Effects of inhaled corticosteroids on clinical and pathological outcomes in COPD - Insights from the GLUCOLD study

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CHAPTER 6

Airway Inflammation in COPD after Long-term Withdrawal of Inhaled Corticosteroids

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ABSTRACT

Background: Long-term treatment with inhaled corticosteroids (ICS) might attenuate lung function decline and decrease airway inflammation in a COPD subset, whereas ICS discontinuation relapses lung function decline. We hypothesized that airway inflammation increases after ICS withdrawal following long-term ICS treatment in COPD.

Methods: In the GLUCOLD-1 study (GL1) 114 patients with moderate to severe COPD were treated randomized to 6-month or 30-month fluticasone propionate (500µg bid), 30-month fluticasone/salmeterol (500/50µg, bid) or placebo. In the follow-up study (GL2), patients were followed prospectively for 5 consecutive years, treated by their physician. Bronchial biopsies and induced sputum were collected at baseline (GL1), 30-month (end GL1) and 7.5-year (end GL2) to assess inflammatory cell counts. Analysis was performed by linear mixed-effects models.

Results: In patients using ICS during GL1 and using ICS 0-50% of time during GL2 (n=61/85), bronchial cells increased in GL2 significantly: $CD3^+$ (fold change/year GL2-GL1 [95%CI] 2.68 [1.87-3.84]), $CD4^+$ (1.91 [1.33-2.75]), $CD8^+$ cells (1.71 [1.15-2.53]) and mast cells (1.91 [1.36-2.68]). Additionally, sputum total cell counts increased significantly in GL2 (1.90 [1.42-2.54]), macrophage (2.10 [1.55-2.86]) neutrophil (1.92 [1.39-2.65]) and lymphocyte counts (2.01 [1.46-2.78]).

Conclusions: ICS discontinuation increases airway inflammation in moderate to severe COPD patients, suggesting that anti-inflammatory effects of ICS in COPD are not maintained after ICS discontinuation.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is characterized by chronic inflammation in the airways, with neutrophils, macrophages and $CD8^+$ T-cells as major inflammatory cell types [1]. More severe airflow limitation is associated with more severe airway inflammation. As the progressive course of the disease continues to more severe airflow limitation, airway inflammation increases over time [2-4]. Except for smoking cessation, there is currently no therapy that halts the inflammatory process in the airways.

According to current guidelines, treatment with inhaled corticosteroids (ICS) is recommended for severe and very severe COPD patients in case of frequent exacerbations. Up to now only a few trials have evaluated the anti-inflammatory effect of ICS in bronchial biopsies and bronchoalveolar lavage (BAL) in COPD. A recent meta-analysis showed that 12- to 26-week ICS treatment in COPD reduced $CD4^+$ and $CD8^+$ cells in bronchial biopsies [5-10]. In addition, ICS reduced neutrophil and lymphocyte counts in BAL, but increased macrophage counts [9, 11-13]. Our study group has previously shown that 30-month treatment with inhaled fluticasone decreased the number of bronchial $CD3^+$, $CD4^+$, $CD8^+$ cells and mast cells, and reduced the sputum neutrophil, macrophage and lymphocyte counts [8].

Discontinuation of ICS may increase the number of exacerbations [14-16] and accelerate lung function decline in patients with COPD [17-22]. However, little is known about the effect of ICS discontinuation on airway inflammation. An increase in percentage sputum neutrophils was found in COPD patients randomized to ICS withdrawal when compared to neutrophils in case of 6-week ICS continuation [23]. We previously showed that discontinuation after 6-month ICS increased bronchial $CD3^+$ cells, mast cells and plasma cells at 2.5 years compared to continued therapy, without significant effect on sputum inflammatory cells [8]. However, the effects of withdrawal of ICS after long-term treatment on airway inflammation have not been investigated, but are highly relevant to evaluate the sustained reductions in bronchial inflammation and possible disease-modifying effects.

We hypothesized that inflammatory cell counts in bronchial biopsies and sputum increase after withdrawal of ICS in COPD patients who had previously been randomized to 30-month ICS treatment during 5 subsequent years of prospective follow-up. Additionally, we examined whether the changes in inflammation after ICS withdrawal were associated with

changes in lung function decline.

