

## **Anti-inflammatory effects of salmeterol/fluticasone propionate in chronic obstructive lung disease**

Neil C Barnes FRCP<sup>1</sup>, Yu-Sheng Qiu PhD<sup>2</sup>, Ian D Pavord FRCP<sup>3</sup>, Debbie Parker BSc<sup>3</sup>, Peter A Davis MBBS<sup>2</sup>, Jie Zhu PhD<sup>2</sup>, Malcolm Johnson PhD<sup>4</sup>, Neil C Thomson MD<sup>5</sup>, Peter K Jeffery DSc<sup>2</sup> on behalf of the SCO30005 study group.

<sup>1</sup>Department of Respiratory Medicine, London Chest Hospital, London, England

<sup>2</sup>Lung Pathology Unit, Imperial College London at the Royal Brompton Hospital, London, England

<sup>3</sup>Institute for Lung Health, Glenfield Hospital, Leicester, England

<sup>4</sup>GlaxoSmithKline Research and Development Ltd, Greenford, Middlesex, England

<sup>5</sup>Department of Respiratory Medicine, University of Glasgow, Glasgow, Scotland

**Requests for reprints:** Professor PK Jeffery FRCPATH, Lung Pathology, Royal Brompton Hospital, Sydney Street, London SW3 6NP, England.

### **Correspondence to:**

Professor PK Jeffery FRCPATH, Lung Pathology, Royal Brompton Hospital, Sydney Street, London SW3 6NP, England.

Telephone: + 44 (0)20 7351 8422. Fax: + 44 (0)20 7351 8435

Email: [p.jeffery@imperial.ac.uk](mailto:p.jeffery@imperial.ac.uk)

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## Abstract

**Rationale:** No currently available treatment is reported to reduce the exaggerated airway wall inflammation of chronic obstructive pulmonary disease.

**Objectives:** We tested the hypothesis that inhaled combined long-acting  $\beta_2$ -agonist (salmeterol) and corticosteroid (fluticasone propionate) will reduce inflammation

**Methods:** Bronchial biopsies and induced sputum were taken from 140 current and former smokers (mean age 64 years) with moderate to severe disease, randomized in a 13-week double-blind study to placebo (n=73) or salmeterol/fluticasone propionate 50/500 $\mu$ g (n=67) twice daily. Biopsies were repeated at 12 and sputa at 8 and 13 weeks. Following adjustment for multiplicity, comparisons between active and placebo were made for median change from baseline in the numbers of biopsy CD8+ and CD68+ cells/mm<sup>2</sup> and sputum neutrophils.

**Measurements and Main Results:** Combination therapy was associated with a reduction in biopsy CD8+ cells of -118 cells/mm<sup>2</sup> (95%CI -209, -42; p=0.02), a reduction of 36% over placebo (p=0.001). CD68+ cells were unaffected by combination treatment. Sputum differential (but not total) neutrophils reduced progressively and, at week 13, significantly with combination treatment (median treatment difference 8.5%; 95% CI 1.75%, 15.25%; p=0.04). The combination also significantly reduced biopsy CD45+, CD4+ and cells expressing genes for tumor necrosis factor- $\alpha$  and interferon- $\gamma$  and sputum total eosinophils (all p $\leq$ 0.03). These anti-inflammatory effects were accompanied by 173mL (95% CI 104, 242; p<0.001) improvement in pre-bronchodilator forced expiratory volume in one second.

**Conclusions:** Salmeterol/fluticasone propionate has a broad spectrum of anti-inflammatory effects in both current and former smokers with chronic obstructive pulmonary disease, which may contribute to clinical efficacy.

**[249 words]**

**Keywords:** airway inflammation, biopsy, sputum, placebo

## Introduction

Chronic obstructive pulmonary disease (COPD) is a leading and rising cause of mortality world-wide<sup>[1]</sup>. Treatments are available that can be used to prevent and control symptoms, reduce exacerbations, increase exercise tolerance and improve health status<sup>[2]</sup>. Long-acting  $\beta_2$ -agonists such as salmeterol combine symptom control with improvement in lung function and provide clinically relevant improvements in health status. Inhaled corticosteroids are recommended for the treatment of patients with more severe disease and frequent exacerbations and inhalation of the combination of long-acting  $\beta_2$ -agonist and inhaled corticosteroid is more effective in improving lung function and symptoms and reducing exacerbations than either drug alone<sup>[3,4,5]</sup>.

Progressive airflow limitation and symptoms in COPD are associated with an exaggerated inflammatory response of the lungs to noxious agents, most commonly cigarette smoke<sup>[2]</sup>. Smoking causes airway inflammation even before there is detectable airflow limitation<sup>[6]</sup> and inflammation persists even after smoking cessation<sup>[7,8]</sup>. Inflammation in COPD is distinct from that in asthma<sup>[9,10]</sup> and is characterized by a predominance of CD8+ cells at all airway levels including the lung parenchyma<sup>[11,12,13,14]</sup>. There is also an increase of CD68+ cells (monocytes/macrophages) in the bronchial sub-epithelium and alveoli<sup>[11,15]</sup> and of B cells in small airways<sup>[14]</sup>. Increased airflow obstruction in COPD is associated with an increase of CD8+ cells in large airways<sup>[11,12]</sup>, CD8+ cells and B-lymphocytes and CD45+ leukocytes in the small airways<sup>[13,14,16]</sup> and CD8+ cells in the lung parenchyma<sup>[13]</sup>. Neutrophils are increased in sputum and bronchoalveolar lavage

fluid<sup>[17,18]</sup> and patients with an accelerated decline in lung function have an increased sputum neutrophil differential count<sup>[19]</sup>. In COPD there is release of pro-inflammatory mediators that include tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interferon- $\gamma$  (IFN $\gamma$ ) and interleukin (IL)-8<sup>[20]</sup>. No currently available treatment has been reported to reduce these key cells and mediators<sup>[2]</sup> in bronchial biopsies and any reported effects in induced sputum are controversial. Therapies that attenuate the ongoing inflammation in COPD may impact on the airway and lung destruction associated with progressive disease.

