Airway inflammation in COPD after long-term withdrawal of inhaled corticosteroids

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ABSTRACT Long-term treatment with inhaled corticosteroids (ICS) might attenuate lung function decline and decrease airway inflammation in a subset of patients with chronic obstructive pulmonary disease (COPD), and discontinuing ICS treatment could result in further lung function decline. We hypothesised that airway inflammation increases after ICS withdrawal following long-term ICS treatment in COPD.

In the GLUCOLD-1 study (GL1), 114 patients with moderate-severe COPD were randomised to 6-month or 30-month treatment with fluticasone propionate (500 μg twice daily), 30-month treatment with fluticasone/salmeterol (500/50 μg twice daily) or placebo. During the 5-year follow-up study (GL2), patients were followed prospectively while being treated by their physician. Bronchial biopsies and induced sputum were collected at baseline, at 30 months (end of GL1) and at 7.5 years (end of GL2) to assess inflammatory cell counts. Data were analysed using linear mixed-effects models.

In patients using ICS during GL1 and using ICS 0–50% of the time during GL2 (n=61/85), there were significant increases in GL2 bronchial $CD3^+$ (fold change per year calculated as GL2 minus GL1 2.68, 95% CI 1.87–3.84), $CD4^+$ (1.91, 95% CI 1.33–2.75) and $CD8^+$ cells (1.71, 95% CI 1.15–2.53), and mast cells (1.91, 95% CI 1.36–2.68). The sputum total cell counts increased significantly in GL2 (1.90, 95% CI 1.42–2.54), as did counts of macrophages (2.10, 95% CI 1.55–2.86), neutrophils (1.92, 95% CI 1.39–2.65) and lymphocytes (2.01, 95% CI 1.46–2.78).

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

Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by chronic inflammation in the airways, with neutrophils, macrophages and $CD8^+$ T-cells as the major inflammatory cell types [1]. As the course of the disease progresses 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 patients with severe and very severe COPD in cases of frequent exacerbations. To date, few trials have used bronchial biopsies and bronchoalveolar lavage (BAL) to evaluate the anti-inflammatory effects of ICS in COPD. A recent meta-analysis showed that 12–26-week ICS treatment in COPD reduced $CD4^+$ and $CD8^+$ cell counts 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 decreases the number of bronchial $CD3^+$, $CD4^+$, $CD8^+$ and mast cells, and reduces 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 the percentage of sputum neutrophils was found in COPD patients randomised to ICS withdrawal when compared to COPD patients randomised to 6-week ICS continuation [23]. We previously showed that discontinuation after 6-month ICS treatment increased bronchial $CD3^+$ cells, mast cells and plasma cells at 2.5 years compared to continued therapy, without a significant effect on sputum inflammatory cells [8]. The effects of withdrawal of ICS after long-term treatment on airway inflammation have not been investigated, but they are highly relevant to evaluate sustained reductions in bronchial inflammation and possible disease-modifying effects.

We hypothesised that, after the withdrawal of ICS in COPD patients who had previously been randomised to 30-month ICS treatment, inflammatory cell counts in bronchial biopsies and sputum would increase during the subsequent 5 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). The total follow-up period was 5 years. Details of the study design were previously described [8, 20]. In GL1, 114 steroid-naive patients with moderate to severe COPD were randomised to one of four treatment arms with Diskus dry-powder inhalers (GlaxoSmithKline, Zeist, the Netherlands), each twice daily for 30 months: 1) fluticasone propionate (FP) 500 µg (F30); 2) FP with salmeterol, 500/50 µg, 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 imply 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 fibre-optic bronchoscopy was performed after 5 years of follow-up (GL2) according to standardised 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 determined by evaluation of sections stained with hematoxylin-eosin. Immunohistochemical staining of 4-µm sections of paraffin-embedded

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bronchial biopsies was 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 previous protocols [25]. Owing 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, Bethesda, MD, USA). Sub-epithelial cells were calculated as weighted means and expressed as number of cells per 0.1 mm^2. The minimal selected area of lamina propria for analysis per biopsy was 20 000 μm^2. Data from bronchial cell counts at baseline and after 30 months (GL1) have previously been reported [8].

