

Sputum Cytokine Repeatability in COPD

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Introduction: Sputum biomarker measurements are used to measure airway inflammation in COPD patients. We have previously validated a Luminex assay able to quantify 15 analytes in COPD sputum supernatant. This assay demonstrated sputum protein expression profiles associated with neutrophilia, airway bacterial colonisation and current smoking in COPD.

Methods: We report repeat sputum supernatant analysis at 6 months from a sub-group of COPD patients who participated in the original study. 48 COPD patients provided a repeat sputum sample at 6 months. 15 panel analytes were detectable.

Results: Repeated sputum cytokine measurements showed a significant positive correlation between baseline and 6 months for all analytes except IL-1RA with intraclass correlation coefficient (ICC) indicating good to excellent repeatability. IL-1 β , IL-2, IL-8, IL-17A, G-CSF, MIP-1 α , MIP-1 β and TNF- α were significantly correlated with sputum neutrophil percentage at 6 months. IL-1 β , IL-4, IL-8 and G-CSF were significantly increased in *Haemophilus influenzae* colonised patients and IL-1 β , IL-4, IL-8, G-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , MIP-1 β and TNF- α were significantly higher in ex-smoking COPD patients.

Discussion: A multiplex immunoassay used at repeated visits in COPD patients showed a high degree of reproducibility for the majority of analytes. There were reproducible inflammatory signatures in sputum associated with clinical characteristics in COPD patients.

Keywords: biomarker, validation, luminex, inflammation, respiratory

Introduction

Chronic obstructive pulmonary disease (COPD) is a complex and heterogenous condition, characterised by airflow obstruction and persistent airway inflammation.¹ The heterogeneity of clinical presentation is accompanied by considerable variation between individuals in the profile of airway inflammation present.² Induced sputum sampling can evaluate airway inflammation in COPD.^{3–5} Subgroups of COPD patients with different characteristics have been identified based on the presence of increased neutrophil or eosinophil cell counts in sputum samples; for example, higher neutrophil counts have been associated with increased presence of *Haemophilus influenzae* in stable state sputum samples,^{3–5} while higher eosinophil counts are associated with a greater corticosteroid response.^{6–8} Sputum sampling therefore identifies subgroups based on inflammation profiles (endotypes) that may guide clinical management strategies.

Sputum supernatant is a matrix that enables the quantification of inflammatory mediators involved in airway inflammation. The use of immunoassays for this purpose requires optimisation and validation, as many commercially available immunoassays were not developed to be used with sputum matrices.^{2,9} Previous COPD sputum supernatant biomarker studies have reported problems with reproducibility which often arise due to insufficient optimisation and/or validation in this matrix.^{2,10} We have previously validated a Luminex assay that quantified 15 analytes in COPD sputum supernatant according to current regulatory standards for assays in clinical trials.¹¹ The application of this validated method demonstrated protein expression profiles in COPD sputum samples associated with different clinical characteristics. For example, current smokers had lower levels of IL-1 β , IL-4, IL-8, G-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , MIP-1 β and TNF- α , while *H. influenzae* colonisation was associated with higher sputum neutrophil counts and increased IL-1 β , IL-4, IL-8, G-CSF and IFN- γ levels. A summary of our previous findings is shown in [Table 1](#), with further details available in [Supplementary Materials, Supplementary Table 1](#).



Table 1 Summary of Previous Key Findings Using Validated Luminex Assay

Sputum Supernatant Cytokine	Correlation: Neutrophil Percentage and Cytokine (n=79)	HI ⁺ ve (n=15) vs HI ⁻ ve (n=34)	Current Smoking (n=31) vs Ex-Smoking (n=48)
IL-1β	X	X	X
IL-1RA			
IL-2	X		
IL-4	X	X	X
IL-6			
IL-8	X	X	X
IL-17A	X		
Eotaxin			
G-CSF	X	X	X
IFN-γ	X	X	X
IP-10	X		X
MCP-1			X
MIP-1α	X		X
MIP-1β	X		X
TNF-α	X		X

Notes: COPD Sputum Neutrophil Percentage and Supernatant Cytokine Correlation analysed using Pearson’s coefficient correlation test for parametric data and Spearman’s rank test for non-parametric data. COPD Haemophilus influenzae Colonised vs COPD Non-Colonised data was analysed using Kruskal-Wallis test with Dunns post hoc test. COPD Current Smoking vs COPD Ex-Smoking data was analysed using Mann Whitney test. X: Statistically significant. **Abbreviations:** HI⁺ve, Haemophilus influenzae positive; HI⁻ve, Haemophilus influenzae negative; IL, Interleukin; RA, Receptor Antagonist; G-CSF, Granulocyte-Colony Stimulating Factor; IFN-γ, Interferon Gamma; IP, Interferon-gamma Inducible Protein; MCP, Monocyte Chemoattractant Protein; MIP, Macrophage Inflammatory Protein; TNF, Tumor Necrosis Factor.

