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Airway mucosal inflammation in COPD is similar in smokers and ex-smokers: a pooled analysis

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ABSTRACT: **Bronchial biopsy specimens from chronic obstructive pulmonary disease (COPD) patients demonstrate increased numbers of CD8+ T-lymphocytes, macrophages and, in some studies, neutrophils and eosinophils. Smoking cessation affects the rate of forced expiratory volume in one second (FEV_1) decline in COPD, but the effect on inflammation is uncertain. Bronchial biopsy inflammatory cell counts were compared in current and ex-smokers with COPD.**

A pooled analysis of subepithelial inflammatory cell count data from three bronchial biopsy studies that included COPD patients who were either current or ex-smokers was performed.

Cell count data from 101 subjects, 65 current smokers and 36 ex-smokers, were analysed for the following cell types: CD4+ and CD8+ T-lymphocytes, CD68+ (monocytes/macrophages), neutrophil elastase+ (neutrophils), EG2+ (eosinophils), mast cell tryptase+ and cells mRNA-positive for tumour necrosis factor-α. Current smokers and ex-smokers were similar in terms of lung function, as measured by FEV_1 (% predicted), forced vital capacity (FVC) and FEV_1/FVC. The results demonstrate that there were no significant differences between smokers and ex-smokers in the numbers of any of the inflammatory cell types or markers analysed.

It is concluded that, in established chronic obstructive pulmonary disease, the bronchial mucosal inflammatory cell infiltrate is similar in ex-smokers and those that continue to smoke.

KEYWORDS: **Chronic obstructive pulmonary disease, inflammation, smoking, smoking cessation**

Smoking *per se* induces inflammation; there are effects on the bone marrow, resulting in peripheral blood leukocytosis, and, in heavy smokers, there is a shift in the balance of peripheral blood lymphocytes from CD4 (T-helper) to CD8 (T-suppressor) predominance [1]. In smokers without lung disease and those with chronic bronchitis (CB) and chronic obstructive pulmonary disease (COPD), this altered balance is also seen in bronchoalveolar lavage fluid [2], bronchial biopsy specimens taken at bronchoscopy [3–5] and airway tissue taken at resection from smokers with lung cancer [6, 7].

In studies of COPD, some protocols include current smokers only, whereas others include both current and ex-smokers. It is unclear whether, once the inflammatory condition has been established in smokers, ex-smokers return to normal after smoking cessation or persist with similar inflammation to that of current smokers. Two published cross-sectional studies indicate that, if symptoms continue once they have developed, then inflammatory changes persist even after smoking cessation. The first study, by TURATO *et al.* [8], examined bronchial biopsy specimens from subjects with the symptoms of CB. Nine current smokers and seven ex-smokers, all with CB, were compared with seven healthy nonsmoking subjects. This study included subjects with and without airflow limitation, and mean forced expiratory volume in one second (FEV_1) 77% predicted. Those with CB were found to have increased numbers of macrophages, interleukin-2-receptor-positive cells, very-late-activation-antigen-1-positive cells, intercellular-leukocyte-adhesion-molecule-1-positive vessels and E-selectin-positive vessels compared with the healthy nonsmoking controls. No significant differences were found between symptomatic

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current and ex-smokers for any cellular marker examined [8]. This study suggested that, in subjects with ongoing CB, inflammation persists regardless of smoking status.

A second study, by RUTGERS *et al.* [9], examined 18 subjects with COPD (mean FEV1 59% pred) and 11 ex-smoking subjects without lung disease. Four subjects in the COPD group had never smoked; all the remaining participants were ex-smokers, having stopped smoking $\geqslant$1 yr before the start of the study. Bronchial biopsy specimens showed that there were more EG2+ cells (eosinophils) and CD68+ cells (monocytes/macrophages) in the COPD group compared with the healthy ex-smoking controls [9]. This study confirmed that, even when smoking has stopped, subjects with COPD continue to show increased numbers of inflammatory cells compared with ex-smokers without the disease. Since bronchial biopsy specimens taken before and after treatment are now used to assess the effects of therapy in COPD [10, 11], it is particularly important to appreciate whether there are significant differences in the numbers of inflammatory cells seen in smokers and ex-smokers with COPD at baseline when treatment commences. If such differences exist, it would be inappropriate to combine current and ex-smokers in such biopsy studies of COPD. In order to answer this question, the present study compares the inflammatory infiltrate in bronchial biopsy specimens from current and ex-smokers with established stable COPD using a pooled analysis of three previously published studies in which a total of 101 patients were included [3, 10, 11].

