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# Clinical characteristics and airway inflammation profile of COPD persistent sputum producers

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**KEYWORDS**
COPD;
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**Summary**
*Background:* COPD patients with chronic bronchitis include a subgroup with persistent sputum production on most or every day. We hypothesized that COPD patients with persistent sputum production have a different profile of airway inflammation, and more severe clinical characteristics.
*Objective:* To compare the airway inflammation profile and clinical characteristics of COPD persistent and non-persistent sputum producers.
*Methods:* COPD persistent sputum producers ($n = 26$) and non-persistent sputum producers ($n = 26$) underwent sputum induction and pulmonary function tests. Exacerbation history was recorded; the St. George's Respiratory Questionnaire, Modified Medical Research Council Dyspnoea scale and COPD Assessment Tool were completed. 33 COPD patients provided sputum for bacteriology.
*Results:* Persistent sputum producers had lower post-bronchodilator $FEV_1$% predicted ($p = 0.01$), diffusion capacity ($p = 0.04$), 6 min walk test distance ($p = 0.05$), and higher closing volume ($p = 0.01$), BODE index ($p = 0.01$), rate of bacterial colonization ($p = 0.004$) and exacerbations ($p = 0.03$) compared to non-persistent sputum producers. The mean SGRQ and CAT scores were higher in persistent sputum producers ($p = 0.01$ and 0.03 respectively). Sputum neutrophil and eosinophil total cell counts were higher in persistent sputum producers ($p = 0.02$ and 0.05 respectively). Sputum levels of eotaxin ($p = 0.02$), MCP-1 ($p = 0.02$), TNF-$\alpha$ ($p = 0.03$) and IL-6 ($p = 0.05$) were higher in persistent sputum producers.

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*Conclusion:* COPD persistent sputum producers have more severe clinical characteristics and increased concentrations of some inflammatory mediators in the airways.


## Introduction

The hallmark features of COPD are poorly reversible airflow obstruction and persistent pulmonary inflammation [1]. It is recognized that disease phenotypes exist, comprising subgroups of patients with distinct clinical or pathological characteristics associated with different prognosis or response to treatment [2].

Chronic bronchitis is defined as a productive cough for $\geq$3 months for $\geq$2 consecutive years. There is evidence that chronic bronchitis is associated with more severe airflow obstruction and breathlessness [3,4], an excessive decline in $FEV_1$ [5], and higher exacerbation [6—8] and mortality rates [9—11]. However, recent large observational studies have not consistently reproduced these findings; The COPD Gene Cohort study showed that chronic bronchitis is associated with more exacerbations but not with other clinical characteristics [12], while the ECLIPSE study only showed that COPD patients with chronic bronchitis had worse general health status [13]. These different outcomes may be due to the broad range of symptom severity covered by the definition of chronic bronchitis; Some COPD patients fall into the category of 'chronic bronchitis' due to a productive cough for a few months, while others have a persistent productive cough for most or every day of the year. The inclusion of patients with mild chronic bronchitis in clinical studies, as opposed to those with severe and persistent sputum production, is likely to reduce the chance of observing positive findings.

Mucins are glycosylated proteins secreted by the airway epithelia that form gels that are key components of mucus in the lungs [14]. MUC5AC and MUC5B are the major mucin subtypes expressed in the lungs, with epithelial MUC5AC expression upregulated in COPD patients [15,16]. A number of cytokines upregulate mucin expression [17], and may cause mucus hypersecretion in COPD. Previous studies evaluating cytokine levels in COPD patients with chronic bronchitis have used healthy control groups [18—20]. However, the optimal study design to investigate cytokines associated with the presence of chronic bronchitis in COPD is to compare COPD patients with and without chronic bronchitis. This approach has been used for airway inflammatory cells; eosinophil numbers were increased in induced sputum and decreased in the bronchial submucosa of COPD patients with chronic bronchitis [21], suggesting that eosinophil chemotaxis is altered.

The primary aim of this study was to investigate clinical characteristics and sputum inflammatory biomarkers in COPD patients with chronic bronchitis. The novel aspects were to focus on COPD patients with symptoms of persistent sputum production, in order to maximize the chance of finding differences between groups, and to use a control group of COPD patients without chronic bronchitis, in contrast to previous studies of sputum cytokines that have used healthy controls [19,20,22]. We processed sputum using a 'two-step' procedure [23], using phosphate buffered saline (PBS) processing first to obtain supernatant followed by dithiothreitol (DTT) to obtain cells. This avoids any effect of DTT on cytokine analysis from sputum supernatant. A secondary aim of this study was to compare results from induced and spontaneous sputum samples from persistent sputum producers, in order to determine which type of sample provides better sample quality and reproducibility in this patient group.

