

**Adding Salmeterol to an Inhaled Corticosteroid:
Long-term Effects on Bronchial Inflammation in Asthma**

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Online Data Supplement

Extended Material and Method

Patients

Patients with documented, mild to moderate persistent, allergic asthma (GINA II and III) [1] were enrolled (Table 1). The following inclusion criteria were used: 1. sensitization to house dust mite (*Dermatophagoides pteronyssinus*) and/or cat dander and/or grass pollen, as determined by Radio-Allergo-Sorbent-Test (RAST) and skin prick test; 2. age between 18 and 60 years; 3. $FEV_1 \geq 70$ % of the predicted value after maximal bronchodilation; 4. bronchial hyperresponsiveness to histamine, $PC_{20} \text{histamine} \leq 8.0$ mg/ml at the end of the run-in period; 5. clinically stable disease, no exacerbations of asthma within 3 months prior to inclusion requiring oral steroids and/or antibiotics; 6. no changes to regular asthma medication during 4 weeks before entry; 7. able to correctly inhale via a Diskus inhaler; 8. able to perform reproducible lung function tests.

Exclusion criteria were as follows: 1. comorbidity likely to interfere with the study; 2. lower respiratory tract infection during 4 weeks before entry; 3. use of theophylline, sodium cromoglycate, nedocromil sodium or antileukotrienes during the study or antibiotics 4 weeks prior to the study; 4. current smoking, regularly smoking within 6 months before entry or a smoking history of more than 10 pack years; 5. pregnant or lactating females; 6. unable to follow the therapy instructions; 7. participation in another clinical trial within 4 weeks prior to the study. The participating patients were recruited via advertisement and via the outpatient department of Pulmonology at the Academic Medical Center (AMC). The Medical Ethical Committee approved the study and all subjects gave written informed consent.

Study design

A schedule of the study design is shown in Figure 1. The study had a double-blind, randomized, two-armed parallel design (Figure 1). After a 2-week steroid wash-out period, a 4-week run-in period with fluticasone propionate 250 µg twice daily and a baseline bronchial allergen challenge, eligible patients were randomized to receive 1 year of twice daily treatment with either fluticasone propionate (250 µg) or twice daily treatment with fluticasone in a combination inhaler with salmeterol (250/50 µg). Patients were provided with rescue salbutamol 200 µg (GlaxoSmithKline, Zeist, The Netherlands) for relieve of symptoms during the study. All drugs were administered via a dry powder inhaler (Diskus). At the start of the wash-out period a full medical history, physical examination and FEV_1 were performed. Baseline values were measured at the end of the run-in period. Other visits were scheduled after the wash-out period and during the randomized treatment period after 1, 6, 9 (no $PC_{20} \text{histamine}$), 11 (no inflammatory parameters) and 12 months of randomization. In

addition, a bronchial allergen challenge was performed one day before randomization and at the end of the randomized treatment period. In order to measure differences in allergen-induced changes between both groups outcomes were determined 24 hours before and 24 hours after bronchial allergen challenges, which were performed at the end of the run-in period (pre-randomization challenge) and at the end of the randomized treatment period (end-of-treatment challenge) (Figure 1). A daily dairy card was completed during two weeks preceding every visit, an additional dairy card was completed after 3 months of randomization. The cards contained peak expiratory flows, rescue-short-acting β_2 -agonist usage and morning and evening symptoms scores (score 0-4 and 0-5 respectively). Before every visit patients abstained from rescue salbutamol (except before the end-wash-out visit) for 8 hours and from the study medication for 12 hours. Before the visit at 11 months of randomization patients abstained from the study medication for 36 hours, to exclude the influence of bronchodilatory effects of salmeterol on the level of PC₂₀histamine. Asthma exacerbations were treated either by increasing the dose of fluticasone (defined as a mild exacerbation) or by oral glucocorticosteroids (moderate exacerbation), as judged by the investigator.

