**SUPPLEMENT TO:**

# 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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MATERIALS AND METHODS

**Human study populations**

Clinical protocols. 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™, 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$^{TM}$; < 7.5 mg intramuscularily or < 5 mg intravenously; Apoteket, Solna, Sweden) and anaestesia with lignocaine (nebulized Xylocaine$^{TM}$ 10 mg/dose; up to 3 times 2 doses oropharyngeally; Apoteket, Solna, Sweden). Additional local anaestesia (Xylocaine$^{TM}$ 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**

*Material 1*. 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™ 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™ 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™, 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.

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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.