## Methods

### Subjects

COPD patients, diagnosed according to current criteria [24], were recruited from the clinical research database of the Medicines Evaluation Unit, University Hospital of South Manchester Foundation Trust. Patients were excluded if they had experienced a respiratory tract infection or exacerbation of COPD in the preceeding 6 weeks. Fifty two COPD patients participated in the main study where clinical characteristics and sputum cytokine levels were assessed. Thirty three COPD patients, including 22 from the main study, participated in a follow-on study where bacterial colonization in sputum was assessed. All patients provided written informed consent, and the local ethics committee approved the study.

Patients were identified as persistent sputum producers using the validated American Thoracic Society questionnaire [25]; Patients bringing up phlegm at least twice a day for four or more days of the week were categorized as persistent sputum producers. Non-persistent sputum producers were defined as patients who did not produce phlegm except during an exacerbation.

### Study design

Measurements of pulmonary function including spirometry, lung volumes, gas transfer (KCO), single breath nitrogen washout were performed, and six minute walk test (6 MWT) and sputum induction. Full details of the methods are in the online data supplement. Exacerbation history using patient recall verified by primary care records in the previous 12 months was recorded. An exacerbation was defined as an acute change in dyspnoea, cough or sputum requiring treatment with antibiotics, oral corticosteroids or both. St. George's Respiratory Questionnaire (SGRQ), Modified Medical Research Council dyspnoea scale (MMRC) and COPD Assessment Tool (CAT) were completed, and the BODE index calculated [26]. High resolution CT scan (HRCT) (GE Medical Systems, Light Speed L52002) had been performed on 26 patients in the previous year; we did not specifically

perform CT scans in this study. The presence and severity of bronchiectasis was based on radiologist assessment. To assess sputum repeatability, 12 patients from the persistent sputum producer group provided a repeat sputum sample after 2 months.

We conducted a further study one year after this main study, where sputum samples were analysed for bacterial colonization in persistent sputum producers compared to non-persistent sputum producers.

### Sputum collection and processing

Patients were asked to blow their nose and rinse their mouth with water prior to providing a spontaneous sputum sample. Spontaneous sputum was collected over a maximum of 30 s. Sputum induction was performed approximately 2 h later using normal saline as previously described [27].

Sputum plugs were selected to separate sputum from saliva in both spontaneous and induced samples, and then processed using a 2 step procedure [23], using PBS processing first to obtain supernatant followed by DTT to obtain cells. Full details are in the online data supplement.

### Supernatant analysis

Cytokines and chemokine levels in sputum supernatants were assayed using a Meso Scale Discovery® (MSD, Gaithersburg—USA) multiplex platform at GSK (Stevenage, UK). The limits of detection (LOD) are stated in the online data supplement.

### Quantitative sputum culture

Sputum culture was conducted and potential pathogenic species identified using standardized methods [28]. Full details of the method are in the online data supplement.

### Statistical analyses

The power of the study was 80%; at least 16 subjects per group were required to detect a neutrophil percentage difference of 15%, based on a within subject standard deviation of 14.4% [29]. Unpaired *t*-tests were performed for parametric data (spirometry, body plethysmography, gas transfer, nitrogen washout, SGRQ and CAT scores and non-eosinophil related sputum data). Mann—Whitney *U* tests were performed on non-parametric data (MMRC, exacerbation history and sputum eosinophil data). Ordinal regression was performed to determine whether sputum producer status was associated with $FEV_1$% predicted and SGRQ total score (independent of age, gender and current smoking status).

Sputum cytokine data were non-parametric, and are presented as medians and analysed using Mann—Whitney *U* tests. Inflammatory mediators could be normalized following logarithmic transformation and were analysed using unpaired *t*-tests. Sputum cytokines levels registering below the LOD were assigned a value of 'zero' for the purpose of statistical analysis. The statistical significance of differences observed in differential cell count data obtained from unmatched spontaneous and induced sputum samples (persistent sputum producers) was assessed using unpaired *t*-tests. Fisher's exact test was used to assess the statistical significance of differences in bacterial colonization rates between groups. Differences in sputum cell counts and protein measurements between individuals with post-bronchodilator $FEV_1 > 50\%$ predicted (GOLD I + II) and $FEV_1 < 50\%$ (GOLD III) predicted were determined using either unpaired *t*-tests or Mann—Whitney *U* tests. Univariate correlations of sputum cell counts and protein measurements were performed using the Spearman Rank test; this analysis involved multiple simultaneous comparisons therefore a $p$ value of $<0.01$ was considered as being significant. Repeatability was assessed by paired *t*-tests, intra-subject standard deviation (SD) and intraclass correlation coefficient (Ri). Statistical analyses were performed using PRISM version 5 (GraphPad Software, San Diego, California, USA) or SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.)

