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

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ABSTRACT (word count: 250)

Background: Adding the long-acting beta(2)-agonist salmeterol to inhaled corticosteroids leads to better symptomatic asthma control than increasing the dose of inhaled corticosteroids. However, little is known about the long-term effects of adding salmeterol on the asthmatic inflammatory process, control of which is considered important for the long-term outcome of asthma.

Methods: After a 4-week fluticasone run-in period, we randomized 54 allergic asthmatics to receive 1 year of twice daily treatment with fluticasone 250 µg with or without salmeterol 50 µg, in a double-blind, parallel group design (total daily dose of fluticasone 500 µg in both treatment groups). Primary outcomes were sputum eosinophil numbers and eosinophil cationic protein concentrations. Secondary outcomes were neutrophil-associated sputum parameters and a respiratory-membrane permeability marker. Effects on allergen-induced changes were determined before and at the end of the randomized treatment period.

Results: Adding salmeterol to fluticasone resulted in improved peak expiratory flows, symptom scores, rescue-medication-usage and bronchial hyperresponsiveness (p-values<0.05). We found no sustained effect on sputum cell differentials and cytokine concentrations, neither throughout the treatment period nor on changes induced by the end-of-treatment allergen challenge (p-values>0.05). However, adding salmeterol significantly reduced sputum ratios of alpha(2)-macroglobulin and albumin throughout the randomized treatment period (p=0.001).

Conclusions: This study shows no sustained effect on (allergen-induced) cellular bronchial inflammation, but a significant improvement in size selectivity of plasma protein permeation across the respiratory membrane by adding salmeterol to fluticasone. Possibly, this phenomenon contributes to the improved clinical outcomes that result from adding a long-acting β2-agonist to inhaled corticosteroids.
INTRODUCTION

Allergic asthma is currently considered primarily a chronic inflammatory disorder and repeated exposure to allergens may be important in the persistence of the disease (1). Besides avoidance of risk factors, the use of inhaled corticosteroids is considered to be the mainstay for the treatment of allergic asthma (1). Regular use of these inhaled corticosteroids in asthma is associated with prolonged improvement in exacerbation frequencies, symptom scores and lung function, as well as with a reduction in inflammatory cells in the airways (2;3). By contrast, β2-agonists are principally regarded as bronchodilators and are advised to be used only in combination with anti-inflammatory therapy, except in case of intermittent use for the mildest form of asthma (1).

Several large, clinical studies have now shown that adding a long-acting β2-agonist to inhaled corticosteroids, leads to better symptomatic asthma control and lower exacerbation frequencies, when compared to increasing the dose of inhaled steroids (3;4). These findings suggest that adding a long-acting β2-agonist to inhaled corticosteroids exerts beneficial effects, beyond smooth muscle relaxation. In this respect it should be mentioned that in vitro studies point to synergistic effects of combining long-acting β2-agonists and corticosteroids (5). Some short-term clinical studies suggest an anti-inflammatory effect, since there were lower numbers of EG1+ eosinophils (6) and submucosal mast cells (7) after 12 weeks of adding salmeterol in these studies. As a consequence, it has been proposed that adding a long-acting β2-agonist influences the underlying inflammatory process. In contrast, a recent study showed no additional anti-inflammatory effect in bronchial biopsies of adding formeterol to low dose budesonide during 16 weeks of treatment (8). It should be emphasized that the long-term clinical studies on adding salmeterol to ICS were limited to clinical outcomes (3;4) and did not evaluate bronchial hyperresponsiveness or surrogate inflammatory biomarkers, such as airway eosinophils. These in turn are considered the ultimate driving force behind symptoms, the disability of asthma, and most importantly the long-term sequelae, such as airway remodelling (9;10). It should be realized that endpoints such as symptoms and lung function may favour the use of long-acting β2-agonists but provide little information about the pathophysiologic hallmarks of the asthmatic inflammatory process (11). In addition, there are indications for pro-inflammatory effects of regular use of short-acting β2-agonists (12;13), which in part formed the basis for concern as to adding a long-acting β2-agonist leads to clinically masking of bronchial inflammation (14).