Lung function and allergy tests

The FEV₁ was measured at each visit with a dry rolling seal spirometer (Sensor Medics BV, The Netherlands) according to standardized guidelines [2]. Values are expressed as the percentage of the predictive value. Bronchial hyperresponsiveness to histamine (PC₂₀histamine) was determined by a 2-minute tidal breathing method [2]. PC₂₀histamine was not performed if FEV₁ was ≤ 60 % of the predicted value or if salbutamol could not be abstained during 8 hours before the measurement. Total and specific Immunoglobulin-E directed against house dust mite were determined, as reported previously [3].

Bronchial allergen challenge

A standardized *Dermatophagoides pteronyssinus*, cat dander or grass pollen extract (containing 50.000 biological units (BU), ALK Abelló, Nieuwegein, The Netherlands) was used for the bronchial allergen challenges. Dilutions of the allergen extract, containing 5000 BU/ml were kept at -20 °C in aliquots of 0.5 ml. A dilution, containing 200 BU/ml, was made freshly from the stock immediately before use, in phosphate buffered saline, 0.03% human serum albumin, 0.5% phenol (ALK Abelló, Nieuwegein, The Netherlands), of which a 0.5 ml sample was nebulized during the bronchial allergen challenge. A reservoir aerosol delivery system was used according to the method described by Sterk and colleagues [2] with

modifications [4]. Briefly, a collapsible reservoir of approximately 30 liter, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA) and filled with dry air, was connected to a nebulizer (Mallinckrodt Diagnostica, Petten, The Netherlands) producing aerosols from a 0.5 ml sample of diluted HDM extract. Previous studies demonstrated a 70% recovery of nebulized allergen. The amount of allergen was not titrated on the decline in FEV₁ during the early asthmatic reaction. For patients convenience all bronchial allergen challenges were preceded 15 minutes before the challenge by the inhalation salbutamol hemisulphate, 500 mg/ml solution, which was nebulized, via a Micro-Mist nebulizer, (Hudson, Terneuzen, California, USA) driven by dry-compressed air, at a flow of 5 L/min during 1.5 min. The dose is equivalent to approximately 400 µg salbutamol via a dry powder inhaler, considering the loss of drug by delivering during both inspiration and expiration [5]. The amount of allergen administered to the patients (70 % of 100 biological units, (for *Dermatophagoides pteronyssinus* equivalent to 42 ng major allergen Der p1) was based on the results of an earlier study which was done in asthma patients with a similar range of severity [6]. However, the patients in that study abstained from anti-inflammatory therapy 6 weeks prior to allergen challenge [6]. The median cumulative dose of allergen that resulted in a decline in FEV₁ of 20 % from baseline was 107 biological units. Baseline FEV₁ (median of three measurements) was measured with a dry rolling seal spirometer (Sensor Medics BV, The Netherlands) as well as with a portable spirometer (Micromedical diarycard, Sensor Medics BV, The Netherlands). After allergen challenge FEV₁ was determined at 10 and 30 minutes with the dry rolling seal and subsequently hourly from 4 to 8 hours after the allergen inhalation with a portable spirometer (best of two measurements). Change in FEV₁ after allergen challenge was expressed as percentage from baseline, as measured with the corresponding spirometer.

Sputum induction

Sputum induction was performed by inhalation of aerosols of hypertonic saline (NaCl) during quiet tidal breathing. An Aerodyne Omega Ultrasonic nebulizer (Kendall, Neustadt/ Donau, Germany) was used with plastic tubes of 30-cm length. This nebulizer generates aerosols with a mass median aerodiameter of 4.5 µm. Before inhalation of the nebulized saline was started, a baseline FEV₁ was recorded. For safety reasons, all subjects received salbutamol (nebulized dose of 400 µg). Each subject inhaled increasing concentrations of nebulized, sterile saline during 21 minutes, namely 3, 4 and 5 % sodium chloride for 7 minutes each. Spirometry was repeated between each concentration step; only if there was no change in FEV₁ or if the fall in FEV₁ was less than 10 % the induction procedure was proceeded to the next inhalation; if the

fall in FEV₁ was between 10 and 20 % the same concentration was inhaled for another 7 minutes. The induction procedure was discontinued if FEV₁ was fallen by more than 20 %, or when patients experienced any discomfort like chest tightness or dyspnea caused by the induction procedure. Before expectorating the sputum subjects were asked to blow their nose and clear their throats and subsequently carefully rinse their mouths with water and swallow the water. Sputum samples were collected in plastic containers on ice. Processing was started within 15 minutes.