## Results

### Clinical characteristics

Table 1 shows that the main study consisted of 26 persistent sputum producers and 26 non-producers, with the majority of patients being GOLD II. No patients had $FEV_1$ reversibility >12%. Some KCO% predicted values were >100% in patients with higher BMI (this has been previously reported) [30,31]. Univariate analysis showed that persistent sputum producers had significantly lower $FEV_1$% predicted ($p = 0.01$) and KCO% predicted ($p = 0.04$), and a higher closing volume ($p = 0.01$) compared to sputum non-producers. Persistent sputum producers also had numerically higher RV, but the difference between groups did not reach statistical significance ($p = 0.13$). Persistent sputum producers had a reduced 6 min walk test distance ($p = 0.05$). Persistent sputum producers had significantly higher SGRQ score ($p = 0.01$), CAT score ($p = 0.03$) and a trend towards significance for MMRC score ($p = 0.07$). The number of exacerbations in the previous year was higher ($p = 0.03$), and the BODE index score was worse in persistent sputum producers ($p = 0.01$).

Ordinal regression showed that persistent sputum production was a significant predictor of lower $FEV_1$% predicted ($p = 0.001$) and higher SGRQ total score ($p = 0.01$), independent of age, gender and current smoking status; see e-Table 1 in online data supplement. High resolution CT scan on 16 persistent sputum producers and 10 non-producers identified only 2 persistent sputum producers with minor secondary bronchiectasis.

### Sputum cell counts

All the persistent sputum producers provided a spontaneous and an induced sputum sample, although induced samples from 2 patients were unsuitable for analysis due to a high proportion of non-viable cells. Induced sputum was obtained in 22/26 non-producers. The total cell count (TCC) in induced samples was increased in persistent sputum

**Table 1** Demographic and clinical details.

| | Persistent sputum producer ($n = 26$) | Non-persistent sputum producer ($n = 26$) | $p$ value |
|---|---|---|---|
| Age (Years) | 65.7 (±6.91) | 66.8 (±6.46) | 0.56 |
| Female gender ($n$) | 14 | 12 | 0.58 |
| Smoking history (Pack years)[#] | 35.30 (12.5–86) | 32.00 (18.50–122.2) | 0.49 |
| Current smokers ($n$) | 15 | 12 | 0.41 |
| BMI (kg/m$^2$) | 28.50 (±4.30) | 27.56 (±5.33) | 0.26 |
| GOLD I ($n$) | 1 | 4 | |
| GOLD II ($n$) | 16 | 17 | |
| GOLD III ($n$) | 9 | 5 | |
| GOLD IV ($n$) | 0 | 0 | |
| SABA ($n$) | 23 | 24 | 0.64 |
| SAMA ($n$) | 2 | 3 | 0.64 |
| LABA ($n$) | 17 | 20 | 0.36 |
| LAMA ($n$) | 12 | 11 | 0.78 |
| ICS ($n$) | 17 | 20 | 0.36 |
| Post bronchodilator $FEV_1$ (% predicted) | 54.5 (±13.09) | 65.1 (±16.25) | 0.01* |
| Post bronchodilator $FEV_1/FVC$ (% predicted) | 46.9 (±6.61) | 50.1 (±4.43) | 0.12 |
| TLC (% Predicted) | 105.7 (±16.75) | 103.2 (±22.17) | 0.55 |
| RV (L) | 3.3 (±1.13) | 2.6 (±0.88) | 0.13 |
| IC (L) | 2.0 (±0.66) | 2.2 (±0.18) | 0.26 |
| IC/TLC | 0.3 (±0.09) | 0.4 (±0.09) | 0.18 |
| KCO (% Predicted) | 78.2 (±20.26) | 93.5 (±21.73) | 0.04* |
| Closing volume (L) | 1.0 (±0.41) | 0.7 (±0.36) | 0.01* |
| N2 Δ/L | 6.5 (±2.54) | 7.1 (±2.82) | 0.77 |
| 6 MWT (m) | 350 (±85.40) | 395.4 (±73.54) | 0.05* |
| BODE Index | 2.4 (±1.77) | 1.3 (±1.44) | 0.01* |
| SGRQ score (Total score/100) | 44.2 (±20.34) | 29.4 (±17.21) | 0.01* |
| SGRQ symptom score (Score/100) | 52.9 (±20.42) | 31.7 (±22.89) | 0.002* |
| SGRQ impact score (Score/100) | 56.3 (±27.70) | 43.5 (±26.49) | 0.01* |
| SGRQ activity score (Score/100) | 34.5 (±19.66) | 21 (±15.54) | 0.07* |
| MMRC score (0–4)[#] | 1.5 (1.0–4.0) | 1 (0–3.0) | 0.07 |
| CAT score (Score/40) | 20 (±07.95) | 14 (±06.53) | 0.03* |
| Exacerbations in last 12 months | 1 (0–4.0) | 0 (0–2.0) | 0.03* |