The beneficial effects of the combination of a long-acting  $\beta_2$ -agonist and inhaled corticosteroid are acknowledged clinically, yet its effects on the airway inflammation that characterizes and forms part of the definition of COPD<sup>[2]</sup> have not been reported. We therefore tested the hypothesis that by comparison with placebo this combination would reduce biopsy CD8+ and CD68+ cells and sputum neutrophils. We also assessed the numbers of biopsy CD45+ leukocytes, CD4+ cells, tryptase + mast cells and cells expressing the pro-inflammatory genes TNF $\alpha$  and IFN $\gamma$ . Sputum eosinophils, macrophages and lymphocytes and the concentrations of IL-8 and eosinophil cationic protein (ECP) were also measured in order to assess inflammation in a complementary airway compartment. Some of the results of this study have been previously reported in the form of abstracts<sup>[21,22,23,24,25,26,27,28]</sup>

## Methods

**[636 words]**

This was a randomized, double-blind, placebo-controlled, parallel-group multicenter study, approved by local research ethics committees or institutional review boards as appropriate. All patients, who were treated as out-patients, gave written informed consent. Following a 4-week run-in, patients with moderate to severe COPD<sup>[2]</sup> were stratified according to whether they were current or former smokers, and randomized to either salmeterol/fluticasone propionate (Seretide<sup>TM</sup>/Advair<sup>TM</sup>/Viani<sup>TM</sup> Diskus<sup>TM</sup>, GlaxoSmithKline, Greenford, England) 50/500 $\mu$ g twice daily or matching placebo for 13 weeks. Patients were not treated with inhaled or oral corticosteroids for at least 4 weeks and long-acting  $\beta_2$ -agonists for at least 2 weeks before the run-in. During the study the only concurrent treatment permitted was ipratropium bromide as required.

Endobronchial biopsies were obtained one week prior to randomization and again after 12 weeks' treatment using standardized procedures with bronchoscopy, biopsy processing (fixation and immunostaining) and quantification performed as described previously<sup>[11,29,30,31]</sup>. A previous study has demonstrated that bronchoscopies may be performed safely in patients with COPD<sup>[32]</sup>. Three biopsies were taken from each of the lobar and sub-segmental carinae. Those from the latter airway generation were used for the analyses described here unless they were of insufficient quality, when they were replaced by biopsies from the more proximal level. All the processing, histological and molecular analyses of paraffin wax-embedded samples were performed centrally (Royal Brompton Hospital, London UK). Induced sputum was collected at randomization, and after 8 and 13 weeks' treatment. Sputum induction

and processing were performed using previously published methods<sup>[33,34]</sup>. Total cell counts were made locally and differential counts and other sputum measurements were made at a central laboratory (Glenfield Hospital, Leicester, UK). Additional details on the methods for biopsy and sputum measurements are provided in an online data supplement.

Measurements of forced expiratory volume in 1 second (FEV<sub>1</sub>) and forced vital capacity (FVC) were made at each clinic visit and safety was assessed by measurement of vital signs and adverse events.

The study was designed by a Steering Committee (NB, IP, MJ, PKJ, NT), who also approved the protocol. The sponsor (GlaxoSmithKline) funded and co-ordinated the study and collected, held and analyzed the data, which was only unblinded when data queries had been resolved, in accordance with ICH E9 guidelines. The Steering Committee and all authors had full access to all the data and vouch for the accuracy of the data and the data analysis. Data interpretation and writing of the manuscript was done predominantly by PKJ, NB and IP with extensive input by all authors. The academic authors had final responsibility for the manuscript.

### **Statistical Methods**

Biopsy CD8+ and CD68+ inflammatory cells and sputum neutrophils were chosen as co-primary endpoints. Sputum neutrophils were expressed as both total and differential counts; consequently p-values obtained were corrected for four co-primary endpoints. As data were not normally distributed, the changes from baseline for numbers of biopsy CD8+ or CD68+ cells and sputum neutrophils for each patient

were ranked and analyzed using the Van Elteren extension to the Wilcoxon-Rank-Sum test<sup>[35,36]</sup> stratified for current and former smokers. Median differences and confidence intervals were constructed using Hodges-Lehman estimates<sup>[37]</sup>.

Comparisons were made using all patients who had both baseline and endpoint values for biopsy and/or sputum. In addition, patients with no endpoint values who had experienced an exacerbation requiring treatment with oral corticosteroids or who were withdrawn due to perceived lack of efficacy were included in the analysis and assigned the lowest rank for change in endpoints. Additional statistical considerations are provided in an online data supplement.

Secondary biopsy and sputum endpoints were analyzed similarly but with no adjustments to nominal significance levels. *Post hoc*, analyses of percentage change were also performed and the effects of smoking status on primary and secondary biopsy and sputum endpoints were investigated in a preliminary fashion, as the study was not powered initially for sub-group analyses. FEV<sub>1</sub> and FVC were analyzed using analysis of covariance.

## **Results**

Details of patients screened, randomized and withdrawn during the study are shown in Figure 1. Sixty-seven patients (55 male; mean age 65years, range 45-77years) were treated with salmeterol/fluticasone propionate and 73 (54 male; mean age 64years,

range 40-80years) with placebo. Active and placebo treatment groups were well-matched for demography, smoking history and baseline lung function (Table 1).

### **Biopsy and Sputum Sample Quality**

Using published criteria<sup>[33]</sup> 98.4% of sputum cytopins were evaluable. Ninety-three percent of biopsies were considered evaluable. Counts were obtained from three biopsies for  $\geq 89\%$  of patients at baseline and  $\geq 84\%$  at the end of treatment. Each marker was counted throughout the study by a single observer. The error of repeat measurement for a single observer for biopsy area and cell counts (expressed as % coefficient of variation) for the primary biopsy parameters was approximately 3% and that between observers was approximately 6%. The majority of biopsies analysed were those from the sub-segmental carinae and samples from the lobar carinae were used from 9 patients only.

### **Primary Endpoints**

For inclusion in biopsy and sputum analyses, patients were required to have a baseline and an endpoint value. Not all randomized patients completed the study and some patients had missing endpoint samples (Figure 1). The numbers used for biopsy and sputum analyses are different since the patients with both evaluable baseline and endpoint samples for biopsy and sputum were not identical. In addition, two patients (one in each treatment group) with no endpoint values were assigned the lowest rank for change in endpoints and included in the analysis since they were withdrawn because of an exacerbation or perceived lack of efficacy.

Compared with placebo, salmeterol/fluticasone propionate significantly reduced the absolute number of biopsy CD8+ cells ( $p=0.015$ , Table 2), a 36% difference (95% CI: 16%, 56%) in favor of the combination treatment ( $p=0.001$ , Figure 2). CD68+ cells increased in number in the placebo group and this was not altered by the combination. There was a progressive reduction in sputum neutrophil differential cell count with active treatment (Figure 3) which was significant compared with placebo at 13 weeks ( $p=0.037$ ). A similar trend was seen for sputum total neutrophils but the difference between treatments was not significant expressed as an absolute change (Table 2). However, expressed as a percentage reduction from baseline the difference in total neutrophils (53%) was significant (95% CI: 1%, 111%;  $p=0.046$ ).

