Inter-relationships between inflammatory markers in patients with stable COPD with bronchitis: intra-patient and inter-patient variability

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ABSTRACT

Background: Measurements of pulmonary biomarkers can be used to monitor airway inflammation in chronic obstructive pulmonary disease (COPD), but the variability of sampled biomarkers and their inter-relationships are poorly understood. A study was undertaken to examine the intra- and inter-patient variability in spontaneous sputum samples from patients in the stable state and to describe the relationship between biomarkers, cell counts and markers of disease.

Methods: Sputum interleukin-1β, tumour necrosis factor α, interleukin 8, myeloperoxidase, leucotriene B4, growth-related oncogene α and differential cell counts were measured in patients with moderate to severe stable COPD (n = 14) on 11 occasions over a 1-month period.

Results: There was significant variability in all inflammatory indices (median intra-patient coefficient of variation (CV) 35% (IQR 22–69), median inter-patient CV 102% (IQR 61–145)). Variability could be reduced by using a rolling mean of individual patient data points. Sample size calculations were undertaken to determine the number of patients required to detect a 50% reduction in neutrophil count. Using a crossover design of a putative effective treatment, the number needed using one data point per patient was 72, reducing to 23 when a mean of three data points was used. Significant correlations were demonstrated both between the inflammatory biomarkers themselves and between inflammatory biomarkers and markers of disease. Some relationships were not apparent when results from a single sample were used. The reliability of inter-relationships improved as more data points were used for each patient.

Conclusions: Clear relationships exist between inflammatory biomarkers in patients with stable COPD. Sequential sampling reduced the variability of individual mediators and the potential number of patients needed to power proof of concept interventional studies.

It is widely accepted that chronic obstructive pulmonary disease (COPD) is an inflammatory condition. and many inflammatory mediators and their corresponding receptors have been highlighted as potential targets for specific anti-inflammatory therapeutic intervention.[1] Some of these agents have been used in limited studies, such as antibodies directed against tumour necrosis factor α (TNFα)[2] and interleukin 8 (CXCL8)[3] and small molecule antagonists of leucotriene B4 (LTB4),[4] while other anti-inflammatory agents are under development with the anticipation of clinical trials in the future.[1]

In drug development for COPD there is a need to conduct small short-term phase 2 trials to identify

biological and efficacy read-outs for novel therapeutic agents. Conventional physiological tests are often inappropriate for the early investigation of drugs with potential disease-modifying anti-inflammatory activity since these trials would be relatively long, involving large patient numbers and hence prohibitively expensive. For this reason, disease-related biomarkers are considered to be the appropriate candidates for initially investigating drug efficacy in disease. Recent studies have listed biomarkers which correlate with disease severity and BODE index,[5] but there is little information about the reproducibility of measurements and the relationship of these indices to other inflammatory mediators, disease development, severity and progression.[6] Only sputum neutrophils, CXCL8, TNFα and C-reactive protein have successfully differentiated between the differing stages of COPD,[6] although other studies have shown associations between different biomarkers both when stable and during exacerbations.[7]

The majority of interventional studies have relied upon a single pre- and post-interventional measurement of a biomarker to ascertain any change.[1] [8] However, without knowledge of the inherent variability of the factor being used, it is difficult to perform the calculations required to ensure that a study has sufficient power to detect a relevant difference, and this will be critical in determining the role of an individual mediator in the inflammatory cascade and the decision to proceed to larger studies to determine clinical benefit.

The purpose of the present study, therefore, was twofold: (1) to describe the variability in inflammatory indices in spontaneous sputum in stable COPD and to assess the effect of multiple sampling to reduce this variability; and (2) to assess the relationship between some mediators that have been implicated in the pathogenesis of COPD.