The secondary outcome was the effect of ICS discontinuation on inflammatory cell counts in induced sputum. A sputum induction was performed in the second and fifth years of GL2. For safety reasons, sputum was only induced in patients with a post-bronchodilator forced expiratory volume in 1 s (FEV_1) $\geqslant$1.2 L. Induced sputum was processed according to the full sample method [27]. Two cytospins per sample were stained with May–Grünwald–Giemsa to obtain differential cell counts. A sputum sample was considered adequate if $\leqslant$80% of cells comprised squamous cells. Differential cell counts were expressed as cell count per 10^4 mL non-squamous nucleated cells. Sputum cell counts at baseline and after 30 months (GL1) have previously been 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, USA). Because there were no differences in inflammatory cell counts after 30 months of treatment between the F6 and placebo groups, nor between the FS30 and F30 groups [8], we combined these into two groups to increase power: F6/placebo and FS30/F30. ICS use during GL2 was retrospectively divided into two groups: patients who used ICS 0–50% of the time, and those who used ICS 50–100% of the time. For the analysis of GL2, we focused on those patients using ICS 0–50% of the time during GL2, because they formed the largest subgroup (61 out of 85 patients). Based on the information from the patients' pharmacies, the daily dose of ICS (μg, in beclomethasone dipropionate (BDP) equivalents) during the 5 years was calculated as the daily sum of the different doses of ICS (μg per 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 the 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 the start of GL1: range 0–7.5 years) and time 2 (time since the 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 that included 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 medical centre) were not included in the model. Smoking was unlikely to be a major confounder, as shown in a previous *post hoc* analysis [8]. We used Spearmans' correlation coefficient to assess whether the changes in inflammation in GL2 *versus* GL1 were associated with change in lung function in the same period.

Because the number of cells decreased during GL1 and increased at the end of GL2, therefore representing 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 intervals, calculated by taking the antilog of estimates from the linear mixed-effects models. Statistical significance was inferred at $p\leqslant0.05$.

Results

Data from 114 patients were used for the analysis of GL1; 92 patients completed GL1, 85 patients started and 61 completed GL2 [8, 20]. Patient characteristics at both baseline and the 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 than in the original combined FS30/F30 groups (table 1). Most patients (61 out of 85) used ICS 0–50% of the time during GL2, with a mean daily ICS dose of 1019±554 μg 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 ICS 0–50% of the time during GL2. Sputum samples suitable for analysis were available from 47 and 33 patients after 2- and 5-year follow-up, respectively (table 3). Figure 1 presents the number of patients per group and available number of samples.

TABLE 1 Patient characteristics at baseline of randomised 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	FS30/F30	F6/placebo	FS30/F30
Subjects n	60	54	46	46
Male/female n	51/9	48/6	41/5	42/4
Age years	61±7.7	62±7.8	64±7.7	64±7.3
Smoking (yes/no) n	36/24	36/18	22/24	26/20
Pack-years	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 two combined groups are derived from the original four treatment groups in GL1 and are presented as mean±SD or median (interquartile range), unless otherwise stated. Data for the four individual treatment groups have been previously reported [20]. 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. FEV_1: forced expiratory volume in 1 s; IVC: inspiratory vital capacity. #: $p \leqslant 0.001$ compared to original combined F6/placebo groups at baseline GL1 (calculated with paired samples t-test).

Bronchial biopsies

Patients within the combined original FS30/F30 groups who used ICS 0–50% of the time during GL2 showed an increase in bronchial $CD3^+$ cells (fold change per year calculated using GL2 minus GL1 (GL2-GL1) 2.68, 95% CI 1.87–3.84, p<0.001), $CD4^+$ cells (1.91, 95% CI 1.33–2.75, p=0.001), $CD8^+$ cells (1.71, 95% CI 1.15–2.53, p=0.008) and mast cells (1.91, 95% CI 1.36–2.68, p<0.001) at the end of GL2 compared with GL1 (figure 2, supplementary figure S1, supplementary table S1). Discontinuation of ICS or using ICS 0–50% of the time during GL2 increased the number of $CD3^+$ cells (fold change per year GL2-GL1 1.78, 95% CI 1.21–2.64, p=0.04), $CD8^+$ cells (1.73, 95% CI 1.05–2.85, p=0.033) and mast cells (1.52, 95% CI 1.06–2.17, p=0.023) in the original combined FS30/F30 groups compared to the original combined F6/placebo groups.