Data presented as mean (± standard deviation). [#] Data represented as median (range). For continuous data, the statistical significance of differences observed between groups was determined using $t$ tests. For categorical data, the statistical significance of differences observed between groups was determined using the $\chi^2$ test. Abbreviations – SABA: short acting β agonist; SAMA: short acting muscarinic antagonist; LABA: long acting β agonist; LAMA: long acting muscarinic antagonist: ICS: inhaled corticosteroid; TLC: total lung capacity; RV: residual volume; IC: inspiratory capacity; KCO: diffusion capacity; N2 Δ/L: slope of nitrogen washout curve; 6 MWT (m): six minute walk test distance (in meters); SGRQ: St George's Respiratory Questionnaire; MMRC: Modified Medical Research Council Dyspnoea score; CAT: COPD assessment tool; BMI: body mass index; BODE: BMI/obstruction ($FEV_1$% predicted)/Dyspnoea (MMRC score)/Exercise capacity (6 MWT distance), *shows $p$ value $\leq 0.05$.

producers, although this did not reach statistical significance ($p = 0.09$), while the total neutrophil and eosinophil counts were significantly increased in persistent sputum producers; see Table 2. The cell differential percentage counts were similar between groups, and the proportion of patients with eosinophils >3% were similar ($p = 0.86$).

The mean viability of all spontaneous sputum samples from persistent sputum producers was reduced and the squamous cell percentage was higher compared to induced samples; see Table 3. There were no other differences between spontaneous and induced samples. Further analysis of paired spontaneous and induced samples is shown in the online supplement (e-Table 2).

## Supernatant cytokines

Persistent sputum producers had significantly higher levels of eotaxin ($p = 0.02$), MCP-1 ($p = 0.02$), TNF-α ($p = 0.03$) and IL-6 ($p = 0.05$), with a trend towards significance for MCP-4 and IL-13 (both $p = 0.06$) compared to non-persistent sputum producers (Fig. 1). There were increased GM-CSF levels in persistent sputum producers ($p = 0.01$), but the levels were low with many samples below the limit of detection (see e-Fig. 1 in the online data supplement). There were no differences ($p > 0.05$) between groups for other inflammatory mediators; see e-Table 3 in the online data supplement. There were no

**Table 2** Induced sputum cell counts in COPD persistent sputum producers & non-producers.

| | Persistent sputum producer ($n = 24$) | Non persistent sputum producer ($n = 22$) | $p$ value |
|---|---|---|---|
| Total cell count ($10^6$) | 3.5 (±3.49) | 2.2 (±3.67) | 0.09 |
| Leucocyte viability % | 74 (±26.13) | 71.1 (±23.22) | 0.66 |
| Squamous cell % | 1.6 (±2.24) | 1.1 (±2.45) | 0.34 |
| Neutrophil % | 73 (±20.75) | 60.9 (±34.54) | 0.61 |
| Macrophage % | 17.5 (±13.09) | 14.4 (±14.83) | 0.11 |
| Eosinophil % | 1.6 (0.50–3.69) | 1 (0.19–2.31) | 0.20 |
| Neutrophil cell count ($10^6$) | 2.9 (±3.32) | 1.6 (±3.98) | 0.02* |
| Macrophage cell count ($10^6$) | 0.7 (±0.42) | 0.3 (±0.55) | 0.24 |
| Eosinophil cell count ($10^6$) | 0.1 (0.03–0.11) | 0.02 (0.01–0.06) | 0.05* |

$n = 24$ in persistent sputum producer group as 2 patients gave induced sputum samples that were non-viable; $n = 22$ in the non-persistent sputum producer group as 4 patients did not produce sputum on induction. Data represented as mean (±SD). Eosinophil % and cell count expressed as median (range). Abbreviation – TCC: total cell count.
* Shows $p$ value ≤0.05.

associations between supernatant proteins and cell counts (data not shown).

We analysed these data according to GOLD stage determined by the degree of airflow obstruction; there were no differences between patients with mild to moderate airflow obstruction (GOLD I/II) versus severe airflow obstruction (GOLD III) for supernatant cytokines; see e-Table 4 in online data supplement. However, sputum total cell counts, neutrophil and eosinophil cell counts and neutrophil percentage were increased in GOLD stage III patients.