METHODS

Study subjects

Patients aged 50–78 years with a diagnosis of GOLD stage III COPD[9] were recruited. They were daily sputum producers with a history of chronic bronchitis as defined by the MRC criteria[10] and were current or ex-smokers. All patients were confirmed to have obstructive airways disease at screening by spirometric measurements and had stable symptoms of COPD for at least 8 weeks prior to recruitment with no changes in medication during this time. Alternative and concomitant lung

disease was excluded clinically and by high-resolution CT scanning.

Study design

Patients were seen on 11 occasions over 4 weeks (daily for 5 days, then twice weekly for 3 weeks). On each visit the patients were reviewed, symptoms were noted and samples of spontaneous sputum and blood were collected. Spirometry was assessed weekly to assess disease stability.

Patients were asked to complete daily diary cards, described previously,[11] throughout the study. Symptoms (including dyspnoea, sputum volume and colour) were recorded using an ordinal scale which allowed differentiation between normal for the patient and better or worse than usual to ensure that there were no changes suggestive of an exacerbation.

Sample collection and processing

Spontaneous sputum samples were collected over 4 h (from rising in the morning) following mouth-washing procedures to minimise salivary contamination. Sputum collection and analysis occurred at the same time on each visit. The samples were divided into two aliquots: the first was ultracentrifuged (50 000 g for 90 min at 4°C) to prepare a sol phase sample to determine mediator concentrations. The second was treated with dithiothreitol to assess total cell numbers and cytospins prepared for total and differential cell counts of squamous cells, neutrophils, eosinophils, macrophages and lymphocytes.[12] Where relevant, patients were asked to abstain from smoking from waking until sputum collection was complete.

Measurement of mediators

Mediators were measured using Enzyme Amplified Sensitivity Immunoassay (R&D Systems, Abingdon, UK) and expressed in molar concentrations. All assays were validated to determine their working range and the variability of mediator measurements both within and between assay plates using methods described previously.[12]

In order to reduce intra-patient variability in biomarkers, the effects of employing a rolling mean were assessed. A 3-day rolling mean was determined for each mediator and cell count in each patient by averaging the results from visits 1, 2 and 3 ("rolling mean 3a"), then from visits 2, 3 and 4 ("rolling mean 3b"), etc, until the final visit was incorporated into a rolling mean ("rolling mean 3i"). A 5-day rolling mean was also determined for each biomarker in each patient by averaging the results from visits 1–5 ("rolling mean 5a"), then from visits 2–6 ("rolling mean 5b"), etc, until the final visit ("rolling mean 5g").

Statistical analysis

Data analysis was performed using SPSS 12.0 for Windows (SPSS, Chicago, Illinois, USA). Normally distributed data are expressed as mean and standard deviations, categorical data as percentages and non-normally distributed data as median and interquartile range (IQR). Non-normally distributed continuous variables (such as mediator concentrations and cell counts) were log transformed to achieve normality and allow calculation of the coefficient of variation (CV). The correlations between logged mediator concentrations and cell counts were evaluated by Pearson's correlation coefficient (PCC).

Sample size calculations were based on both a two-group parallel comparison and a paired (crossover) comparison in clinical trials. A parallel design used the formula:

$$n = 1 + 2C\left(\frac{S}{D}\right)^2$$

and a crossover design used the formula:

$$n = 1 + C\left(\frac{S}{D}\right)^2$$

where D was the smallest difference to be detected (a 50% decrease in mediator concentration or cell count was used as an arbitrary effect size for illustrative purposes) and S represented the standard deviation of the observations. C = 7.85 to provide an 80% power of detecting a reduction in mean mediator concentration or cell counts at the 5% level of significance.[13]

RESULTS

Baseline characteristics

Fourteen patients were enrolled and their baseline characteristics are summarised in table 1. The medications being taken by each patient during the study are listed in table E1 in the online supplement. Exacerbations, defined clinically,[14] were excluded on each visit by review of daily dairy cards, clinical examination, confirmation of unchanged therapy[15] and weekly spirometry. None of the patients experienced an exacerbation during the study period and there were no significant changes in diary scores or lung function between visits.

