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Clinical and inflammatory determinants of bronchial hyperresponsiveness in COPD

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Key message: Bronchial hyperresponsiveness in COPD does not merely reflect airway obstruction. It is also independently associated with both airway inflammation and RV/TLC, a measure of air trapping. Our findings indicate that BHR is an independent trait in COPD and provides important information on phenotype heterogeneity and disease activity.

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Abstract

Background: Bronchial hyperresponsiveness (BHR) is regarded as a hallmark of asthma, yet it is also present in a considerable number of COPD patients. Epidemiological studies have shown that BHR provides complementary information to FEV_1 for development and progression of COPD. We hypothesized that the severity of BHR and its longitudinal changes associate with both clinical and airway inflammation measures in COPD. *Methods: O*ur hypothesis was tested in 114 COPD patients (median age 62.9 years, packyears 45.9) participating in the GLUCOLD study, previously showing an improvement in BHR with fluticasone and fluticasone/salmeterol. At baseline, and 6 and 30 months after treatment, we investigated lung function, including body plethysmography, PC_{20} methacholine, sputum induction, and bronchial biopsies. *Results:* By performing both cross-sectional and longitudinal analyses, we showed that BHR in COPD is predominantly associated with residual volume/total lung capacity, a measure of air trapping, and airway inflammation reflected by the number of neutrophils, macrophages, and lymphocytes in sputum and bronchial biopsies. *Conclusions:* Our findings indicate that BHR is an independent trait in COPD and provides important information on phenotype heterogeneity and disease activity.

Keywords: Bronchial hyperresponsiveness, methacholine, COPD, sputum, bronchial biopsies, neutrophils

Introduction

Chronic Obstructive Pulmonary Disease (COPD) has a major health impact throughout the world [1;2]. Patients with COPD generally show a progressive lung function loss with a concomitant reduced health status and increase in symptoms. Part of these symptoms, like sudden increase in cough and dyspnea when inhaling cold air are due to bronchial hyperresponsiveness (BHR). Bronchial hyperresponsiveness is often thought to be a hallmark of asthma, yet it has been shown to occur in up to two thirds of patients with COPD as well [3]. In asthma, BHR is associated with both baseline level of forced expiratory volume in one second (FEV_1) and eosinophilic airway inflammation measured in peripheral blood, sputum, BAL, or bronchial biopsies [4;5]. Thus far, the factors underlying BHR in COPD remain largely unknown. Since patients with COPD invariably have airway obstruction that is often quite severe, it has been argued that the presence of BHR in COPD merely reflects a lower pre-challenge FEV_1 and is not of pathophysiological importance [6;7]. An argument against this assumption is the observation that the presence of BHR precedes the development of COPD-like symptoms in the general population [8]. In addition, a more severe BHR is associated with an accelerated decline in lung function in COPD patients even after adjusting for baseline FEV_1 [9;10]. Further, the severity of BHR is an independent predictor of improvement in FEV_1 after smoking cessation in patients with mild to moderate COPD participating in the Lung Health Study [11]. Given these observations, it is important to further explore the underlying physiology of BHR in COPD.

The GLUCOLD (Groningen Leiden Universities Corticosteroids in Obstructive Lung Disease) study has shown an improvement in FEV_1 and BHR after treatment with fluticasone or fluticasone/salmeterol for up to 30 months, and at the same

time improvements in inflammatory parameters in patients with mild-to-moderate COPD [12]. We hypothesized that the severity of BHR and its longitudinal changes are not only associated with lung function, but also with the extent of airway inflammation in patients with COPD. The GLUCOLD study provides an excellent opportunity to investigate this, since all patients were extensively characterized before, 6 and 30 months after treatment with either inhaled corticosteroids with or without a long-acting beta-agonist or placebo.

Methods

Patients

One-hundred-and-fourteen patients with COPD participating in the GLUCOLD study were included [12]. The GLUCOLD study enrolled patients with COPD GOLD stages II and III who were aged 40-75 years and current or former smokers with at least 10 pack years smoking. Exclusion criteria were a history of asthma and the use of ICS and oral corticosteroids within 6 months prior to the start of the study.

