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Anti-inflammatory effects of salmeterol/fluticasone, tiotropium/fluticasone or tiotropium on COPD

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Short title: Salmeterol/fluticasone and tiotropium on COPD

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ABSTRACT

We investigated the anti-inflammatory effects of salmeterol/fluticasone (SFP), tiotropium/fluticasone (Tio+FP) and tiotropium (Tio) alone on the inflammatory cells and mediators in sputum induced from COPD patients.

Subjects were either newly-diagnosed or had not taken any medication for 3 months prior to the study. Subjects (N=99) were randomized (not double blinded) and received either SFP (100/1000 μg daily), Tio+FP (18 μg/1000 μg daily) or Tio (18 μg daily) for 12 weeks. Induced sputum and serum C-reactive protein (CRP) were analyzed prior to and at the end of treatment.

The results showed that treatment with SFP caused a significant reduction in interleukin-8 (IL-8) and matrix metalloprotease (MMP)-9 in induced sputum as compared with treatment with Tio alone. There were no treatment differences between the SFP and Tio+FP groups in decreasing IL-8 and MMP-9 levels. The reduction in IL-8 showed significant association with the reduction in MMP-9. All treatment groups failed to significantly reduce the numbers of total cells, neutrophils, macrophages and eosinophils in induced sputum; in addition, there were no treatment differences in terms of improvement of FEV_1, FVC, CRP or quality of life between three groups.

The anti-inflammatory effects of SFP likely contribute to the clinical benefits seen in COPD patients.

Keywords: chronic obstructive pulmonary disease, inflammation, interleukin-8, salmeterol/fluticasone, matrix metalloprotease, tiotropium.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by the airflow limitation associated with an abnormal inflammatory response [1]. The inflammatory cells present in COPD are heterogeneous; neutrophils, macrophages, eosinophils, mast cells and $CD8^+$ lymphocytes have been shown to play important roles in inflammatory processes in COPD [2–5]. Neutrophils are one of the major inflammatory cell types in the airways of patients with COPD [6], and increased numbers of neutrophils in sputum were found to be correlated with a rapid decline in patients' FEV_1 (forced expiratory volume in one second) in a fifteen-year follow-up study [7]. In stable COPD patients, FEV_1 showed a significant inverse correlation with the number of neutrophils in induced sputum [8] and in the sub-epithelium from bronchial biopsies [9], suggesting that neutrophilic inflammation of the airways may contribute to the pathogenesis of COPD.

The combinations of an inhaled corticosteroid and a long-acting β_2-agonist or an inhaled long-acting anti-cholinergic are currently used in COPD treatment to improve lung function and quality of life, prevent exacerbation and reduce hospitalization [10–12]. Inhaled corticosteroid alone has limited anti-inflammatory effect in COPD patients [3,13,14]. Treatment with a combination of salmeterol and fluticasone has

been shown to reduce the numbers of macrophages [15], neutrophils [16] and $CD8^+$ T lymphocytes [15,16] in bronchial biopsy tissue and in induced sputum from patients with COPD. M_3-selective muscarinic antagonists have been shown to inhibit the release of chemoattractant for neutrophils from alveolar macrophages, which were stimulated by acetylcholine [17]. However, the anti-inflammatory effect of tiotropium on the airways of COPD patients remains to be elucidated.

The aim of this study was to investigate the anti-inflammatory effects of salmeterol/fluticasone (SFP), tiotropium/fluticasone (Tio+FP) and tiotropium (Tio) alone on inflammatory cells and mediators in induced sputum from patients with COPD. The changes in levels of neutrophils, macrophages, eosinophils, interleukin (IL)-8 and matrix metalloprotease (MMP)-9 in sputum were assessed after 12 weeks' therapy; furthermore, lung function was also measured and health-related quality of life was assessed using the St. George Respiratory Questionnaire (SGRQ).

MATERIALS AND METHODS

Patient population

One hundred and thirteen subjects with a clinical diagnosis of COPD [1] were enrolled between July 2006 and July 2007 from outpatient clinics in our medical center, Taipei Veterans General Hospital. Subjects were eligible for this study if they were aged between 40 and 85 years; were a current or former smoker (history ≥ 20 pack-years); had a post-bronchodilator FEV_1 < 80% of the predicted value and FEV_1/FVC < 70%; and had no history of asthma, atopy (as defined by a positive reaction to one or more allergen in a fluoroenzyme immunoassay) or any other active lung disease. Subjects were either newly-diagnosed or had not taken corticosteroids (either oral or inhaled), nor any other bronchodilators or theophylline, for a minimum of 3 months prior to the commencement of the study. All subjects had been free from respiratory tract infections or COPD exacerbation for at least 12 weeks prior to the pulmonary function tests and sputum induction. The hospital's ethics committee approved the study and written informed consent was obtained from all subjects before the study commenced.