The present paper presents the results of a long-term randomized clinical trial, which was designed to investigate whether the improved clinical outcomes resulting from adding salmeterol to fluticasone, are accompanied by a prolonged effect on the underlying bronchial inflammatory process in asthma. After a run-in period with fluticasone 250 µg twice daily, we randomized 54 mild to moderate persistent, allergic asthma patients to receive 1 year of twice daily treatment with either fluticasone (250 µg) or fluticasone/salmeterol (250/50 µg). Moreover, to investigate the effect on allergen-induced bronchial inflammation of maintenance treatment with fluticasone/salmeterol versus fluticasone, we performed bronchial allergen challenges 1 day before randomization and at the end of the randomized treatment period. Primary outcomes were sputum eosinophil numbers and eosinophil cationic protein concentrations. Secondary outcomes were serum interleukin-5, size-selectivity of the respiratory membrane as measured by the ratio of sputum α2-macroglobulin to albumin, sputum neutrophil numbers, interleukin 8 and myeloperoxidase. In addition, we measured lung function parameters, peak expiratory flows, symptom scores and rescue-medication-usage. We analyzed overall differences in outcome parameters over the 1-year treatment period as well as differences in allergen-induced changes between the two treatment groups.
METHODS

The extended version of the methods section is available in the online data supplement. Non-smoking patients with mild to moderate persistent, allergic asthma (1) were enrolled (Table 1). The participating patients were recruited via advertisement and via the Outpatient Department of Pulmonology at the Academic Medical Center (AMC). The study was approved by the institutional Ethics ad Research Committees and all subjects gave written informed consent. 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 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 (250 µg) or fluticasone in a combination inhaler with salmeterol (250/50 µg). Patients were provided with rescue salbutamol 200 µg (GlaxoSmithKline, Zeist, The Netherlands). All drugs were administered via a dry powder inhaler (Diskus). Primary outcomes were: sputum eosinophil numbers and eosinophil cationic protein concentrations in sputum. Secondary outcomes were: neutrophil-associated sputum parameters and a respiratory-membrane permeability marker. In addition, lung function parameters, peak expiratory flows, symptom scores and rescue-medication-usage were measured throughout the study. At the start of the wash-out period a full medical history, physical examination and FEV₁ were performed. Baseline values of lung function parameters as well as primary and secondary outcomes were measured at end of run-in (time point 0 months). In addition, outcomes were measured after wash-out and during the randomized treatment period at 1, 3, 6, 9, 11 and 12 months after randomization. Furthermore, 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). Two-week daily dairy cards were completed prior to and throughout the randomized treatment period, containing peak expiratory flows, symptom scores and rescue-salbutamol usage. Before every visit patients abstained from rescue salbutamol for 8 hours and from the study medication for 12 hours; except at 11 months of randomization (abstaining from study medication for 36 hours).

FEV₁ and PC20histamine were measured according to guidelines (15). Standardized allergen extracts were used for the allergen challenges, which were performed as described (15), with modifications. A single dose of allergen was administered containing 100 biological units and was preceded by the inhalation of nebulized salbutamol. Levels of total and specific Immunoglobulin-E were determined in serum (16). Sputum induction and processing of whole sputum samples were performed as described (17). Differential cell counts were expressed as number and percentage of cells, excluding squamous epithelial cells. Sputum samples containing more than 80 % squamous cells on differential cell counting were excluded from analysis. Levels of eosinophil cationic protein (ECP) (18), myeloperoxidase (MPO) (19) and interleukin (IL)-8 (20) were measured in sputum, and levels of IL-5 in serum (21). Size selectivity of the respiratory membrane was analyzed by measuring the relative coefficient of excretion in induced sputum (RCEs), which is the ratio of sputum concentrations of α2-macroglobulin to albumin (17;22).

SAS (SAS Institute Inc., Cary, NC, USA version 8.2) was used for statistical analyses. The study was designed to have a 80 % power to be able to detect a 50% difference in geometric means of the primary outcomes 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 mixed model ANOVA, adjusted for differences at baseline. Differences in allergen-induced changes were determined using ANCOVA and
adjusted for baseline allergen-induced changes. All p-values are two-tailed and p-levels of less than 0.05 were considered significant.

RESULTS

Patients

Sixty patients agreed to participate in this study. Four did not fulfill the inclusion criteria after the run-in period and two withdrew during the run-in period for personal reasons. Fifty-four patients were randomized to receive either of the two treatment regimens. Patient characteristics, including age, dose of inhaled corticosteroids prior to the study and lung function parameters, were not significantly different between the treatment groups at baseline (end of run-in) (Table 1).