We now report repeat sputum supernatant analysis at 6 months from a sub-group of COPD patients who participated in the original validation study. The aim was to investigate the stability over time of sputum supernatant cytokine levels. We evaluated whether the cytokine expression profiles previously associated with neutrophilia, airway bacterial colonisation and current smoking were reproducible after 6 months.

Materials and Methods

Subjects

From the 81 COPD patients who previously provided a baseline sample (see our previous publication for full results),¹¹ 48 individuals agreed to return for a repeat sample at 6 months. All COPD patients had a smoking history of >10 pack years, age ≥40 years old and a diagnosis of COPD according to Global Initiative for Obstructive Lung Disease (GOLD) criteria, including a post bronchodilator first second of forced expiration/forced vital capacity (FEV₁/FVC) ratio of <0.7.¹² Patients with a current or previous diagnosis of asthma, lung cancer or occurrence of a COPD exacerbation or respiratory tract infection within 2 months prior to the baseline visit were excluded from the study. All patients provided written informed consent using protocols that comply with the Declaration of Helsinki and are approved by the local Greater Manchester East and Northwest Preston Research Ethics Committees (05/Q1402/41, 10/H1016/25 and 16/NW/0836).

Study Design

Retrospective analysis of sputum samples that had previously been collected during stable state, defined as no symptom-defined exacerbation or respiratory illness within 4 weeks of sampling. Spirometry was performed at both visits

according to guidelines^{13,14} (using the EasyOn PC Sensory, NDD, Intermedical) and symptoms were assessed using COPD Assessment Test (CAT) and Modified Medical Research Council Questionnaire (mMRC) scores and health-related quality of life using the St George's Respiratory Questionnaire (SGRQ-C).¹⁵

Sputum Processing

Induced sputum was processed using a 2-step method consisting of a Dulbecco's phosphate-buffered saline (D-PBS) wash step followed by a dithiothreitol (DTT) step as previously described.¹⁶ Further details are available within [Supplementary Materials](#). PBS supernatant was stored at -80°C for later analysis. Cytospin preparations (Cytospin 4, Shandon, Runcorn, UK) were stained with RapiDiff II (Atom Scientific, Hyde, UK), and 400 non-squamous cells were counted, and differential cell counts (DCC) obtained as a percentage of non-squamous cells. Cell viability was analysed by trypan blue exclusion method. Sputum supernatant samples were removed from the freezer and allowed to thaw completely prior to sample dilution. Samples were diluted within one hour of removal from freezer storage.

Quantitative PCR Detection of *H. influenzae* in Sputum

A subgroup of samples ($n = 30$) were processed for real-time quantitative polymerase chain reaction (qPCR) detection of absolute abundance for *H. influenzae*. Prior to the processing method outlined above, a proportion of the sputum sample was homogenised with PBS and glass beads to provide a sample for qPCR. Quantification of *H. influenzae* was performed as per Beech et al,¹⁷ further details are available within [Supplementary Materials](#). We used the previously reported normal range for *H. influenzae* abundance obtained from healthy non-smokers (upper threshold 3.22×10^5 genome copies/mL) to classify COPD patients as *H. influenzae* positive ($\text{HI}^{+\text{ve}}$) or *H. influenzae* negative ($\text{HI}^{-\text{ve}}$), where *H. influenzae* abundance was above or below the threshold, respectively.¹⁷

Sputum Supernatant Biomarker Analysis

Sputum supernatants were analysed for 15 mediators previously validated and detectable in COPD sputum supernatant; interleukin (IL)-1b, IL-1 receptor antagonist (RA), IL-2, IL-4, IL-6, IL-8, IL-17A, Eotaxin, Granulocyte-colony stimulating factor (G-CSF), Interferon (IFN)- γ , Interferon gamma-induced protein (IP)-10, Monocyte chemoattractant protein (MCP)-1, Macrophage inflammatory protein (MIP)-1 α , MIP-1 β and TNF- α by Luminex multiplex assay (Bio-Rad, Hertfordshire, UK). Lot-specific assay quantitative ranges are available in [Supplementary Materials](#), [Supplementary Table 2](#). Samples were prepared at a 1:8 dilution using proprietary diluent and analysed in duplicate. Values below the assay LLOQ were reported as half the value of the LLOQ. The mean was used for statistical analysis. Inter-assay and analyst repeatability assessments formed part of the original assay validation and met the required acceptance criteria.

