Online data supplement

Systemic signs of neutrophil mobilization during clinically stable periods and during exacerbations in smokers with obstructive pulmonary disease

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Methods

Study subjects

Recruitment. The 60 smokers with OPD-CB were recruited from the outpatient clinic at the Section of Respiratory Medicine at Sahlgrenska University Hospital, Gothenburg, Sweden, and by advertising in the local press. The in total 20 control subjects were recruited through an advertisement. All subjects were included during stable clinical conditions at a designated inclusion visit (see below).

Smokers with OPD-CB. All smokers with OPD-CB were current and long-term smokers. These smokers with OPD-CB certified that they had not suffered from any respiratory tract infection during the 4 weeks preceding inclusion (see below). We classified these smokers in accordance with GOLD criteria (stage I–IV). All of the smokers with OPD-CB also fulfilled the criteria for chronic bronchitis. Thus, they all confirmed a history of productive cough every morning during at least 3 months and 2 consecutive years and they responded in typical manner to an interview and a symptom questionnaire (specified below). We excluded patients with asthma, atopy, or lung diseases other than OPD-CB, as well as patients with regular use of oral glucocorticoids. Patients with alpha$_1$-antitrypsin deficiency, cancer, clinically significant heart failure, documented immunodeficiency, known mental disorder or obvious abuse of alcohol or drugs were also excluded.

Four smokers with OPD-CB did not complete the study. Of these individuals, one died from an overdose of antidepressant drugs, another two were excluded owing to compliance problems and use of oral steroids, respectively. The fourth individual finished participation in the study prematurely (after Visit 4, specified below) when he was diagnosed with a neurological disease.

Control subjects. We included 10 AS subjects (current smokers with >10 pack-years) and 10 NS subjects with verified normal lung function as controls. These subjects only attended one visit, at the time of inclusion, when samples were collected.

Ethics. The study protocol was approved after ethical review by the Regional Ethics Committee for Medical Research at the University of Gothenburg (diary No. S233-03, T286-04 and T521-06). Informed consent was obtained with both oral and written information from all participating subjects, all in accordance with the *Helsinki declaration.*

Study design

Inclusion. For inclusion (at Visit 1), all subjects underwent physical examination by the study physician and an evaluation of lung function tests, pulmonary x-ray, blood tests, urine cotinine test to check for tobacco use. In addition, all subjects underwent a structured interview and filled out a symptom questionnaire; a modified version of the European Community Respiratory Health Survey to secure a correct diagnose of chronic bronchitis[1]. The investigated smokers with OPD-CB also donated a spontaneous sputum sample. This sputum sample was obtained during stable clinical conditions at the time of inclusion and during exacerbations, whenever these occurred, for the subsequent culture and identification of bacteria (see below).

Sampling. During the subsequent 60 weeks after inclusion (Visit 1), the smokers with OPD-CB donated blood tests every 15th week (thus at Visit 2-5). If the patient had an exacerbation (EXA), an extra visit was arranged. At this occasion, the smokers with OPD-CB was medically examined and received adequate clinical treatment immediately after study sampling. We used the definition of exacerbations based on criteria described by Wedzicha and Donaldson[2], in turn modified from those described by Anthonisen et al[3].

Lung function tests

Ventilatory lung function. Forced expired volume in one second (FEV_1) and forced vital capacity (FVC) were obtained utilizing a calibrated spirometer (Jaeger® Masterscope, VIASYS Healthcare GmbH, Hoechberg, Germany and Sensormedics® Vmax 22, VIASYS Healthcare, Yorba Linda, CA, USA). Spirometry was performed without prior bronchodilation (hence the term OPD) to avoid selection of smokers with non-reversible obstructive pulmonary disease. The European Respiratory Society reference values for spirometry were utilized for evaluation[4].

Gas diffusion capacity. Diffusion capacity test (D_LCO) was assessed by the single breath method with the standard equipment (SensorMedics® 2200, SensorMedics Co, Bilthoven, the Netherlands) and the reference values according to Salorinne *et al.* were utilized[5].

Blood

Sampling. Samples of venous blood were collected from smokers with OPD-CB and control subjects as described above. Thus, plasma and serum samples were prepared for analysis of soluble protein markers and cell differential counts were performed (see below). The concentrations of leukocytes were determined with the means of an accredited and automated blood differential count analysis (ADVIA® 2120i Hematology system, Siemens, Duisburg, Germany) in this laboratory.