MATERIALS AND METHODS

Study design and participants

Patients of the GLUCOLD (Groningen and Leiden Universities Corticosteroids in Obstructive Lung Disease) study (GL1) were enrolled in the observational, follow-up study (GL2). Details of the study design were previously described [8, 21]. In short, in GL1 114 moderate to severe, steroid-naive COPD patients were randomized to one of the four treatment arms, with Diskus dry-powder inhalers (GlaxoSmithKline, Zeist, The Netherlands) each twice daily for 30 months: 1) fluticasone propionate (FP) 500µg (F30); 2) FP with salmeterol, 500/50µg, single inhaler (FS30); 3) 6-month FP followed by 24-month placebo (F6); and 4) placebo. During GL2, patients were treated by their own physician according to current guidelines [24], which implied that the majority of patients intermittently used or did not use ICS. At the end of GL2, a list of delivered medications was provided by the patients' pharmacy. The ethics committees of Leiden University Medical Center and University Medical Center Groningen approved both GL1 and GL2. Separate written informed consent for GL2 was provided by all patients.

Outcomes and measurements

The primary outcome of the present study was the effect of ICS withdrawal on inflammatory cell counts in the lamina propria of bronchial biopsies. Therefore, a fiberoptic bronchoscopy was performed after 5 years of follow-up (GL2) according to standardized protocols, consistent with bronchoscopies in GL1 [25]. Processing of bronchial biopsies was performed according to present recommendations [26], and two biopsies per patient were selected based on the largest lamina propria by evaluaton of hematoxylin-eosin stained sections. Immunohistochemical stainings on 4µm sections of paraffin-embedded bronchial biopsies were performed with specific antibodies against T-lymphocytes (CD3, CD4, and CD8), macrophages (CD68), neutrophil elastase (NE), mast cell tryptase (AA1) and eosinophils (EG2), according to the previous protocols [25]. Due to a lack of significant ICS-induced changes in plasma cells in GL1, we did not include these cells in our current analysis. Bronchial cells were counted using image analysis software (ImageJ, version 1.48i, National Institutes of Public Health). Subepithelial cells were calculated as weighted means and expressed as number of cells per 0.1mm^2. The minimal selected area of lamina propria for analysis per biopsy was 0.02mm^2. Data from bronchial cell counts of baseline and after 30 months (GL1) were

previously reported [8].

The secondary outcome was the effect of ICS discontinuation on inflammatory cell counts in induced sputum. A sputum induction was performed at year two and five of GL2. For safety reasons, sputum was only induced in patients with a post-bronchodilator FEV_1 ≥1.2l. Induced sputum was processed according to the full sample method [27]. Two cytospins per sample were stained with May-Grünwald Giemsa (MGG) to obtain differential cell counts. A sputum sample was considered adequate if ≤80% squamous cells were present. Differential cell counts were expressed as cell count per 10^4ml non-squamous nucleated cells. Sputum cell counts at baseline and after 30 months (GL1) were previously reported [8].

Statistical analysis

Data from all patients were used for the analysis and the statistical analysis was performed with SPSS 22.0 software (SPSS Inc., Chicago, IL). Because there were no differences in inflammatory cell counts after 30 months of treatment in the F6 and placebo groups, and in the FS30 and F30 groups [8], we combined these to increase power into two groups: F6/placebo and FS30/F30. ICS use during GL2 was retrospectively divided into the following groups: patients who used ICS 0-50% or 50-100% of the time. For the analysis of GL2, we focused on those patients using 0-50% ICS during GL2, being the largest subgroup (61 out of 85 patients). Based on the information of the patients' pharmacy, the daily dose of ICS (in µg, in beclomethasone dipropionate [BDP] equivalents) during 5 years was calculated as daily sum of the different doses of ICS (in µg/day) divided by the total time that ICS were used (days).