Table 1. Characteristics of randomized patients

<table>
<thead>
<tr>
<th></th>
<th>fluticasone</th>
<th>fluticasone/salmeterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Age * (years)</td>
<td>32 (19-57)</td>
<td>32 (21-59)</td>
</tr>
<tr>
<td>M/F</td>
<td>8/19</td>
<td>10/17</td>
</tr>
<tr>
<td>Inhaled corticosteroids prior to study (µg/day)*</td>
<td>593 (200-1200)</td>
<td>619 (200-1000)</td>
</tr>
<tr>
<td>Total IgE † (IU/ml)</td>
<td>127 (3.4)</td>
<td>172 (2.7)</td>
</tr>
<tr>
<td>SpecIgE † (IU/ml)</td>
<td>5.2 (6.7)</td>
<td>8.4 (3.1)</td>
</tr>
<tr>
<td>PC20 histamine start run-in † (mg/ml)</td>
<td>0.4 (1.6)</td>
<td>0.5 (1.5)</td>
</tr>
<tr>
<td>PC20 histamine end run-in † (mg/ml)</td>
<td>1.0 (1.5)</td>
<td>1.6 (1.3)</td>
</tr>
<tr>
<td>FEV1 start run-in † (%pred)</td>
<td>89.9 (14)</td>
<td>88.8 (18)</td>
</tr>
<tr>
<td>FEV1 end run-in † (%pred)</td>
<td>92.6 (16)</td>
<td>93.1 (16.1)</td>
</tr>
<tr>
<td>Morning PEF end run-in † (l/min)</td>
<td>422 (102)</td>
<td>418 (102)</td>
</tr>
<tr>
<td>Evening PEF end run-in † (l/min)</td>
<td>435 (110)</td>
<td>431 (106)</td>
</tr>
<tr>
<td>Morning symptom score end run-in † (scale 0-4)</td>
<td>0.2 (0.3)</td>
<td>0.3 (0.5)</td>
</tr>
<tr>
<td>Evening symptom score end run-in † (scale 0-5)</td>
<td>0.6 (0.6)</td>
<td>0.6 (0.7)</td>
</tr>
<tr>
<td>Short-acting β2-agonist usage ‡§ (puffs/day)</td>
<td>1.4 (1.8)</td>
<td>1.0 (1.3)</td>
</tr>
</tbody>
</table>

No significant differences in patient characteristics were observed at the end of the run-in period (defined as baseline) between the fluticasone/salmeterol and the fluticasone group. Over the run-in period FEV1 and PC20 histamine improved significantly (p-values < 0.001). Definition and abbreviations: values are expressed as *median (range); †geometric mean (geometric standard deviation) or ‡mean (standard deviation); §β2-agonist usage was recorded during the two weeks preceding the end run-in visit. IgE=Immunoglobulin E, SpecIgE=serum level of specific IgE directed against the type of allergen that was used during allergen challenge. PEF=peak expiratory flow.

Four patients in the fluticasone group failed to complete the study. Two of these patients withdrew 1 month after randomization, one patient because of worsening of asthma symptoms and one was lost to follow-up. The other two patients withdrew after 6 and 9 months of treatment respectively, because of personal reasons. All patients in the fluticasone/salmeterol group completed the study.
Run-in period
FEV1 and PC20histamine improved significantly over the run-in period, which was preceded by a steroid wash-out period (p values < 0.001, Table 1). Mean values of peak expiratory flow rates, asthma symptom scores, as well as short-acting β2-agonist usage, recorded during the two weeks preceding the end of run-in visit (baseline) are depicted in Table 1. Levels of the inflammatory parameters before and after the run-in period are shown in Table 2.