Assay variability and sputum characterisation

In all cases the mediator intra- and inter-assay variability was less than 10%. The median percentage of squamous cells in the samples was 0.6% (IQR 0–7.9%) and the median percentage of viable cells (assessed by trypan blue exclusion) was 83% (IQR 63–92%) which is comparable to that seen with induced sputum.[16]

Intra-patient variability in mediator concentrations and neutrophils

There was considerable variability in the concentrations of mediators and cells within individual patients over the study period. Figure 1 demonstrates the variability seen for one

Table 1 Baseline characteristics of the study patients

Characteristic	
Age (years)	65.5 (8.5)
Male (%)	6 (43%)
Diagnosis (years)	6.4 (5.4)
FEV$_1$ (l)	0.89 (0.3)
FEV$_1$ (% of predicted)	40 (8.3)
FVC (l)	2.28 (0.61)
FEV$_1$/FVC	42 (11)
Increase in FEV$_1$ following 400 µg inhaled salbutamol	
ml	132 (77)
% predicted	5 (3.3)
BMI (kg/m²)	23.8 (4.5)
Current smokers (%)	6 (43%)
Pack year history	45 (20.7)
Taking inhaled steroids at enrolment (%)	11 (79%)

FEV$_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; BMI, body mass index.
Results are presented as mean (SD) or n (%).

Table E1 The list of medications taken by each patient during the study period

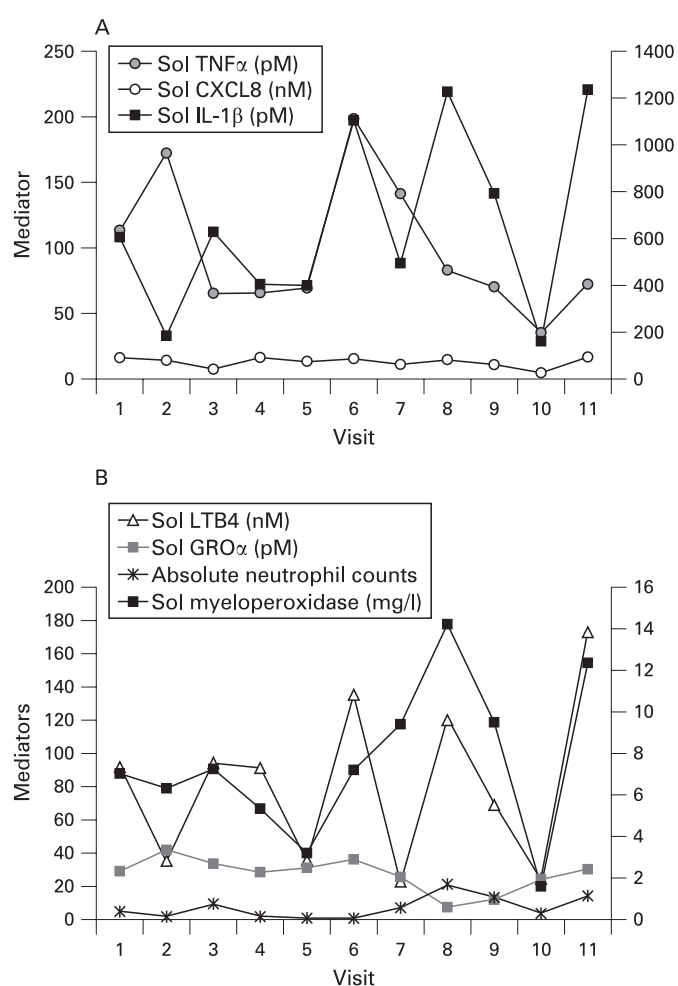| Patient	Medications	Patient	Medications
1	Salbutamol 100 mcg inh bd	8	Salbutamol 100 mcg inh qds
	Tiotropium 18 mcg inh od		Iprtropium 20 mcg inh bd
	Beclometasone dipropionate 200 mcg inh bd		Theophylline modified release 175 mg po bd
	Aspirin 75 mg po od		Seretide (250 mcg/50 mcg) inh bd
			Gliclazide 80 mg po od
2	Salmeterol 50 mcg inh bd		
	Salbutamol 100 mcg inh prn	9	Salbutamol 100 mcg inh prn
	Budesonide 200 mcg inh bd		
	Ipratropium 40 mcg inh bd		
	Theophylline modified release 250 mg po bd		
3	Salmeterol 100 mcg inh bd	10	Salmeterol 50 mcg inh bd
	Salbutamol 100 mcg inh bd		Salbutamol 100 mcg inh prn
	Ipratropium 40 mcg inh bd		Fluticasone propionate 200 mcg inh bd
	Fluticasone propionate 100 mcg inh bd		
	Ranitidine 300 mg po od		
4	Seretide (250 mcg/50 mcg) inh bd	11	Salbutamol 100 mcg inh qds
	Salbutamol 100 mcg inh prn		Ipratropium 20 mcg inh bd
			Fluticasone propionate 250 mcg inh bd
5	Salmeterol 50 mcg inh bd	12	Salmeterol 50 mcg inh bd
	Salbutamol 100 mcg inh prn		Salbutamol 100 mcg inh prn
	Ipratropium 20 mcg inh bd		Fluticasone propionate 250 mcg inh bd
	Beclometasone dipropionate 20 mcg inh bd		
	Paracetamol 1 g po QDS		
6	Salmeterol 50 mcg inh bd	13	Combivent inh (100 mcg/20 mcg) inh qds
	Salbutamol 100 mcg inh prn		Beclometasone dipropionate 200 mcg inh bd
	Ipratropium 20 mcg inh qds		
7	Ipratropium 20 mcg inh bd		
	Fluticasone propionate 250 mcg inh bd	14	Formoterol fumarate 4.5 mcg inh bd
	Lansoprazole 15 mg po od		Tiotropium 18 mcg inh od
	Warfarin 3 mg po od (variable dose)		
	Furosemide 20 mg po od		