Statistical Analysis

Associations between baseline and 6-month sputum cytokine measurements and differential cell counts were assessed using Pearson's coefficient correlation test for parametric data and Spearman's rank test for non-parametric data (GraphPad Prism version 10.4.0, San Diego, CA, USA). Sputum cytokine repeatability was assessed using intraclass correlation coefficient (ICC) analysis, using log transformed data and based on an absolute agreement, two-way mixed effects model¹⁸ (SPSS 25.0, IBM, Armonk, NY, USA). ICC values are interpreted as excellent (>0.75), fair to good ($0.40-0.75$) or poor (<0.40).¹⁹

Comparisons between COPD current and ex-smokers and colonised and non-colonised patients were performed using Mann-Whitney tests (GraphPad Prism version 10.4.0, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

The baseline characteristics and sputum cell count data for the cohort ($n = 48$) are presented in [Table 2](#); the mean post-bronchodilator forced expiratory volume in 1 second (FEV_1) was 62.5% predicted, with 58.4% of patients having ≥ 1

Table 2 Baseline Demographics

Characteristic	n=48
Gender (Male/Female)	28/20
Age	65.2 (7.9)
Smoking Statug	43.2 (21.4)
BMI (kg/m ²)	28.7 (5.8)
Exacerbations (1 year period)	1.1 (1.3)
0 (%)	41.7
1 (%)	31.3
≥ 2 (%)	27.1
Post FEV _i (L)	1.8 (0.5)
Post FEV _i (%)	62.5 (16.7)
Gold Category (%)	
1	2.1
2	25.0
3	58.3
4	14.6
CAT	22.6 (5.3)
mMRC	4.0 [2.0–4.0]
SGRQ-C (Total)	52.6 (15.9)
ICS Use (%)	72.9 ⁺
Sputum Characteristics	
Total cell count (x10 ⁶ /g)	2.1 [0.2–56.5]
Neutrophil (%)	69.1 [21.5–97.8]
Macrophage (%)	20.6 [1.0–68.0]
Eosinophil (%)	1.0 [0.0–10.0]
Lymphocyte (%)	0.3 [0.0–4.8]
Epithelial (%)	2.0 [0.0–60.3]
Neutrophil cell count (x10 ⁶ /g)	5.3 [0.3–98.1]
Macrophage cell count (x10 ⁶ /g)	1.3 [0.2–7.6]
Eosinophil cell count (x10 ⁶ /g)	0.1 [0.0–2.5]
Lymphocyte cell count (x10 ⁶ /g)	0.0 [0.0–0.6]
Epithelial cell count (x10 ⁶ /g)	0.2 [0.0–2.5]

Notes: Data is presented as percentages, mean (SD) or median [range] as appropriate (n=48). + Further details on ICS use are available in the [Supplementary Materials, Supplementary Table 3](#).

Abbreviations: BMI, Body Mass Index; FEV, Forced Expiratory Volume; CAT, COPD Assessment Test; mMRC, Modified Medical Research Council; SGRQ-C, St. George's Respiratory Questionnaire – Condensed; ICS, inhaled Corticosteroid.

exacerbation in the previous 12 months. Mean CAT and total SGRQ scores were 22.6 and 52.6, respectively, with a median mMRC of 4.

Associations Between Sputum Measurements at Baseline and 6 Months

Repeated sputum neutrophil, macrophage, eosinophil and epithelial cell counts showed significant positive correlations between baseline and 6 months with ICC indicating good to excellent repeatability; for example, ICC for neutrophils and eosinophils were 0.73 and 0.77, respectively (Table 3 and Supplementary Materials, Supplementary Figure 1).