Study design

The study design of the GLUCOLD study has been described in detail before [12]. In brief, patients were randomly assigned to receive 1 of 4 double-blind treatments: fluticasone 500 μg twice daily (b.i.d.) for 30 months, fluticasone/salmeterol 500/50 μg b.i.d. for 30 months, fluticasone 500 μg b.i.d. for the first 6 months followed by placebo b.i.d. for 24 months, or placebo b.i.d. for 30 months (see figure 1 for CONSORT flow diagram of the study). At baseline and after 6 and 30 months treatment, the following investigations were performed: spirometry, body plethysmography, provocative concentration of methacholine causing the FEV_1 to drop by 20% (PC_{20}), blood collection, sputum induction, and bronchoscopy with bronchial biopsies. The study was carried out in two Dutch centers (University Medical Center Groningen and the Leiden University Medical Center). Both centers' ethics committees approved the study and all patients provided written informed consent.

Lung function and bronchial hyperresponsiveness

FEV_1 was measured with a daily-calibrated pneumotachograph (Masterscreen Pneumo, Jaeger, Wurzburg, Germany) according to standardized guidelines. PC_{20} methacholine

was measured by 2-minute tidal breathing method as described previously [13]. Patients were considered to be hyperresponsive when they had a PC_{20} methacholine bromide ≤ 9.6 mg/ml corresponding to a PC_{20} methacholine chloride < 8 mg/ml on a molar base [13]. Total lung capacity (TLC),residual volume (RV), and inspiratory capacity (IC) were measured using a constant volume body plethysmograph, according to standardized guidelines [14].

Sputum induction and sputum processing

Sputum was induced by inhalation of hypertonic saline aerosols as previously described [15]. Fifteen minutes after salbutamol (200 µg) inhalation, 4.5% hypertonic saline was nebulized for 3 times during 5 minutes. Whole samples were processed according to the method described by Fahy et al [16].

Bronchoscopic biopsy analyses

The methods for biopsy processing, staining, and analysis have been described in detail previously [17]. In short, 4 µm thick paraffin-embedded sections were stained using specific antibodies against T-lymphocytes (CD4, CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1), eosinophils (EG2), and plasma cells (CD138). Digital images per coded biopsy section were prepared using a colour camera (Basler A101fc-le) and a dedicated software program (RVC Software, Amersfoort, The Netherlands). These images were united into one large image consisting of the entire biopsy section (100 mm=115.7 pixels). Numbers of subepithelial positively staining inflammatory cells were counted within the largest possible area, of maximal 125 µm deep beneath the basement membrane, per biopsy section, and expressed as the mean number of cells per 0.1 mm^2 of two tissue samples per patient.

Statistical analysis

Means and standard deviations or medians with interquartile ranges (IQR) of variables were calculated. When appropriate, variables were normalized by logarithmic transformation before statistical analysis. Calculations of PC_{20} were performed with the base-2 logarithm (log_2) as this reflects doubling concentrations and normalizes the distribution [18]. We performed analyses on the level of PC_{20} at baseline and on the changes in PC_{20} during the first 6 months of treatment and between 6 and 30 months of treatment. Univariate analyses were performed in all patients from all treatment groups. Subsequently, multivariate linear regression analysis was performed in the full cohort with baseline or change in PC_{20} methacholine as dependent variable and age, gender, treatment group, and smoking status (current or ex) as covariates. In addition, we included those variables with the most significant univariate regression coefficients in each of the following categories: a) lung function, b) blood cell differential count, c) sputum cell differential count, d) inflammatory cells in bronchial biopsies.

Results

Participants

A total of 114 patients with COPD were included in the study. A PC_{20} methacholine was not performed in 4 patients, because their baseline FEV_1 was below 1.2 liters. The baseline characteristics of the remaining 110 patients are presented in table 1. The sputum sample was discarded in 8 patients since it contained > 80% squamous cells. A bronchoscopy was performed in all patients, 1 patient had no adequate sample. Blood was collected in all patients. A complete dataset was available for 74 of the 114 patients at baseline. After treatment, 19 patients were withdrawn from further analyses because they did not meet the predefined criteria for treatment compliance or withdrew their consent (n=6). Of the remaining 95 patients, a sputum sample of sufficient quality was obtained in 87 and 80 patients after 6 and 30 months treatment and a bronchoscopy performed in 90 and 77 patients respectively.