Table 2. Inflammatory parameters at start and end of the run-in period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Start run-in</th>
<th>End run-in</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum eosinophils (x 10⁴/gr)</td>
<td>2.1 (1.4)</td>
<td>0.6 (1.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sputum eosinophils (%)</td>
<td>3.2 (1.3)</td>
<td>0.8 (1.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sputum ECP (ng/ml)</td>
<td>97 (1.3)</td>
<td>65 (1.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum IL-5 (pg/ml)</td>
<td>1.1 (1.2)</td>
<td>0.6 (1.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sputum neutrophils (x 10⁴/gr)</td>
<td>28.1 (1.3)</td>
<td>29.1 (1.3)</td>
<td>0.98</td>
</tr>
<tr>
<td>Sputum IL-8 (ng/ml)</td>
<td>400 (1.4)</td>
<td>338 (1.8)</td>
<td>0.62</td>
</tr>
<tr>
<td>Sputum MPO (µg/ml)</td>
<td>1308 (1.3)</td>
<td>1273 (1.3)</td>
<td>0.95</td>
</tr>
<tr>
<td>RCEs (x10³)</td>
<td>76 (1.2)</td>
<td>52 (1.2)</td>
<td>0.006</td>
</tr>
<tr>
<td>Sputum α2-macroglobulin (ng/ml)</td>
<td>1388 (1.3)</td>
<td>984 (1.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Sputum albumin (µg/ml)</td>
<td>24 (1.3)</td>
<td>21 (1.3)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Definition and abbreviations: All values are expressed as geometric mean (SEM). ECP=eosinophil cationic protein, IL=interleukin, MPO=myelo-peroxidase, RCEs=relative coefficient of excretion, representing the ratio of α2-macroglobulin to albumin in induced sputum.

Randomized treatment period
Lung function.
Morning and evening peak expiratory flows, asthma symptom scores and short-acting β2-agonist usage were significantly improved throughout the randomized treatment period in the fluticasone/salmeterol group, relative to the fluticasone group (Table 3, Figure 2).

Table 3. Improvements in clinical and lung function parameters in the fluticasone/salmeterol group relative to the fluticasone group: mean differences over the 1-year treatment period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean difference over 1-year</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEF morning (l/min)*</td>
<td>29 (9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PEF evening (l/min)*</td>
<td>36 (9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Symptom score morning (scale 0-4)*</td>
<td>-0.1 (0.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Symptom score evening (scale 0-5)*</td>
<td>-0.2 (0.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Short-acting β2-agonist usage (puffs/day)*</td>
<td>-0.9 (0.3)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>FEV1 (%predicted)*</td>
<td>2.7 (1.5)</td>
<td>0.07</td>
</tr>
<tr>
<td>PC20histamine (doubling doses)†</td>
<td>0.7 (0.3)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Definition and abbreviations: Values are expressed as * mean or †geometric mean (SEM). For a study schedule see Figure 1. Definition of abbreviation: S/FP=fluticasone/salmeterol, FP=fluticasone, PEF=peak expiratory flow.
In the fluticasone/salmeterol group there was a trend for a higher FEV₁ (Table 3). PC₂₀histamine was significantly higher in the fluticasone/salmeterol group over the 1-year treatment period, as measured on visits after abstaining for 12 hours from the study medication (Table 3), however there was no significant difference in PC₂₀histamine at the visit at 11 months of randomization, when patients abstained from the study medication for 36 hours (geometric mean baseline-adjusted PC₂₀histamine [SEM]: 1.6 [1.2] in fluticasone vs 1.9 [1.3] mg/ml in fluticasone/salmeterol group, p = 0.53). There were no differences in numbers or severity of exacerbations between the two treatment groups (results not shown).

Sputum eosinophils and ECP

The geometric mean number of eosinophils in the fluticasone/salmeterol group expressed as percentage difference from that in the fluticasone group varied significantly between visits (p=0.019). After one month of randomized treatment the number of sputum eosinophils in the fluticasone/salmeterol group was -71% (95% CI: -92% to -1.2%) compared to the fluticasone group (p = 0.048, Figure 3). Other timepoints, however, did not show a significant difference between groups (6 months: 56% [95%CI: -71 to 741]; 9 months: -0.14 [95%CI: -77 to 335], 12 months: 61% [955CI: -43 to 358]). Overall during the 1 year treatment period there were no significant differences between the two treatment groups, neither in numbers or percentages of sputum eosinophils (p values 0.72, and 0.85 Figure 3). Geometric mean ECP level in the fluticasone/salmeterol group was 5.7% lower than in the fluticasone group (p=0.88). There were no indications that the difference varied between visits (p=0.73). The 95%CI of the difference was -57% to +108% indicating that the true geometric mean ECP in the fluticasone/salmeterol group should have been more than 57% lower than in the fluticasone group to be detected as significant, given the results of the study.

Sputum neutrophils, IL-8, MPO and serum IL-5.

Sputum neutrophil numbers, sputum IL-8, sputum MPO and serum IL-5 were not significantly different between the two treatment groups throughout the randomized treatment period (p-values 0.87, 0.70 and 0.23 respectively, data not shown).

