalsATS.201512-797OC
25 Henke, M.O., John, G., Germann, M., Lindemann, H. and Rubin, B.K. (2007) MUC5AC and MUC5B mucins increase in cystic fibrosis airway secretions during pulmonary exacerbation. *Am. J. Respir. Crit. Care Med.* **175**, 816–821, https://doi.org/10.1164/rccm.200607-1011OC
26 Kirkham, S., Sheehan, J.K., Knight, D., Richardson, P.S. and Thornton, D.J. (2002) Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem. J.* **361**, 537–546, https://doi.org/10.1042/bj3610537
27 Young, H.W., Williams, O.W., Chandra, D., Bellinghausen, L.K., Perez, G., Suarez, A. et al. (2007) Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5′ elements. *Am. J. Respir. Cell Mol. Biol.* **37**, 273–290, https://doi.org/10.1165/rcmb.2005-0460OC
28 Koeppen, M., McNamee, E.N., Brodsky, K.S., Aherne, C.M., Faigle, M., Downey, G.P. et al. (2013) Detrimental role of the airway mucin Muc5ac during ventilator-induced lung injury. *Mucosal Immunol.* **6**, 762–775, https://doi.org/10.1038/mi.2012.114
29 Bonser, L.R. and Erle, D.J. (2017) Airway mucus and asthma: the role of MUC5AC and MUC5B. *J. Clin. Med.* **6**, https://doi.org/10.3390/jcm6120112
30 Bonser, L.R., Zlock, L., Finkbeiner, W. and Erle, D.J. (2016) Epithelial tethering of MUC5AC- rich mucus impairs mucociliary transport in asthma. *J. Clin. Invest.* **126**, 2367–2371, https://doi.org/10.1172/JCI84910
31 Kim, V., Zhao, H., Boriek, A.M., Anzueto, A., Soler, X., Bhatt, S.P. et al. (2016) Persistent and newly developed chronic bronchitis are associated with worse outcomes in chronic obstructive pulmonary disease. *Ann. Am. Thorac. Soc.* **13**, 1016–1025, https://doi.org/10.1513/AnnalsATS.201512-800OC
32 Chen, E.Y., Sun, A., Chen, C.S., Mintz, A.J. and Chin, W.C. (2014) Nicotine alters mucin rheological properties. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **307**, L149–L157, https://doi.org/10.1152/ajplung.00396.2012
33 Livraghi-Butrico, A., Grubb, B.R., Wilkinson, K.J., Volmer, A.S., Burns, K.A., Evans, C.M. et al. (2017) Contribution of mucus concentration and secreted mucins Muc5ac and Muc5b to the pathogenesis of muco-obstructive lung disease. *Mucosal Immunol.* **10**, 395–407, https://doi.org/10.1038/mi.2016.63
34 Ermund, A., Meiss, L.N., Rodriguez-Pineiro, A.M., Bahr, A., Nilsson, H.E., Trillo- Muyo, S. et al. (2017) The normal trachea is cleaned by MUC5B mucin bundles from the submucosal glands coated with the MUC5AC mucin. *Biochem. Biophys. Res. Commun.* **492**, 331–337, https://doi.org/10.1016/j.bbrc.2017.08.113
35 Linden, S.K., Sutton, P., Karlsson, N.G., Korolik, V. and McGuckin, M.A. (2008) Mucins in the mucosal barrier to infection. *Mucosal Immunol.* **1**, 183–197, https://doi.org/10.1038/mi.2008.5
36 Sharba, S., Navabi, N., Padra, M., Persson, J.A., Quintana-Hayashi, M.P., Gustafsson, J.K. et al. (2019) Interleukin 4 induces rapid mucin transport, increases mucus thickness and quality and decreases colitis and Citrobacter rodentium in contact with epithelial cells. *Virulence* **10**, 97–117, https://doi.org/10.1080/21505594.2019.1573050
37 Milara, J., Diaz-Platas, L., Contreras, S., Ribera, P., Roger, I., Ballester, B. et al. (2018) MUC1 deficiency mediates corticosteroid resistance in chronic obstructive pulmonary disease. *Respir. Res.* **19**, 226, https://doi.org/10.1186/s12931-018-0927-4
38 Ishikawa, N., Mazur, W., Toljamo, T., Vuopala, K., Ronty, M., Horimasu, Y. et al. (2011) Ageing and long-term smoking affects KL-6 levels in the lung, induced sputum and plasma. *BMC Pulmon. Med.* **11**, 22, https://doi.org/10.1186/1471-2466-11-22
39 Zheng, Z., Qi, Y., Xu, X., Jiang, H., Li, Z., Yang, Q. et al. (2017) Sputum mucin 1 is increased during the acute phase of chronic obstructive pulmonary disease exacerbation. *J. Thorac. Dis.* **9**, 1873–1882, https://doi.org/10.21037/jtd.2017.06.63
40 Gustafsson, J.K., Navabi, N., Rodriguez-Pineiro, A.M., Alomran, A.H., Premaratne, P., Fernandez, H.R. et al. (2013) Dynamic changes in mucus thickness and ion secretion during Citrobacter rodentium infection and clearance. *PLoS ONE* **8**, e84430, https://doi.org/10.1371/journal.pone.0084430
41 Li, Y., Dinwiddie, D.L., Harrod, K.S., Jiang, Y. and Kim, K.C. (2010) Anti-inflammatory effect of MUC1 during respiratory syncytial virus infection of lung epithelial cells in vitro. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **298**, L558–L563, https://doi.org/10.1152/ajplung.00225.2009
42 McAuley, J.L., Corcilius, L., Tan, H.X., Payne, R.J., McGuckin, M.A. and Brown, L.E. (2017) The cell surface mucin MUC1 limits the severity of influenza A virus infection. *Mucosal Immunol.* **10**, 1581–1593, https://doi.org/10.1038/mi.2017.16
43 Choi, S., Park, Y.S., Koga, T., Treloar, A. and Kim, K.C. (2011) TNF-α is a key regulator of MUC1, an anti-inflammatory molecule, during airway Pseudomonas aeruginosa infection. *Am. J. Respir. Cell Mol. Biol.* **44**, 255–260, https://doi.org/10.1165/rcmb.2009-0323OC
44 Kuwahara, I., Lillehoj, E.P., Hisatsune, A., Lu, W., Isohama, Y., Miyata, T. et al. (2005) Neutrophil elastase stimulates MUC1 gene expression through increased Sp1 binding to the MUC1 promoter. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **289**, L355–L362, https://doi.org/10.1152/ajplung.00040.2005