Study design

This study was a randomized (not double blinded) clinical trial, with subjects allocated to receive treatment with one of the following: SFP, 25/250 μg/puff, 2 puffs twice daily by Evohaler (GlaxoSmithKline, Ware, UK); Tio, 18 μg once daily by Handihaler (Boehringer Ingelheim Pharma, Ingelheim, Germany) plus FP 250 μg/puff, 2 puffs twice daily by Acuhaler (GlaxoSmithKline); or Tio alone, 18 μg once daily. After screening, subjects were randomized to one of the treatment groups: randomization was performed using a computer-generated list of random numbers. The treatment duration was 12 weeks, with clinical visits in weeks 4, 8, and 12. Pulmonary function, serum C-reactive protein (CRP), sputum induction and assessment of health-related quality of life were carried out in the morning at screening and in week 12. Health status was assessed using the SGRQ, and the total score and scores from the three categories (symptoms, activity, and impact) [18] were calculated. Induced sputum was processed as described previously [8] and the supernatant was aspirated and frozen at –80°C prior to measurement of inflammatory mediators IL-8 and MMP-9. The levels of IL-8 and MMP-9 in supernatants were assayed by ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Statistics

In light of the results of our previous studies [8,19,20], IL-8 was selected as the reference for calculation of the estimated sample size. We assumed that the mean difference in IL-8 was 300 pg/ml between groups, with a standard deviation of 420 pg/ml; the level of significance was 0.05, the power of the test was 0.9, and the calculated sample size was 21 per treatment group. The differences between the results (pre-treatment minus post-treatment), including FEV_1, FVC, serum levels of CRP and cortisol, and all cells and mediators measured in the induced sputum, passed the test for normality. Data are expressed as means ± SD. Statistical analysis for multiple comparisons was performed using ANOVA, and the post-hoc test applied for pairwise comparison following ANOVA was the LSD (least significance difference) test. Association between IL-8 and MMP-9 in treatment differences (pre-treatment–post-treatment) was measured using Spearman's rank correlation test. *P* values <0.05 were considered significant for all tests.

RESULTS

The details of the 113 eligible patients screened, randomized and withdrawn during the study are shown in Figure 1. Thirty-three patients were treated with SFP, 32 with Tio+FP and 34 with Tio alone. Demographic details, smoking history, baseline lung function and health-related quality life were matched among treatment groups (Table 1). Table 2 reveals the characteristics of the inflammatory cells and mediators in the induced sputum: no differences in baseline inflammatory cells and mediators were found among the groups. The overall yield rate of sputum induction was 72.7% (72/99) at the end of study.

Inflammatory cells and markers

The mean treatment differences in total cell numbers (Fig. 2A), neutrophils (Fig. 2B), macrophages (Fig. 2C) and eosinophils (Fig. 2D) between the SFP, Tio+FP and Tio groups failed to reach statistical significance. The levels of serum CRP showed significant association with total cell numbers (r=0.273, p=0.02) and with the numbers of neutrophils (r=0.247, p=0.037) in induced sputum. IL-8 concentrations showed strong association with those of MMP-9 before (r=0.639, p<0.001) and following (r=0.704, p<0.001) treatment. The decrease in CRP was 0.3 ± 0.2, 0.6 ± 0.5 and 0.3 ± 0.2 in the SFP, Tio+FP and Tio groups, respectively; there were no statistically

significant differences between the three groups. In contrast, there was a significant reduction in IL-8 and MMP-9 in the SFP group as compared with patients treated with Tio alone (p=0.03 and p=0.004, respectively, Fig. 3). Interestingly, IL-8 (p=0.041) and MMP-9 (p=0.02) levels increased in the Tio group, but no difference was found between the SFP and Tio+FP groups. In addition, the reduction in IL-8 was found to be strongly associated with that in MMP-9 (Fig. 4).

Lung function and health-related quality of life

The improvement in FEV_1, FVC and health-related quality of life as assessed by the SGRQ in each treatment group is shown in Table 3. No treatment differences were found between the groups in terms of FEV_1, FVC and SGRQ score.

DISCUSSION

This is the first randomized (not double blinded) clinical trial to compare the anti-inflammatory effects of SFP, Tio+FP and Tio alone on the airways of patients with COPD. This study showed that 12 weeks of treatment with SFP caused a significant reduction in the levels of inflammatory mediators IL-8 and MMP-9 as compared with treatment with tiotropium alone. There were no differences between the SFP and Tio+FP groups in terms of decreasing IL-8 and MMP-9 levels. The reduction in IL-8 was significantly associated with the reduction in MMP-9. Although treatment with SFP resulted in a mean reduction of 1.53 x 10^6 neutrophils/g sputum in our study, no treatment group showed a reduction in the numbers of total cells, which included neutrophils, macrophages and eosinophils, of statistical significance. In addition, there were no differences in terms of improvement in FEV_1, FVC and health-related quality of life between the three groups.