Repeated sputum supernatant cytokine measurements showed a significant positive correlation between baseline and 6 months for all cytokines except IL-1RA, with ICC values indicating good to excellent repeatability (Table 3 and

Table 3 Association Between Baseline and 6-Month Measures of Sputum Supernatant Cytokines and Sputum Differential Cell Counts (%) in COPD

Sputum Supernatant Cytokine (pg/mL)	Baseline-6-Month Correlations (n=48)		Baseline-6-Month ICC (n=48)
	Rho [Confidence Interval]	P value	
IL-1β	0.62 [0.40–0.77]	p<0.0001	0.774
IL-1RA	0.26 [−0.04–0.51]	0.08	0.415
IL-2	0.67 [0.47–0.80]	p<0.0001	0.801
IL-4	0.54 [0.29–0.72]	p<0.0001	0.631
IL-6	0.50 [0.24–0.69]	p<0.001	0.722
IL-8	0.57 [0.34–0.74]	p<0.0001	0.705
IL-17A	0.55 [0.30–0.74]	p<0.0001	0.861
Eotaxin	0.50 [0.25–0.69]	p<0.001	0.665
G-CSF	0.64 [0.42–0.78]	p<0.0001	0.854
IFN-γ	0.37 [0.09–0.60]	p<0.01	0.410
IP-10	0.72 [0.54–0.83]	p<0.0001	0.833
MCP-1	0.44 [0.17–0.65]	p<0.01	0.549
MIP-1α	0.64 [0.43–0.79]	p<0.0001	0.786
MIP-1β	0.72 [0.54–0.84]	p<0.0001	0.860
TNF-α	0.57 [0.34–0.74]	p<0.0001	0.794
Sputum Cell Count (%)			
Neutrophils	0.74 [0.57–0.85]	p<0.0001	0.731
Macrophages	0.78 [0.63–0.87]	p<0.0001	0.877
Eosinophils	0.61 [0.38–0.76]	p<0.0001	0.774
Lymphocytes	0.09 [−0.21–0.37]	0.54	0.287
Epithelial	0.48 [0.21–0.67]	p<0.001	0.718

Notes: Data is presented as rho [confidence interval] and p values and intraclass correlation coefficient. Results analysed using Pearson's coefficient correlation test for parametric data and Spearman's rank test for non-parametric data. Non-parametric data was log-transformed for ICC analysis. P-values <0.05 were considered statistically significant.

Abbreviations: IL, interleukin; ICC, intraclass correlation coefficient; RA, Receptor Antagonist; G-CSF, Granulocyte-Colony Stimulating Factor; IFN-γ, Interferon Gamma; IP, Interferon-gamma Inducible Protein; MCP, Monocyte Chemoattractant Protein; MIP, Macrophage Inflammatory Protein; TNF, Tumor Necrosis Factor.

[Supplementary Materials, Supplementary Figure 2](#)); for example, excellent repeatability ($ICC > 0.75$) was observed for IL-1 β , IL-2, IL-17A, G-CSF, IP-10, MIP-1 β and TNF- α .

Sputum Supernatant Measurements at 6 Months

Correlation with Sputum Neutrophils

IL-1 β , IL-2, IL-8, IL-17A, G-CSF, MIP-1 α , MIP-1 β and TNF- α were significantly correlated with sputum neutrophil percentage at 6 months ([Figure 1A–H](#)), with the rho values being between 0.29 and 0.48.

HI^{+ve} Compared to HI^{-ve} Samples

30 patients had a sufficient sample for bacterial qPCR at 6 months, with 8 being classified as HI^{+ve}. IL-1 β , IL-4, IL-8 and G-CSF were significantly increased in HI^{+ve} compared to HI^{-ve} ($p < 0.01$ for all comparisons, [Figure 2](#)). Sputum neutrophil percentage was significantly higher while sputum macrophage and eosinophil percentages were significantly lower in HI^{+ve} versus HI^{-ve} patients (shown in the [Supplementary Materials, Supplementary Figure 3](#)).

Current versus Ex-Smokers

31 patients were ex-smokers (35.4%); IL-1 β , IL-4, IL-8, G-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , MIP-1 β and TNF- α were significantly higher in ex-smokers compared to current smokers ($p < 0.01$ for all comparisons, [Figure 3](#)). Sputum neutrophil percentage was significantly higher ($p < 0.01$, [Supplementary Materials, Supplementary Figure 4](#)) and macrophages were significantly lower in ex-smokers compared to current smokers ($p < 0.01$, [Supplementary Materials, Supplementary Figure 4](#)).