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45 Karlicic, K. (2014) Cellular composition of bronchoalveolar lavage (BAL) as inflammation indicator in patients with chronic obstructive pulmonary disease (COPD). *Eur. Respir. J.* **44**, P70
46 Hodge, S.J., Hodge, G.L., Holmes, M. and Reynolds, P.N. (2004) Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. *Cytometry B Clin. Cytom.* **61**, 27–34, https://doi.org/10.1002/cyto.b.20020
47 Wen, Y., Reid, D.W., Zhang, D., Ward, C., Wood-Baker, R. and Walters, E.H. (2010) Assessment of airway inflammation using sputum, BAL, and endobronchial biopsies in current and ex-smokers with established COPD. *Int. J. Chron. Obstruct. Pulmon. Dis.* **5**, 327–334

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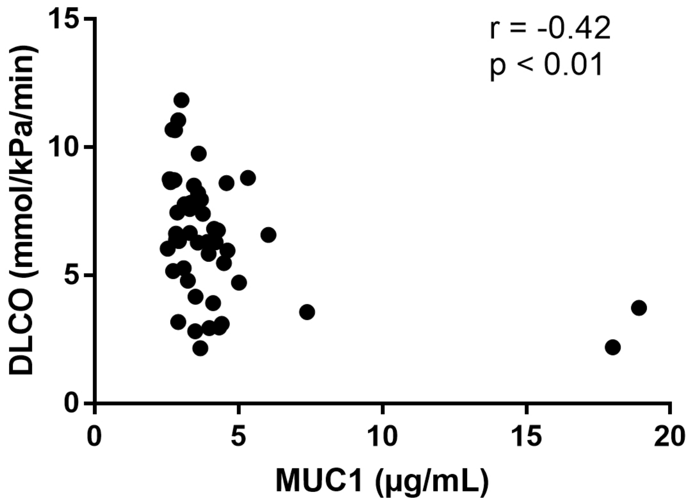
r = -0.42
p < 0.01
DLCO (mmol/kPa/min)
MUC1 (µg/mL)
0
5
10
15
20

Figure S1. Association of MUC1 in bronchoalveolar lavage (BAL) fluid with DLCO for all study subjects in *Material 1*, including long-term smokers with (LTS+) and without (LTS-) COPD as well as healthy non-smokers (HNS). Analytical statistics are shown according to Spearman's rank order correlation.