Bourbeau *et al*. reported that 3 months of treatment for COPD with SFP (50/500 μg, twice daily) significantly reduced $CD8^+$ T cells and $CD68^+$ macrophages in bronchial biopsy tissue as compared with a placebo, but that this treatment did not significantly affect the numbers of neutrophils and eosinophils [15]. In contrast, the same dose with a similar treatment duration was reported as significantly reducing neutrophils, eosinophils and $CD8^+$ T cells in biopsies by Barnes *et al*. [16]. $CD68^+$ macrophages

were not affected in this study. In conjunction with our results, these studies show that heterogeneity exists in the COPD patient population and that treatment response varies. Therefore, the factors that influence anti-inflammatory response deserve urgent investigation.

Long-term use of Tio has been shown to increase sputum IL-8, although it decreases the frequency of exacerbation in COPD patients [21]. In our study, both IL-8 and MMP-9 levels significantly increased in the group treated with Tio alone. It is difficult to link the increases in inflammatory mediators with decreased exacerbation rates in COPD. Presumably, tiotropium may influence the depth of airway surface liquid through inhibition of cholinergic stimulation [22]. Serous and mucous-secreting cells in the airways may reduce secretion under such motor control, therefore leading to increased concentrations of mediators in the airways. Neither IL-8 nor MMP-9 increased in the tiotropium/fluticasone arm of the study. SFP treatment significantly reduced sputum IL-8 and MMP-9 levels; such anti-inflammatory effects may be attributed partly to the action of salmeterol. Introduction of salmeterol into the airways of asthmatics was found to reduce IL-8 and myeloperoxidase levels in bronchoalveolar lavage fluid; in the same study, high-dose inhaled corticosteroid treatment increased rather than decreased airway neutrophils [23]. However, *in vitro* studies have suggested that there exist dose-dependent effects of fluticasone on inhibition of IL-8 and other mediators

released from various types of cells, including airway epithelial cells [24], smooth muscle cells [25] and fibroblasts [26]. A synergistic effect of salmeterol and fluticasone on the inhibition of mediator release has also been demonstrated [27–30].

The reductions in IL-8 and MMP-9 levels may explain the clinical benefit of SFP in decreasing airway inflammation. IL-8 is a potent chemoattractant for neutrophils, and MMP-9 is expressed and released from neutrophils and alveolar macrophages and is present in the airways of COPD patients at an increased level [31]. The level of MMP-9 has been found to be associated with emphysema formation [32] and lung remodelling [33]. The relationship between decreasing MMP-9 and the slowing of lung function decline by SFP [10,34] deserves further investigation.

Elevated CRP levels have been associated with a higher probability of cardiac infarction [35]. Inhaled and oral corticosteroids may reduce serum CRP levels in patients with COPD, suggesting a potential application for corticosteroids in improving cardiovascular outcomes in COPD [36]. However, a recent study demonstrated that neither FP nor SFP had a significant effect on serum CRP levels [37]. In our study, neither SFP, Tio+FP nor Tio treatment resulted in reduced serum CRP levels at the end

of treatment. The levels of CRP may be too low to reflect the differences between groups.

The limitations of this study were that the number of study subjects was small and there was no placebo group for comparison. A placebo group was not included because of ethical considerations. All three treatments are believed to be beneficial to COPD patients. The improvement in terms of FEV_1, FVC and SGRQ score showed no differences between groups, and the potential benefit of adding an inhaled corticosteroid to tiotropium is not clear. This study may provide some information that can be used as a basis for further investigation of the anti-inflammatory effects of tiotropium with or without inhaled corticosteroid.

In conclusion, the anti-inflammatory effects of SFP likely contribute to the clinical benefits seen in COPD patients. A long-term, large-scale study is needed in order to determine whether adding inhaled corticosteroid to either a long-acting β_2 agonist or a long-acting anticholinergic or both can modify the progressive nature of COPD.

ACKNOWLEDGEMENT

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Figure Legends

Figure 1. Flow diagram of patients in the study.