## Sputum culture

The demographics of patients who provided samples for sputum culture are shown in Table 4. Bacteria were detected at a significantly higher rate ($p = 0.004$) in persistent sputum producers (12 out of 20 patients; 60%) compared to non-persistent sputum producers (1 out of 13; 7.7%). The most frequent micro-organisms isolated in the persistent producer group were Haemophilus influenzae [$n = 7$; mean count: 1.96 (SD 2.36) × $10^8$ CFU/g] and Streptococcus pneumoniae [$n = 3$; mean count: 4.39 (SD 4.91) × $10^8$ CFU/g]. One sputum sample was positive for bacteria in non-persistent sputum-producers (*Haemophilus influenzae*). The individual sputum bacterial cell counts are shown in e-Table 5 in the online data supplement.

## Sputum repeatability

The repeatability of samples from 12 persistent sputum producers is shown in Table 5. The mean values of repeated samples were not different ($p > 0.05$) for induced or spontaneous sputum cell counts. There was much better agreement between visits for induced sputum, with Ri values indicating moderate to good agreement, compared to spontaneous sputum where Ri values were generally 0.3 or less, indicating slight or poor agreement. The neutrophil percentage within subject SD for induced samples was 13%.

## Discussion

We have demonstrated that COPD patients with persistent sputum production have worse airflow obstruction, reduced exercise capacity, greater symptoms and more

**Table 3** Induced and spontaneous sputum cell counts in COPD persistent sputum producers.

| | Induced sample ($n = 24$) | Spontaneous sample ($n = 18$) | $p$ value |
|---|---|---|---|
| Total cell count ($10^6$) | 3.5 (±3.49) | 4.5 (±10.2) | 0.65 |
| Leucocyte viability % | 74 (±26.13) | 51.2 (±14.5) | <0.0001* |
| Squamous cell % | 1.6 (±2.24) | 3.7 (±4.9) | 0.06 |
| Neutrophil % | 73 (±20.75) | 68.1 (±18.1) | 0.31 |
| Macrophage % | 17.5 (±13.09) | 23.7 (±15.8) | 0.25 |
| Eosinophil % | 1.6 (0.50–3.69) | 1.3 (0–31) | 0.42 |
| Neutrophil cell count ($10^6$) | 2.9 (±3.32) | 3.6 (±9.4) | 0.24 |
| Macrophage cell count ($10^6$) | 0.7 (±0.42) | 1.8 (±5.2) | 0.76 |
| Eosinophil cell count ($10^6$) | 0.1 (0.03–0.11) | 0.02 (0–0.5) | 0.29 |

$n = 24$ in persistent sputum producer (induced) group as 2 patients gave induced sputum samples that were non-viable; $n = 18$ in persistent sputum producer (spontaneous) group as 8 slides in this group were not countable due to high number of squamous cells (>10%); Data represented as mean (±SD). Eosinophil % and cell count expressed as median (range). Abbreviation - TCC: total cell count.
* Shows $p$ value ≤0.05.

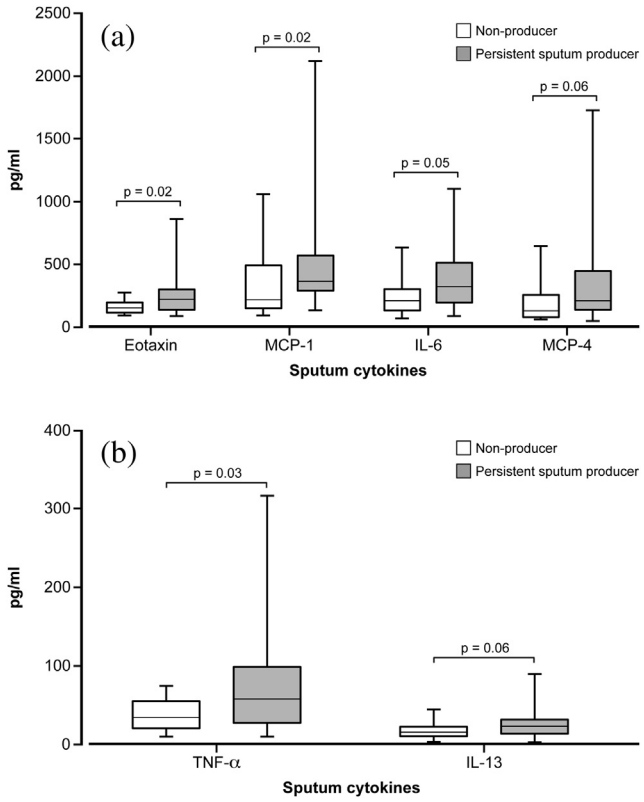


**Figure 1** Graphical representation of difference in (a) sputum eotaxin, MCP-1, IL-6 and MCP-4 and (b) sputum TNF-α and IL-13 levels, in COPD non-producers and persistent sputum producers. Boxes represent inter-quartile range and the horizontal line is the median.

exacerbations. Furthermore, COPD persistent sputum producers had increased concentrations of certain inflammatory mediators also present in COPD sputum non-producers; eotaxin, IL-6, MCP-1, and TNF-α levels in induced sputum samples were increased. IL-6, MCP-1 and TNF-α are known to upregulate mucin gene transcription [32—34], and may therefore drive mucus hypersecretion. Eotaxin is an eosinophil chemoattractant [35], and we also found increased sputum eosinophil total cell numbers in persistent sputum producers.