Table S1

Parameter [median (range)]		*Material 1*	
A	**HNS**	**LTS-**	**LTS+**
Number of subjects	20	20	16
Gender (M/F)	14/6	10/10	5/11
Age (years)	54 (41-72)	54 (41-66)	62 (48-73)
BMI	21 (20-31)	24 (20-33)	24 (19-30)
Current smoking (cigarettes/day)	0 (0-0)	5 (5-20)	20 (2-35)
Pack-years (years)	0 (0-0)	35 (5-60)	40 (30-60)
FVC	4.66 (2.74-7.72)	4.16 (2.8-7.3)	2.73 (1.39-5.33)
FVC (% predicted)	116 (85-157)	109 (86-146)	93 (63-128)
FEV1	3.58 (2.2-4.85)	3.03 (1.97-5.16)	1.62 (0.94-3.06)
FEV1 (% predicted)	111 (75-142)	96 (72-120)	63 (43-91)
FEV1/FVC	76 (56-86)	72 (61-82)	55 (45-68)
DLCO (mmol/kPa/min)	8,51 (5.85-11.84)	6.65 (3.92-8.89)	3.57 (2.19-7.59)
DLCO (% predicted)	90 (67-124)	75 (50-90)	45 (27-78)

		Material 2	
B	**HNS**	**LTS-**	**LTS+**
Number of subjects	5	9	4
Gender (M/F)	4/1	4/5	2/2
Age (years)	45 (41-66)	67 (45-72)	57 (52-64)
BMI	24 (22-29)	24 (22-26)	25 (19-30)
Current smoking (cigarettes/day)	0 (0-0)	10 (10-40)	14 (8-20)
Pack-years (years)	0 (0-0)	38 (21-51)	32.5 (20-40)
FVC	5.7 (3.5-7.0)	3.2 (2.5-5.4)	4.4 (3.5-6.3)
FVC (% predicted)	123 (109-127)	116 (74-125)	119 (97-130)
FEV1	4.7 (2.6-5.1)	2.4 (2.0-3.9)	2.9 (2.1-3.2)
FEV1 (% predicted)	108 (100-127)	100 (68-116)	81 (75-105)
FEV1/FVC	74 (68-83)	73 (70-82)	61 (50-68)
DLCO (mmol/kPa/min)	9.5 (6.0-10.2)	6.1 (4.8-8.9)	7.7 (5.6-10.0)
DLCO (% predicted)	83 (68-115)	79 (62-88)	75 (70-98)

Footnote.
HNS = healthy non-smokers
LTS- = long-term smokers without COPD
LTS+ = long-term smoker with COPD

Table S2

Material 2

Parameter, median (range)	Study group		
	HNS	LTS-	LTS+
Number of subjects	5	9	4
Blood Neutrophil (million cell/mL)	2.85 (2.3-3.6)	3.55 (2.8-6)	4.3 (2.7-4.6)
Blood Lymphocyte (million cell/mL	1.8 (1.2-2.8)	2.05 (1.5-2.9)	2.6 (1.8-3.2)
Blood Monocyte (million cell/mL)	0.4 (0.2-0.5)	0.45 (0.3-0.6)	0.5 (0.3-0.7)
Blood Eosinophil (million cell/mL)	0.2 (0.1-0.4)	0.2 (0.1-0.4)	0.3 (0.1-0.6)
Blood Basophil (million cell/mL)	0 (0-0)	0 (0-0.1)	0.1 (0-0.1)

Footnote.

HNS = healthy non-smokers

LTS- = long-term smokers without COPD

LTS+ = long-term smoker with COPD

SUPPLEMENT TO:

Increased MUC1 plus a larger quantity and complex size for MUC5AC in the peripheral airway lumen of long-term tobacco smokers

Médea Padra, Anders Andersson, Bettina Levänen, Pushpa Premaratne, Helga Asgeirsdottir, Sara Tengvall, Karin Christenson, Marit Stockfelt, Steven Bozinovski, Shigemi Yoshihara, Ann-Sofie Lantz, Lena Palmberg, Barbro Dahlén, Kjell Larsson, Ingemar Qvarfordt, Sara K. Lindén and Anders Lindén