Medications taken during the study period. Medications did not change for 8 weeks prior to the study or during the study period.

Figure 1 Changes in the concentrations of sputum sol phase mediators for one patient during the study period. Each data point represents the measured concentration of one mediator for a single patient on a single visit (in units as per figure legend). (A) Changes in raw data over 1 month for patient 1 for the sputum sol mediators IL-1β, TNFα and CXCL8. IL-1β concentrations are given on the right hand axis, TNFα and CXCL8 concentrations are given on the left hand axis. (B) Changes in raw data over 1 month for patient 1 for the sputum sol phase mediators MPO, LTB4, GROα and absolute neutrophil counts. MPO concentrations are given on the right hand axis, LTB4, GROα concentrations and absolute neutrophil counts (10^6/ml) are given on the left hand axis. IL, interleukin; TNF, tumour necrosis factor; CXCL8, interleukin 8; MPO, myeloperoxidase; LTB4, leucotriene B4; GRO, growth-related oncogene.

patient and table E2 (in the online supplement) describes the intra-patient variability for all mediators and sputum neutrophil and macrophage counts for each patient.

The variability in sputum inflammatory markers seen within patients was wide, irrespective of the inflammatory mediator or cell type measured. The median intra-patient CV for each mediator (calculated from each patient's CV) is shown in table 2. This variability was not related to any changes in individual symptoms as recorded in the patients' diary card scores, clinical features, sputum microbiology or lung function (all of which remained stable).

Reducing intra-patient variability

Table 3 compares the effects of a rolling mean on intra-patient variability for all patients and all mediators (expressed as CVs). When comparing a single mediator within patients, a significant reduction in variability of the raw data (as determined by the CV) was obtained using a 3-day rolling mean in all biomarkers except

for sputum neutrophil counts. A further reduction in variability was seen using a 5-day rolling mean in all biomarkers including sputum neutrophil counts. Figure 2A–C (available online) compares graphically the variability in mediator concentrations and the effect of rolling means for one representative patient.