Previous studies have shown significantly higher levels of some cytokines in the airways of subjects with chronic bronchitis compared to healthy controls [18—20]. We have now elucidated cytokines that are specifically associated with the presence of chronic bronchitis in COPD patients, as our control group comprised COPD patients without chronic bronchitis. Persistent sputum producers had significantly elevated levels of eotaxin, IL-6, MCP-1 and TNF-α compared to non-producers, with a trend towards significance for MCP-4 and IL-13. These inflammatory mediators all have biological roles that may be of importance in COPD and mucus hypersecretion; TNF-α is a potent neutrophil stimulant [36], while IL-6, IL-13, MCP-1 and TNF-α all induce mucin gene upregulation, [17,37]. Eotaxin and MCP-4 are eosinophil chemo-attractants [35] that may play an important role in causing increased eosinophil numbers in induced sputum of persistent sputum producers.

It is known that a subgroup of COPD patients have increased numbers of eosinophils in induced sputum [38]. Furthermore, a subset of patients with chronic bronchitis also have increased eosinophil numbers in induced sputum [39], and eosinophils are preferentially distributed towards the airway lumen in COPD patients with chronic bronchitis [21]. Eotaxin levels are increased in exacerbations of chronic bronchitis [40]; our findings further support a potential role for eosinophils in the pathophysiology of chronic bronchitis. Eosinophils may contribute to mucus hypersecretion through the action of TGF-α [41], or by releasing mediators that stimulate degranulation of mucus-producing cells [42].

There was evidence of increased neutrophil and eosinophil cell counts on patients with more severe airflow obstruction (GOLD stage III). This is compatible with the increase in the number of inflammatory cells in the airways

**Table 4** Demographics of patients undergoing bacteriology.

| | Persistent sputum producer ($n$ = 20) | Non-persistent sputum producer ($n$ = 13) |
|---|---|---|
| Age (Years) | 66.0 (±6.76) | 68.7 (±4.70) |
| Sex (M:F) | 11/8 | 7/6 |
| Smoking history (Pack years)* | 39.2 (14—83) | 47.5 (21.50—119.18) |
| Current smokers ($n$) | 12/8 | 5/8 |
| BMI (kg/m$^2$) | 26.4 (±4.36) | 28.5 (±4.04) |
| GOLD I ($n$) | 1 | 3 |
| GOLD II ($n$) | 12 | 7 |
| GOLD III ($n$) | 7 | 3 |
| GOLD IV ($n$) | 0 | 0 |
| SABA ($n$) | 19 | 13 |
| SAMA ($n$) | 3 | 2 |
| LABA ($n$) | 11 | 9 |
| LAMA ($n$) | 9 | 7 |
| ICS ($n$) | 11 | 9 |
| Post bronchodilator $FEV_1$ % predicted | 59 (±15.06) | 71.5 (±8.85) |

Data presented as mean (±standard deviation). * Data represented as median (range). Abbreviation - SABA: short acting β agonist; SAMA: short acting muscarinic antagonist; LABA: long acting β agonist; LAMA: long acting muscarinic antagonist; ICS: inhaled corticosteroid.