MATERIALS AND METHODS

Human study populations

<u>Clinical protocols.</u> The study subjects of *Material 1* included healthy non-smokers (HNS), long-term tobacco smokers with (LTS+) and without (LTS-) chronic obstructive pulmonary disease (COPD). These subjects were recruited either through an advertisement in a regional newspaper or via the outpatient clinic of the Department of Respiratory Medicine and Allergy, Karolinska University Hospital in Stockholm. Details of the clinical protocol for this study material have been published elsewhere (1-3). Briefly, each subject attended a first visit for clinical characterization including clinical history, medical examination, lung function including dynamic spirometry with reversibility test and gas diffusion capacity (4-6), radiology of the chest (common X-ray), electrocardiography (ECG) and results from standard blood tests, analyzed at the Department of Clinical chemistry at Karolinska University Hospital. These investigations were performed prior to bronchoscopy. As inclusion criteria for all subjects, we required a negative history of atopy. For all the subjects of the LTS+ and the LTS- group (all LTS), we required a historic exposure to tobacco smoke corresponding to a tobacco load of ≥ 5 pack-years and current smoking. For the LTS- group, we required a ventilatory lung capacity with a FEV_1/FVC ratio of ≥ 0.7 and a FEV_1 % predicted of ≥ 80%, determined with dynamic spirometry as previously described (1-3). For this study group, a history of lung disease constituted an exclusion criterion. In contrast, for the LTS+ group, we required a post bronchodilatory FEV_1/FVC ratio of < 0.7 in accordance with GOLD criteria (4). For all LTS, any lung disease other than COPD, chronic bronchitis or emphysema constituted exclusion criteria. The inclusion criteria for clinical pharmacotherapy (medication) have been published elsewhere (1-3). For reasons of safety during bronchoscopy, an FEV_1% predicted of ≥ 40% post bronchodilation was required for subjects in the LTS+ group. For the subjects in the HNS group, we required that they were non-smokers and lacked history of lung disease of any kind. These

control subjects were required to have a FEV_1 of ≥ 80% of the predicted value and a FEV_1/FVC ratio of ≥ 70% after bronchodilation, determined with dynamic spirometry. For all subjects, the gas diffusion capacity was recorded but it did not constitute an inclusion/exclusion criterion. Clinical or laboratory signs of infection within four weeks prior to bronchoscopy resulted in a re-scheduled investigation. For reasons of clinical safety, a transcutaneous arterial oxygen saturation (SaO_2) of ≥ 92% was required for all included subjects prior to bronchoscopy. For the *Material 1* only, certain data sets, including data on demography, clinical and BAL sample characteristics, have been published elsewhere (2, 3). Here, the principal clinical characteristics for the study subjects included in the current study only are presented in **Table S1A**.

The subjects of the HNS, LTS- and LTS+ group of *Material 2* were recruited through an advertisement in a regional newspaper or via the outpatient clinic of Respiratory Medicine, Sahlgrenska University Hospital in Gothenburg. Each subject attended a first visit for recording of clinical history, medical examination, standard blood tests including coagulation parameters, hemoglobin and leukocyte counts, a pregnancy test when applicable, a standard panel of specific IgE (see below), virus serology (HIV, hepatitis B and C), all analyzed at the Department of Clinical Chemistry and Department of Microbiology, Sahlgrenska University Hospital. Each subject also underwent investigation of ventilatory lung function with dynamic spirometry including reversibility test (7) and assessment of gas diffusion capacity (8), as well as electrocardiography (ECG) at the Department of Clinical Physiology at Sahlgrenska University Hospital, and, finally, radiology of the chest (common pulmonary X-ray) at the Department of Radiology at Sahlgrenska University Hospital. All these investigations were performed prior to bronchoscopy. As inclusion criteria, we required a negative history of atopy as well as a negative screening test for specific IgE against inhaled allergens (Phadiatop®, Phadia™, Uppsala, Sweden). Chronic diseases other than treated and controlled depression, epilepsy,

heart failure or hypertension, substituted hypothyreosis, treated osteoporosis and past cancer (successfully treated >5 years ago) constituted exclusion criteria.