Sputum

Patients in the original FS30/F30 groups who used ICS 0–50% of the time during GL2 had a higher total sputum cell count (fold change per year GL2-GL1 1.90, 95% CI 1.42–2.54, p<0.001) as well as higher counts of sputum macrophages (2.10, 95% CI 1.55–2.86, p<0.001), neutrophils (1.92, 95% CI 1.39–2.65, p<0.001) and lymphocytes (2.01, 95% CI 1.46–2.78, p<0.001) at the end of GL2 compared to during GL1 (figure 3, supplementary figure S2, supplementary table S2). Discontinuation of ICS or use of ICS 0–50% of the time during GL2 increased the total number of sputum cells (expressed as fold change per year GL2-GL1 1.66, 95% CI 1.12–2.46, p=0.012), sputum neutrophils (1.68, 95% CI 1.09–2.58, p=0.018), macrophages (1.90, 95% CI 1.26–2.85, p=0.002) and lymphocytes (1.73, 95% CI 1.09–2.74, p=0.020) in the original combined FS30/F30 groups compared to the original combined F6/placebo groups.

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

Original treatment group	No ICS use	⩽50% use of ICS	>50% use of ICS	100% use of ICS	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

Data are presented as n or mean±SD. ICS: inhaled corticosteroids; 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. #: the daily dose of ICS (in beclomethasone dipropionate equivalents) during the 5 years of follow-up was calculated by summing the different doses of ICS per day and dividing by the total time that ICS were used (in days). Doses were based on data provided by the patients' pharmacy.

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	<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

Data are presented as n. Sputum samples were collected from 63 and 51 patients after 2 and 5 years of follow-up (47 and 33 suitable for analysis), respectively. ICS: inhaled corticosteroids; F6: 6 months treatment with fluticasone, followed by 24 months of placebo; FS30: 30-month fluticasone with salmeterol; F30: 30-month fluticasone.

Relationship 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 in the original FS30/F30 groups (Rs= −0.63, p=0.04), with a trend towards an increase in bronchial neutrophil counts (Rs= −0.60, p=0.07) (figure 4a and b, respectively).

Discussion

The present study shows that withdrawal of ICS after previous long-term ICS treatment in patients with moderate-severe COPD is accompanied by an increase in bronchial T-lymphocytes and mast cells as well as several types of sputum cells. In addition, we found a significant association between the accelerated rate of lung function decline and the 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 discontinuation of long-term ICS treatment.

We observed that counts of several inflammatory cell types in bronchial biopsies and sputum significantly increased during a 5-year follow-up in patients with moderate-severe COPD who did not use or only intermittently used ICS after previous randomisation to a 30-month ICS treatment. These unique data confirm and extend previous findings by our study group, which showed that withdrawal of ICS after 6-month ICS treatment increases the numbers of bronchial $CD3^+$ cells, mast cells and plasma cells over a 30-month follow-up in comparison with patients who continued ICS therapy, without a significant effect on sputum inflammatory cells [8]. Another open-label pilot study showed an increase in the percentage of sputum neutrophils with only 6-week ICS withdrawal compared to ICS continuation [23]. Taken together,