METHODS

Baseline bronchial biopsy inflammatory cell count data from three previously published studies were used to give a stable COPD group comprising 101 patients [3, 10, 11]. All of the studies contained both smokers and ex-smokers as part of the inclusion criteria. The inclusion and exclusion criteria were similar for the three studies. Table 1 shows the demographic and lung function data of the smoking and ex-smoking groups. Of the 101 patients, 87 (86%) met UK Medical Research Council criteria for the presence of CB [12]. The subjects had moderate-to-severe COPD according to Global Initiative for Chronic Obstructive Lung Disease criteria [13]. Patients were recruited from chest clinics and by advertisement. Ethics committee approval for each study was obtained from the local research ethics committees and subjects gave written informed consent.

TABLE 1 Demographic characteristics

	Smokers	Ex-smokers
Males/females n	55/10	30/6
Age yrs	61 (40–77)	65 (49–78)
Smoking pack-yrs	46±25	59±36
Chronic bronchitis n	55	32
FEV1 L	1.55±0.45	1.58±0.49
FEV1 % pred	51±12	54±13
FVC L	3.09±0.77	3.01±0.81
FEV1/FVC %	51±11	53±10
Reversibility# %	6.7±5.9	8.1±5.6

Data are presented as median (range) or mean±SD, unless otherwise stated. FEV1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. #: salbutamol reversibility as percentage change from pre-bronchodilator FEV1.

Smoking status was recorded on inclusion into the studies and confirmed at subsequent interviews. Smoking history was quantified in pack-years and is given in table 1. In the study by HATTOTUWA *et al.* [10], exhaled carbon monoxide was measured in order to verify smoking status, and it was found to be significantly higher in current smokers ($p=0.0002$). There was confidence, therefore, that smoking status was being accurately reported. In the study by HATTOTUWA *et al.* [10], ex-smokers had stopped smoking on average 8 yrs (range 1–25 yrs) previously. Such information was not available for the other two studies.

Bronchoscopy was performed according to American Thoracic Society guidelines, as previously described elsewhere [3]. Bronchial biopsy specimens were processed and immunostained as described in each article. Briefly, in the studies by O'SHAUGHNESSY *et al.* [3] and HATTOTUWA *et al.* [10], biopsy specimens were fixed and snap-frozen. Positively stained cells within the subepithelium were counted to a depth of 100 μm below the reticular basement membrane. In the study by GAMBLE *et al.* [11], biopsy specimens were fixed and paraffin-embedded. Positively stained cells within all of the available subepithelium were counted. All biopsy processing and evaluation were performed in the same laboratory (Lung Pathology, Imperial College London, London, UK). Since there were methodological differences in biopsy processing and counting between the individual protocols, as outlined previously, absolute cell counts were not directly comparable between the studies. However, using the standardised mean differences (SMDs) in cell counts between smokers and ex-smokers from each study, it was possible to combine the data and perform a pooled analysis. Results regarding the following cells and a key marker of inflammation, which were included in all three studies, are reported in the present study: CD4+ and CD8+ T-lymphocytes, CD68+ (monocytes/macrophages) and neutrophil elastase+ (neutrophils) by immunostaining; and mRNA-positive cells for tumour necrosis factor (TNF)-α by *in situ* hybridisation. Numbers of EG2+ (eosinophils) and mast cell tryptase+ cells (mast cells), available from two of the three studies, were also analysed. The repeatability of cell counts was assessed in each study. The coefficients of variability for repeat counts for each study were 1.7–11.4% for O'SHAUGHNESSY *et al.* [3], 6.5% for HATTOTUWA *et al.* [10] and 2.7% for GAMBLE *et al.* [11].

Statistical analysis

Data from all three trials were pooled using Review Manager 4.2.8 (The Cochrane Collaboration, Oxford, UK). Outcomes of interest were continuous variables; therefore, all data were pooled using either weighted mean difference and 95% confidence interval, when outcomes were measured using the same scale (*e.g.* FEV1 % pred), or SMDs when outcomes were measured using different scales (*e.g.* CD8 count measured in cells·mm^{-1} or cells·mm^{-2}). The SMD is used as a summary statistic when all studies assess the same outcome but measure it in a variety of ways. In this circumstance, it is necessary to standardise the results of the trials to a uniform scale before they can be combined. The SMD expresses the size of the

treatment effect in each trial relative to the variability observed within that trial. Thus, studies for which the difference in means is the same proportion of the SD have the same SMD, regardless of the scales used to make the measurements. Heterogeneity among pooled estimates was tested using the method of DERSIMONIAN and LAIRD [14]. To adjust for multiple comparisons, the Bonferroni correction was applied; therefore, a p-value <0.01 was considered significant herein. Results are reported using the fixed-effect model when no heterogeneity was observed, and the random-effects model when heterogeneity was present. No heterogeneity was present for the current end-points. Further explanation of the statistical analysis is supplied in figure 1 and table 2.