A linear mixed-effect model with a random intercept for each subject was applied, using all natural-log transformed inflammatory measurements from GL1 and GL2 as outcome variable and an identity covariance matrix. The analysis was stratified for original combined treatment groups and ICS use during GL2. The change in inflammatory cell counts in GL2 compared to GL1 (GL2 minus GL1) was assessed by two time variables in the models: time 1 (time since start of GL1: range 0-7.5 years) and time 2 (time since start of GL2: range 0-5 years; during GL1 this value is zero). To assess the change in cell counts between the original FS30/F30 groups compared to the original F6/placebo groups, a linear mixed-effect model with the same time variables was used including an interaction term between these time variables with the original combined treatment groups. Given the limited sample size of patients completing the 5 years of prospective follow-up, possible confounders (age, sex and center) were not included in the model. Smoking was unlikely to be a major confounder as shown in

a previous post-hoc analysis [8]. By Spearmans' correlation coefficient, we assessed whether the changes in inflammation in GL2 versus GL1 were associated with change in lung function in the same period.

Since the number of cells decreased during GL1 and increased at the end of GL2, and therefore represent a difference in slope, we calculated the rate of change. Therefore, data are presented as fold change per year between GL2 minus GL1 with 95% confidence interval (CI), calculated by taking the antilog of estimates from the linear mixed-effects models. Statistical significance was inferred at P≤0.05.

Table 1. Patient characteristics at baseline of randomized treatment (GL1) and at the start of 5 years of follow-up (GL2) for the original FS30/F30 and F6/placebo groups.

	Baseline GL1		**Start of GL2**	
	F6/placebo (n=60)	FS30/F30 (n=54)	F6/placebo (n=46)	FS30/F30 (n=46)
Gender (M/F) (n)	51/9	48/6	41/5	42/4
Age (yr)	61 (7.7)	62 (7.8)	64 (7.7)	64 (7.3)
Smoking (y/n) (n)	36/24	36/18	22/24	26/20
Packyears (yr)	41 (31-54)	47 (31-56)	43 (31-58)	49 (34-57)
Post-bronchodilator FEV_1 (% pred)	64 (8.3)	62 (9.3)	61 (11.1)	63 (11.7)
Post-bronchodilator FEV_1 (L)	2.05 (0.5)	2.06 (0.4)	1.92 (0.6)*	2.03 (0.5)
Post-bronchodilator FEV_1/IVC (%)	49 (8.5)	47 (8.6)	46 (10.6)	47 (9.9)

Data of the 2 combined groups are derived from the original 4 treatment groups in GL1 are presented as means and standard deviation, median with interquartile range (packyears) or numbers. F6: 6 months treatment with fluticasone, followed by 24 months of placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone. Data for the individual 4 treatment groups have been previously reported [21].

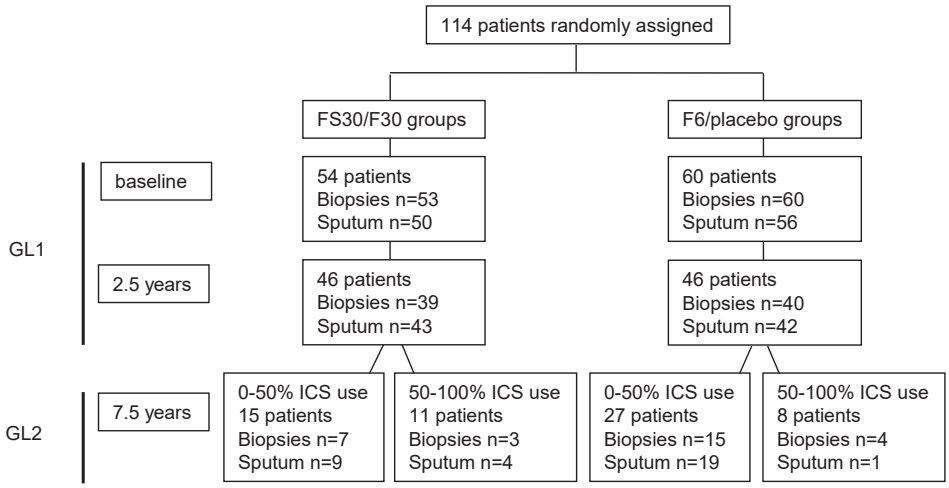
* P≤0.001 compared to original combined F6/placebo groups at baseline GL1 (calculated with paired samples T-test).

RESULTS

Data from 114 patients were used for the analysis of GL1, 92 patients completed GL1, 85 patients started and 61 completed GL2 [8, 21]. Patient characteristics at both baseline and at start of GL2 were similar among the original combined treatment groups, except for post-bronchodilator FEV_1, which was significantly lower at the start of GL2 in the original combined F6/placebo groups compared to the original combined FS30/F30 groups (Table 1).