Discussion

We report good to excellent repeatability over 6 months of sputum supernatant biomarkers quantified using a validated immunoassay in COPD patients. We observed cytokine expression profiles associated with neutrophilia, airway bacterial colonisation and current smoking at 6 months which were highly similar to the profiles previously reported at baseline. Overall, our findings demonstrate that these repeated sputum immunoassay measurements are stable over time, including cytokine profiles associated with neutrophilia, airway bacterial colonisation and current smoking.

Repeated sputum biomarker measurements showed a significant positive correlation between baseline and 6 months for 14 out of 15 analytes with ICC values indicating good to excellent repeatability. Similarly, sputum cell counts also demonstrated significant positive correlations at repeat sampling, while ICC values indicated good to excellent repeatability for all cell types except lymphocytes. Previous studies have demonstrated significant variability in repeat measurements of sputum biomarkers in both spontaneous and induced sputum collected over 4-to-6-week periods.^{20–23} Assay validation and standardisation of the sputum induction procedure can improve variability in single-plex immunoassay measurements.^{24,25} We focus on multiplex analysis and show how using a rigorously validated analytical method in sputum supernatant can lead to a high degree of repeatability.

Biomarkers are often used to evaluate the pharmacological effects of novel drugs in early phase clinical trials;^{26,27} this study supports the use of this immunoassay panel for COPD clinical trials up to a duration of 6 months. In such studies, the use of sequential sampling and calculation of an average biomarker concentration can reduce variability of sputum biomarker measurements.^{22,23} However, repeated visits for sample collection may not be practical for some patients. The immunoassay reported here has potential for use in clinical trials without the need for multiple sampling. Longer repeatability studies could support the use of this immunoassay in clinical trials of longer duration, eg, 1 year. While the current results are from COPD patients in the stable state, exacerbation sampling with increased inflammation may show changes in various cytokines.^{11,28,29}

Here, we observed that IL-1 β , IL-2, IL-8, IL-17A, G-CSF, MIP-1 α , MIP-1 β and TNF- α levels were significantly correlated to sputum neutrophil percentage at 6 months. Using the baseline sample in a larger sample size ($n=81$), we previously reported associations between these 8 proteins and sputum neutrophil percentage, in addition to associations for IL-4, IFN- γ and IP-10 ([Supplementary Materials, Supplementary Table 4](#)). The high degree of similarity between the baseline and 6-month samples indicates that this signature of inflammatory proteins is associated with upregulation of