In order to investigate the individual variability in response, the proportions of patients achieving a 10%, 25% or 50% change from baseline in CD8+ and CD68+ cells or a 10% or 35% decrease in neutrophils were tabulated (Table 3). The thresholds applied for differential neutrophil counts were defined by reference to data from Stanescu and colleagues<sup>[19]</sup> and those for biopsy endpoints were based on the distribution of the data since no clinically relevant limits have been defined. The results showed considerable individual variability, although the proportion of patients showing decreases in sputum neutrophils and in CD8+ cells was greater for salmeterol/fluticasone propionate than for placebo.

### **Secondary endpoints**

Compared with placebo, there were significantly greater reductions by salmeterol/fluticasone propionate in numbers of biopsy CD45+ cells, an overall measure of the total number of leukocytes. CD4+ cells, TNF $\alpha$  mRNA+ cells and

IFN $\gamma$  mRNA<sup>+</sup> cells were reduced whether expressed as absolute changes (Table 2) or percentage changes (Figure 2). There were significant positive associations at baseline between the number of CD8<sup>+</sup> cells and the numbers of TNF $\alpha$  mRNA<sup>+</sup> and IFN $\gamma$  mRNA<sup>+</sup> cells at baseline ( $\rho=0.404$ ,  $p=0.002$  and  $\rho=0.407$ ,  $p=0.002$ , respectively). Positive associations were also found between the reduction of CD8<sup>+</sup> cells and the relative reductions of cells expressing genes for these two pro-inflammatory mediators following combination treatment ( $\rho=0.386$ ,  $p=0.004$  and  $\rho=0.443$ ,  $p=0.001$ , respectively).

There was a strong trend towards a reduction in absolute numbers of mast cells by the combination but this did not achieve statistical significance ( $p=0.083$ ). However, there was a significant difference when data were expressed as percentage change ( $p=0.022$ ). While the ratio was highest in the group given the combination, no significant difference was seen between treatments for the ratio of CD8<sup>+</sup> to CD4<sup>+</sup> cells (median ratio 3.1:1 for placebo, 3.5:1 for salmeterol/fluticasone propionate) as both CD8<sup>+</sup> and CD4<sup>+</sup> cells had been reduced by active treatment compared with placebo.

Reductions in sputum total cell counts were seen with salmeterol/fluticasone propionate after 8 weeks' treatment and at endpoint but there was no significant difference between active and placebo treatments (Table 2). The change in total numbers of sputum eosinophils was significant in favor of active treatment but changes in eosinophil differential counts did not reach significance. IL-8 and ECP concentrations were not significantly changed by salmeterol/fluticasone propionate in comparison with placebo.

The directions of the response to combination treatment were generally similar in smokers and former smokers, albeit the reductive effect was generally greater in the former smokers (Figure E1 of the online supplement).

### **Clinical Endpoints**

Patients treated with salmeterol/fluticasone propionate showed increases in mean pre-bronchodilator FEV<sub>1</sub> at each visit which were significantly greater ( $p < 0.001$ ) than with placebo (Figure 4). The mean treatment difference at the end of the study was 173mL (95% CI 104mL, 242mL;  $p < 0.001$ ). Similar results were seen for FVC (mean treatment difference at end of study 170mL; 95% CI 41mL, 299mL;  $p = 0.010$ ).

*Post hoc* evaluation of correlations between lung function (change in FEV<sub>1</sub> as percentage of predicted normal and change in FVC) and primary and secondary biopsy parameters showed no convincing or significant correlations. A reduction in total biopsy inflammation (i.e. CD45+ cells expressed as absolute or percentage change) corresponded to an improvement in FEV<sub>1</sub> percent of predicted ( $\rho = 0.25$ ,  $p = 0.065$ , and  $\rho = 0.26$ ,  $p = 0.057$ , respectively), but these relationships appeared mainly to be driven by the outlier values. For salmeterol/fluticasone propionate, reduction in sputum percentage neutrophils showed a significant association with an increase in absolute FVC ( $\rho = -0.30$ ;  $p < 0.05$ ).

The bronchoscopy procedure was well accepted by most patients and only four patients (3 at baseline and 1 at endpoint) experienced adverse events (nose bleed, cough and sore throat, dyspnea, high blood pressure) associated with this procedure.

Both active and placebo treatments were well-tolerated. Five patients (7%) treated with placebo and 15 (22%) treated with salmeterol/fluticasone propionate experienced events that were considered to be drug-related with no event reported by more than four patients and all events reported were predictable effects of treatment. Oral candidiasis was the most frequently recorded adverse event on active treatment (6% vs 1%). Fewer patients in the salmeterol/fluticasone propionate group than in the placebo group experienced a worsening of COPD symptoms that required any change in normal treatment (11 [16%] vs 24 [33%];  $p=0.025$ ). In six patients (9%) in the combination group and eight patients (11%) in the placebo group, antibiotic treatment was required to treat the worsening symptoms, with one patient in the combination group also hospitalised to treat the worsening symptoms. A further one patient (1%) in the combination group and two patients (3%) in the placebo group received oral corticosteroids to treat worsening symptoms.

## **Discussion**

This is the first demonstration that a currently available treatment can reduce the exaggerated bronchial inflammation in COPD. The combination of inhaled salmeterol and fluticasone propionate significantly reduced the absolute numbers of biopsy (CD45+) leukocytes, CD8+ cells and CD4+ cells together with decreases in cells expressing genes for the pro-inflammatory mediators IFN $\gamma$  and TNF $\alpha$ . In the complementary airway compartment sampled by induced sputum, combination treatment significantly reduced sputum differential neutrophils and total eosinophils. Considering the only published longitudinal data for the relationship between sputum

neutrophils and lung function<sup>[19]</sup>, the difference of 8.5% in favor of combination treatment is likely to be clinically significant.

The broad-spectrum of anti-inflammatory effects was accompanied by significant improvements in lung function. The magnitude of the improvements seen in FEV<sub>1</sub> were of a similar or greater magnitude to those seen in other studies of anti-inflammatory treatment used in COPD<sup>[3,30,38,39]</sup>.

There are a number of potential mechanisms that may contribute to such improvement. Experimental studies have shown that CD8+ cells are able to recognize viral antigen expressed on the surface of lung epithelial cells in a class I-restricted manner and these cells may destroy host cells by apoptosis directly via release of perforins and granzymes<sup>[40]</sup> or indirectly via release of TNF $\alpha$  and IFN $\gamma$ . TNF $\alpha$  can stimulate epithelial cells to release chemoattractants for macrophages<sup>[41]</sup> or for neutrophils, acknowledged effector cells in COPD<sup>[42]</sup>. IFN $\gamma$  is also associated with generation of emphysematous lesions experimentally<sup>[43]</sup>. We speculate that the effects of combination treatment most likely to be associated with clinical benefit are centered on its reduction of the sputum neutrophil differential count and biopsy CD8+ cells and the associated mediators TNF $\alpha$  and IFN $\gamma$ .