Most patients (61/85) used ICS 0-50% of time during GL2, with a mean daily ICS dose of 1019µg (SD 554µg) in BDP equivalents during GL2 (Table 2), which was not significantly different between the original combined treatment groups. Bronchial biopsies of 29 patients were available at the end of GL2 (Table 3), and 21 of these 29 patients used 0-50% of time ICS during GL2. Sputum samples suitable for analysis were available from 47 and 33 patients after 2 and 5-year follow-up, respectively (Table 3). A diagram with the number of patients per group and available number of samples is presented in Figure 1.

Figure 1: Diagram presenting the number of patients and available bronchial biopsies and sputum samples in GL1 and GL2 in the original combined treatment groups.



GL1: GLUCOLD 1 study, first part of the study. GL2: GLUCOLD 2 study, follow-up study. F6: 6 months treatment with fluticasone, followed by 24 months of placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone.

Table 2: Number of patients at the start of the GLUCOLD follow-up study (GL2) using ICS and daily dose of ICS (in µg) during 5 years in those patients who used ICS during GL2.

Original treatment group	No ICS use (n)	≤50% use of ICS (n)	>50% use of ICS (n)	100% use of ICS (n)	Daily dose ICS (µg)
F6/placebo	20	12	10	1	875 (479)
FS30/F30	15	14	8	5	1141 (591)
Total	35	26	18	6	1019 (554)

The daily dose of ICS (in µg, in BDP equivalents) during 5 years was calculated by the sum of the different doses of ICS per day (in µg/day), divided by the total time that ICS were used (in days). Doses were based on data provided by the patients' pharmacy.

Daily dose of ICS is presented as means with standard deviations. F6: 6 months treatment with fluticasone and 24 months with placebo; FS30: 30 months treatment with fluticasone and salmeterol; F30: 30 months treatment with fluticasone.

Bronchial biopsies

Compared to GL1, patients within the combined original FS30/F30 groups, who used ICS 0-50% of time during GL2 showed an increase in bronchial $CD3^+$ [GL2-GL1, expressed a fold change/year: 2.68 (1.87-3.84); P<0.001], $CD4^+$ [1.91 (1.33-2.75); P=0.001], $CD8^+$ cells [1.71 (1.15-2.53); P=0.008] and mast cells [1.91 (1.36-2.68); P<0.001] at the end of GL2 (Figure 2). Discontinuation of ICS or use of ICS 0-50% of time during GL2 in the original combined FS30/F30 groups increased the number of $CD3^+$ cells (expressed as fold change/year) [1.78 (1.21-2.64); P=0.04], $CD8^+$ cells [1.73 [1.05-2.85); P=0.033] and mast cells [1.52 (1.06-2.17); P=0.023] compared to the original combined F6/placebo groups.

Table 3: Sputum samples and bronchial biopsies at year 2 and year 5 of GL2, presented by original combined treatment groups and use of ICS during GL2.

			No ICS use	<50% ICS use	>50% ICS use	100% ICS use	total
Sputum	Year 2	F6/placebo	10	10	3	1	24
		FS30/F30	8	7	5	3	23
	Year 5	F6/placebo	12	7	1	0	20
		FS30/F30	4	5	3	1	13
Bronchial biopsies	Year 5	F6/placebo	10	5	3	1	19
		FS30/F30	3	3	4	0	10

Sputum samples were collected from 63 and 51 patients after 2 and 5 years of follow-up (suitable for analysis 47 and 33), respectively. Data presented as number of samples.

GL2: follow-up study; ICS: Inhaled corticosteroids. F6: 6-month ICS followed by 24-month placebo. FS30: 30-month fluticasone with salmeterol; F30: 30-month fluticasone.