Inter-patient variability and the effects of a rolling mean

There was marked variability in inflammatory biomarkers between patients with COPD using data from a single day. Using a 3-day or 5-day rolling means for each patient did not alter the inter-patient variability, which suggests that it relates to individual differences in the degree of airway inflammation.

When all mediators were considered together, the median variability between patients using daily data (expressed as the CV) was 101% (IQR 90–123%) and the median variability

Table E2 The within patient variability in pulmonary inflammatory mediators and cells seen in stable patients with severe COPD over one month

Patient		IL-1ß (pM)	TNFα (pM)	CXCL8 (nM)	MPO (mg/ml)	LTB4 (nM)	GROα (nM)	Neutrophils (10⁶/ml)	Macrophages 10⁶/ml
1	Median	606	72.2	14.27	7.2	91.2	29.1	4.8	0.09
	IQR	399–1104	66–141	10.9–16.1	5.34–9.5	35–120	24–33	1.8–13.4	0.06–0.17
	CV	11	11.3	15.9	32.9	16.9	16	77.3	31.8
2	Median	19.1	0.4	4.8	1.8	1.4	14.7	1.3	0.18
	IQR	9.3–26.6	0.19–0.69	3.85–6.68	1.24–1.8	0.85–1.96	12–20.4	0.4–1.95	0.14–0.34
	CV	19.6	87	25.4	162.5	194	9.6	471	37.1
3	Median	5.8	0.3	1.4	1.7	5.9	18.1	0.9	0.12
	IQR	3.4–6.6	0.2–0.6	1.2–1.5	1.4–1.9	5–6.5	16.6–19.7	0.7–1.4	0.07–0.13
	CV	30.3	45.3	85	61.2	22.2	12.3	804	44
4	Median	501	52.2	24.4	2.41	28	16.8	5.35	0.2
	IQR	417–865	48.5–68.8	19.8–27.5	1.6–4.5	20.5–42	14.7–18.7	2.5–7.4	0.13–0.42
	CV	8.3	6.6	20.7	84.3	13.5	9.5	44.9	51.7
5	Median	697	74.6	26	4.13	10.23	3.16	6.25	0.4
	IQR	375–775	33.2–105	18.4–43	2–7.2	7.4–23.4	1.5–3.8	5.2–25.9	0.26–0.81
	CV	10	31	13.9	74.3	25.1	96.8	44.8	81.7
6	Median	120.2	4.32	5.37	4.27	11.17	12.3	3.4	0.27
	IQR	65.9–343	0.4–42	2.5–6.9	3.4–6.4	2.2–17.6	10.2–16.5	2.2–5.9	0.16–0.3
	CV	21.2	159	44.3	35.4	47.9	18.5	38.7	30.4
7	Median	606	253	40.1	5.79	49.04	12.3	18.1	0.46
	IQR	504–1128	212–301	27.7–61.4	3.4–13.8	21.7–75.7	10.6–21.2	12.7–24.9	0.31–0.73
	CV	12	5.6	12.5	59.2	21.9	16.4	17.3	98.3
8	Median	5.1	0.1	0.33	1.15	0.95	4.87	0.4	0.03
	IQR	3.1–5.9	0.03–0.14	0.24–0.41	1.04–1.43	0.6–1.5	3.5–6.4	0.3–0.8	0.03–0.06
	CV	39.8	29.8	29	224	1299	189	92.5	31.3
9	Median	9.26	0.25	1.04	1.93	0.49	8.12	0.4	0.33
	IQR	3.5–43.4	0.02–1.1	0.5–2.6	1.1–2.6	0.32–0.7	4.7–9.8	0.2–1.6	0.17–0.65
	CV	55.5	113	633	70.5	159.4	20.6	236.5	86.7
10	Median	156.9	5.25	2.11	2.03	10.81	5.04	2.15	0.7
	IQR	88–417	3–10.4	1.7–3	1.9–2.4	7.1–15.1	3.9–5.3	0.95–12.6	0.31–2.07
	CV	19	37.4	49.2	22.7	27.9	11.6	181	152.4
11	Median	10.6	0.06	0.11	1.38	6.74	0.3	1.65	0.35
	IQR	7.5–11.8	0.04–0.2	0.06–0.14	1.2–2.6	3.8–11.7	0.1–0.4	1.1–2.2	0.09–0.68
	CV	11.4	29.2	24.3	85.7	32	54.1	92.4	104.7
12	Median	31	4.89	3.7	0.88	3.07	24	5.1	3.31
	IQR	18–41.9	2.7–10	2.8–4.3	0.6–1.1	2.3–7.6	21.4–28.4	4.1–6.7	2.3–3.9
	CV	16.3	87	32.7	186.7	111.2	4.4	20	44.5
13	Median	13.8	0.19	2.05	1.28	1.77	16.8	1.8	1.92
	IQR	6.7–30.8	0.1–0.3	1.3–3.7	1–1.4	0.9–1.8	7.8–21.8	0.8–5.3	0.28–2.56
	CV	31.2	29.3	73.8	107.9	111.8	4.4	178	1100
14	Median	3.12	0.16	2.03	0.78	0.49	19.7	2.65	1.12
	IQR	2–4.9	0.1–0.2	1.4–2.6	0.3–0.9	0.2–1.6	18.4–22	2.2–5.2	1–1.53
	CV	46.9	13.4	60.1	129.5	180	5.5	50.1	159.7