Cross-sectional analysis on PC_{20} methacholine at baseline

From the 110 patients with a PC_{20} methacholine available at baseline, a total of 103 (94%) were hyperresponsive. Patients with BHR were more often female, had a higher RV/TLC (% predicted), a higher number of sputum eosinophils and tended to have a lower FEV_1/IVC (%) than patients without BHR.

Univariate associations with clinical and inflammatory parameters <u>at baseline</u>

At baseline, higher PC_{20} methacholine values, i.e. less severe BHR was associated with a higher postbronchodilator FEV_1 %predicted, FEV_1/IVC (%), $FEF_{50\%}$ % predicted and $FEF_{75\%}$ %predicted and a lower reversibility and RV/TLC %predicted

(figure 2 and online repository table E1). As an example, for every percent increase in RV/TLC % predicted the severity of BHR increased with 0.04 doubling concentration and for every 10 * 10^4 increase in the number of neutrophils per ml, the severity of bronchial hyperresponsivess increased with 1.87 doubling concentration (see online repository table 1).

A lower PC_{20} methacholine, i.e. more severe BHR, was associated with higher numbers of sputum neutrophils, macrophages, lymphocytes and eosinophils.

Multivariate linear regression with clinical and inflammatory variables

Females had more severe BHR than males, independent of their baseline level of airway obstruction, age, or smoking status. In addition, more severe BHR was associated independently with both a lower FEV_1/IVC (%) and a higher number of sputum neutrophils (table 2). When replacing the number of sputum neutrophils by other sputum cell counts that associated with PC_{20} methacholine with a p-value < 0.1, a higher number of sputum lymphocytes was the single factor contributing independently to the severity of PC_{20} methacholine (β -0.27 (CI -4.55 - -0.81)).

Analysis on change in PC_{20} methacholine during the first 6 months

No significant associations existed between the change in PC_{20} methacholine during 6-month treatment and the change in postbronchodilator FEV_1 (% predicted) or postbronchodilator FEV_1/IVC (%) (online repository table E2). Improvement in PC_{20} methacholine was associated with reduction in RV/TLC %predicted, and increase in percentage of blood monocytes. Further, improvement in PC_{20} methacholine was associated with a decrease in the number of sputum neutrophils, but not with other

numbers of inflammatory cells in sputum. Finally, improvement in PC_{20} methacholine was associated with reduction in the number of $CD4^+$ cells in bronchial biopsies.

Multivariate linear regression with changes in clinical and inflammatory variables

Improvement in PC_{20} methacholine was significantly and independently associated with a decrease in RV/TLC %predicted and an increase in the percentage of blood monocytes (table 3). In addition, improvement in PC_{20} methacholine tended to associate with a decrease in the number of neutrophils in sputum (p=0.076, table 3).

Analysis on changes in PC_{20} methacholine between 6 and 30 months of treatment with changes in clinical and inflammatory parameters

A larger improvement in PC_{20} methacholine associated with more reduction in RV/TLC %predicted (figure 2 and online repository table E2). Further, improvement in PC_{20} methacholine associated with decreases in the number of sputum total cells, neutrophils, eosinophils, macrophages, and lymphocytes.

Multivariate linear regression with changes in clinical and inflammatory variables

Multivariate analysis showed that ex-smoking, higher reduction in RV/TLC (%predicted) and sputum macrophages independently associated with better improvement in PC_{20} methacholine. Findings were corroborated by the observation that the severity of PC_{20} methacholine increased when patients were switched from fluticasone to placebo (table 4). When replacing the change in the number of sputum macrophages with changes in other sputum cells that were significantly associated with changes in PC_{20} methacholine in the univariate analyses, reductions in the number of sputum lymphocytes and neutrophils, but not total sputum cell counts were independently associated with improvements in BHR (β

= -1.56 (CI -2.69 - -0.42), β = -1.33 (CI -2.40 - -0.27), and β = -1.11 (CI -2.30 - 0.08) respectively).