Size selectivity of the respiratory membrane: relative coefficient of excretion (RCEₚ)

Overall during the 1-year treatment period the RCEₚ was significantly lower in the fluticasone/salmeterol group relative to the fluticasone group (p = 0.001, Figure 4).

**Effect of adding salmeterol on allergen-induced changes in bronchial inflammation**

There were no significant differences between the treatment groups in the change in FEV₁ after the allergen challenges, which were preceded by the inhalation of salbutamol (p = 0.81).

Sputum eosinophils and ECP

Sputum eosinophils (Figure 5) and ECP concentrations increased significantly after the pre-randomization allergen challenge as well as after the end-of-treatment allergen challenge (Table 4).
Table 4. Allergen-induced increase factors of inflammatory parameters

<table>
<thead>
<tr>
<th></th>
<th>Pre-randomization allergen challenge</th>
<th>End-of-treatment allergen challenge</th>
<th>End-of-treatment allergen challenge</th>
<th>p value</th>
<th>FP vs FP/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>54</td>
<td>23</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp EO number</td>
<td>3.7 (1.9; 7.1)</td>
<td>4.4 (2.4; 8.1)</td>
<td>2.6 (1.0; 6.8)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Sp EO %</td>
<td>2.4 (1.5; 3.8)</td>
<td>3.4 (1.9; 6.1)</td>
<td>1.8 (0.9; 3.8)</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>Sp ECP</td>
<td>2.0 (1.2; 3.4)</td>
<td>2.1 (1.0; 4.4)</td>
<td>1.4 (0.7; 2.5)</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>RCEs</td>
<td>1.4 (1.0; 1.8)</td>
<td>0.8 (0.5; 1.3)</td>
<td>1.3 (-0.9; 1.8)</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Sp α2-m</td>
<td>1.3 (1.0; 1.7)</td>
<td>1.2 (-0.7; 2.0)</td>
<td>1.0 (-0.7; 1.5)</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Sp albumin</td>
<td>-0.9 (-0.6; 1.3)</td>
<td>1.5 (-0.9; 2.5)</td>
<td>-0.8 (-0.5; 1.3)</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>

Increase factors in geometric mean values (95% confidence interval) are shown. Increase factors were calculated from levels at 24 hours after, relative to 24 hours before the bronchial allergen challenges. Definition of abbreviations: no=number, sp= sputum, eo=eosinophils, ECP= eosinophil cationic protein, RCEs= relative coefficient of excretion, representing the ratio of α2-m to albumin in induced sputum, α2-m=α2-macroglobulin.

There was a trend for a smaller increase in the percentage of sputum eosinophils in the fluticasone/salmeterol group after the end-of-treatment allergen challenge (p = 0.09), but there were no significant differences between the two treatment groups after the end-of-treatment allergen challenge in the changes in numbers of sputum eosinophils and ECP concentrations (Table 4).

Sputum neutrophils, IL-8, MPO and serum IL-5
The number of sputum neutrophils, sputum IL-8, sputum MPO, and serum IL-5 increased after the pre-randomization allergen challenge (p-values < 0.001, 0.001, 0.005 and < 0.001 respectively) as well as after the end-of-treatment allergen challenge (p values 0.02, 0.06, 0.02, and 0.02 respectively, data not shown). There were no significant differences between the two treatment groups in the changes in these parameters after the end-of-treatment allergen challenge (p values 0.92, 0.85, 0.93 and 0.89 respectively, data not shown).

Size selectivity of the respiratory membrane: relative coefficient of excretion (RCEs)
There was no significant difference between the two treatment groups in the changes in the RCEs after the end-of-treatment allergen challenge (p = 0.15, Table 4). Likewise, there were no significant differences between the two treatment groups in the changes in sputum α2-macroglobulin or albumin after the end of treatment allergen challenge (Table 4).

DISCUSSION
This is the first randomized clinical trial to investigate over a 1-year treatment period whether the improved clinical outcomes resulting from adding salmeterol to fluticasone are accompanied by an additional effect on bronchial inflammation. In concordance with earlier studies peak expiratory flows, symptom scores, rescue-medication-usage and bronchial hyperresponsiveness were significantly improved in the fluticasone/salmeterol group relative to the fluticasone group. With respect to inflammatory parameters we found similar levels of sputum eosinophils, sputum eosinophil cationic protein, sputum interleukin-8, sputum myeloperoxidase and serum interleukin-5 in both groups throughout the treatment period, as well as after the bronchial allergen challenges. However, we did find significantly reduced ratios of sputum α2-macroglobulin and albumin in the fluticasone/salmeterol group relative to...
the fluticasone group. This secondary outcome, the relative coefficient of excretion (RCEs) was chosen as a marker of size selectivity of plasma protein permeation across the respiratory membrane.