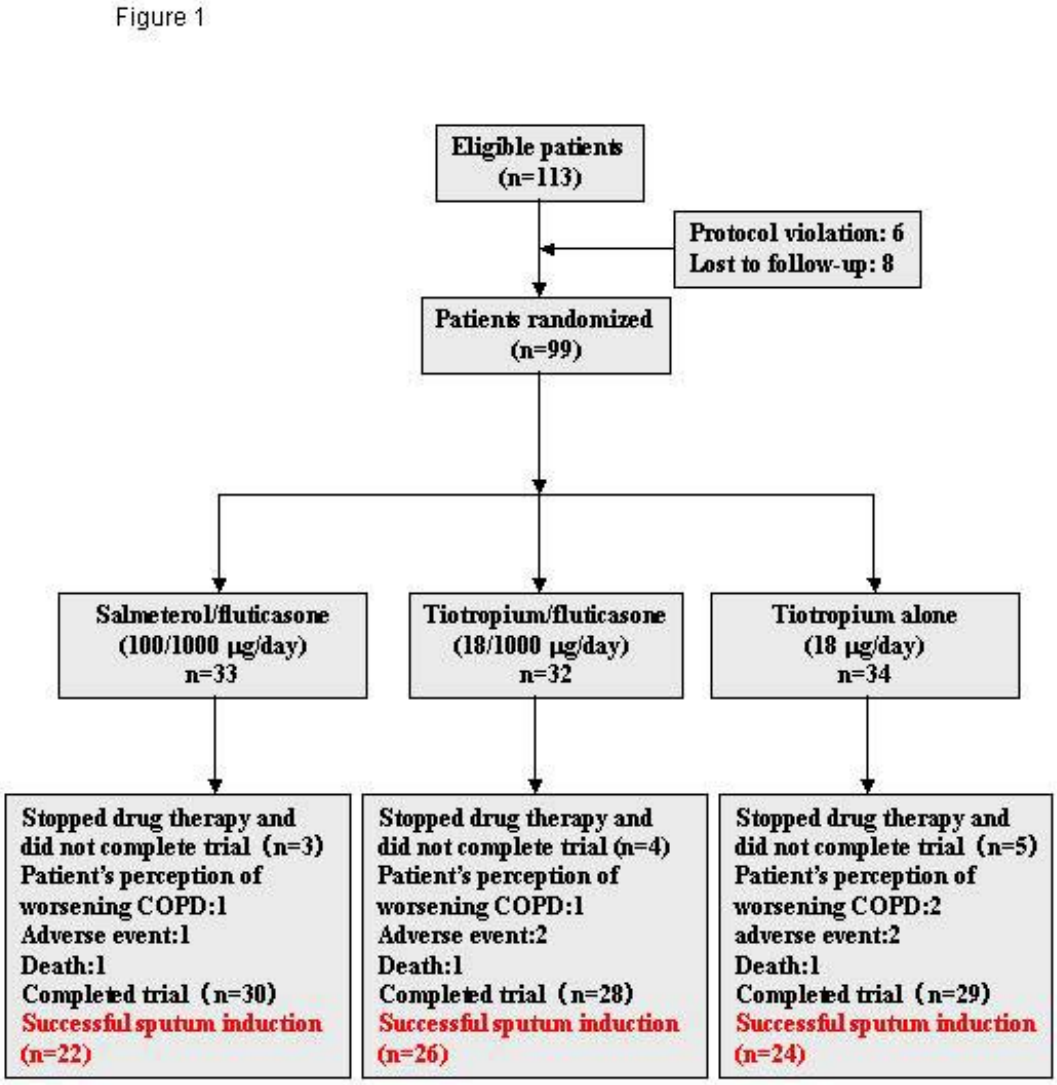

Figure 2. Treatment differences in terms of numbers of total cells (A), neutrophils (B), macrophages (C), and eosinophils (D). Data are expressed as mean difference in the

number of cells with 95% confidence intervals. SFP, salmeterol/fluticasone; Tio+FP, tiotropium/fluticasone; Tio, tiotropium.

Figure 2

A

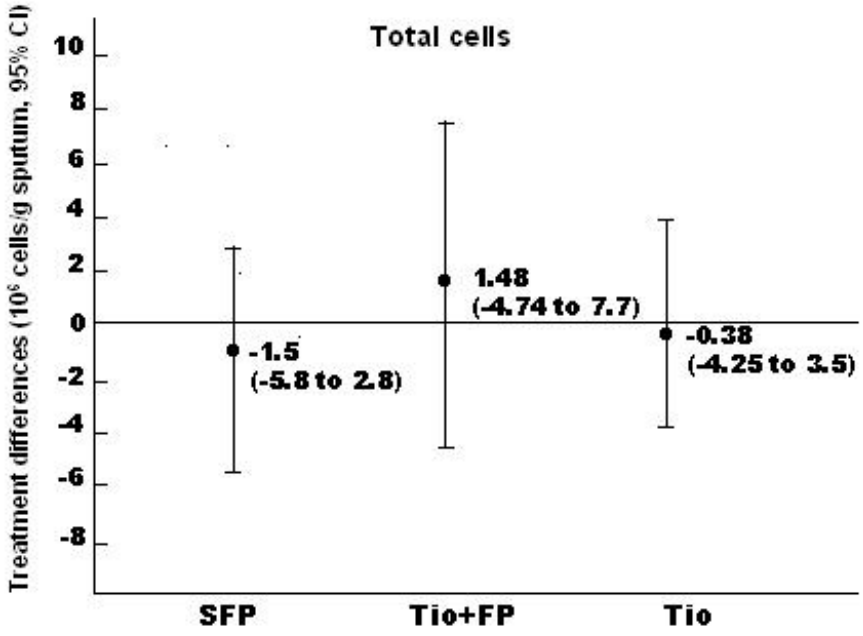
Total cells
Treatment differences (10^6 cells/g sputum, 95% CI)
10
8
6
4
2
0
-2
-4
-6
-8
-1.5
(-5.8 to 2.8)
1.48
(-4.74 to 7.7)
-0.38
(-4.25 to 3.5)
SFP
Tio+FP
Tio