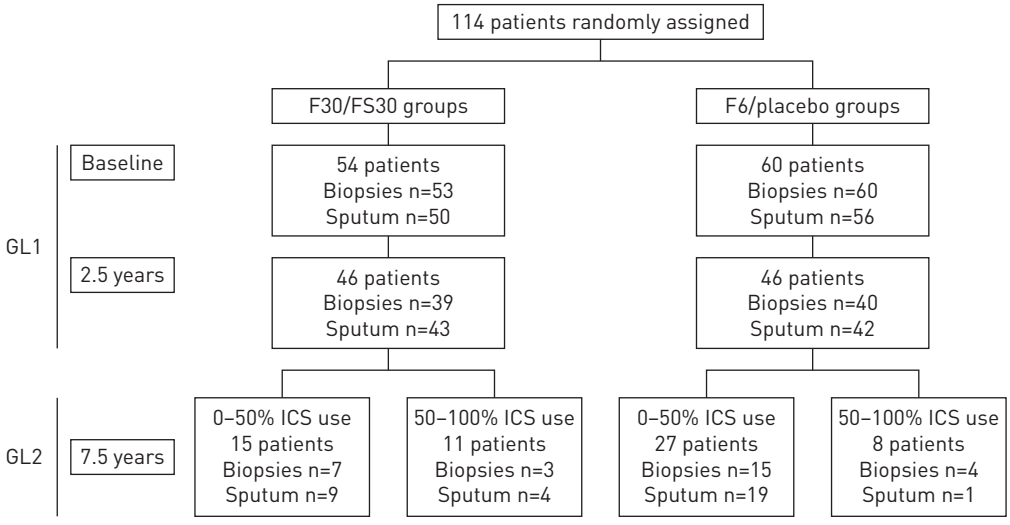

FIGURE 1 The number of patients and available bronchial biopsies and sputum samples in GL1 and GL2 in the original combined treatment groups. GL1: GLUCOLD-1 study, first part of the study; GL2: GLUCOLD-2 study, follow-up study; F6: 6 months treatment with fluticasone, followed by 24 months of placebo; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol; ICS: inhaled corticosteroids.

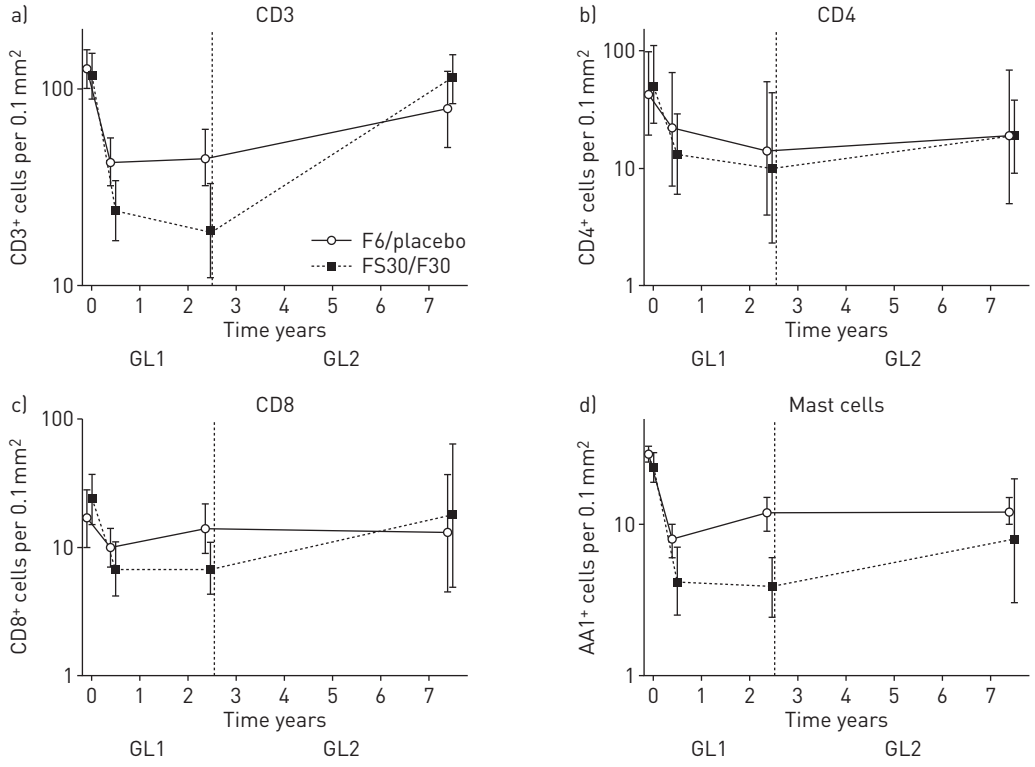

FIGURE 2 Geometric mean cell counts in bronchial biopsies (per 0.1 mm^2 lamina propria) for those patients who used inhaled corticosteroids (ICS) 0–50% of the time during GL2 in the original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% CI. The dotted line indicates the separation between GL1 and GL2. Data of bronchial a) $CD3^+$ cells, b) $CD4^+$ cells, c) $CD8^+$ cells and d) mast cells ($AA1^+$) are presented. Data were calculated by taking the antilog of the means of the natural log-transformed number of cells. The group of patients who used ICS 50–100% of the time during GL2 was too small, and is therefore not shown in the figures. 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; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol.

the present study provides novel data on the relapse of airway inflammation after prolonged ICS discontinuation following previous long-term ICS use in COPD.