**Table 5** Visit 1 (baseline) and Visit 2 (8 week) induced and spontaneous sputum cell counts.

| | Visit 1 (baseline) ($n = 12$) | Visit 2 (8 weeks) ($n = 12$) | $p$ value | Ri ($p$ value) |
|---|---|---|---|---|
| Induced sputum indices | | | | |
| Total cell count, ($10^6$) | 3.5 (±3.11) | 4.4 (±2.82) | 0.34 | 0.6 (0.008)* |
| Neutrophil % | 73.4 (±20.71) | 76.1 (±21.67) | 0.32 | 0.6 (0.003)* |
| Macrophage % | 11.7 (±22.64) | 13.3 (±19.84) | 0.68 | 0.6 (0.004)* |
| Eosinophil % | 1.7 (0.54–2.94) | 1.7 (0.59–2.98) | 0.12 | 0.7 (0.001)* |
| Neutrophil cell count, ($10^6$) | 2.1 (±3.96) | 2.3 (±4.14) | 0.54 | 0.8 (0.003)* |
| Macrophage cell count, ($10^6$) | 0.3 (±0.39) | 0.4 (±0.38) | 0.25 | 0.7 (0.022)* |
| Eosinophil cell count, ($10^6$) | 0.08 (0.05–0.12) | 0.09 (0.07–0.15) | 0.34 | 0.7 (0.026)* |
| Spontaneous sputum indices | | | | |
| Total cell count, ($10^6$) | 3.3 (±2.89) | 2.5 (±3.61) | 0.56 | 0.4 (0.267) |
| Neutrophil % | 68.7 (±17.94) | 78.4 (±15.69) | 0.60 | 0.1 (0.367) |
| Macrophage % | 20 (±14.44) | 12.3 (±12.97) | 0.56 | 0.3 (0.146) |
| Eosinophil % | 1.8 (0.61–2.92) | 1.0 (0.45–1.79) | 0.62 | −0.3 (0.513) |
| Neutrophil cell count, ($10^6$) | 2.6 (±3.77) | 3.2 (±3.21) | 0.87 | 0.3 (0.156) |
| Macrophage cell count, ($10^6$) | 0.3 (±0.27) | 0.5 (±0.45) | 0.38 | 0.3 (0.180) |
| Eosinophil cell count, ($10^6$) | 0.06 (0.02–0.10) | 0.01 (0.00–0.04) | 0.32 | −0.1 (0.515) |

Data represented as mean (±SD). Eosinophil % and cell count expressed as median (range). Abbreviation - TCC: total cell count. * Shows $p$ value $\leq 0.05$. Ri and $p$ value denote intraclass correlation coefficient and its statistical significance.

with more severe disease [1]. There were no differences for any of the supernatant proteins measured, which is perhaps surprising given the difference for inflammatory cells. This may be due to insufficient sample size of severe COPD patients ($n = 13$) for this subanalysis.

Persistent sputum producers had higher total sputum neutrophil and eosinophil counts, but there were no differences in differential percentages between groups. This suggests increased influx of both of these cell types into the airways of persistent sputum producers, rather than selective recruitment of one cell type. An increase in total cell number, despite no change in percentage, is likely to be of clinical relevance as it indicates an increase in the overall burden of inflammatory cells within the airways. Sputum neutrophil percentages in COPD show only very weak correlations with clinical characteristics [29]. We show that the neutrophil percentage is not changed in COPD persistent sputum producers, but that these patients display an increased overall total burden of neutrophils (and eosinophils) in the airways.

Our results agree with previous data showing that induced sputum has a higher number of viable cells and less squamous cells compared to spontaneous sputum [43]. The reduced viability of sputum samples can create practical difficulties in cell identification and supernatant protein measurements [44]. The reduced quality of spontaneous samples was associated with worse reproducibility compared to induced sputum. This has important implications for sequential sputum sampling, such as during clinical trials, as more variable methods have less statistical power. However, such variability can be reduced by using mean values from samples collected on different days; this has been shown to improve variability from spontaneous sputum samples [45].

Mucus hypersecretion can cause plugging of the small airways, predisposing to distal airway collapse and air trapping. Persistent sputum producers had an increased closing volume, possibly due to small airway mucus plugging, and a trend towards increased residual volume, suggestive of hyperinflation. KCO, an indicator of emphysema, was reduced in persistent sputum producers. It is possible that persistent sputum producers had two causes of hyperinflation; emphysema due to alveolar destruction, and small airways mucus plugging. CT scanning to quantify emphysema would have been a valuable tool to validate this observation.

It has previously been reported that mucus hypersecretion in COPD patients is associated with increased exacerbation rates [3,6–8], and we also observed this finding. This may be due to increased colonization of pathogenic bacteria in patients with mucus hypersecretion as observed in this study, or mucus impaction in the small airways leading to worsening symptoms compatible with an exacerbation. Prospective collection is the preferred method to evaluate exacerbation rates; we used retrospective history, which is known to be a good predictor of future exacerbation rates [46].

COPD persistent sputum producers had evidence of more severe clinical characteristics compared to non-sputum producers, including reduced quality of life using the SGRQ and CAT scores, worse airflow obstruction, reduced exercise capacity and a worse prognostic BODE score. The association between sputum production and both airflow obstruction and SGRQ scores were confirmed by ordinal regression. However, this analysis does not tell us whether persistent sputum production is the cause of worse clinical characteristics, or whether it arises as a consequence of progression to severe disease. Furthermore, it is possible that some of the effects of these markers of COPD severity are mediated through exacerbations. However, it seems plausible that persistent mucus hypersecretion *per se* affects health status.

The proportion of persistent sputum producers with coexistent bronchiectasis was low, and so was not associated with persistent sputum production. Bronchiectasis rates in COPD have varied greatly between studies with some

publications observing rates of 29—57% [47—49]. However, the low rate reported here is compatible with observations from the ECLIPSE study where 4% of COPD patients had bronchiectasis [13]. This wide variation between studies is difficult to explain, and may be due to differences in inclusion criteria for patient recruitment, geographical variation or methodology for assessment. Our sample size of HRCT scans was limited, and hence we are not able to make robust deductions about the associations between persistent sputum production and the presence of bronchiectasis.