Figure 2

B

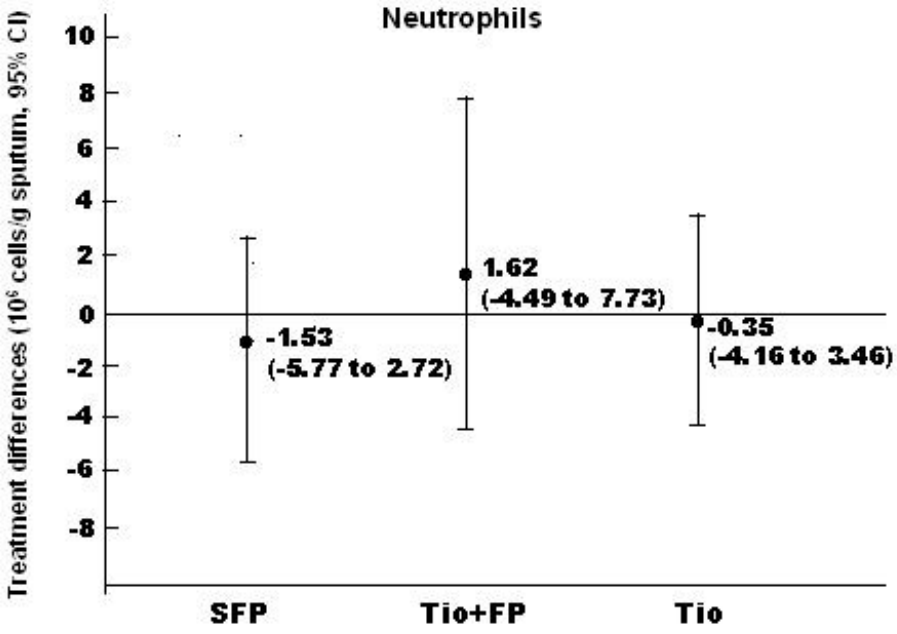
Neutrophils
Treatment differences (10⁶ cells/g sputum, 95% CI)
10
8
6
4
2
0
-2
-4
-6
-8
1.62
(-4.49 to 7.73)
-1.53
(-5.77 to 2.72)
-0.35
(-4.16 to 3.46)
SFP
Tio+FP
Tio

Figure 2

C

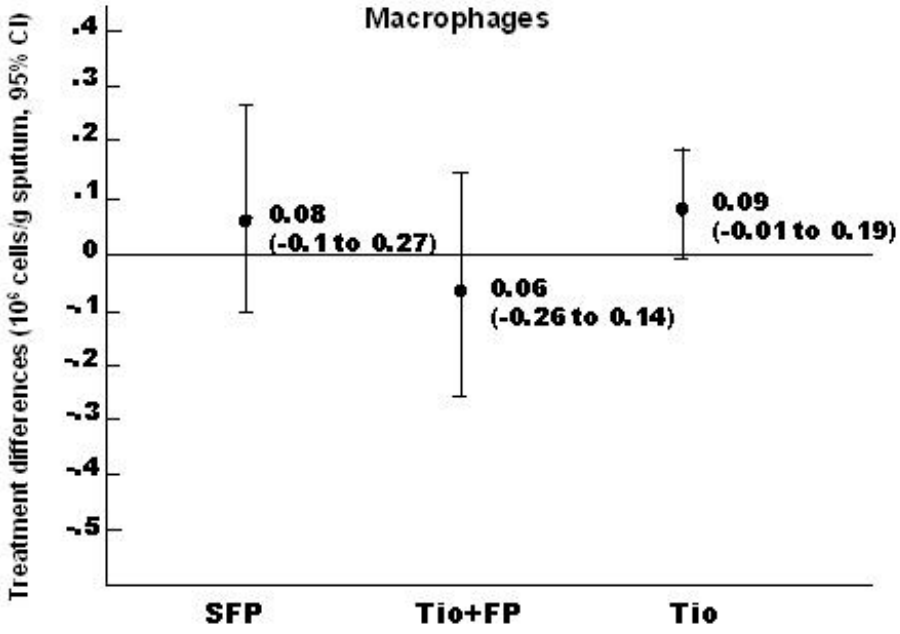
Macrophages
Treatment differences (10^6 cells/g sputum, 95% CI)
.4
.3
.2
.1
0
-.1
-.2
-.3
-.4
-.5
0.08
(-0.1 to 0.27)
0.06
(-0.26 to 0.14)
0.09
(-0.01 to 0.19)
SFP
Tio+FP
Tio