Sputum processing

The volume of the whole sputum sample was determined and an equal volume of dithiotreitol (10 mM DTT in 135 mM Tris buffer, pH 8.0) was added. The samples were then mixed gently at 4 °C for 15 min. In case a sample was still not homogenized after this procedure, DNase 1:1000 was added and the sample was mixed at 4 °C for another 15 min. The homogenized sputum was centrifuged at 1640 rpm at 4 °C for 10 min. The supernatants were aspirated and frozen at –20 °C for later analysis. The cell pellets were resuspended in 1 ml 2 % Human Serum Albumin (HSA) in phosphate buffered saline (PBS). Total cell number was determined by counting manually in a Bürker counting chamber. Cells were cytocentrifuged for 2 minutes at 550 rpm in Shandon Cytocentrifuge and stained with Romanovsky (Diff-Quick) and Jenner-Giemsa. One investigator, blinded for the subject's history, counted 200 non-squamous cells on each sputum slide; squamous and non-squamous epithelial cells, macrophages, lymphocytes, neutrophils and eosinophils were identified. Differential cell counts were expressed as number per gram sputum and as percentage of cells, excluding squamous epithelial cells. In case the percentage of eosinophils was less than 10 % but more than 1 %, a total of 500 non-squamous cells was counted; 1000 non-squamous cells were counted in case the eosinophil percentage was less than 1 %. Sputum samples containing more than 80 % squamous cells on differential cell counting were excluded from cell differential analysis.

Protein assays

Levels of ECP were determined with an ELISA [7]. The detection limit was 15 pg/ml. Assay reagents (Rabbit-anti-human ECP antiserum and anti-ECP antibody-biotin) were kindly donated by dr. A. Zuurbier (CLB Sanquin, Amsterdam, The Netherlands). A series of standard dilutions of ECP was obtained from Pharmacia & Upjohn (Uppsala, Sweden). Major basic protein (MPO) [8] and interleukin (IL)-8 [9] were measured with an ELISA as described earlier. Size selectivity of plasma protein leakage across the respiratory membrane, into the

airway lumen was analyzed by measuring the relative coefficient of excretion (RCE_s), which is defined as the ratio of α 2-macroglobulin and albumin in valid sputum samples [10,11]. The levels of albumin were measured by an immunoturbidimetric assay with a Cobas Bio analyzer (Roche Diagnostics, Inc.). Antiserum for albumin was obtained from Dako (code A001, Glostrup, Denmark). As a standard we used N protein standard serum for nephelometry (Behring, Marburg, Germany). The levels of A2M were measured with an ELISA [11].

Statistical analysis

SAS (SAS Institute Inc., Cary, NC, USA version 8.2) was used for statistical analyses. The study had 80 % power to detect a 50% difference in change from baseline between the groups with a sample size of 54 subjects. Changes over the run-in period were determined using Wilcoxon signed ranks test, or in case of normally distributed data with t-test. Differences within and between the treatment-groups were determined using a mixed model ANOVA, adjusted for differences at baseline (end of run-in). Differences in allergen-induced changes were determined using ANCOVA and adjusted for baseline allergen-induced changes. Differences between the treatment groups in changes from baseline in PC₂₀histamine at the visit at 11 months of randomization were analyzed by multiple linear regression analysis, including baseline PC₂₀histamine as co-variable. All p-values are two-tailed and p-levels of less than 0.05 were considered significant.

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