RESULTS

Demographic and baseline lung function data are presented in table 1. There were no significant differences between smokers and ex-smokers in lung function, as measured by FEV_1, forced vital capacity (FVC) and FEV_1/FVC (table 3). Figure 1 shows weighted mean differences between current and ex-smokers for FEV_1 (% pred) for each individual study (table 2) and the overall mean for all three studies. There were no significant differences between current and ex-smokers for any of the cell counts or markers (CD8, CD4, CD68, neutrophil elastase, EG2, mast cells and TNF-α). Table 3 details all the outcomes measured.

Subgroup analyses were performed to compare subjects with and without CB. No differences in any cell or marker were found between subjects with and without CB in either the ex-smoking or current smoking group.

DISCUSSION

Data from three studies performed in the same laboratory were combined in a pooled analysis using primary data to investigate whether there is any difference in airway inflammation between smokers and ex-smokers with COPD. The results show no differences between current and ex-smokers in counts of CD4+ and CD8+ T-lymphocytes, neutrophils, CD68+ monocytes/macrophages, EG2+ eosinophils and mast cells and gene expression of TNF-α. In addition, no difference in cell counts between subjects with and without CB was shown. The present results concur with and further the findings of TURATO *et al.* [8], who showed no difference in inflammatory cell counts between current and ex-smokers who continue to be productive of sputum. RUTGERS *et al.* [9] showed increased eosinophil and macrophage numbers in ex-smokers with COPD compared with healthy ex-smokers, indicating continuing inflammation in the symptomatic ex-smoking group, in agreement with the present findings. PESCI *et al.* [15] reported a trend towards a reduction in the number of subepithelial mast cells in ex-smokers compared with current smokers with CB (mean FEV_1 73% pred) [15]. No difference was found in mast cell numbers between ex-smokers and continuing smokers. A recent cross-sectional study comparing bronchial inflammation in COPD between smokers and ex-smokers [16] showed that ex-smokers with COPD have higher CD3+, CD4+ and plasma cell numbers than current smokers with COPD. Furthermore, short-term ex-smokers had higher CD4+ and CD8+ cell numbers than current smokers, whereas long-term ex-smokers had lower CD8+ cell numbers and CD8/CD3 ratios and higher plasma cell numbers [16]. The present study did not show a difference in CD4+ cell numbers between ex-smokers and current smokers with COPD. Duration of smoking cessation was not analysed, but this would be an interesting focus for future work using a prospective longitudinal study.

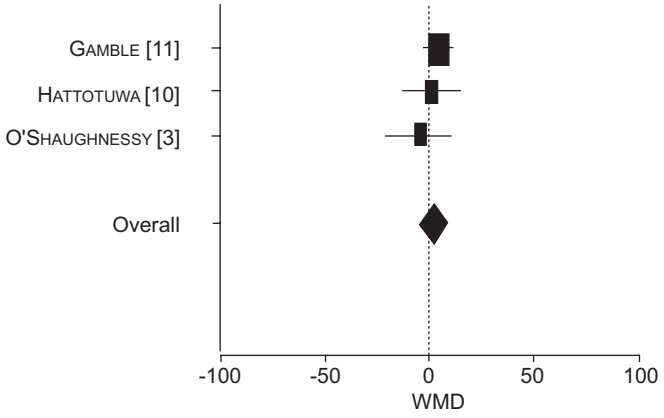

FIGURE 1. Pooled analysis of smokers *versus* ex-smokers for forced expiratory volume in one second. Data are presented as weighted mean difference (WMD) using a fixed-effect model (■ (size reflects weighting)), and 95% confidence interval (horizontal bars). ·····: line of no effect. The centre of the diamond indicates the combined mean effect of the three studies and its extremities the 95% confidence interval. If the WMD is positive, it is higher in ex-smokers and, if it is negative, it is higher in smokers. In testing for heterogeneity of the combined results that contributed towards the overall mean result, Chi-squared=1.61 (2 degrees of freedom; p=0.45) and I^2=0%. In testing for overall effect, z=0.79 (p=0.43).

TABLE 2 Forced expiratory volume in one second (FEV_1) in chronic obstructive pulmonary disease patients from the trials used in the pooled analysis and weighting of trials

First author [Ref.]	Ex-smokers		Smokers		Weight %
	Subjects n	FEV_1% pred	Subjects n	FEV_1 % pred	
GAMBLE [11]	21	57.54±11.18	30	53.65±9.87	64.84
HATTOTUWA [10]	10	46.60±15.56	27	45.89±12.44	19.92
O'SHAUGHNESSY [3]	5	56.76±12.19	8	61.52±8.68	15.24

Data are presented as mean±SD, unless otherwise stated. The percentage weighting is based on the precision and sample size of the mean estimate of each trial. % pred: % predicted.