Figure 2

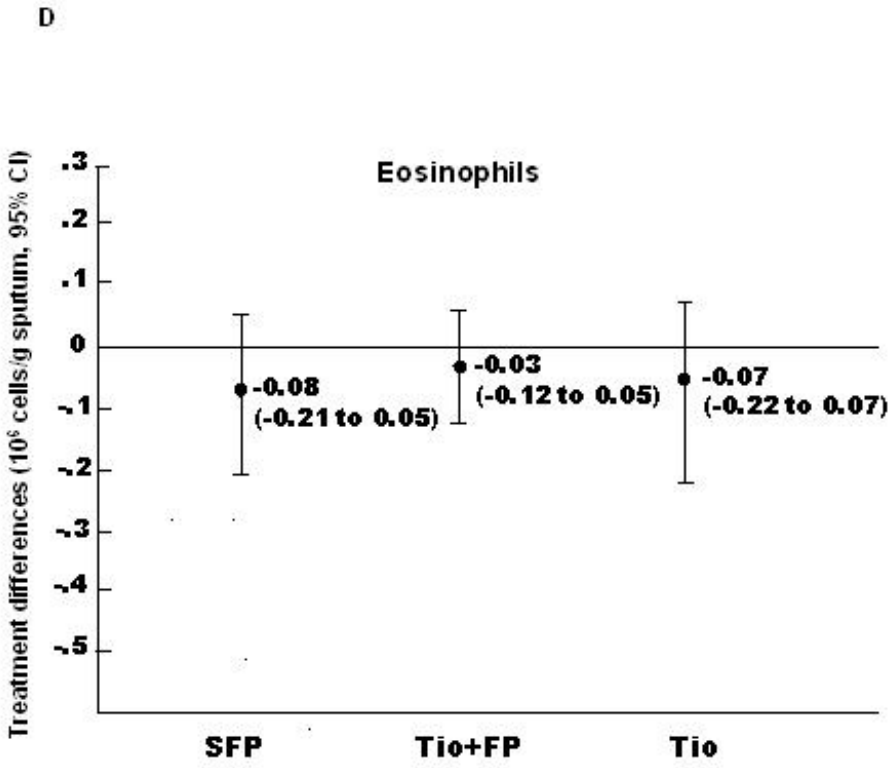

Figure 3. Effects of treatments on interleukin (IL)-8 and matrix metalloprotease (MMP)-9. Data are expressed as mean difference in the concentration of induced sputum with 95% confidence intervals. SFP, salmeterol/fluticasone; Tio+FP, tiotropium/fluticasone; Tio, tiotropium.

Figure 3

A

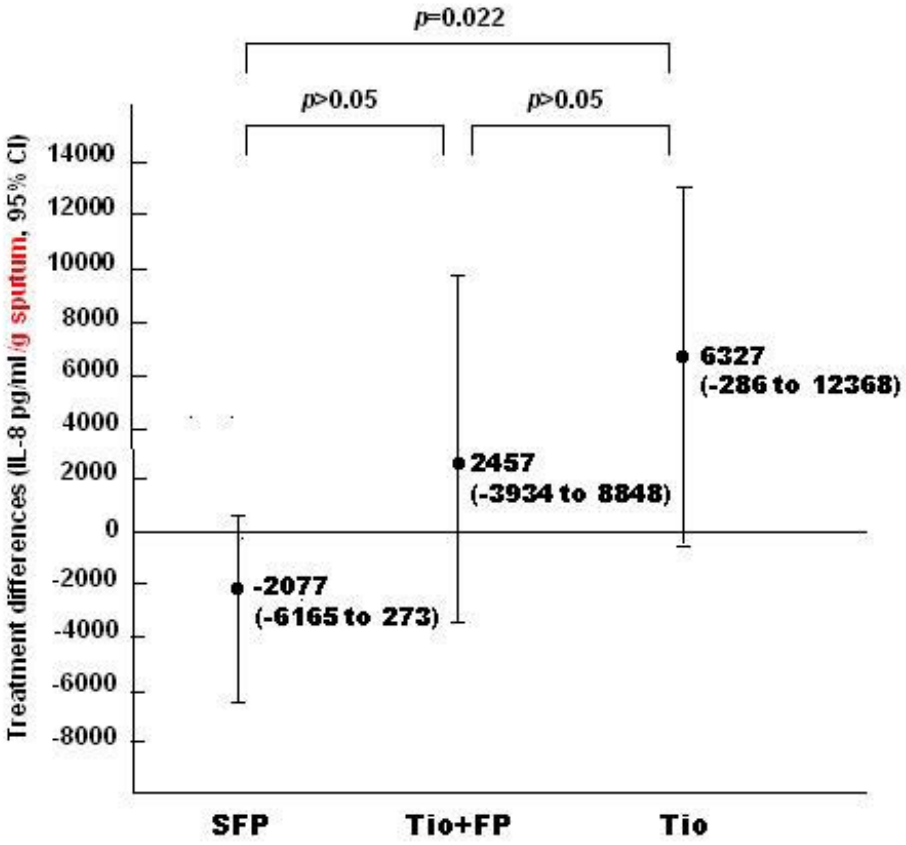
p=0.022
p>0.05
p>0.05
Treatment differences (IL-8 pg/ml/g sputum, 95% CI)
14000
12000
10000
8000
6000
4000
2000
0
-2000
-4000
-6000
-8000
6327
(-286 to 12368)
2457
(-3934 to 8848)
-2077
(-6165 to 273)
SFP
Tio+FP
Tio