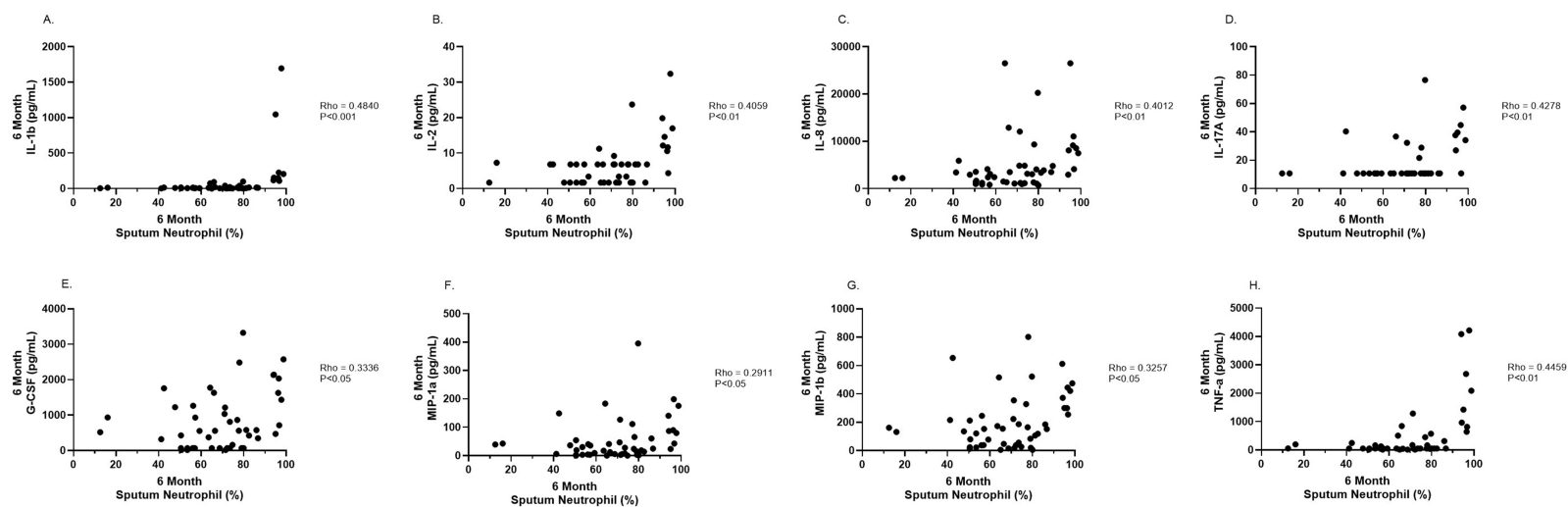


Figure 1 Sputum neutrophil percentage and sputum supernatant cytokine correlations at 6 months in COPD, n=48. **(A)** Sputum neutrophil percentage and sputum IL-1 β correlation at 6 months in COPD. **(B)** Sputum neutrophil percentage and sputum IL-2 correlation at 6 months in COPD. **(C)** Sputum neutrophil percentage and sputum IL-8 correlation at 6 months in COPD. **(D)** Sputum neutrophil percentage and sputum IL-17A correlation at 6 months in COPD. **(E)** Sputum neutrophil percentage and sputum G-CSF correlation at 6 months in COPD. **(F)** Sputum neutrophil percentage and sputum MIP-1 α correlation at 6 months in COPD. **(G)** Sputum neutrophil percentage and sputum MIP-1 β correlation at 6 months in COPD. **(H)** Sputum neutrophil percentage and sputum TNF- α correlation at 6 months in COPD. Data is presented as rho and p values. Results analysed using Pearson's coefficient correlation test for parametric data and Spearman's rank test for non-parametric data.