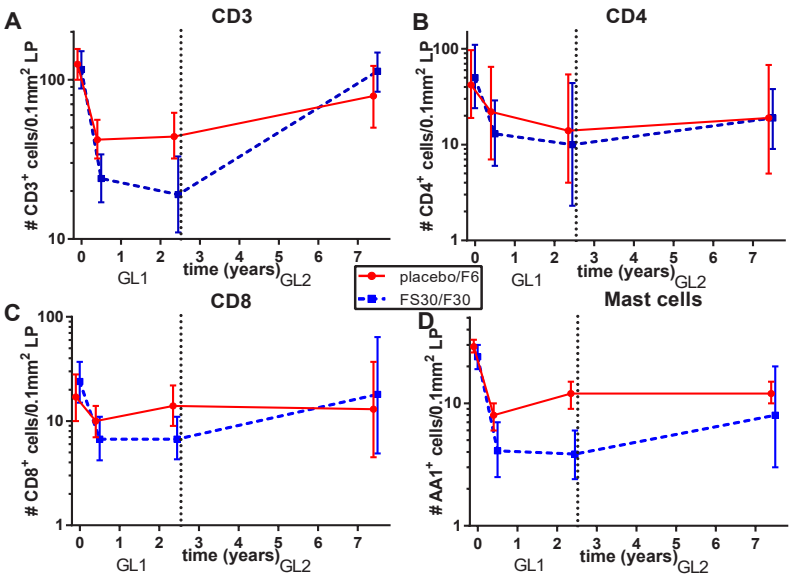


Figure 2: Geometric mean cell counts in bronchial biopsies (per 0.1 mm^2 lamina propria) for those patients who used ICS 0-50% of the time during GL2 in the original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% confidence interval. Data of bronchial CD3$^+$ cells (Figure 2A), CD4$^+$ cells (2B), CD8$^+$ cells (2C) and mast cells (2D) are presented. Data were calculated by taking the antilog of the means of natural log-transformed number of cells. The group of patients who used ICS 50-100% of time during GL2 was too small, and is therefore not shown in the figures.

Sputum

At the end of GL2, patients of the original FS30/F30 groups who used 0-50% of time during GL2 had a higher total sputum cell count [GL2-GL1, expressed as fold change/year: 1.90 (1.42-2.54); P<0.001], as well as higher counts of sputum macrophages [2.10 (1.55-2.86)]; P<0.001), neutrophils [1.92 (1.39-2.65)]; P<0.001) and lymphocytes [2.01 (1.46-2.78)]; P<0.001), at the end of GL2 compared to during GL1 (Figure 3). Discontinuation of ICS or use of ICS 0-50% of time during GL2 in the original combined FS30/F30 groups increased the total number of sputum cells (expressed as fold change/year) [1.66 (1.12-2.46); P=0.012], sputum neutrophils [1.68 [1.09-2.58); P=0.018], macrophages (1.90 [1.26-2.85]; P=0.002) and lymphocytes [1.73 (1.09-2.74); P=0.020] compared to the original combined F6/placebo groups.

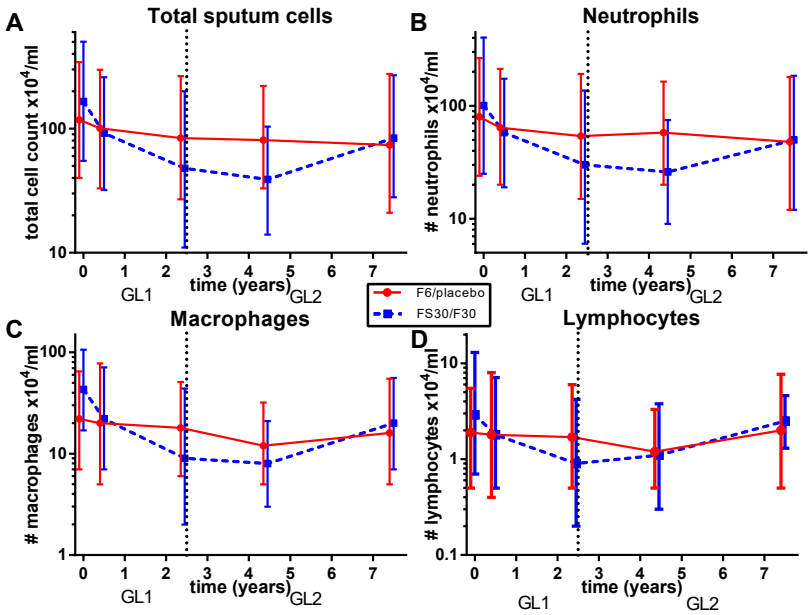


Figure 3: Geometric mean sputum cell counts (x 10^4 per ml) for original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% confidence interval. Data of patients who used ICS 0-50% of the time during GL2 are presented for total sputum cells (Figure 3A), neutrophils (3B), macrophages (3C) and lymphocytes (3D). Data were calculated by taking the antilog of the means of natural log-transformed number of cells. The group of patients who used ICS 50-100% of time during GL2 was too small, and is therefore not shown in the figures.