Discussion

Our data show that a more severe BHR in COPD is associated with a higher degree of airway obstruction as reflected by lower FEV_1 and FEV_1/IVC values. This can be explained by the simple fact that the same bronchoconstrictor response results in a larger drop in FEV_1 in a subject with more severe airway obstruction [3;18-20]. Of interest, we additionally show that a more severe BHR is independently associated with airway inflammation in COPD as reflected by higher numbers of sputum neutrophils, even after adjusting for age, sex, smoking status and baseline level of airway obstruction. Moreover, we performed a longitudinal analysis, which revealed that both short-term (6 months) and long-term (between 6 and 30 months) treatment-induced improvements in BHR were independently associated with reductions in numbers of sputum neutrophils, macrophages, and lymphocytes.

Many researchers and clinicians consider BHR to be a hallmark of asthma, but not of COPD. Our data show that BHR is also present in a considerable proportion of COPD patients. This is in agreement with the findings two earlier studies. First, the Lung Health study reported a prevalence of BHR of approximately 60% in COPD [3]. Second, Walker and colleagues investigated the effects of methacholine inhalation on respiratory mechanics and found hyperresponsiveness to be present in all 25 included COPD patients [21]. Taking these findings into account, the high prevalence of 94% for BHR in the GLUCOLD study may not be surprising. Importantly, we specifically excluded patients with asthma by carefully reviewing family charts for earlier diagnosis of asthma and an interview and physical examination by a pulmonary physician. In addition, the diagnosis of COPD was verified by including only patients older than 45 years, with a smoking history $\geq$ 10 packyears.

In asthma, several studies have shown that the severity of bronchial hyperresponsiveness and its treatment-induced improvement is associated with (reduction in) eosinophilic airway inflammation [4;18;22]. This contrasts with our findings in COPD. We observed a strong and independent association between BHR and neutrophilic airway inflammation as reflected by the number of sputum neutrophils both in cross-sectional and longitudinal analyses. This is in agreement with our earlier observations that increased superoxide anion production in peripheral blood neutrophils associates with more severe BHR in patients with COPD.[23] There is extensive evidence that neutrophils are important effector cells in COPD. In this context, the findings of Baraldo and coworkers are of interest [24]. They found that neutrophils infiltrate the airway smooth muscle of patients with COPD to a greater extent than in healthy subjects and this higher degree of neutrophilic infiltration was associated with a lower FEV_1. The latter is not surprising, since neutrophils are able to release a variety of pro-inflammatory mediators, amongst others elastase, leukotriene B4, myeloperoxidase, defensins, cathepsin G, and tumor necrosis factor-alpha. This may lead to damage of the epithelium and lung extracellular matrix, increased mucus secretion, increased permeability of the bronchial mucosa with associated airway wall thickening, and an increased contractile status of airway smooth muscle cells all contributing to a lower FEV_1 and more severe BHR [25].

Further, improvements in BHR in our study during the last 2 years of treatment were independently associated with decreases in the number of sputum macrophages and lymphocytes, indicating that these cells are also important factors contributing to BHR. Taken together, we have now clearly shown that BHR is not merely a surrogate marker for airway obstruction, but also reflects the inflammatory process underlying COPD.

Interestingly, improvement in BHR in patients with COPD was not associated with reduction in eosinophilic airway inflammation as is the case in asthma, again reflecting that the mechanisms underlying BHR are very different between asthma and COPD.

To our surprise, we did not find an independent association between improvement in BHR and reduction in airway obstruction as reflected by FEV_1, or FEV_1/IVC. Of interest, reduction in RV/TLC %predicted was of importance. This could be either due to a decrease in airway resistance or a reduction in air trapping due to closure of the large and/or small airways. In line with this, Salome *et al* showed that an increased RV was a significant independent predictor of BHR in older patients with asthma [26]. In addition, Wagers *et al* showed in a mouse model that airway closure is a central factor contributing to BHR in asthma [27;28]. The findings of our study suggest that airway closure also contributes to bronchial hyperresponsiveness in COPD.

We have previously demonstrated that fluticasone and fluticasone/salmeterol significantly improve BHR both after 6-month treatment and 6-30-month treatment [12]. However, in the current multivariate regression analysis treatment with fluticasone or fluticasone/salmeterol was not independently associated with improvement in BHR, suggesting that treatment-induced improvement is, at least partly, mediated *via* reduction in both hyperinflation and airway inflammation.