The lack of a sustained effect of adding salmeterol with regard to cellular bronchial inflammation, as we found in induced sputum, is in concordance with a recent study by Overbeek et al (8) who were unable to show an additional anti-inflammatory effect in bronchial biopsy histology of adding formeterol to low doses of budesonide during 16 weeks of treatment. Earlier in vitro (23;24) and short-term in vivo studies (6;7) did report an initial anti-inflammatory effect which may be in line with our observation of a transient decrease in the number of sputum eosinophils one month after randomization in the fluticasone/salmeterol group. This difference in sputum eosinophils at 1 month after the pre-randomization allergen challenge may be explained by a slower recovery from the allergen-induced increase in bronchial inflammation in the fluticasone group. Bronchial allergen challenge increases levels of inflammatory markers (25), which may increase the sensitivity to detect differences in levels of inflammatory markers between the treatment groups. On the other hand it should be noted that the medium-high daily dose of fluticasone resulted in relatively low baseline levels of the inflammatory parameters. This may have obscured a modest long-term anti-inflammatory effect on cellular bronchial inflammation of adding salmeterol. The study was powered to detect a 50% difference in primary outcomes (sputum eosinophils and ECP) between the groups, since such a difference was considered to be clinically relevant. Therefore, adding salmeterol to an inhaled corticosteroid under the conditions chosen does neither cause a clinically relevant, deleterious or masking long-term effect on cellular bronchial inflammation nor significantly improves it. It should be emphasized that this finding may be unique to the administration of the combination product of salmeterol and fluticasone as patients may be tempted to leave off the latter when using both medications separately. It was reported previously that reducing the dose of inhaled corticosteroids in patients on salmeterol can mask increasing inflammation and delay awareness of worsening asthma (14).

With respect to the effects on allergen-induced bronchial inflammation, it should be stressed that patients abstained from the study medication for 12 hours before every visit, including the allergen challenge visits. This may have obscured an acute effect of adding salmeterol to fluticasone, but the aim of the study was to investigate the long-term, in stead of acute, effects of fluticasone/salmeterol versus fluticasone on allergen-induced bronchial inflammation. We, like others, found indications in vivo for an acute short-term anti-inflammatory effect of the addition of salmeterol to ICS in a bronchial allergen challenge model (in press).

A concomitant characteristic of the bronchial cellular inflammatory process is the presence of increased permeability of the respiratory membrane (26). In healthy subjects the permeation of large plasma proteins is restricted, but during inflammation there is an apparent loss of size selectivity of the respiratory membrane (22). This will affect the sputum concentration of large plasma proteins more than of smaller proteins. The ratio of sputum levels of a large plasma protein (α₂-macroglobulin) to levels of a smaller plasma protein (albumin) is considered a marker of size selectivity of the barrier between the blood and the airway lumen (13). We particularly chose this ratio since it is not influenced by variable dilution of the sputum samples. Over the run-in period, when all patients received fluticasone 250 µg twice daily, we found a significant decrease in the RCEs. This finding is in keeping with an earlier study (13). Moreover, we found a continued decrease in the RCEs in the fluticasone/salmeterol group throughout the randomized treatment period, whereas the RCEs stabilized in the fluticasone group. This finding may point to increased size selectivity of the respiratory membrane caused by adding salmeterol. This is in keeping with earlier studies that
showed anti-exudative, short-term effects of long-acting β_2-agonists in animal (27;28) as well as in human models (29). Such an anti-exudative effect may be desirable in the treatment of asthma, considering that the extra-vasated plasma contains potent adhesive and leukocyte activating proteins (such as fibrinogen and fibronectin) and inflammatory peptides including bradykinins and complement factors (30). Analyzing the sputum concentrations of the individual serum proteins suggested however an increase in albumin rather than a decrease in α2-macroglobulin in the fluticasone/salmeterol arm throughout the randomized treatment period. This might be explained by increased water clearance by salmeterol, which has been shown in animal models (31;32), in addition to increased size-selectivity. An alternative explanation for the decrease in RCEs may be provided by the occurrence of selective transport of serum albumin to the airway lumen. \textit{In vitro}, active transport of albumin across the ferret trachea has been reported previously (33) and was increased by the short-acting β_2-agonist salbutamol (33). Moreover, specific serum albumin-binding proteins have been identified (34). The role of albumin in the airway secretions is presently unclear. There is evidence that albumin may bind various mediators such as leukotrienes (35) and may therefore render potentially active luminal agents less effective. Albumin may also act as a luminal antioxidant, preventing the formation of oxygen free radicals (36). In this respect elevated levels of albumin in sputum may have physiological advantages.