Relation between lung function decline and inflammatory cells

The accelerated rate of decline in post-bronchodilator FEV_1 during GL2 was associated with an increase in sputum macrophages in patients of the original FS30/F30 groups (Rs=-0.63, P=0.04) and with a trend towards an increase in bronchial neutrophil counts (Rs=-0.60, P=0.07) (Figures 4A and B, respectively).

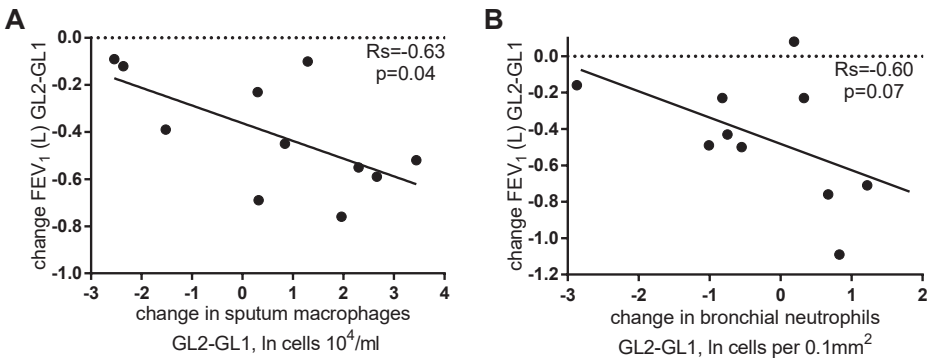


Figure 4: Correlation between change (end GL2-end GL1) in post-bronchodilator FEV_1 (L) and change in natural log-transformed sputum macrophages (end GL2-end GL1, expressed as number of cells x 10^4 per ml) (Figure 4A, left panel) and changes in natural log-transformed bronchial neutrophils (per 0.1 mm^2 lamina propria, Figure 4B, right panel). Each dot represents a single patient.

DISCUSSION

The present study shows that withdrawal of ICS after previous long-term ICS treatment in patients with moderate to severe COPD, is accompanied with increased bronchial T-lymphocytes and mast cells as well as several sputum cell counts. In addition, we found an association between the accelerated rate of lung function decline and increase in sputum macrophages during GL2, and a trend with bronchial neutrophils. These results suggest that airway inflammation is suppressed during active treatment with ICS and might relapse after long-term discontinuation of ICS.

We observed that several inflammatory cells in bronchial biopsies and sputum significantly

increased during 5-year follow-up in patients with moderate to severe COPD who did not use or only intermittently used ICS after previous 30-month randomized ICS treatment. These unique data confirm and extend previous findings of our study group, showing that withdrawal of ICS after 6-month ICS treatment increases bronchial $CD3^+$ cells, mast cells and plasma cells when followed up to 30 months compared to those who continued ICS therapy, without a significant effect on sputum inflammatory cells [8]. Another open-labelled pilot study with only 6-week ICS withdrawal showed an increase in the percentage of sputum neutrophils compared to ICS continuation [23]. Taken together, the present study provides novel data on a relapse of airway inflammation after prolonged ICS discontinuation with previous long-term ICS use in COPD.

In this longitudinal study, we found that lung function decline seems to be related to an increase in sputum macrophage counts in moderate to severe COPD patients, the majority being without ICS treatment. Furthermore, a trend for an association with higher bronchial neutrophils was found. A previous study showed that higher sputum neutrophils were associated with faster FEV_1 decline in patients with severe COPD using ICS [3]. Furthermore, a weak association between sputum percentage neutrophils and FEV_1 percentage predicted was found in a cross-sectional study [4]. These findings in sputum neutrophils are likely not only explained by differences in study design, but also by differences among the studies in number of patients, severity or phenotype of COPD, and duration of treatment and withdrawal of ICS. A recent study by Barnes et al. found that COPD patients treated with fluticasone and a high percentage blood eosinophils at baseline have a slower rate of FEV_1 decline compared to placebo treated patients [28]. However, we could not detect a relation between baseline blood eosinophilia and lung function decline during GL2 in those who stopped or continued using ICS during GL2. Taken together, until now, this is the only long-term study that suggests an association between lung function decline and change in inflammation after prolonged ICS withdrawal.