TABLE 3 Details of outcomes measured in the three studies presented in the pooled analysis of smokers *versus* ex-smokers

	Subjects n	Mean difference (95% CI)	p-value#
FEV_1 pred %¶	101	1.94 (-4.35–8.32)+	0.43
FVC¶ L	101	-0.18 (-0.50–0.14)	0.26
FEV_1/FVC¶ %	101	0.02 (-0.20–0.06)	0.31
CD8 count§	101	-0.18 (-0.50–0.14)	0.26
CD68 count§	101	0.02 (-0.02–0.06)	0.31
Neutrophil count§	101	0.13 (-0.28–0.54)	0.54
CD4 count§	100	-0.25 (-0.67–0.16)	0.23
TNF-α count§	101	0.03 (-0.38–0.45)	0.88
EG2 count§	45	0.38 (-0.04–0.80)	0.07
Mast cell count§	50	0.38 (-0.05–0.81)	0.09

Cell counts were expressed in cells·mm^{-2} in the study by GAMBLE *et al.* [11] and in cells·mm^{-1} in the other two studies [3, 10]. CI: confidence interval; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; TNF: tumour necrosis factor. #: smokers *versus* ex-smokers; ¶: weighted mean difference; +: 99% CI; §: standardised mean difference.

Smoking cessation has previously been shown to improve survival [17] and reduce hospital admissions [18] and the rate of FEV_1 decline [19, 20], at least in mild-to-moderate COPD. Since airway inflammation has been shown to be related to lung function in COPD [3, 21], an improvement in inflammation might be expected after smoking cessation. No reduction in airway inflammation was found in the present population of ex-smokers compared with current smokers with similar lung function. The degree of benefit derived from smoking cessation may be determined by the severity of the disease at the point of smoking cessation. Therefore, it is suggested that, in established COPD, inflammation is determined by disease severity rather than by smoking status. In support of these proposals, persistence of inflammation in bronchial biopsy specimens and sputum has been shown in 12 subjects with COPD 1 yr after smoking cessation by WILLEMSE *et al.* [22]. In contrast, inflammation was reduced in asymptomatic smokers 1 yr after smoking cessation. The Lung Health Study [20] showed that the degree of improvement in FEV_1 after smoking cessation diminished as lung function declined. Thus, it might be informative to examine the effect of smoking cessation on airway inflammation in subjects with milder disease. A prospective longitudinal study is required in order to further examine the effects of smoking cessation on inflammation, FEV_1 decline, exacerbations and survival.

Given the apparent dissociation between the beneficial effects of smoking cessation on FEV_1 decline [19, 20], exacerbations [18] and survival [17] and the apparent lack of effect on inflammation, an alternative explanation for the present findings could be that bronchial mucosal inflammation does not play a role in the development of COPD. However, previous cross-sectional studies have shown a correlation between airflow obstruction and airway and parenchymal CD8 T-lymphocyte counts [3, 7, 21], thus linking inflammation with disease severity. It is possible that smoking cessation may affect markers other than those examined in the present study, *e.g.* plasma cells, interleukin-8 or structural elements, and this is an area for future work. Although it has been shown that smokers and ex-smokers with established COPD exhibit similar levels of bronchial inflammation, a longitudinal study is required in order to definitively show the effects of smoking cessation on bronchial inflammation.

In any study of biopsy cell counts, it is important to consider the biological variability of such a signal and to ensure that the repeatability of cell counts is satisfactory. If noise from poorly repeatable measurements is present, it may prevent a signal, such as a difference in a cell count between study groups, from being detected. The repeatability of cell counts within the three pooled studies forming the present analysis was satisfactory. There is marked biological variability in inflammatory cell counts in subjects with COPD, and sampling strategies can affect the sample size required [23]. The sample size of the present analysis is large compared with those of previous similar studies [8, 9], and the present authors believe that it is of sufficient power to show a difference in all the cell types analysed in the present study, with the exception of neutrophils, which show greater variability than other cell types [23].

When planning a hypothesis-testing study that involves bronchial biopsies in chronic obstructive pulmonary disease, it is important to know whether mucosal inflammation is affected by smoking status. The present results support the inclusion of both current and ex-smokers with chronic obstructive pulmonary disease in a study group for the quantification of the immunomarkers CD8, CD68, CD4, EG2+ (eosinophils), mast cells and tumour necrosis factor-α gene expression. The present authors suggest that the characteristic inflammatory changes, although initially induced by smoking, are fundamental to the disease process rather than to smoking *per se*. It is necessary to understand more about the basic mechanisms of cigarette-smoke-induced inflammation, how they are triggered and how they might be switched off. It is concluded that bronchial biopsy specimens from patients with chronic obstructive pulmonary disease who are ex-smokers show persisting mucosal inflammation similar to that seen in continuing smokers.

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