For *Material 2*, all included subjects displayed a negative virus serology. No included subject was pregnant. To be included, the subjects were allowed a maximum of three (3) respiratory infections the last year prior to the study inclusion. All the smokers in the LTS group were current smokers that were required to have a historic exposure to tobacco smoke corresponding to a tobacco load of ≥ 20 pack-years and to have smoked ≥ 5 cigarettes per day during the last 5 years. Current smoking was recorded (cigarettes per day) during the screening visit. For the subjects in the LTS- group, we required an FEV_1/FVC ratio of ≥ 70% and a FEV_1 of >80% of predicted after bronchodilation (3 doses of inhaled terbutaline; 0.5 mg/dose; using Bricanyl Turbuhaler^{TM}, AstraZeneca Ltd, Södertälje, Sweden), as determined with dynamic spirometry. For all LTS, the subjects were allowed to have chronic bronchitis as defined by standard criteria (4); this was merely recorded. In accordance with established criteria, the subjects in the LTS+ group were required to have a post-bronchodilatory FEV_1/FVC ratio < 70% (4), corresponding to GOLD stage I-III. For the LTS+ group, any lung disease other than COPD, chronic bronchitis or emphysema constituted exclusion criteria. For inclusion, inhaled short-acting but not long-acting bronchodilators were allowed in the LTS+ group only. No subject was treated with inhaled or oral glucocorticoids (steroids) during the course of the study. For reasons of clinical safety during bronchoscopy, these subjects were also required to have a post-bronchodilatory FEV_1 ≥ 1.0L, a gas diffusion capacity of ≥ 50% predicted and a transcutaneous SaO_2 of ≥ 93%. The subjects of the HNS group had to be never-smokers without any history of occasional smoking or lung disease. These subjects were required to have a normal ventilatory lung capacity, with a post-bronchodilatory FEV_1/FVC ratio of ≥ 70% and a FEV_1 of > 80% of predicted; with one exception. One subject included in the HNS group displayed a FEV_1/FVC ratio of 68% and a FEV_1% predicted of 106%. However, this subject had large absolute lung

volumes and displayed no signs or history arguing for disease (apart from the spirometry results). For all subjects, gas diffusion capacity was recorded but the results did not constitute an inclusion criterion. Clinical or laboratory signs of infection within four weeks prior to bronchoscopy resulted in a re-scheduled investigation. No results from this material (*Material 2)* have been published previously. The principal clinical characteristics for the study subjects included in the current study are presented in **Table S1B**.

Bronchoscopy

For both *Material 1* and *Material 2*, the bronchoscopy investigation was performed by an experienced pulmonologist during stable clinical conditions, in accordance with clinical routine at Karolinska and Sahlgrenska University Hospital, respectively.

Material 1. For this material, the bronchoscopy procedure has previously been described in detail (1-3). In summary, the study subject obtained premedication with morphine scopolamine and local anaesthesia with lignocaine. The bronchoscopy was performed primary via the nasal route using a flexible bronchoscope. The bronchoscope was positioned and wedged in a sub-segmental bronchus (middle lobe). The BAL was then performed by instilling five aliquots of 50 mL (a total of 250 mL) sterile phosphate-buffered saline (PBS with pH of 7.4) at 37°C. The BAL samples were aspirated after every 50 mL portion, in a sterile and siliconized plastic bottle and kept on ice until transportation to the laboratory for processing.

Material 2. For this material, a peripheral venous blood sample of totally 60 mL was drawn immediately before the bronchoscopy. The study subject then obtained premedication with ketobemidone hydrocloride (Ketogan™; < 7.5 mg intramuscularily or < 5 mg intravenously; Apoteket, Solna, Sweden) and anaestesia with lignocaine (nebulized Xylocaine™ 10 mg/dose; up to 3 times 2 doses oropharyngeally; Apoteket, Solna, Sweden). Additional local anaestesia (Xylocaine™ 20 mg/mL, < 14 mL) was given as needed through the bronchoscope. The bronchoscopy was performed through the mouth using flexible bronchoscopes of several brands

and models. The bronchoscope was positioned and wedged in a segmental bronchus (mid lobe or lingula). The BAL was performed by instilling three aliquots of 50 mL PBS at 37°C (a total of 150 mL). The BAL samples were aspirated after every 50 mL portion, collected and pooled in a plastic container (SERRES® Polypropylen measuring cup 250 ml, No. 6057257; Mediplast AB, Malmö, Sweden) and kept on ice until transportation to the laboratory for processing.

Bacterial colonization

For the subjects in *Material 2* only, a lower airway sample (a BAL or brush biopsy sample) was secured and sent to the Department of Microbiology at Sahlgrenska University Hospital, for a qualitative and quantitative analysis of the growth of aerobic bacteria in accordance with clinical standard procedures within the accredited laboratory. These samples were also evaluated morphologically to ascertain that they were representative for the lower airways using light microscopy. For representative samples (< 1% squamous epithelial cells), species were determined using MALDI-TOF (if > 100 CFU/mL) for the 10 most common species in each sample. Non-representative samples were examined with reference to bacterial pathogens (> 1,000 CFU/mL) only. The principal bacteriological results are presented in **Table 2**.