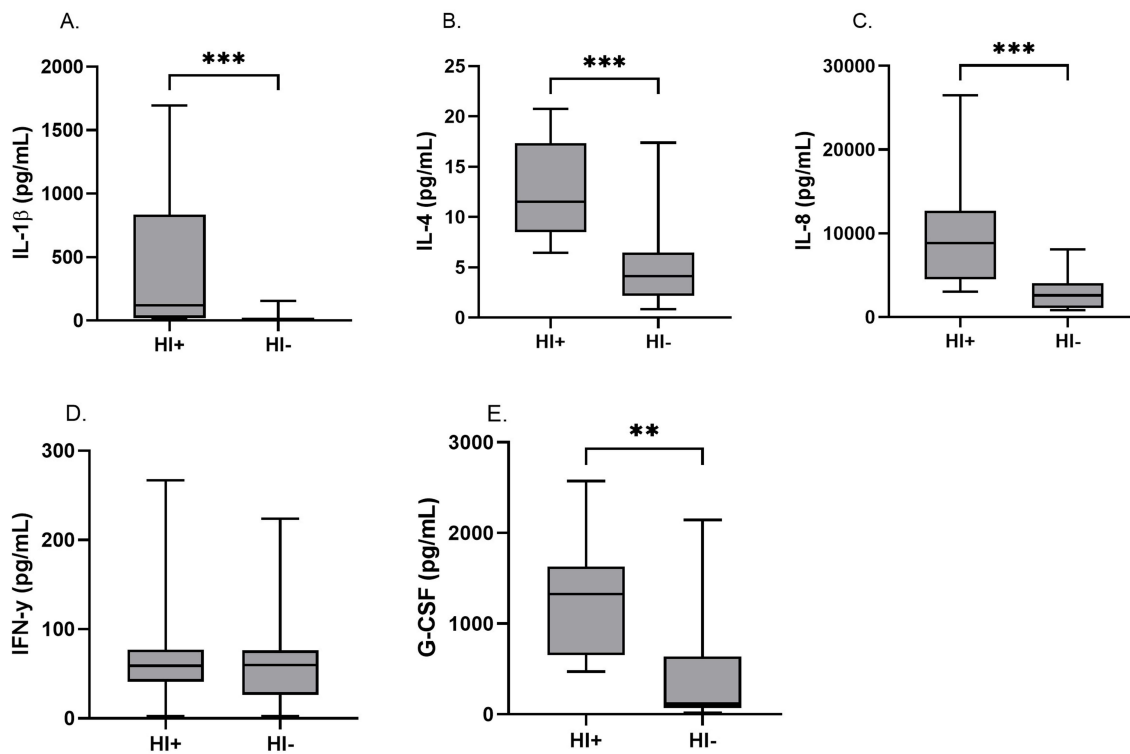


Figure 2 Sputum cytokines in COPD HI⁺ve (n=8) and COPD HI⁻ve (n=22) at 6 months. (A) IL-1β in COPD HI⁺ve and COPD HI⁻ve at 6 months. (B) IL-4 in COPD HI⁺ve and COPD HI⁻ve at 6 months. (C) IL-8 in COPD HI⁺ve and COPD HI⁻ve at 6 months. (D) IFN-γ in COPD HI⁺ve and COPD HI⁻ve at 6 months. (E) G-CSF in COPD HI⁺ve and COPD HI⁻ve at 6 months. Data is presented as minimum, maximum and median concentrations. Results analysed using Mann-Whitney t-test. **p<0.01, ***p<0.001. Haemophilus influenzae positive COPD patients (HI⁺ve), Haemophilus influenzae negative COPD patients (HI⁻ve).

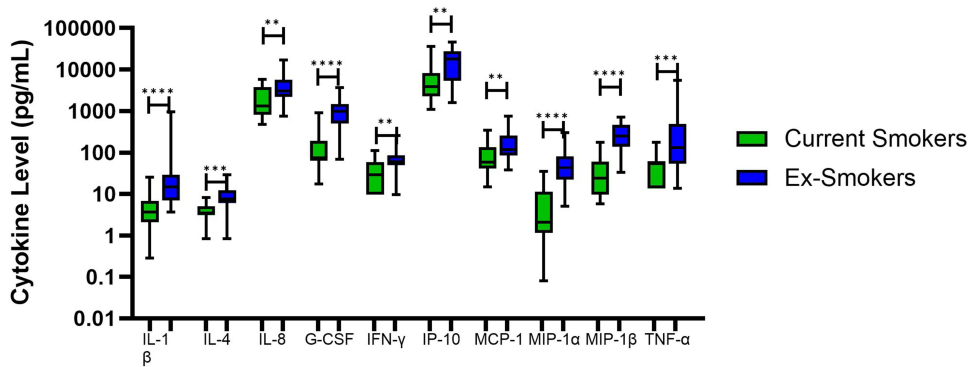


Figure 3 Sputum cytokines in COPD current smokers (n=17) and COPD ex-smokers (n=31) at 6 months. Data is presented as minimum, maximum and median concentrations. Results analysed using Mann-Whitney t-test. **p<0.01, ***p<0.001, ****p<0.0001.

neutrophilic inflammation. We suggest that the core set of 8 proteins that were significantly associated at both visits are the most closely related to neutrophilic inflammation, with the smaller sample size at 6 months possibly contributing to the loss of significance for 3 proteins. Neutrophil recruitment and activation are key functions of some of these proteins. IL-8 plays key roles in neutrophil chemotaxis^{30,31} and the release of neutrophil extracellular traps (NETs),³² while MIP-1α is involved in neutrophil chemotaxis and transmigration through the endothelium.³³ IL-17 acts on the airway epithelium to induce the secretion of various neutrophil chemokines including IL-8,³⁴ while IL-1β increases transcription of multiple neutrophil recruiting chemokines from bronchial epithelial cells.³⁵ Previous studies have also reported correlations between IL-8 and TNF-α and sputum neutrophils in COPD.^{28,36,37}

We observed significantly increased levels of IL-1 β , IL-4, IL-8, G-CSF and sputum neutrophils in HI^{+ve} COPD patients at 6 months. These differences were also previously observed in the baseline samples, in addition to higher IFN- γ levels. Previous studies have demonstrated associations between *H. influenzae* colonisation and IL-1 β levels,^{38–41} IL-8 levels^{38,40,42} and sputum neutrophilia.⁴ Wang et al reported that *H. influenzae* dominance in the sputum microbiome of COPD patients was associated with elevated supernatant IL-1 β and TNF- α concentrations.³⁹ Similarly, Winslow et al identified that COPD patients with *H. influenzae* colonisation had increased sputum neutrophils and a supernatant cytokine signature indicating upregulation of IL-6 trans-signalling (IL-6TS) with higher levels of IL-1 β , IL-6, soluble IL-6 receptor, MIP-1 β and IL-8.³ While there are some differences between our current results and these previous studies, there is a common theme that *H. influenzae* colonisation is associated with upregulation of innate immune signalling including mediators such as IL-1 β and IL-8. *H. influenzae* induces secretion of innate immune mediators from alveolar macrophages including IL-8, thus facilitating neutrophil chemotaxis.⁴³ *H. influenzae* colonisation appears to skew the airway inflammatory profiles towards neutrophilic inflammation and away from a ICS responsive eosinophilic endotype.⁴⁴

The limitations of this study include a moderate sample size (n = 48), which was further reduced following subgroup analyses, particularly HI^{+ve} patients (n=8). Additionally, longer-term follow-up data would provide valuable insights and could support the use of this validated immunoassay in clinical trials of longer duration; however, this is often challenging in COPD cohort studies due to participant dropout over time, commonly because of declining health. Lastly, environmental exposure factors were not assessed in this study, although they may influence cytokine expression profiles.