Figure 3

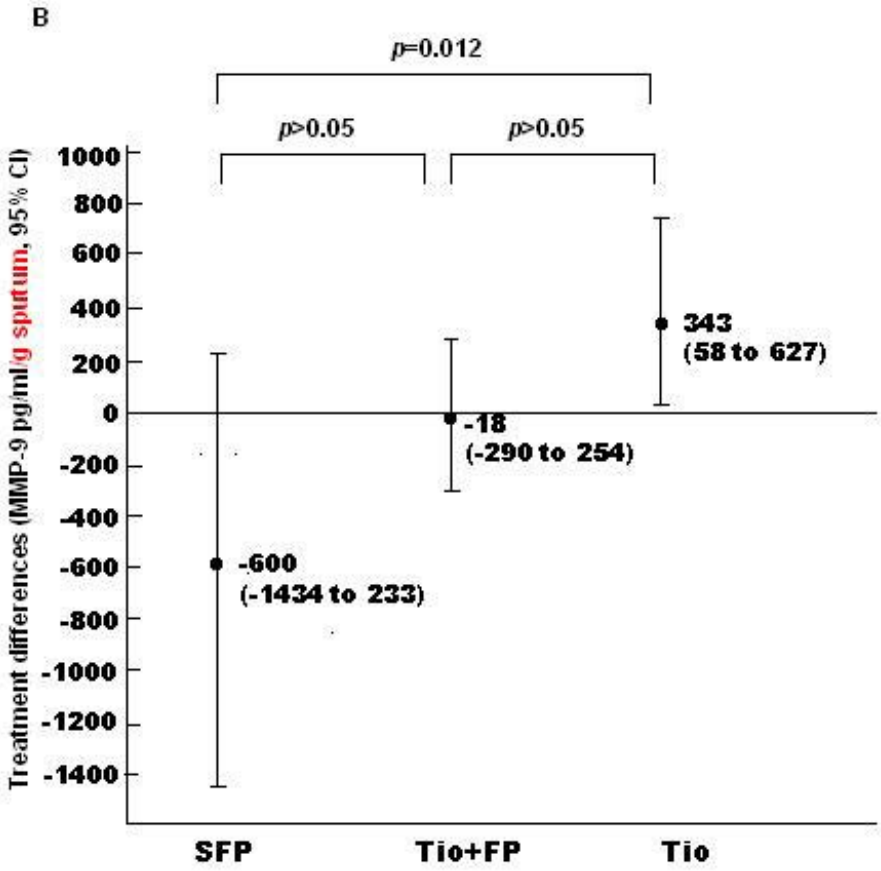

Figure 4. Associations between the changes in levels (pre-treatment–post-treatment) of IL-8 and MMP-9 in induced sputum from the COPD patients (n=72) who completed the treatment course.

Figure 4

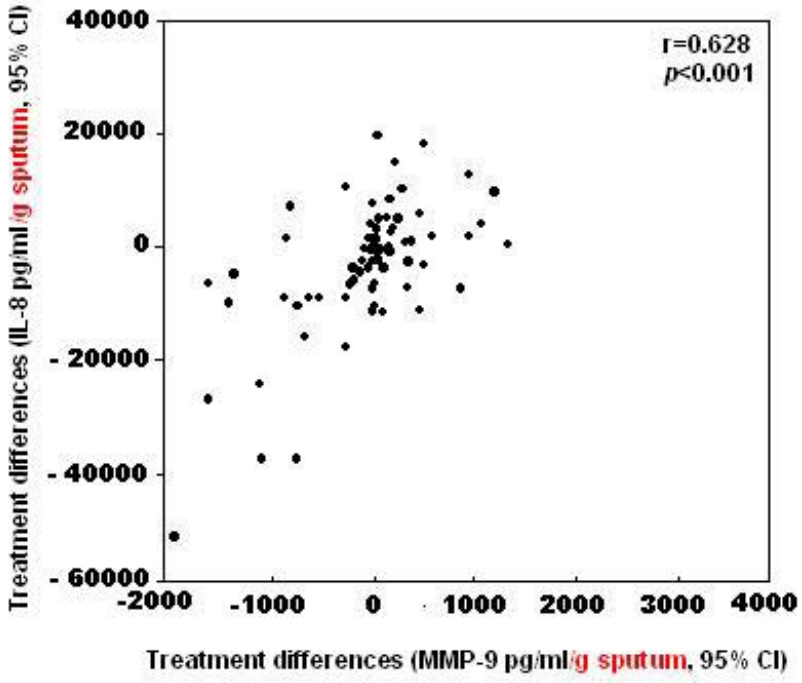
r=0.628
p<0.001
40000
20000
0
- 20000
- 40000
- 60000
-2000
-1000
0
1000
2000
3000
4000
Treatment differences (IL-8 pg/ml/g sputum, 95% CI)
Treatment differences (MMP-9 pg/ml/g sputum, 95% CI)

Table 1. Patient characteristics for (A) all completed cases and (B) completed cases with successful sputum induction.