Similar to findings by Kanner and colleagues, we found that female patients with COPD have more severe BHR than males even after adjustment for baseline airway obstruction [29]. This is especially remarkable given the low number of women in our study. Thus far, the reason why women have more severe BHR is unclear. A possible explanation might be

that females have a different type of COPD. It has been shown using CT-imaging and histologic examination of resected lung specimens that female patients have less emphysema, but thicker bronchiolar airway walls with disproportionately reduced lumens compared to males [30]. Alternatively, hormone-related events may play a role in the development and severity of BHR in COPD [31]. We have extended the findings of Kanner and colleagues by also investigating the longitudinal changes in BHR after 6- and 30-month treatment. In this way, we were able to show that the level of BHR improved to a similar extent after treatment in males and females with COPD.

The observation that an increase in peripheral blood monocytes after 6-month treatment was independently associated with a decrease in PC_{20} methacholine was unexpected and intriguing. In this context, our previous findings in asthma are of interest, showing that a higher percentage of peripheral blood monocytes associates with less severe BHR [4]. Although it has been suggested that peripheral blood monocytes may play a role in the immune responses, relatively little is known about their relation to BHR in asthma or COPD. Therefore, our finding merits further investigation.

Correlations between treatment-induced changes of PC_{20} methacholine and airway inflammation in patients participating in the GLUCOLD study have been presented before [12] Changes in mast cells and CD4+ cells associated with change in PC_{20} in univariate analyses. The previous report only included subgroups of COPD patients using fluticasone or placebo for 30 months, whereas we have now analyzed patients from all four treatment groups. In addition, the previous report assessed the change in BHR between 0-30 months, while we now analyzed the change between 0-6 and 6-30 months. The latter time points were chosen, because a subgroup of patients participating in the GLUCOLD study was

treated with fluticasone for the first 6 months followed by placebo during the second 24 months. Compatible with our previous report, univariate regression analysis showed that improvement in PC_{20} methacholine after 6 months was associated with a reduction in the number of $CD4^+$ cells in bronchial biopsies and tended to associate with reduction in mast cells (p=0.002 and p=0.06 respectively, online repository table E2). However, the current multivariate analyses did not show any associations between the number of CD4+ lymphocytes or mast cells and the severity of BHR at baseline, or between reductions in CD4+ lymphocytes or mast cells and improvements in BHR between 6- and 30- month treatment. In addition, the number of CD4+ lymphocytes or mast cells did not contribute independently to improvement of BHR. Thus, in contrast to asthma, our findings do not suggest a large contribution of CD4+ lymphocytes or mast cells to the severity of BHR in COPD, but rather highlight the contribution of sputum neutrophils, lymphocytes and macrophages [32;33].

In conclusion, the results of our study improve the knowledge on BHR in COPD even though the dispersion of our data is such that it leaves room for additional mechanisms/interactions explaining the mysterious relationship between lung function and biology in COPD. We show that BHR is not only a hallmark of asthma, but also occurs in many patients with moderately severe COPD who do not use inhaled corticosteroids. Nevertheless, the factors underlying BHR seem to be different in asthma and COPD. In asthma, the severity of bronchial hyperresponsiveness and its treatment-induced improvement have been shown to be associated with (reduction in) the number of eosinophils and mast cells in sputum and bronchial biopsies [4;18;22]. This contrasts with our findings in COPD. By performing both cross-sectional and longitudinal analyses, we were able to show, for the first time, that BHR in COPD is predominantly associated with

airway inflammation reflected by numbers of neutrophils, lymphocytes and macrophages in sputum and bronchial biopsies. In addition, the longitudinal analysis showed that especially RV/TLC, a measure of air trapping, rather than airflow obstruction contributes importantly to BHR in COPD. Our data indicate that BHR is an independent trait in COPD and provides additional information on phenotype and disease activity. The role of BHR in COPD deserves further investigation in epidemiological, pathological and pharmacological studies.