A potential criticism of our study is that comparison with the effects of corticosteroid or long-acting  $\beta_2$ -agonist alone would have been additionally informative. Whilst we considered the merits of a four-arm study design, we rejected this for several reasons. We wished to conduct a proof of principle study to ascertain whether inhaled therapy can modify inflammation in COPD. This has never been shown previously. From a

practical perspective, sufficiently powered biopsy studies are extremely demanding and, based on our power calculations using the data we had obtained previously in COPD, a four-arm study would have taken several years to complete. To be adequately powered, and to ensure sufficient patients with both baseline and end of treatment samples, each additional treatment group would have required the recruitment of a further 65 patients. As designed, the present study represents the largest biopsy study ever to be completed in COPD. Our rejection of a four-arm study design was also influenced by clear evidence from a randomized study in COPD patients of similar severity (and in which analyses were carried out in the same biopsy central laboratory) that inhaled fluticasone propionate alone at the same dose and for the same duration as that used here had no effect on the number of biopsy subepithelial CD8+, CD68+ or CD4+ cells (key outcome measures in the current study), although significant reductions of biopsy mast cells were seen as were differences in the epithelial CD8+:CD4+ cell ratio<sup>[44,45]</sup>. Similarly, most studies have shown no effect of either inhaled or oral corticosteroids on sputum neutrophils<sup>[20,34,46]</sup>.

We acknowledge that we cannot exclude an effect of salmeterol alone. While no biopsy studies of long-acting  $\beta_2$ -agonist monotherapy have been conducted in patients with COPD, non-bronchodilator effects of salmeterol are well established *in vitro*<sup>[47]</sup>, and, in asthma, salmeterol, and more recently formoterol, have been shown to reduce biopsy and sputum neutrophils and associated markers<sup>[48,49]</sup>. We chose to study the salmeterol/fluticasone propionate combination rather than salmeterol alone since combination treatment has been shown clinically to have greater effects than monotherapy in patients with COPD and salmeterol has been considered to be effective clinically because of its bronchodilator rather than anti-inflammatory effects.

Therefore we considered that salmeterol/fluticasone propionate was the inhaled intervention most likely to have anti-inflammatory effects *in vivo*. It is possible, however, that the long-acting  $\beta_2$ -agonist may have contributed to the anti-inflammatory effect and, we acknowledge that further research is needed to elucidate the anti-inflammatory effects of  $\beta_2$ -agonists alone in COPD.

Synergy between corticosteroid and long-acting  $\beta_2$ -agonist might have enhanced the  $\beta_2$ -agonist effect<sup>[50]</sup> resulting in a general increase of intracellular cAMP, such as that achieved by the selective PDE4 inhibitor, cilomilast, which has been shown to decrease CD8+ cells and macrophages in patients with COPD<sup>[30]</sup>. Considering our present results and those of previous studies, it appears that combination therapy has anti-inflammatory effects not seen with inhaled corticosteroids alone. Compared with monotherapy, enhanced effects for the combination are consistent not only with the clinical data<sup>[3,4,5]</sup> but also with *in vitro* data, which demonstrate the synergistic interaction between salmeterol and fluticasone propionate<sup>[51,52]</sup>. For example, rhinovirus-induced chemokine production is decreased with the combination<sup>[51]</sup> and there is reduced production of TNF $\alpha$  from alveolar macrophages<sup>[52]</sup> compared with corticosteroid alone. In these studies, salmeterol alone had no effect in either model. Similar interactions between fluticasone propionate and salmeterol *in vivo* are a likely explanation for the effects seen in our study. Thus, salmeterol could be enhancing the effect of the inhaled corticosteroid, or alternatively the  $\beta_2$ -agonist effect could be enhanced by fluticasone propionate<sup>[50]</sup>.

Bronchoscopy and biopsy, and sputum access the proximal bronchi only and we have not been able to assess the effects of combination therapy on small airways, the site

considered to contribute most to reduced lung function in COPD<sup>[14]</sup>. However, the predominance of CD8+ cells is seen in both proximal and small airways and the correlation between this cell type and impaired lung function is similar in both large and small airways and lung parenchyma<sup>[11,12,13,14]</sup>. This, together with recent data<sup>[53]</sup>, suggest that similar processes of inflammation and airway wall thickening appear to be taking place in both large and small airways. Thus, biopsy samples of large airways may be a reasonable surrogate for assessing the potential effects of treatment on small airway inflammation and remodelling. Furthermore, it is likely that the formulation of the combination treatment we used would have reached the small airways as the Diskus device delivers approximately 20% of its dose as a fine particle, respirable fraction (i.e. particles <5 microns)<sup>[54,55]</sup>, that would be delivered throughout the lung. While the associations between the extent, type and site of inflammation and lung function decline remain to be determined<sup>[56]</sup>, the improvements in lung function seen in our study also indicate that sites affecting resistance to airflow were accessed by treatment.

Increases in sputum neutrophils, biopsy CD68+ cells (monocyte/macrophage) and other cells were observed in the placebo control group. An increase of CD68+ cells with placebo was also seen in a previous biopsy study of patients with stable COPD<sup>[30]</sup>. The reasons for these increases are presently unclear. As patients were recruited when their disease was stable, deterioration in their condition was a likely outcome when no active treatment was given. This requires further study, as does the effects of smoking status on the results seen. The present study was not powered to investigate effects by smoking status, but a preliminary *post hoc* analysis indicated that there are anti-inflammatory effects seen in both current and former smokers, with

a trend for the magnitude of the effect to be greater in former smokers, although there is variability in response.

In summary, we report here results of the largest multicenter clinical trial yet in COPD involving repeat bronchial biopsy and sputum samples in patients with disease of severity GOLD stage II or III. The data demonstrate broad-spectrum anti-inflammatory effects of a currently available treatment, salmeterol/fluticasone propionate combination. We prospectively identified key parameters, biopsy CD8+ cells and sputum neutrophils, shown previously to relate to severity and disease progression and show anti-inflammatory effects of the combination of a magnitude likely to be clinically significant. These findings may support the consideration that combination treatment applied earlier than currently proposed in guidelines may be helpful. They provide a rationale for further investigation of salmeterol/fluticasone propionate in disease progression and survival. The TORCH (TOwards a Revolution in COPD Health) trial<sup>[57]</sup> is currently investigating the effect of salmeterol/fluticasone propionate combination and its components on all cause mortality. Its results will determine whether the same combination that we have shown attenuates the exaggerated inflammation of COPD will also improve the prognosis of patients with this chronic condition.