Processing of BAL samples

<u>*Material 1*</u>. After arrival to the laboratory, the entire BAL sample yield was filtered through a sterile filter (VWR 732-2758 Cell Sterile Non-Pyrogenic, DNase/RNase-free Nylone Strainer 70 µm; VWR International™, Spånga, Sweden). The filtered BAL sample was then centrifuged (400 g; during 5 minutes (min) at 4°C) to divert extracellular fluid from cells. The obtained supernatant was separated from the cell pellet. The obtained cell-free BAL fluid was frozen (-70°C) until further analysis. The cell pellet was re-suspended in PBS and the cells were counted in a Bürker chamber to determine the concentration of all leukocytes (total leukocyte count). Smears for differential cell counts were prepared with centrifugation (1,000 rpm; 246 g; for 5

min at RT) using a cytocentrifugation machine (Cytospin™ 4, Thermo Fischer Scientific™, Shandon, MA, USA). The obtained preparations were stained using May-Grünwald-Giemsa followed by the counting of the % of different populations of leukocytes (300 cells per sample). Cell viability was assessed in a subset of samples covering all study groups (96.0 (88.0-98.0) %; n=20) using trypan blue exclusion. The basic data on BAL samples from *Material 1* is included in **Table 1A**.

Material 2. After arrival to the laboratory, a 50-100 mL portion of BAL sample yield was filtered (Woven mesh spacers, Dacron® 124 mm diameter, No. AP3212450; Merck Chemicals and Life Science™ AB, Solna, Sweden) to separate debris from the viable leukocytes and fluid in the BAL samples. In addition, from each BAL sample, 2 mL of BAL sample was saved and stored at -80°C without filtering and centrifugation procedure (ie. "unprocessed BAL sample"), to allow assessment of large mucin complexes (see below). This collection of unprocessed BAL samples was unique for *Material 2*. The filtered BAL samples were collected in a plastic tube (Falcon® 50 mL Conical centrifugation tubes; VWR collection, Radnor, PA, USA) and centrifuged (1,400 rpm; 378 g; 10 min at 4°C) in order to separate extracellular fluid from cells. The supernatant was transferred to a new tube and centrifuged (2,000 rpm; 771 g; 10 min at 4°C) to separate debris from the cell-free BAL fluid. The obtained cell-free BAL fluid was collected in new plastic tubes (Falcon 50 mL Conical centrifugation tubes®) and frozen (-80°C) until further analysis. The cell pellet was re-suspended in PBS and the cells were counted in a Bürker chamber to determine the concentration of all leukocytes (total leukocyte count). Smears for differential cell counts were prepared using cytocentrifugation as follows: two times 100 µL cell suspension (600 cells/µL) was loaded to tissue slides and were then subjected to centrifugation (1,000 rpm; 246 g; for 5 min at RT) using a cytocentrifugation machine (Cytospin™ 4, Thermo Fischer Scientific™, Shandon, MA, USA). The slides were dried in air overnight, then wrapped in plastic cling film and stored at -20°C until use. The obtained

cytospin slides were stained using May-Grünwald-Giemsa followed by the counting of subpopulations of leukocytes (200 cells per sample). Cell viability was assessed in a subset of samples covering all study groups (99.7% (97.2-100.0 %); n=9) using Türks solution (Cat. No. 93770, Sigma-Aldrich Sweden AB, Stockholm, Sweden). The basic data on BAL samples from *Material 2* is included in **Table 1B**.

Processing of blood samples

For *Material 2*, a venous blood sample was collected as plasma in four ethylene diamine-tetra-acetic acid tubes (EDTA; 4 mL; #102770195; Hettich Labinstrument AB, Sollentuna, Sweden) vacutainer and as serum in four serum separator tubes with coagulation factors (SST; 10 mL; #104790597; Hettich Labinstrument AB,) tubes, respectively. Blood cell differential counts were determined utilizing an automatic flow cytometer (Advia^{TM} model 2120I, Siemens Healthcare GmbH, Erlangen, Germany) according to clinical routine. The whole blood samples were centrifuged (1,500 rpm; 443 g; 10 min at RT) and the supernatant (plasma or serum) was removed and stored (-80°C) until further analysis.