The median biomarker concentration (with corresponding inter-quartile range) is given for each patient. The co-efficient of variation (CV) for each patient was determined from log transformed data and is expressed as a percentage.

between patients using the first 5-day rolling mean was only slightly reduced to 91% (IQR 73–115%) (table 4).

Determining the sample size required to power interventional studies

Using a crossover design, the number of patients needed to confirm a 50% reduction in sputum neutrophil counts with 80% power and at the 5% significance level using a single data point per patient would be 72. However, if a 3-day mean is used, the number needed to confirm the same reduction would be 23, and 20 with a 5-day mean. For a parallel design, the number needed to confirm a 50% reduction in sputum neutrophil counts using a single data point would be 140 (70 per group), which would be reduced to 106 and 103 using a 3-day or 5-day mean, respectively.

Table E3 (available online) summarises the mean (SE) number of patients required to detect similar changes in other mediators

Table 2 Median intra-patient coefficient of variation (CV) for each mediator

Mediator	CV (%)	IQR
IL-1β (pM)	19.4	11–33
TNFα (pM)	30.3	13–88
LTB4 (nM)	39.9	22–165
CXCL8 (nM)	30.8	19–63
GROα (nM)	16.2	10–29
Absolute neutrophil count (10⁶/ml)	77.3	42–180
Absolute macrophage count (10⁶/ml)	74.2	41–102
MPO (mg/ml)	79.3	53–138

The median intra-patient CV is derived from each individual patient's CV. Each patient's mediator-specific CV was calculated using data from 11 sequential visits.
IL-1β, interleukin 1β; TNFα, tumour necrosis factor α; LTB4, leucotriene B4; CXCL8, interleukin 8; GROα, growth-related oncogene α; MPO, myeloperoxidase; IQR, interquartile range.

Table 3 Comparison of the median intra-patient coefficient of variation (CV) for each mediator using daily data, a 3-day rolling mean and a 5-day rolling mean

	IL-1β (pM)	TNFα (pM)	CXCL8 (nM)	MPO (mg/l)	LTB4 (nM)	GROα (pM)	Sputum neutrophils (10^6/ml)	Sputum macrophages (10^6/ml)
Raw data								
Median CV (%)	19.4	30.3	30.8	79.3	39.9	16.2	77.3	74.2
IQR	11–33	13–88	19–63	53–138	22–165	10–29	42–180	41–102
3-day rolling mean								
Median CV (%)	11.2*	20.6*	17.3*	44.5*	22.5*	7.6*	46.7	33.9*
IQR	6–17	8–50	7–23	20–60	10–96	4–11	13–218	26–59
5-day rolling mean								
Median CV (%)	6.9*†	12.2*†	9.5*†	22*†	13.8*†	4.1*†	13.4*†	20*
IQR	4–9	2–37	4–20	13–39	4–53	2.6–6.8	8–124	13–32