In this longitudinal study, we found that lung function decline was related to an increase in sputum macrophage counts in patients with moderate-severe COPD, the majority of whom were not receiving ICS treatment. Furthermore, a trend for an association with higher bronchial neutrophils was found. A previous study showed that a higher sputum neutrophil count is associated with a faster decline in FEV_1 in patients with severe COPD using ICS [3]. Furthermore, a weak association between the percentage of sputum 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 with a high percentage of blood eosinophils at baseline who are treated with fluticasone have a slower rate of FEV_1 decline compared with placebo-treated patients [28]. However, we did not detect a relationship between baseline blood eosinophilia and lung function decline during GL2 in patients who stopped or continued using ICS during GL2. To date, 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 lung function monitoring and the availability of sputum and bronchial biopsies during a 5-year follow-up. This is unique, because no previous studies have had such a prolonged treatment period as well as a 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 of not only studying sputum cell counts when investigating COPD.

Nevertheless, our study had some limitations. First, expectedly, the number of patients that finished the complete study was limited, particularly when considering the original treatment groups separately.

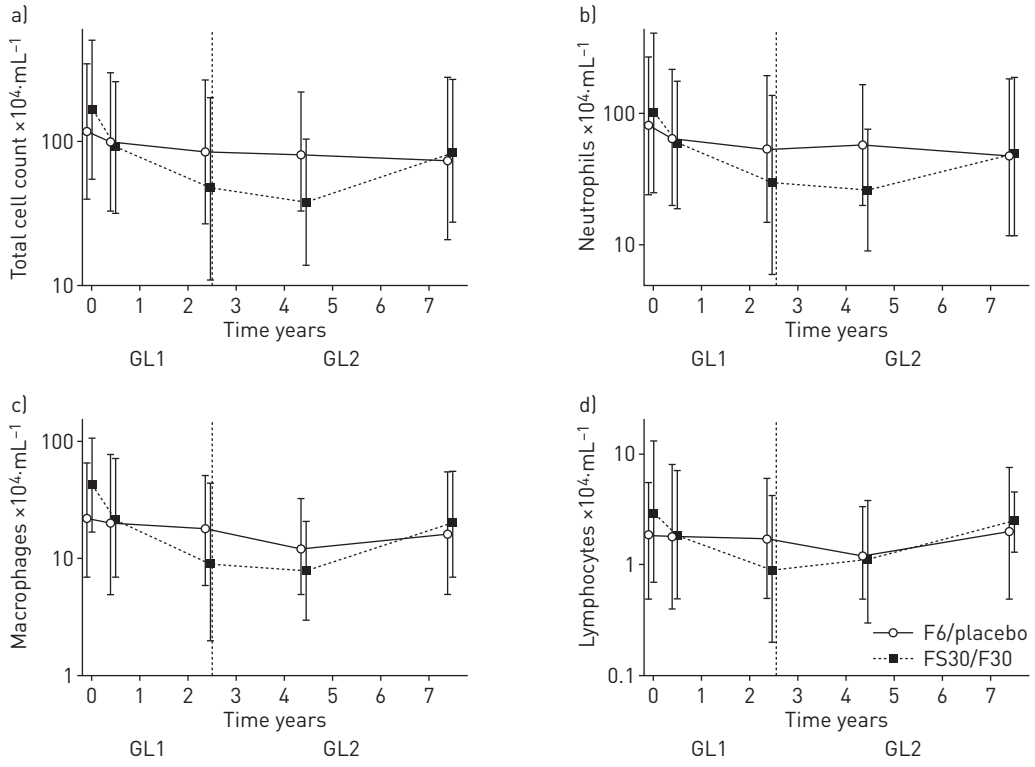

FIGURE 3 Geometric mean sputum cell counts (×10^4 per mL) for original combined treatment groups FS30/F30 and F6/placebo. Error bars represent 95% CI. Data of patients who used inhaled corticosteroids (ICS) 0–50% of the time during GL2 are presented for a) total sputum cells, b) neutrophils, c) macrophages and d) lymphocytes. Data were calculated by taking the antilog of the means of the natural log-transformed number of cells. The group of patients who used ICS 50–100% of the time during GL2 was too small, and is therefore not shown in the figures. 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; F30: 30 months treatment with fluticasone; FS30: 30 months treatment with fluticasone and salmeterol.