We report increased levels of the same 10 sputum cytokines and increased sputum neutrophils in COPD ex-smokers at baseline and at 6 months.¹¹ Sputum neutrophil percentage and IL-8 have previously been shown to be increased in COPD ex-smokers versus current smokers.^{45,46} In-vitro studies have highlighted potential mechanisms to explain these observations; acute exposure to cigarette smoke extract causes cell death and downregulation of gene expression of multiple pro inflammatory cytokines.^{47–49}

Conclusion

In conclusion, a multiplex immunoassay used at repeated visits in COPD patients showed a high degree of reproducibility for the majority of analytes. Furthermore, highly similar protein signatures associated with the presence of *H. influenzae* colonisation and smoking status were observed at baseline and 6-month visits. These results highlight reproducible inflammatory signatures in sputum associated with clinical characteristics in COPD patients. This multiplex assay has potential for monitoring levels of airway inflammation over time, for example during clinical trials.

Abbreviations

COPD, Chronic Obstructive Pulmonary Disease; HI^{+ve}, Haemophilus influenzae positive; HI^{-ve}, Haemophilus influenzae negative; IL, Interleukin; GOLD, Global Initiative for Obstructive Lung Disease; CAT, COPD Assessment Test; mMRC, Modified Medical Research Council Questionnaire; SGRQ-C, St George's Respiratory Questionnaire; D-PBS, Dulbecco's phosphate-buffered saline; DTT, Dithiothreitol; DCC, Differential Cell Counts; qPCR, Quantitative Polymerase Chain Reaction; RA, Receptor Antagonist; G-CSF, Granulocyte-colony stimulating factor; IFN, Interferon; IP-10; Interferon Gamma-Induced Protein; MCP-1, Monocyte Chemoattractant Protein; MIP, Macrophage Inflammatory Protein; ICC, Intra-Class Correlation Coefficient; FEV₁, forced expiratory volume in 1 second; ICS, Inhaled Corticosteroid.

Data Sharing Statement

The datasets supporting the conclusions of this article are included within the article (and its additional files).

Acknowledgment

The abstract of this paper was presented at the “ERS International Congress” as a poster presentation with interim findings. The poster’s abstract was published in ‘Poster Abstracts’ in European Respiratory Journal: <https://doi.org/10.1183/13993003.congress-2024.PA2994>.

Dave Singh and Simon Lea are supported by the National Institute for Health and Care Research (NIHR) Manchester Biomedical Research Centre (BRC) (NIHR203308).

Funding

This study was supported by AstraZeneca.

Disclosure

Dr Dave Singh reports personal fees from Adovate, personal fees from Aerogen, personal fees from Almirall, personal fees from Apogee, personal fees from Arrowhead, personal fees from AstraZeneca, personal fees from Bial, personal fees from Boehringer Ingelheim, personal fees from Chiesi, personal fees from CIPLA, personal fees from Connect Biopharm, personal fees from Covis, personal fees from CSK Behring, personal fees from DevPro Biopharma LCC, personal fees from Elpen, personal fees from Empirico, personal fees from EpiEndo, personal fees from Genentech, personal fees from Generate Biomedicines, personal fees from GlaxoSmithKline, personal fees from Glenmark, personal fees from Kamada, personal fees from Kinaset Therapeutics, personal fees from Kymera, personal fees from Menarini, personal fees from MicroA, personal fees from OM Pharma, personal fees from Orion, personal fees from Peris Pharmaceuticals, personal fees from Pulmatrix, personal fees from Revolo, personal fees from Roivant Sciences, personal fees from Sanofi, personal fees from Synairgen, personal fees from Tetherex, personal fees from Teva, personal fees from Theravance Biopharma, personal fees from Upstream, personal fees from Verona Pharma, outside the submitted work. The authors report no other conflicts of interest in this work.

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