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## Figure Legends

Figure 1 - Flowchart of Patient Disposition through the Study

Figure 2 - Median Treatment Differences in Percentage Change from Baseline for Biopsy Endpoints.

Hodges-Lehmann Estimator of median treatment difference and 95% confidence interval presented.

Figure 3 - Median (inter-quartile ranges) Sputum Neutrophil Differential (%) through the Study

□ Baseline

■ 8 Weeks

■ 13 Weeks

Week 13  $p=0.037$  for treatment difference (between salmeterol/fluticasone propionate and placebo) in change from baseline. Week 8  $p=ns$ .

Figure 4 - Adjusted mean changes from baseline in pre-bronchodilator FEV<sub>1</sub>.

...◆... Placebo

—■— Salmeterol/fluticasone propionate

Treatment differences analysed by ANCOVA (\*\*\*) =  $p<0.001$ )

## Tables

**Table 1: Baseline subject characteristics**

	<b>Placebo</b>	<b>Salmeterol/ Fluticasone propionate</b>
	<b>N=73</b>	<b>N=67</b>
Mean [sd] age (years)	63.9 [8.9]	64.9 [7.6]
Male/female (%)	74/26	82/18
Race: White/Asian (%)	100/0	99/1
Current/Former smokers (%)	59/41	63/37
Mean [sd] pack years	44.0 [21.7]	40.3 [24.5]
Median (range) pack years	40 (10 -150)	35 (12 - 177)
Mean [sd] % predicted* FEV <sub>1</sub>	59 [10.8]	58 [12.0]
Mean [sd] pre-bronchodilator FEV <sub>1</sub> (L)	1.68 [0.47]	1.67 [0.44]
Mean [sd] post-bronchodilator FEV <sub>1</sub> /FVC (%)	58 [9.1]	54 [8.9]
Mean [sd] reversibility to salbutamol <sup>†</sup> %	3.9 [3.4]	3.9 [3.1]

\*European Community for Coal and Steel normal values

<sup>†</sup> Calculated as percentage of predicted value

**Table 2: Results of Analyses of Biopsy and Sputum Endpoints (Absolute Counts)**

	Placebo		Salmeterol/Fluticasone Propionate		Treatment difference (95% CI) ‡	p- value
	Baseline	Week	Baseline	Week		
	Median	12/13 <sup>†</sup>	Median	12/13 <sup>†</sup>		
	[Range*]	Median	[Range*]	Median		
		[Range*]		[Range*]		
<b>Primary endpoints</b>						
CD8+ cells/mm <sup>2</sup> (biopsy)	315.6 [174.9 - 473.6]	285.6 [187.8 - 422.8]	274.1 [173.6 - 538.1]	180.9 [102.8 - 256.1]	-117.9 (-208.6, -41.9)	0.015 <sup>§</sup>
CD68+ cells/mm <sup>2</sup> (biopsy)	64.7 [36.8 - 123.6]	107.4 63.0 - 158.7]	68.2 [35.7 - 142.6]	105.2 [54.1 - 150.5]	-17.9 (-50.0, 13.1)	0.255 <sup>§</sup>
Neutrophils (sputum):						
- differential (%)	81.00% [64.25 - 88.88]	83.75% [76.00 - 90.50]	80.25% [66.75 - 88.50]	76.00% [55.00 - 93.00]	-8.50%; (-15.25, -1.75%)	0.037 <sup>§</sup>
- total numbers x10 <sup>6</sup>	1.040 [0.367 - 4.802]	2.069 [0.545 - 3.968]	0.942 [0.324 - 2.704]	0.779 [0.372 - 3.235]	-0.606 (-1.554, 0.038)	0.130 <sup>§</sup>

**Secondary biopsy endpoints**

CD45+ cells/mm <sup>2</sup>	706.1	659.3	782.2	482.2	-285.3	0.006
	[470.6 -	[465.4 -	[424.9 -	[294.9 -	(-488.9, -100.1)	
	1186.0]	975.9]	1098.5]	657.5]		
CD4+ cells/mm <sup>2</sup>	74.7	94.2	95.4	42.8	-45.0	0.017
	[36.0 -	[45.2 -	[47.6 -	[23.2 -	(-85.5, -4.8)	
	255.2]	176.1]	235.1]	85.0]		
Mast cells/mm <sup>2</sup>	155.8	152.6	128.6	114.2	-28.6	0.083
	[87.5 -	[102.0 -	[94.9 -	[67.0 -	(-59.4, 2.2);	
	225.3]	208.4]	200.1]	153.3]		
IFN $\gamma$ mRNA+	38.7	49.9	37.3	16.1	-29.7	0.026
cells/mm <sup>2</sup>	[16.6 -	[11.1 -	[14.7 -	[8.4 - 32.1]	(-55.8, -3.6).	
	102.2]	94.7]	83.1]			
TNF $\alpha$ mRNA+	31.7	32.6	21.2	14.2	-18.9	0.003
cells/mm <sup>2</sup>	[14.2 -	[16.0 -	[12.7 -	[5.6 - 23.8]	(-34.3, -6.1)	
	64.5]	60.6]	75.1]			

**Secondary sputum endpoints**

Total cells x10 <sup>6</sup>	1.745	2.190	1.310	1.220	-0.701	0.130
	[0.619 -	[0.800 -	[0.480 -	[0.559 -	(-2.062, 0.204)	
	6.334]	4.960]	4.026]	3.900]		
Eosinophils						
- differential (%)	1.63%	1.50%	2.25%	0.75%	-0.75%	0.065
	[0.63 -	[0.50 -	[0.75 -	[0.25 -	(-1.75, 0.00%)	
	3.25]	3.25]	5.00]	2.50]		
- total numbers	0.031	0.029	0.027	0.017	-0.026	0.030
x10 <sup>6</sup>	[0.007 -	[0.007 -	[0.009 -	[0.001 -	(-0.054, -0.002)	
	0.075]	0.090]	0.078]	0.048]		
IL-8 (ng/mL)	74.3	46.2	43.4	26.8	-13.3	0.400
	[14.9 -	[18.6 -	[16.3 -	[8.0 -	(-40.2, 22.8)	
	226.5]	223.6]	159.3]	169.3]		
ECP (ng/mL)	539.1	669.2	743.4	494.1	-109.8	0.327
	[255.6 -	[230.4 -	[190.8 -	[184.5 -	(-466.2, 123.3)	
	1287.0]	1512.0]	1602.0]	1017.0]		

All cells in bronchial biopsies expressed as cells/mm<sup>2</sup> subepithelium

Numbers of subjects in analyses are shown in Figure 1.