The study was powered on sputum cell counts, and the sample size is similar to other induced sputum studies in COPD patients investigating inflammatory cell counts and cytokines [45,50,51]. The significant findings in sputum reported here are biologically plausible or have strong support from the literature [32—35]. Further studies of the function of the inflammatory mediators increased in persistent sputum producers are warranted. The sample size for assessing clinical characteristics was small, and it would be of value for the sputum and clinical findings here to be validated in a larger population. Our supernatant protein findings could also be further validated by studies using other techniques on sputum samples, such as gene expression analysis [52].

We focused on measuring inflammation in induced sputum samples. It would also have been informative to measure mucins in the sputum samples, as cytokines are known to increase mucin gene expression [33,37], which could account for increased sputum production in patients with chronic bronchitis.

In conclusion, persistent sputum production in COPD patients is associated with different clinical characteristics and increased concentrations of certain cytokines in induced sputum. Increased levels of IL-6, MCP-1 and TNF-$\alpha$ in the airways of persistent sputum producers may be a cause of increased mucin gene expression, while increased eotaxin may contribute to excessive eosinophilic inflammation. Novel therapies that target these cytokines may reduce the burden of mucus production and airway inflammation in this COPD subgroup.

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## Author contributions

Conception and Design: SK, DS.

Acquisition analysis and Interpretation of Data: SK, AR, RM, RW, JV, DS.

Drafting the manuscript and revising it critically for important intellectual content: SK, AR, JP, JV, DS.

Final approval of the version to be published: SK, DS, JV.

Responsible for overall content: SK, DS.

## Summary of conflict of interest

Professor Dave Singh has received lecture fees, research grants, consultancy fees and support for conference attendance from various pharmaceutical companies including AstraZeneca, GlaxoSmithKline, Chiesi, Boehringer Ingelheim, Roche, Novartis, Cipla, Almirall and Merck. Professor Jorgen Vestbo has received lecture fees and consultancy fees from various pharmaceutical companies including GlaxoSmithKline, Novartis, Astra Zeneca, Boehringer Ingelheim, Nycomed, Chiesi, Syntaxin and Bioxydyn. Roberta Milone and Rick Williamson work for GlaxoSmithKline (Stevanage, UK). Dr Shruti Khurana, Dr Arjun Ravi, Dr Justyna Sutula and Dr Jonathan Plumb have no conflicts of interest to declare.

## Notation of prior publication

None.

## Abbreviations

| | |
|---|---|
| 6 MWT | six minute walk test |
| ATS | American Thoracic Society |
| BMI | body mass index |
| CAT | COPD assessment tool |
| $X^2$ | Chi squared test |
| CV | closing volume |
| CFU | colony forming unit |
| DCC | differential cell count |
| DTT | dithiothreitol |
| ECLIPSE | evaluation of COPD longitudinally to identify predictive surrogate end-points |
| $FEV_1$ | forced expiratory volume in 1 s |
| FRC | functional residual capacity |
| FVC | forced vital capacity |
| GOLD | Global initiative for Chronic Obstructive Lung Disease |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| HRCT | high resolution computerised tomography |
| GSK | GlaxoSmithKline |
| IC | inspiratory capacity |
| ICS | inhaled corticosteroid |
| IFN-$\gamma$ | interferon-gamma |
| IL- | interleukin |
| IP-10 | IFN- $\gamma$ activated protein-10 |
| KCO | carbon monoxide diffusion capacity |
| L/sec | litres per second |
| LABA | long acting beta 2 agonist |
| LAMA | long acting muscarinic antagonist |
| LOD | limit of detection |
| MCP | monocyte chemo attractant protein |
| MDC | macrophage derived chemokine |
| MIP | macrophage inflammatory protein |
| MMRC | Modified Medical Research Council |
| MUC | mucin |
| MSD | meso-scale discovery |
| N2$\Delta$/L | slope of nitrogen washout curve |
| PBS | phosphate buffered saline |
| $p$ | probability value |
| Ri | intraclass correlation coefficient |
| RV | residual volume |
| SABA | short acting beta 2 agonist |
| SAMA | short acting muscarinic antagonist |
| SGRQ | St. George's respiratory questionnaire |

| | |
|---|---|
| TCC | total cell count |
| TCC/g | total cell count per gram |
| TGF | transforming growth factor |
| TLC | total lung capacity |
| TNF-α | Tumour necrosis factor-α |
| VC | vital capacity |

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2014.09.020.

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