In summary, we have shown that improved clinical outcomes resulting from adding a long-acting β_2-agonist to maintenance treatment with inhaled corticosteroids are accompanied with similar levels of markers of chronic as well as allergen-induced cellular bronchial inflammation. However, size selectivity of plasma protein permeation across the respiratory membrane appeared to be significantly improved by adding salmeterol to fluticasone. Possibly, this phenomenon contributes to the improved clinical outcomes that result from adding a long-acting β_2-agonist to inhaled corticosteroids.

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ETHICS APPROVAL: The Medical Ethical Committee of the Academic Medical Center, Amsterdam, The Netherlands approved the study and all subjects gave written informed consent.

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LEGENDS TO THE FIGURES

**Figure 1**
Study schedule. After a 2-week steroid wash-out period and a 4-week run-in period with fluticasone 250 µg twice daily, eligible asthma patients were randomized to receive 1 year of twice daily treatment with fluticasone (250 µg) with or without salmeterol (50 µg). Baseline values were determined at the end of the run-in period. Bronchial allergen challenges were performed one day before randomization and at the end of the randomized treatment period. Primary and secondary outcomes were measured after wash-out, pre-randomization, at 1, 6, 9, and 12 months of randomization, as well as 24 hours before and 24 hours after the bronchial allergen challenges (depicted by asterisks (*)).

**Figure 2**
Morning peak expiratory flow rate (A) and short-acting β2-agonist usage (B) (mean values ± SEM). There were significant differences in morning peak flow recordings and short-acting β2-agonist usage between the two treatment groups (p-values < 0.01). For a study schedule see Figure 1. Definition of abbreviation: PEF=peak expiratory flow rate.

**Figure 3**
Number of sputum eosinophils (A) and sputum ECP concentration (B). Baseline-adjusted, geometric mean values ± SEM are shown. Overall during the 1 year treatment period there were no significant differences between the two treatment groups, neither in the number of sputum eosinophils, nor in the sputum ECP concentration (p values 0.72 and 0.88 respectively). For a study schedule see Figure 1. Definition of abbreviation: ECP=eosinophil cationic protein.

**Figure 4**
RCEs during the run-in and the randomized treatment period. Baseline-adjusted, geometric mean values (± SEM) are shown. Over the run-in period there was a significant decrease in the RCEs (p = 0.006). Moreover, overall during the 1-year treatment period the RCEs was significantly higher in the fluticasone/salmeterol group relative to the fluticasone group (p = 0.001). For a study schedule see Figure 1. Definition of abbreviation: RCEs=relative coefficient of excretion which is the ratio of α2-macroglobulin to albumin in induced sputum.

**Figure 5**
Numbers of sputum eosinophils before and after bronchial allergen challenges, pre-randomization (A) and at end-of-treatment (B). Geometric mean numbers of sputum eosinophils increased significantly after the pre-randomization as well as at the end-of-treatment allergen challenge. There was no significant difference between the increases in sputum eosinophils after the end-of-treatment allergen challenge between the fluticasone and the fluticasone/salmeterol treatment groups (p = 0.33). For a study schedule see Figure 1.
2 weeks

<table>
<thead>
<tr>
<th>wash-out</th>
<th>run-in fluticasone 250μg bid</th>
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4 weeks

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1-year treatment period

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<tr>
<td>fluticasone/salmeterol 250/50μg bid</td>
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* * *

month:

1 6 9 12

** *

pre-randomization allergen challenge

end-of-treatment allergen challenge
A

- fluticasone/salmeterol
- fluticasone
- run-in

peak flow rate
morning (L/min)

550

500

450

400

0 1 3 6 9 11 12

time (month)
p<0.01

A allergen challenge

B

- fluticasone/salmeterol
- fluticasone
- run-in

short-acting β₂-agonist
usage (puffs)

2

1.5

1

0.5

0

0 1 3 6 9 11 12

time (month)
p<0.01

B allergen challenge