The median coefficient of variation (CV) has been derived from the marker-specific CV from each patient. Differences in CVs were assessed using the Friedman matched samples test and the Wilcoxon matched pairs signed rank test.
IL-1β, interleukin 1β; TNFα, tumour necrosis factor α; CXCL8, interleukin 8; MPO, myeloperoxidase; LTB4, leucotriene B4; GROα, growth-related oncogene α; IQR, interquartile range.
*Significant reduction in mean CV from raw data (p<0.05).
†Significant reduction in mean CV from 3-day rolling mean (p<0.05).

when a single sample is collected or when the mean of three or five consecutive samples is used for power calculations.

Relationship between sol phase inflammatory mediators and sputum cell counts

The strength of the correlations between mediators and cells (and the significance of these correlations) increased as more data points were used for each patient. Figure 3A–C (available online) demonstrates the correlations seen between sol phase LTB4 and sputum neutrophil counts when the first day's data were used for each patient (fig 3A) compared with the mean of the first 3 days (fig 3B) or all 11 days (fig 3C). Figure 4A–C (available online) demonstrates the correlations seen between IL-1β and the absolute sputum neutrophil count, TNFα and CXCL8, respectively, using the mean value for all 11 samples for each patient.

Tables E4 and E5 (available online) describe the correlations seen between all mediators and neutrophils using a similar approach. In general there was an increase in the correlation coefficient using the mean of 3 days' data compared with 1 day's data (mean difference in Pearson's correlation coefficient between 1 day's data and the mean of 3 days' data = 0.11 (95% confidence interval 0.04 to 0.18), p = 0.003), but there was no further increase in the strength of the correlation using the mean of more data points.

All biomarkers measured showed inter-relationships with each other of varying significance (see table E4 in the online supplement) with the exception of GROα which showed no correlation with neutrophils or any other mediator measured in sputum, apart from CXCL8.

Relationship between sol phase inflammatory mediators, cell counts and disease

Correlations were seen between inflammatory mediators and markers of disease but, as with the inter-relationships between mediators, the strength of the correlation (and the significance value) increased as more data points were used for each patient. Using the first day's data for each patient, only IL-1β correlated with body mass index (BMI; r = −0.5, p = 0.03). Using the mean of the first 3 days for each patient, significant correlations were seen between BMI and IL-1β (r = −0.5, p = 0.02) and TNFα (r = −0.48, p = 0.03). However, using the mean of 11 data points per person, significant correlations were present between BMI and IL-1β (r = −0.41, p = 0.05), TNFα (r = 0.4, p = 0.05) and LTB4 (r = −0.51, p = 0.03); smoking history and sputum neutrophils (r = −0.61, p = 0.008) and sputum macrophages (r = 0.46, p = 0.05); and forced expiratory volume in 1 s (FEV$_1$) and TNFα (r = −0.45, p = 0.04) and sputum neutrophils (r = −0.6, p = 0.01). Figure 5A–C (available online) summarises the correlation between FEV$_1$ and sputum neutrophil counts for a single sample (day 1) for each patient (fig 5A) compared with the mean for the first 3 days (fig 5B) or 11 days (fig 5C).