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Table 1. Baseline characteristics and cell count data of the study population.[*]

Age (years)	62.9	(57.0 – 68.0)
Gender M/F	96/14	
Current smoker (%)	63	
Packyears, number	41.8	(31.2 – 54.8)
Body Mass Index (kg/m^2)	25.3	(22.4 – 27.8)
PC_{20} methacholine (mg/ml)[#]	1.8	(0.2 – 2.4)
FEV_1 (% predicted)[**]	64.2	(57.1 – 69.3)
FVC (% predicted)**	volgt	
FEV_1/IVC (%)[**]	45.2	(39.3 – 52.7)
RV/TLC (% predicted) [**]	125.6	(112.6 – 140.3)
IC (% predicted)	volgt	
$TLCO_{VA}$ (% predicted)	74.4	(57.7 – 90.0)
Blood (% leukocytes)		
Neutrophils	59.2	(52.3 – 66.3)
Eosinophils	2.2	(1.1 – 3.3)
Monocytes	8.7	(7.4 – 10.1)
Basophils	0.5	(0.3 – 0.8)
Lymphocytes	28.2	(22.3 – 34.4)
Sputum (10^4/mL)		
Total cells[&]	2.4	(2.1 – 2.6)
Macrophages[&]	1.5	(1.3 – 1.8)
Lymphocytes[&]	0.50	(0.3 – 0.9)
Neutrophils[&]	2.0	(1.7 – 2.4)
Eosinophils[&]	0.4	(0.1 – 0.7)
Bronchial epithelial cells[&]	0.4	(0.2 – 0.6)
Bronchial Biopsies (n/0.1 mm^2)		
Macrophages[&]	1.0	(0.8 – 1.1)
Neutrophils[&]	0.6	(0.3 – 0.9)
Eosinophils[&]	0.4	(0.2 – 0.7)
$CD4^+$ cells[&]	1.7	(1.4 – 1.9)
$CD8^+$ cells[&]	1.3	(1.1 – 1.6)
Mast cells[&]	1.4	(1.3 – 1.5)

[*] *Data are expressed as median with interquartile ranges unless stated otherwise*

[**] *Postbronchodilator values.*

[#] *Geometric mean with interquartile range between brackets.*

[&] *Log transformed.*

Table 2. Multivariate regression analysis on the association between PC_{20} methacholine and clinical and inflammatory variables in blood, sputum and bronchial biopsies at baseline.

	^{2}**Log PC_{20} methacholine**		
	Beta	**95%CI**	
Current smoker	0.10	(-1.00 - 1.19)	p = 0.862
Age, yrs	-0.05	(-0.11 - 0.02)	p = 0.187
Female gender	**-2.86**	**(-4.47 - -1.26)**	**p = 0.001**
FEV_1/IVC (%)**	**0.10**	**(0.03 - 0.16)**	**p = 0.004**
$TLCO_{VA}$ (% predicted)	0.002	(-0.02 – 0.026)	p = 0.846
Blood monocytes (%)	-0.16	(-0.37 - 0.05)	p = 0.138
Sputum neutrophils (10^4/ml)$^{\&\#}$	**-1.18**	**(-2.25 - -0.11)**	**p = 0.032**
Bronchial $CD4^+$ cells (n/0.1 mm^2)$^{\&}$	-0.50	(-2.15 - 1.15)	p = 0.546

$^{\&}$Log transformed.

** *Postbronchodilator value.*

Beta = -1.67 (CI -3.13 - -0.22) for sputum lymphocytes, p = 0.025.

Table 3. Multivariate regression analysis on the association of the change in PC_{20} methacholine after 6-month treatment with the change in clinical and inflammatory variables in blood, sputum and bronchial biopsies.

	Δ^2Log PC_{20} methacholine		
	Beta	**95%CI**	
Current smoker	-0.66	(-1.83 - 0.52)	p = 0.268
Age, yrs	-0.30	(-1.04 - 0.04)	p = 0.423
Female gender	0.26	(-1.67 - 1.72)	p = 0.976
Treatment group fluticasone/salmeterol	0.07	(-1.70 - 1.85)	p = 0.936
Treatment group fluticasone	0.36	(-1.09 - 1.81)	p = 0.622
ΔRV/TLC (% predicted)	-0.03	(-0.06 - 0.00)	**p = 0.050**
ΔBlood monocytes (%)	0.35	(0.10 - 0.59)	**p = 0.006**
ΔSputum neutrophils (10^4/ml)[&]	-0.98	(-2.06 - 0.11)	p = 0.076
Δ Bronchial $CD4^+$ cells[†] (n/0.1 mm^2)[&]	-0.93	(-2.14 - 0.27)	p = 0.128

[&]Log transformed, Δ = change.