Quantification and characterization of mucins

A complete protease inhibitor cocktail (Sigma p8340; Sigma-Aldrich^{TM} Inc., St. Louis, MO, USA) was added to the BAL samples (10 µL to 1ml of sample) during thawing. Samples were diluted 1/100 in reduction buffer (6 M GuHCl, 5 mM EDTA, 0.1 M Tris/HCl, pH 8.0). To expose the MUC5B and MUC2 epitopes, the BAL samples were reduced (2 mM 1,4-dithiothreitol, at 37ºC during 1 hour (h)) and then alkylated (5 mM iodoacetamide, during 1 h at RT). The analyses of other mucins were not preceded by reduction and alkylation. Each sample (100 µL) was loaded onto a polyvinylidene difluoride membrane (PVDF transfer membrane; 0.45µm pore size, No. IPVH0010, Millipore^{TM}, Bedford, MA, USA) using a Slot Blot apparatus (Minifold–II, Schleicher & Schuell Bioscience GmbH, Dassel, Germany). In addition, nine serial dilutions of the mucin standards were also loaded (MUC5AC from human

stomach; MUC2 from pig intestine, recombinant MUC1 from cell culture; MUC5B from one BAL sample in *Material 2* (5)). The standard curves used to calculate the level (ie. concentration) of most mucins were prepared from tissues other than that from the airways. Since it is uncertain whether the immunoreactivity and glycosylation are identical between these tissues and airway mucins, the respective level shown in the graphs should be viewed as an estimate for comparisons between groups, and not as an absolute quantification. Vacuum was applied to attach the mucins to the membrane. The membranes were then air-dried (1 h), pre-wetted briefly (100% methanol) and rinsed (ultrapure water) and incubated in phosphate buffered saline (PBS: 0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^3, during 10 min). Membranes to be analyzed for MUC1 were analyzed in duplicates, whereof one of the membranes was subject to periodate oxidation (20 mM periodic acid prepared in 0.05 M acetate buffer, at pH 5.0 during 45 min). After 3 washes (3 min each) in PBS containing 0.1% Tween 20 (PBS-T), incubated in glycine (1%, 30 min), to neutralize acidic groups before being washed again in PBS-T. Unspecific binding was blocked by incubating in Odyssey blocking buffer (LI-COR Biosciences™, Lincoln, NE, USA) for 1 h at 22 ºC. Membranes were then incubated with rabbit serum against MUC5B (LUM5B-2 (6) diluted 1:16,000) or MUC2 (LUM2-3 (7) diluted 1:1,000) or with mouse monoclonal antibodies against MUC5AC (clone 45MI diluted 1:1,000; Sigma-Aldrich™) or MUC1 antibody (BC-2 (8) diluted 1:1,000) in Odyssey blocking buffer containing 0.1% Tween 20 overnight at 4 ºC with gentle shaking. Membranes were washed in PBS-T (4 times 5 min, at 22 ºC). The membranes were thereafter incubated with labeled secondary antibody (Goat anti-rabbit IR dye 680 or Goat anti-mouse IR dye 800 (LI-COR Biosciences™) diluted 1:10,000 in Odyssey blocking buffer (0.1% Tween-20 and 0.01% SDS, during 30 min at 22 ºC) in the dark. Membranes were washed again in PBS-T (four times 5 min) and the blots were subsequently visualized (Odyssey infrared imaging system, LI-COR Biosciences™) and quantified (ImageJ software®, National Institutes of Health, Bethesda, MD,

USA). The size of mucin large complexes (% of total mucin) was calculated based on the difference in the measured integrated density (quantity) of mucin samples between filtered and unfiltered material.

Quantification of C-reactive protein

Concentrations of C-reactive protein (CRP) in serum were determined with a high sensitivity (HS) assay (CRPHS; Roche/Cobas, No. 04628918190; Roche Diagnostics Scandinavia AB, Solna, Sweden) utilizing the agglutination of CRP to latex particles that are subsequently exposed to anti-CRP antibodies and then detected by a turbidimetric analysis instrument (Cobas® 8000 Roche Diagnostics Scandinavia AB), in accordance with the manufacturer's instructions. This analysis was performed at the accredited laboratory of the Department of Clinical Chemistry at Sahlgrenska University Hospital, Gothenburg, Sweden.