A strength of our study is the long-term follow-up with monitoring of lung function and availability of sputum and bronchial biopsies during 5-year follow-up. This is unique, since no previous studies had such a prolonged treatment period as well as long follow-up period with treatment according to the current guidelines in a real life setting. It needs to be noted that the effect of ICS withdrawal in the present study was more pronounced in bronchial biopsies than in sputum, stressing the importance to not only study sputum cell counts when investigating COPD. Nevertheless, our study has some limitations. First, expectedly, the number of patients that finished the complete study is limited, particularly when considering the original treatment groups separately. Therefore, we chose to combine the original FS30/

F30 and F6/placebo groups to increase power as there were no differences between the F6 and placebo groups at the start of the GL2 but only following first 6-months treatment during GL1. The small groups of patients from whom bronchial biopsies and sputum samples were available, make the correlation with lung function decline less strong. Despite these relatively low numbers, we still detected associations between clinical and histological outcomes. Second, GL2 was a prospective, (non-randomized) observational study and the majority of patients (61 of 85) discontinued their ICS or used 0-50% of time of observation ICS during GL2 (Table 2). Adherence to medication was not checked, reflecting daily practice. This could have led to a misclassification of ICS use during GL2 and therefore might have influenced out outcomes. Nevertheless, when only compliant patients during GL1 were included in the current analysis, similar results were found (data not shown). Due to the limited number of patients in whom a bronchoscopy was performed (Figure 1), a comparison between those with continuous ICS use versus ICS withdrawal during GL2 was not possible. Third, inflammatory cells were stained by immunohistochemistry with different batches of antibodies and counted using a different camera and image analysis software compared to GL1 [28]. We cannot rule out that these differences could have influenced our data. However, the bronchial inflammatory cell counts found in GL1 and GL2 were in a comparable range and cannot explain the observed difference found between and within the groups. Finally, bronchial inflammation is unequally distributed along the airways [29]. Since we were only able to collect samples from the central airways, the effect of ICS withdrawal in the small airways could not be investigated in this study. Taken into account these considerations, we are nevertheless confident that our data provide a novel view on relevant changes in airway inflammation after long-term cessation of prolonged ICS treatment.

How can we interpret our results? In the first part of the study (GL1), we found a reduction in bronchial inflammation and attenuation of lung function decline during 30-month ICS treatment in patients with moderate to severe COPD [8]. In the current study (GL2), we observed the expected opposite in that ICS withdrawal after previous long-term treatment resulted in an increase in $CD3^+$, $CD4^+$, $CD8^+$ T-cells and mast cells in this selected group of patients, which further extends our previous observation that withdrawal of ICS relapses lung function decline [21]. The opposite outcome of ICS therapy in the first phase when compared to the effects of ICS withdrawal on airway inflammation in the second observational phase of our study can be regarded as a validation and strongly supports the plausibility of our findings. Taken together, our data suggest that during active treatment with ICS effects on lung function and inflammation are transient, without persistent disease modification.

During the past 25 years numerous studies have been published concerning the question whether or not ICS are beneficial in COPD patients [30]. Our study shows that in this group of moderate to severe COPD, patients experience transient positive effects on lung function and on airway inflammation during ICS therapy, which are not maintained after ICS withdrawal [21]. The present selection of COPD patients may be representative of a particular phenotype that is responsive to steroid treatment [31], in whom long-term ICS therapy may need to be continued to maintain the observed beneficial effects on the course of lung function and airway inflammation over time.

CONCLUSION

In conclusion, the present data indicate that ICS discontinuation during 5 years following 30 months use of ICS, in a group of moderate to severe COPD patients, induces a relapse in bronchial and sputum inflammatory cells which is partially accompanied by a more rapid decline in lung function. These data suggest that ICS do not have a sustained disease modifying activity after ICS withdrawal in this group of COPD patients which is in line with observations in asthmatic patients.

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