Table 4. Multivariate regression analysis on the association between the changes in PC_{20} methacholine between 6- and 30- month treatment with the changes in clinical and inflammatory variables in sputum and bronchial biopsies.

	Δ^2**Log PC_{20} methacholine**		
	Beta	**95%CI**	
Current smoker	-1.96	(-3.35 - -0.58)	**p = 0.006**
Age, yrs	-0.05	(-0.13 - 0.04)	p = 0.259
Female gender	0.11	(-2.48 - 2.69)	p = 0.934
Treatment group fluticasone/salmeterol	-0.94	(-2.77 – 0.90)	p = 0.310
Treatment group fluticasone 6 months	-2.43	(-4.14 - -0.73)	**p = 0.006**
Treatment group fluticasone 30 months	-0.12	(-1.80 - 1.56)	p = 0.887
ΔRV/TLC (% predicted)	-0.04	(-0.08 - -0.07)	**p = 0.021**
ΔSputum macrophages (10^4/ml)[†#]	-1.72	(-2.78 - -0.68)	**p = 0.002**

[†] Log transformed, Δ = change.

[#] Beta = -1.56 (CI -2.69 - -0.42) for sputum lymphocytes, p = 0.008

[#] Beta = -1.33 (CI -2.40 - -0.27) for sputum neutrophils, p = 0.015

[#] Beta = -1.11 (CI -2.30 - 0.08) for sputum total number of cells, p = 0.066

Legends to figures:

Figure 1 Total number of randomized patients who adhered to therapy (> 70% medication use) per treatment group. Adapted with permission from reference 12.