\* Interquartile range

† Biopsies performed at Week 12, sputum samples taken at Week 13.

‡ Median difference in median changes from baseline for salmeterol/ fluticasone propionate minus placebo.

§ Bonferroni-Holm adjustment made to p-values for co-primary endpoints

**Table 3: Number (%) of Patients with Changes from Baseline that Exceeded Defined Limits after 12 Weeks (Biopsy) or 13 Weeks (Sputum) of Treatment**

	<b>Placebo</b>	<b>Salmeterol/fluticasone propionate</b>
<b>Primary Endpoints</b>		
<b>Neutrophils (sputum)</b>	<b>N=59</b>	<b>N=50</b>
At least 10% reduction	6 (10%)	19 (38%)
At least 35% reduction	2 (3%)	5 (10%)
<b>CD8 + cells (biopsy)</b>	<b>N=68</b>	<b>N=54</b>
Any reduction	38 (56%)	38 (70%)
At least 10% reduction	34 (50%)	38 (70%)
At least 25% reduction	24 (35%)	35 (65%)
At least 50% reduction	9 (13%)	24 (44%)
<b>CD68+ cells (biopsy)</b>	<b>N=68</b>	<b>N=54</b>
Any increase	49 (72%)	35 (65%)
At least 10% increase	47 (69%)	33 (61%)
At least 25% increase	41 (60%)	30 (56%)
At least 50% increase	34 (50%)	27 (50%)
<b>Selected Secondary Endpoints</b>		
<b>IFN<math>\gamma</math> mRNA+ cells (biopsy)</b>	<b>N=68</b>	<b>N=53</b>
Any reduction	38 (56%)	38 (72%)
At least 10% reduction	37 (54%)	37 (70%)
At least 25% reduction	32 (47%)	34 (64%)
At least 50% reduction	27 (40%)	28 (53%)
<b>TNF<math>\alpha</math> mRNA+ (biopsy)</b>	<b>N=68</b>	<b>N=54</b>
Any reduction	38 (56%)	42 (78%)
At least 10% reduction	36 (53%)	41 (76%)

At least 25% reduction	32 (47%)	35 (65%)
At least 50% reduction	21 (31%)	29 (54%)

## Figures

**Figure 1**

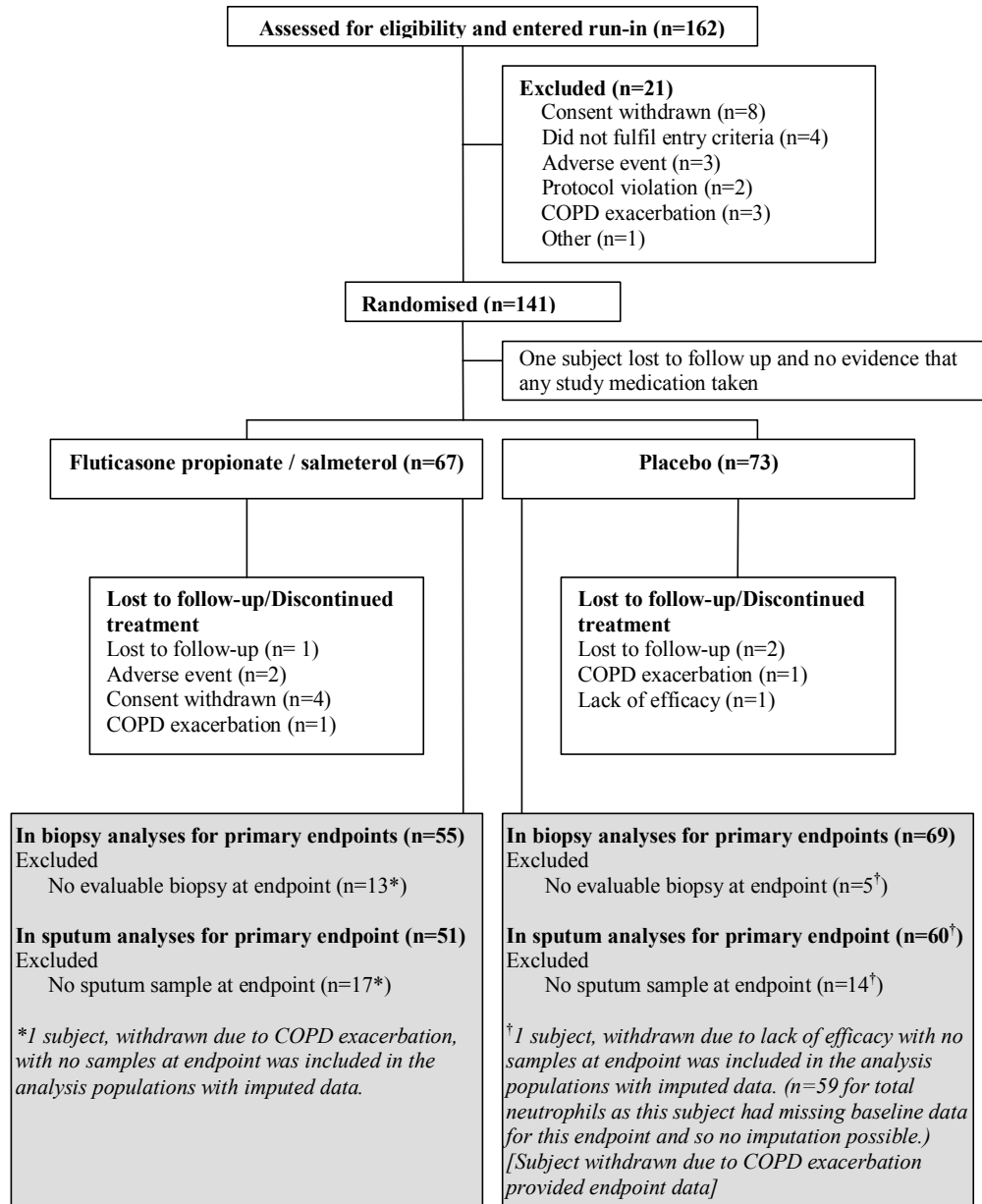
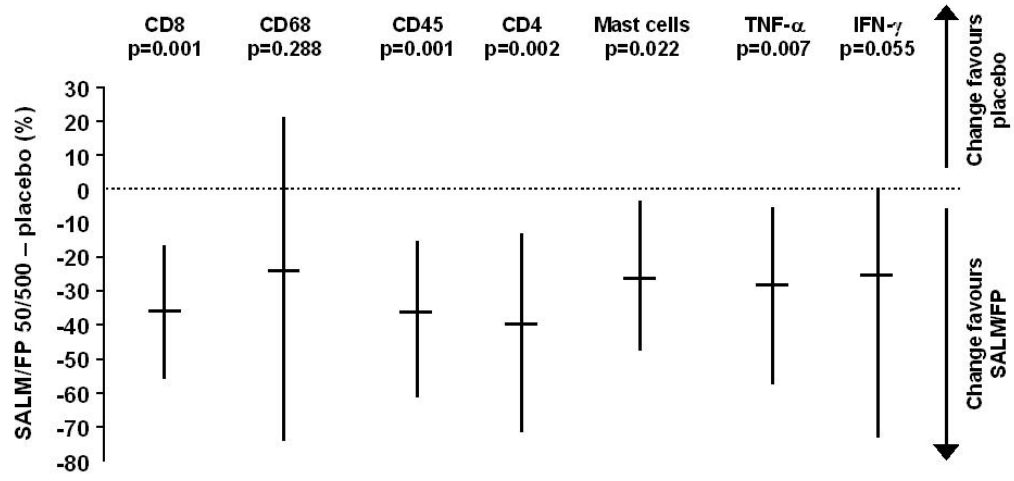
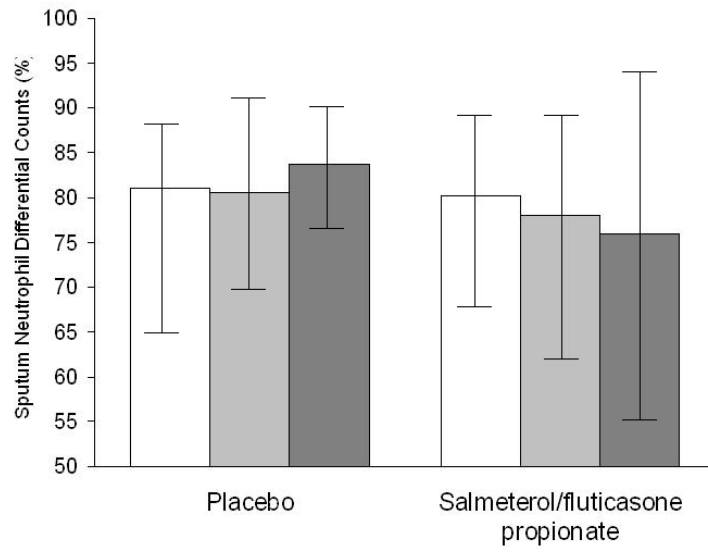