A

	SFP N=33	Tio+FP N=32	Tio N=34
Mean age	72.0 (8.0)	74.1 (8.1)	74.3 (7.2)
Male/female	31/2	32/0	32/2
Current smoker, %	57	60	65
Pack-years smoked	49.5	50.4	47.4
Pre-bronchodilator FEV_1 (L)	1.24 (0.34)	1.28 (0.34)	1.18 (0.28)
Pre-bronchodilator FEV_1 (% predicted)	53.4 (11.1)	57.8 (13.6)	53.1 (11.0)
Pre-bronchodilator FVC (L)	2.35 (0.47)	2.43 (0.56)	2.28 (0.54)
Pre-bronchodilator FVC (% predicted)	72.2 (13.9)	76.5 (16.3)	73.5 (14.5)
Reversibility (% predicted FEV_1)	6.8 (4.5)	5.5 (4.6)	6.8 (5.6)
Serum C-reactive protein, mg/L	0.8 (0.3)	1.0 (0.5)	0.8 (0.2)
SGRQ score	44.3 (13.2)	47.9 (10.5)	47.8 (15.7)

Data are presented as means (SD).

B

	SFP N=22	Tio+FP N=26	Tio N=24
Mean age	74.3 (8.0)	76.5 (7.9)	76.1 (4.3)
Male/female	21/1	26/0	22/2
Current smoker, %	55	58	60
Pack-years smoked	48.5	48.4	49.5
Pre-bronchodilator FEV_1 (L)	1.21 (0.28)	1.30 (0.24)	1.25 (0.27)
Pre-bronchodilator FEV_1 (% predicted)	52.8 (10.5)	58.7 (9.1)	54.1 (10.3)
Pre-bronchodilator FVC (L)	2.32 (0.56)	2.31 (0.41)	2.36 (0.59)
Pre-bronchodilator FVC (% predicted)	70.5 (16.0)	72.2 (10.1)	74.1 (16.2)
Reversibility (% predicted FEV_1)	7.2 (4.2)	6.5 (4.3)	6.8 (4.6)
Serum C-reactive protein, mg/L	0.6 (0.3)	0.9 (0.2)	0.7 (0.3)
SGRQ score	45.3 (11.3)	46.7 (11.2)	45.7 (10.5)

Data are presented as means (SD).

Table 2. Baseline of cells and mediators in induced sputum.

	SFP N=22	Tio+FP N=26	Tio N=24	*p* value
Total cells, $\times 10^6$/g sputum	7.0 ± 1.6	7.9 ± 3.6	6.3 ± 1.6	Ns
Macrophages (%)	9.7 ± 2.3	14.8 ± 3.2	10.2 ± 2.1	Ns
Neutrophils (%)	84.2 ± 3.7	81.8 ± 3.6	86 ± 2.3	Ns
Eosinophils (%)	3.3 ± 2.5	1.9 ± 1.6	2.3 ± 0.9	Ns
Lymphocytes (%)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	Ns
Epithelial cells (%)	0.6 ± 0.1	1.3 ± 0.6	1.2 ± 0.3	Ns
MMP-9, ng/ml/g sputum, CV %	352.2 ± 38.7 10.9 %	366.3 ± 51.1 13.9 %	373.2 ± 49.1 13.1 %	Ns
IL-8, ng/ml/g sputum, CV %	7.9 ± 1.9 24.6 %	7.4 ± 1.6 21.9 %	7.6 ± 1.7 22.6 %	Ns

Data are presented as means ± SD; Ns, no significance; CV, coefficient of variation.

Table 3. Effect of treatment on lung function and qualify of life for (A) all completed cases and (B) completed cases with successful sputum induction.

A.

	SFP N=33	Tio+FP N=32	Tio N=34
Baseline			
FEV_1 (L)	1.24	1.28	1.18
FVC (L)	2.35	2.43	2.28
SGRQ score	44.3	47.9	47.8
Treatment difference			
FEV_1 (ml)	129 (19–238)	115 (–6–216)	128 (67–229)
FVC (ml)	194 (–30–237)	222 (–42–303)	179 (–32–324)
SGRQ score	12 (6–19)	11 (4–18)	9 (5–14)

Data are presented as means (95% CI).

B

	SFP N=22	Tio+FP N=26	Tio N=24
Baseline			
FEV_1 (L)	1.21	1.30	1.25
FVC (L)	2.32	2.31	2.36
SGRQ score	45.3	46.7	45.7
Treatment difference			
FEV_1 (ml)	121 (29–243)	115 (–16–223)	124 (58–248)
FVC (ml)	204 (–15–221)	212 (–34–293)	189 (–42–302)
SGRQ score	11 (5–19)	10 (3–16)	12 (4–17)

Data are presented as means (95% CI).