Figure 1

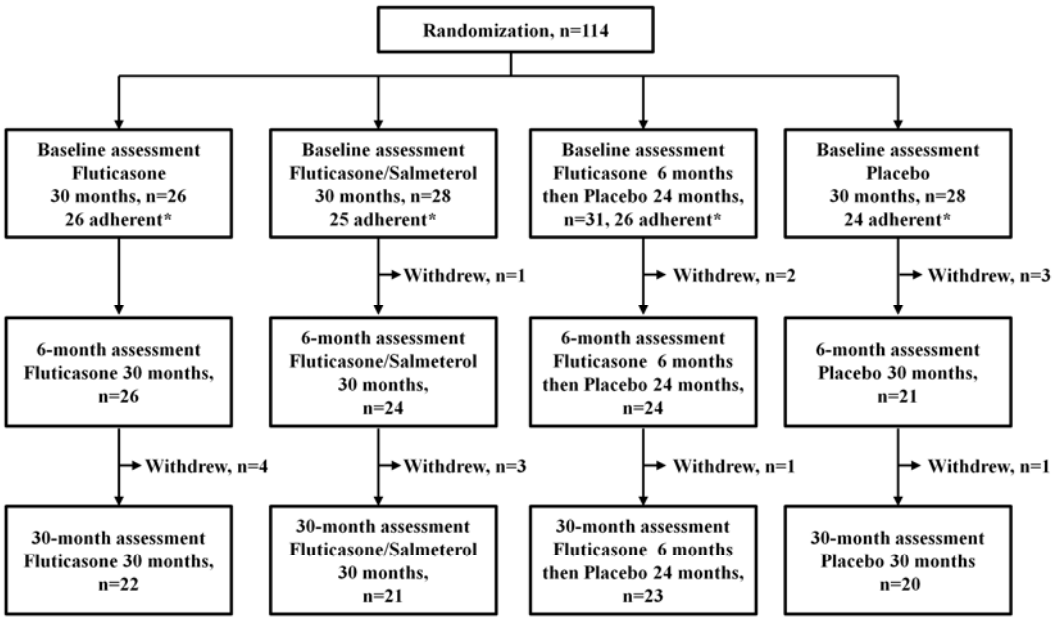
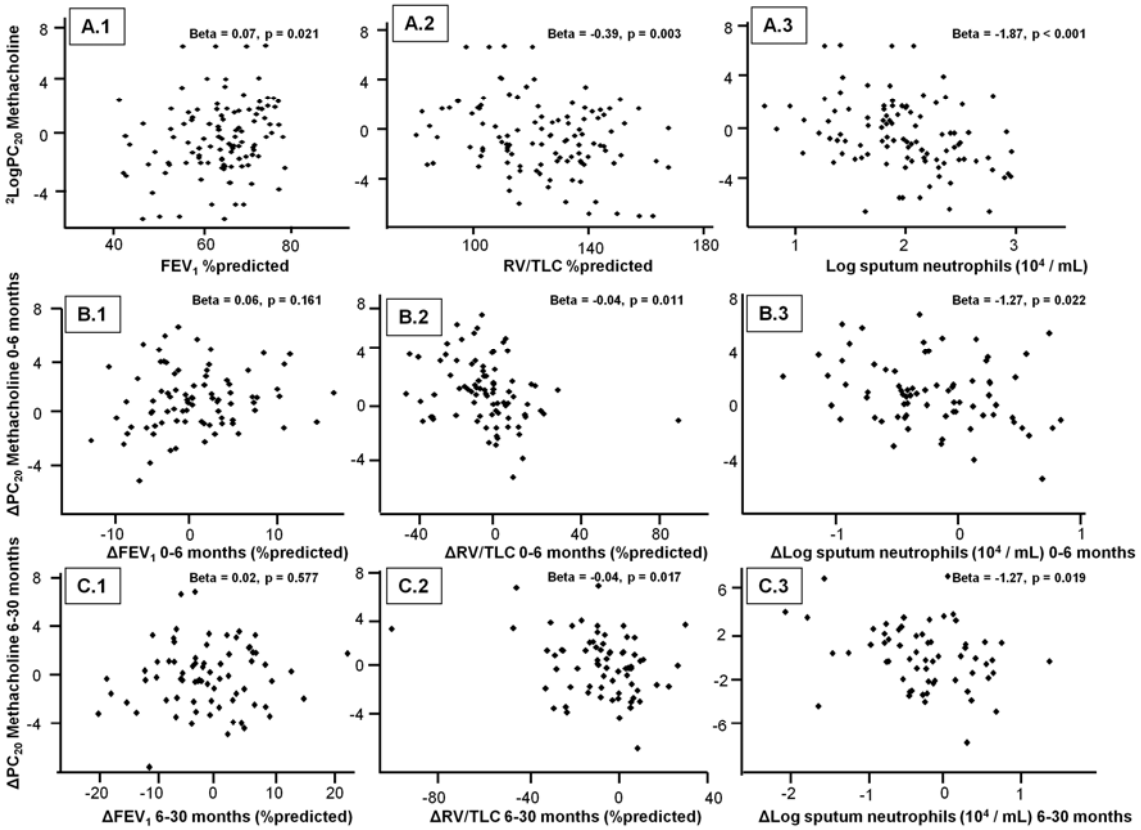


Figure 2 Univariate associations between: A1-A3) Baseline PC_{20} methacholine and FEV_1, RV/TLC, or the number of sputum neutrophils. B1-B3) Changes in PC_{20} methacholine between 0 and 6 months of treatment and changes in FEV_1, RV/TLC or the number of sputum neutrophils. C1-C3) Changes in PC_{20} methacholine between 6 and 30 months of treatment and changes in FEV_1, RV/TLC or the number of sputum neutrophils.


A.1
Beta = 0.07, p = 0.021
A.2
Beta = -0.39, p = 0.003
A.3
Beta = -1.87, p < 0.001
2LogPC20 Methacholine
FEV1 %predicted
RV/TLC %predicted
Log sputum neutrophils (10⁴ / mL)
B.1
Beta = 0.06, p = 0.161
B.2
Beta = -0.04, p = 0.011
B.3
Beta = -1.27, p = 0.022
ΔPC20 Methacholine 0-6 months
ΔFEV1 0-6 months (%predicted)
ΔRV/TLC 0-6 months (%predicted)
ΔLog sputum neutrophils (10⁴ / mL) 0-6 months
C.1
Beta = 0.02, p = 0.577
C.2
Beta = -0.04, p = 0.017
C.3
Beta = -1.27, p = 0.019
ΔPC20 Methacholine 6-30 months
ΔFEV1 6-30 months (%predicted)
ΔRV/TLC 6-30 months (%predicted)
ΔLog sputum neutrophils (10⁴ / mL) 6-30 months