Quantification of cotinine

Concentrations of the nicotine metabolite cotinine were determined in serum samples utilizing a competitive immunochemical method (Immulite 2000XPi Nicotine metabolite; No. L2KNM2 (200 test); Siemens Medical Diagnostic™, Siemens Healthcare, Upplands-Väsby, Sweden) with detection by a chemiluminescence instrument (Immulite 2000 XPi®; Siemens Medical Diagnostic™), as recommended by the manufacturer. The detection limit for cotinine is 10 pg/mL. This analysis was performed at the accredited laboratory of the Department of Clinical Chemistry at Sahlgrenska University Hospital, Gothenburg, Sweden.

Quantification of net gelatinase and serine proteinase activity

The methods for quantification of net gelatinase and serine proteinase activity using substrate assays have been described elsewhere (9-11). In summary, fluorometric substrates were used to measure net gelatinase and serine proteinase (elastase) activity in the bronchoalveolar lavage (BAL) fluid as previously described, where the increase in fluorescence is proportional to

proteolytic activity. Briefly, the net gelatinase or elastase activity was quantified using fluorescein-labeled DQ gelatin or elastin EnzChek® molecular probes respectively (Thermo Fisher Scientific™, Waltham, MA USA). The substrates were incubated with cell-free BAL fluid (during 16 h at 37 ºC) and the fluorescence intensity was measured using a multimode microplate reader (CLARIOStar®; BMG Labtech Pty. Ltd™, Ortenberg, Germany). Digested products from DQ Gelatin or Elastin were assessed at their respective absorption maxima of 495 nm and emission maxima of 515 nm.

Quantification of neutrophil elastase. The methods for quantification of neutrophil elastase have been described elsewhere (12). Briefly, the protein concentrations of the neutrophil elastase-α-1 proteinase inhibitor complex in cell-free BAL samples were determined utilizing a commercial sandwich ELISA (Human PMN Elastase ELISA; No. 191021100; BioVendor® Laboratorni Medicina A.S., Brno, Check Republic) as recommended by the manufacturer.

REFERENCES

1. von Scheele I, Larsson K, Dahlen B, Billing B, Skedinger M, Lantz AS, et al. Toll-like receptor expression in smokers with and without COPD. Respiratory medicine. 2011;105(8):1222-30.
2. Bergström J, Cederlund K, Dahlén B, Lantz AS, Skedinger M, Palmberg L, et al. Dental health in smokers with and without COPD. PloS one. 2013;8(3):e59492.
3. Levanen B, Glader P, Dahlen B, Billing B, Qvarfordt I, Palmberg L, et al. Impact of tobacco smoking on cytokine signaling via interleukin-17A in the peripheral airways. International journal of chronic obstructive pulmonary disease. 2016;11:2109-16.
4. Global Initiative for Chronic Obstructive Lung Disease [Available from: https://goldcopd.org/.
5. Padra M, Adamczyk B, Benktander J, Flahou B, Skoog EC, Padra JT, et al. Helicobacter suis binding to carbohydrates on human and porcine gastric mucins and glycolipids occurs via two modes. Virulence. 2018;9(1):898-918.
6. Wickström C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I. MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. The Biochemical journal. 1998;334 (Pt 3)(Pt 3):685-93.
7. Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, et al. Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. J Biol Chem. 1999;274(22):15828-36.
8. Price MR, Rye PD, Petrakou E, Murray A, Brady K, Imai S, et al. Summary report on the ISOBM TD-4 Workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17-23, 1996. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 1998;19 Suppl 1:1-20.
9. Prause O, Bozinovski S, Anderson GP, Linden A. Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. Thorax. 2004;59(4):313-7.
10. Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA, et al. Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. American journal of respiratory and critical care medicine. 2010;182(1):34-40.
11. Smith ME, Bozinovski S, Malmhall C, Sjostrand M, Glader P, Venge P, et al. Increase in net activity of serine proteinases but not gelatinases after local endotoxin exposure in the peripheral airways of healthy subjects. PloS one. 2013;8(9):e75032.
12. Laing SJ, Jackson AR, Walters R, Lloyd-Jones E, Whitham M, Maassen N, et al. Human blood neutrophil responses to prolonged exercise with and without a thermal clamp. Journal of applied physiology (Bethesda, Md : 1985). 2008;104(1):20-6.

LEGENDS

Table S1. Clinical characteristics for *Material 1* and *Material 2*

Table S2. Differential counts for blood leukocytes in *Material 2*

Figure S1. Association of MUC1 in bronchoalveolar lavage (BAL) fluid with DLCO for all study subjects in *Material 1*, including long-term smokers with (LTS+) and without (LTS-) COPD as well as healthy non-smokers (HNS). Analytical statistics are shown according to Spearman's rank order correlation.