Therefore, to increase power we combined the original FS30 and F30 groups, and the F6 and placebo groups, as there were no differences between the F6 and placebo groups at the start of the GL2 but only following the first 6-month treatment during GL1. The small size of the 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-randomised) observational study and the majority of

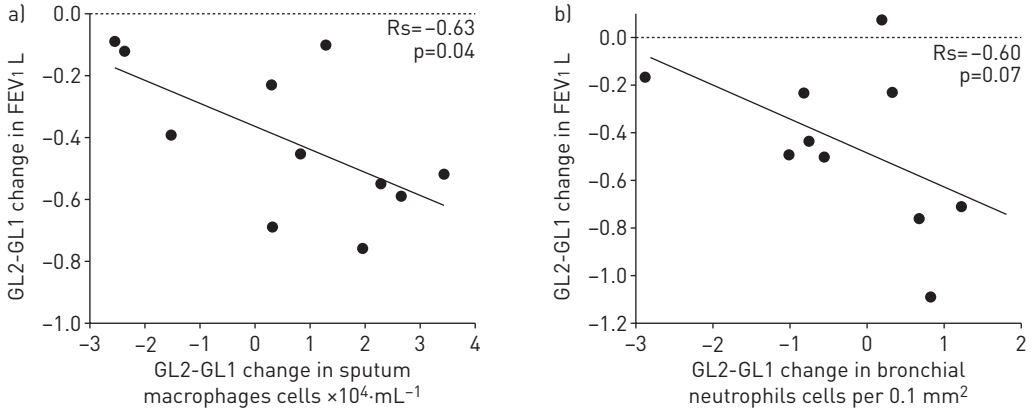

FIGURE 4 Correlation between change (end GL2 minus end GL1 (GL2-GL1)) in post-bronchodilator FEV_1 and a) change in natural log-transformed sputum macrophages (GL2-GL1, expressed as number of cells ×10^4 per mL) and b) changes in natural log-transformed bronchial neutrophils. Each dot represents a single patient. FEV_1: forced expiratory volume in 1 s; GL1: GLUCOLD-1 study, first part of the study; GL2: GLUCOLD-2 study, follow-up study.

patients (61 out of 85) discontinued their ICS or used ICS 0–50% of the time during GL2 (table 2). Adherence to medication was not checked, reflecting daily practice. This could have led to misclassification of ICS use during GL2, which could have affected our outcomes. Nevertheless, when only patients who were compliant during GL1 were included in the current analysis, similar results were found (data not shown). Owing 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 in GL2 than in GL1 [29]. We cannot rule out that these differences 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 [30]. Because 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 these considerations into account, 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-severe COPD [8]. In the current study (GL2), we observed the expected opposite effect in that ICS withdrawal after previous long-term treatment resulted in an increase in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells and mast cells in this selected group of patients, which further extends our previous observation that withdrawal of ICS causes a relapse in lung function decline [20]. 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 validation and strongly supports the plausibility of our findings. Taken together, our data suggest that the effects of ICS on lung function and inflammation are transient, without persistent disease modification.

During the past 25 years numerous studies have been published concerning the question of whether or not ICS are beneficial in COPD patients [31]. In our study, patients with moderate-severe COPD experienced transient positive effects on lung function and on airway inflammation during ICS therapy, which were not maintained after ICS withdrawal [20]. The present selection of COPD patients may represent a particular phenotype that is responsive to steroid treatment [32], 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.

In conclusion, the present data indicate that ICS discontinuation during 5 years following 30 months of ICS use in a group of patients with moderate-severe COPD induces a relapse in the production of 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 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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