Inflammatory proteins in blood. The extracellular concentration of MPO protein (µg/mL) in plasma was measured in diluted samples 1:10 using ELISA (HK324; Hycult® Biotechnology bv, Uden, the Netherlands). After the measurement of each protein concentration, the result was corrected for dilution. The extracellular concentration of NE protein (µg/mL) was assessed using the latex bead

concentration method[6,7]. The measurement of CRP was conducted using Tina–quant C-reactive protein high sensitive assay (HS No. 1972944001 Roche® Diagnostics, Mannheim, Germany) at the accredited laboratory at the Department of Clinical Chemistry, Sahlgrenska University Hospital, in accordance with the manufacturer's instructions.

Messenger RNA for inflammatory proteins in blood. For quantitative (q) PCR analysis blood samples were collected in PAXgene Blood RNA Tubes and stored in -70º C until analyses were performed. Total RNA was isolated using PAXgene Blood RNA Kit (QIAGEN®, Hilden, Germany) according to the protocol. Copy (c)DNA was synthesized utilizing the High Capacity cDNA Reverse Transcription Kit with random hexamer primers (Life Technologies® Corporation, Carlsbad, CA,USA) and RNase inhibitor (QIAGEN®) using 300 ng RNA in a 30 µl reaction. The qPCR analysis was performed using TaqMan® Universal PCR Master Mix kit and gene expression assays Hs00275607_m1 for carboxylesterase 1 (monocyte/macrophage serine esterase 1), Hs00236952_m1 for NE 2 and Hs00924296_m1 for MPO (Life Technologies Corporation). Human β-actin was used as the reference gene (part no 4333762F, Life Technologies® Corporation). All reactions were run in duplicate with 2 µl cDNA per reaction. The reactions were analyzed on a 7500 Real-Time PCR system (Life Technologies® Corporation).

Sputum

Sampling. The samples of spontaneous sputum were conducted and sent to the accredited Laboratory of bacteriology at Sahlgrenska University Hospital for semi-quantative analysis of the growth of aerobic bacteria. Samples were analyzed morphologically to ascertain that they were representative for the peripheral airways using light microscopy. Thus, samples were squamous epithelial cells dominated

were judged as not representative and were consequently excluded. All samples were cultured aerobically and isolates (10^5 cfu/ml concentration) were further analyzed according to standard bacteriological methods. A traditional threshold concentration (10^6 CFU/ml) was used to define significant growth of pathogens.

Evaluation of current smoking

Cotinine test. Cotinine, the major and short-lived metabolite of nicotine in human urine, was targeted as a marker of ongoing tobacco smoking. For the qualitative detection of systemic cotinine, we used *The One-Step Cotinine Test* 008A086 (Ulti Med Products® Ahrensburg, Germany) in accordance with the manufacturer's instructions.

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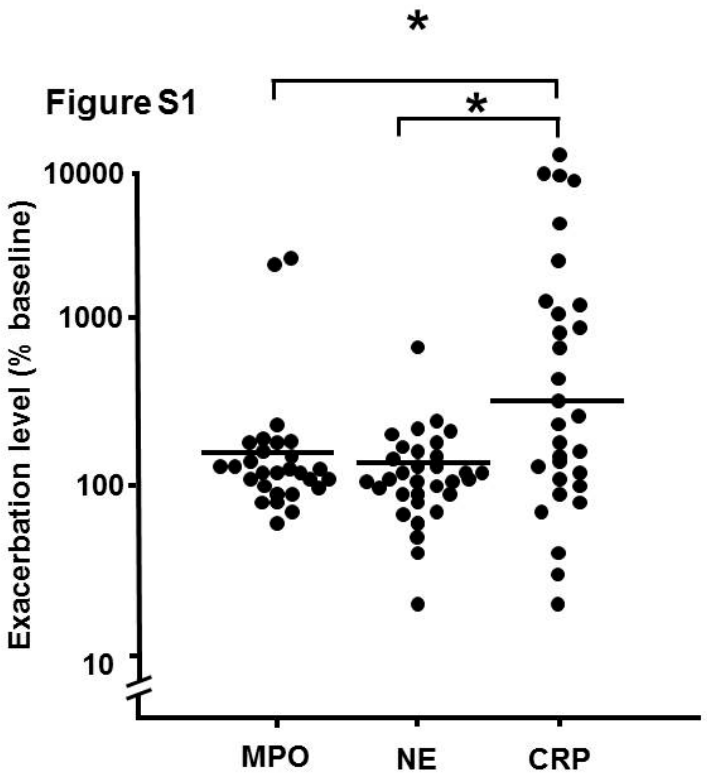

Figure S1. Blood concentrations of myeloperoxidase (MPO), neutrophil elastase (NE) and C-reactive protein (CRP) protein in smokers with obstructive pulmonary disease and chronic bronchitis (OPD-CB) during exacerbations, in relation to stable clinical conditions at the time of inclusion (% of baseline). Data presented as individual (circles) and median (bold lines) values (*Mann-Whitney U-test: $p< 0.05$, n=38).