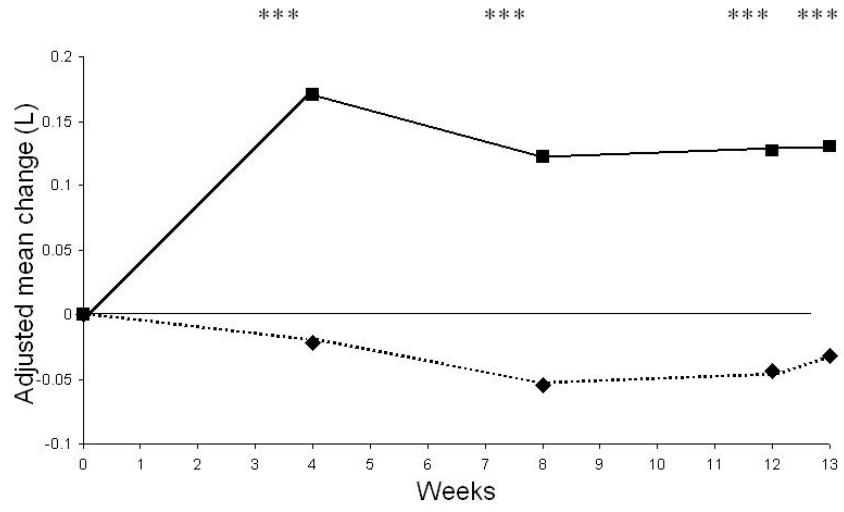
Figure 2



**Figure 3**



**Figure 4**



## **Online Data Supplement**

### **Anti-inflammatory effects of salmeterol/fluticasone propionate in chronic obstructive lung disease**

Neil C Barnes FRCP, Yu-Sheng Qiu PhD, Ian D Pavord FRCP, Debbie Parker BSc, Peter A Davis MBBS, Jie Zhu PhD, Malcolm Johnson PhD, Neil C Thomson MD, Peter K Jeffery DSc on behalf of the SCO30005 study group.

## Methods

### Study Design

This randomised, double-blind, placebo-controlled, parallel-group multicenter study, was designed by a Steering Committee (NB,IP,MJ,PJ) that included an independent adviser (NT). It was carried out in 18 centers in 8 European countries. Following a 4-week run-in period, patients were stratified according to whether they were current or former smokers and were randomised to either salmeterol/fluticasone propionate (Seretide™/ Advair™/ Viani™ Diskus™, GlaxoSmithKline) 50/500µg twice daily or matching placebo for 13 weeks using a computer-generated randomization schedule with a block size of four. All analyses were performed by a statistician according to a predefined analysis plan, and data were only unblinded when data queries had been resolved, in accordance with ICH E9 guidelines. The progress and impartiality of the entire study was monitored by the Steering Committee.

### Patients

Current or former smokers (with a smoking history of  $\geq 10$  pack-years) with moderate to severe COPD<sup>[E1]</sup> were enrolled and treated as out-patients. Requirements included an oxygen saturation  $\geq 88\%$  at room air, pre-bronchodilator FEV<sub>1</sub> 40-80% of predicted normal value, FEV<sub>1</sub>/FVC ratio  $\leq 70\%$  predicted, and demonstration of poor reversibility in airflow obstruction ( $< 10\%$  increase in FEV<sub>1</sub> as a percentage of predicted normal value or  $< 200$ ml absolute improvement 30 minutes after inhalation of 400µg albuterol). Patients with significant respiratory disorders other than COPD or with  $\alpha 1$ -antitrypsin deficiency were excluded, as were those who experienced an

exacerbation or chest infection in the 6 weeks before the start of the run-in period. Patients had not been treated with inhaled or oral corticosteroids for at least 4 weeks and long-acting  $\beta_2$ -agonists for at least 2 weeks before the 4-week run-in. During the run-in and 13-week study treatment period, the only concurrent treatment permitted was ipratropium bromide as required.

## **Bronchial Biopsies**

Bronchoscopy, biopsy and processing (fixation and immunostaining) for quantification of these tissues were performed as described previously<sup>[E2,E3,E4]</sup>. Training sessions were run to ensure consistency between centers and maintain biopsy quality which was validated by examination of two sets of pilot biopsy samples prior to the study start. At each visit, in order to take account of within-patient variability, three biopsies were taken from each of the lobar and sub-segmental carinae. Those from the latter airway generation were used for the analyses described here unless they were of insufficient quality, when they were replaced by biopsies from the more proximal level. We have previously shown no statistically significant differences in the numbers of inflammatory cells between these two airway levels<sup>[E2]</sup>. All the processing, histological and molecular analyses of paraffin wax-embedded samples were carried out at the Royal Brompton Hospital, London UK (under the supervision of PKJ). Inflammatory cells were identified using monoclonal antibodies directed against CD8+, CD68+, CD4+ and CD45+ cells and mast cell tryptase (DakoCytomation, Denmark). Digoxigenin-labelled antisense complementary ribonucleic acid (RNA) probes were used for *in situ* hybridization to detect TNF $\alpha$  messenger (m)RNA and IFN $\gamma$  mRNA<sup>[E5]</sup>. In stable COPD biopsy neutrophils are relatively infrequent. For this reason, we planned to assess biopsy neutrophils only after the results of the analysis of sputum neutrophils were known to be informative of

an effect of the treatment. Areas of subepithelium excluding muscle, gland and large vessels were measured using computerized image analysis<sup>[E5]</sup>. Inflammatory cell phenotypes were identified by their specific immuno-positivity and those with a nucleus in the plane of the tissue section were counted using light microscopy. Each phenotype was counted throughout the study by a single observer (YQ, JZ or PD), with counts obtained from three biopsies for each biopsy occasion where possible.

### **Induced Sputum**

Sputum induction and processing were performed using a previously published method<sup>[E6]</sup>. Training sessions were run to standardize sputum procedures across sites and test slides were sent to the central laboratory before the study start. All processing was done at 4°C. Total cell counts were made locally and differential counts (neutrophils, eosinophils, macrophages and lymphocytes) were made (by DP) at a central laboratory (Glenfield Hospital, Leicester, UK, under the supervision of IP). Concentrations of IL-8 and ECP in supernatant were measured as previously described<sup>[E7]</sup>.

### **Statistical Considerations**

All statistical analyses were performed using SAS version 8.2 (SAS, Cary, North Carolina, USA). Biopsy CD8+ and CD68+ inflammatory cells and sputum neutrophils were chosen as co-primary endpoints. The sample size calculation was made *a priori* and was based on 90% power to detect a difference between salmeterol/fluticasone propionate and placebo on any one of three primary endpoints at  $\alpha=0.0167$  to preserve an overall  $\alpha=0.05$ , using the Bonferroni-Holm multiplicity adjustment<sup>[E8]</sup> in the analysis. If the true probability of a combination

subject having a lower cell count than a placebo subject was greater than 0.75 or less than 0.25, then 39 evaluable subjects per treatment group (78 in total) would give 90% power. (Thus, the number of patients required in each group precluded a 4-arm study being performed within a reasonable time period.) Randomization was planned for 130 subjects to ensure sufficient subjects with both baseline and endpoint samples. Shortly after study initiation, a protocol amendment specified that sputum neutrophils would be expressed as both total and differential counts; consequently p-values obtained were corrected for four co-primary endpoints.

For the analysis of covariance of FEV<sub>1</sub> and FVC, the covariates included were center, age, sex, smoking status (current or former smoker) and baseline.

The effects of smoking status on primary and secondary biopsy and sputum endpoints were investigated in *post hoc* analyses, although the study was not powered to detect treatment differences in subgroups.

## **RESULTS**

The results of the *post hoc* analyses of the effects of smoking status on the biopsy and sputum endpoints are summarized in Figure E1. Results were generally similar for smokers and former smokers. There was a trend for the magnitude of the effect to be greater in former smokers, although there was much variability in the responses.

## **List of Participating Centers**

Dr V Backer (Denmark)

Dr H Harving (Denmark)

Prof A Altraja (Estonia)

Dr R Sepper (Estonia)

Prof L Laitinen (Finland)

Prof G Bonsignore (Italy)

Dr A Bagdonas (Lithuania)

Dr A Blaziene (Lithuania)

Dr R Sakalauskas (Lithuania)

Dr V Chalkova (Slovakia)

Dr R Alvarez Sala (Spain)

Dr C Disdier (Spain)

Prof N Barnes (United Kingdom)

Dr J Corne (United Kingdom)

Prof A Knox (United Kingdom)

Prof A Morice (United Kingdom)

Dr I Pavord (United Kingdom)

Dr C Ramsay (United Kingdom)

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## Figure Legends

Figure E1 (a) Biopsy endpoints by smoking status

(b) Sputum endpoints by smoking status

Current smokers 

Former smokers 

\* Median difference for salmeterol/fluticasone propionate (SALM/FP) minus placebo.

p-values for the median treatment difference are provided for current smokers and for

former smokers. (Lower limit of 95% confidence limit for CD45+ cells in former

smokers is -867 cells/mm<sup>2</sup>)

# Figures

Figure E1 (a)

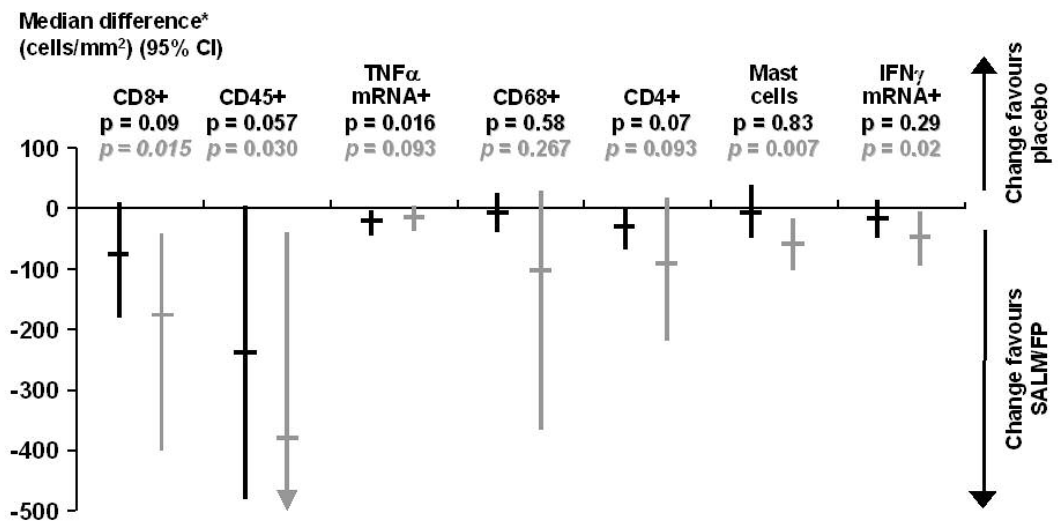


Figure E1 (b)