DISCUSSION

In this well characterised group of patients with GOLD stage III COPD and chronic bronchitis, marked variability in inflammatory mediators and cells was found, both between patients and within each patient over the course of a 4-week period. Given the heterogeneous nature of COPD, it is perhaps unsurprising that inflammatory biomarkers vary between individuals and, indeed, this finding is in agreement with a wealth of previously published data.[17 18]

The variability of the raw data within individuals was also marked, even in samples taken on consecutive days, and the fluctuations did not relate to changes in symptom scores, clinical findings or changes in FEV$_1$ (all of which remained stable). It is likely that the variability reflects changes in sampling methodology or sputum humidification over the study period rather than individual patients showing wide fluctuations in the daily inflammatory load of the lungs without this impacting on daily symptoms. It may be that only when changes in lung inflammatory load are sustained at a higher magnitude over consecutive days does it relates to changes in clinical status. This relationship has been demonstrated in patients with prednisone-dependent asthma where clinical indices deteriorate much later than the inflammatory changes in sputum.[19]

Sampling techniques vary between studies of inflammation in COPD, but the intra- and inter-patient variability of measured mediators is unknown for most methodologies. The variability of sequential bronchial biopsies appears significant despite this technique having the advantage of directly sampling airway
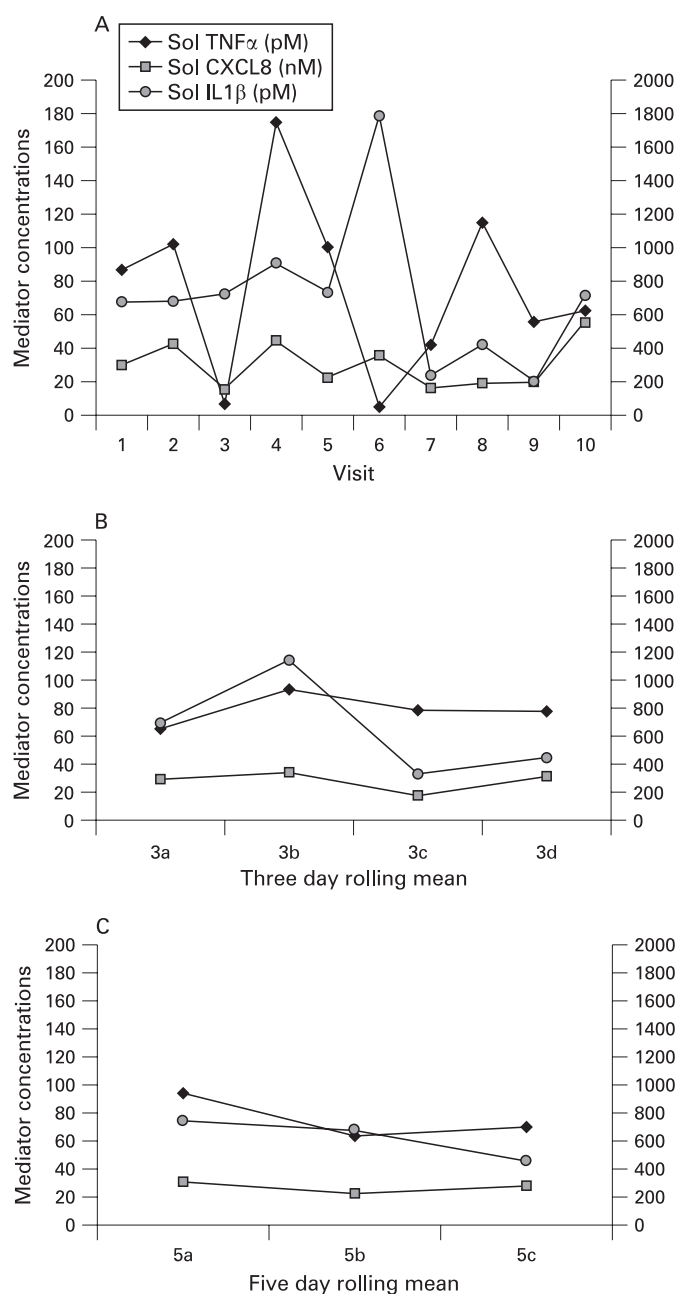
Figure 2 Comparison of intra-patient variability in three mediators using either data from 10 sequential visits or a rolling mean. For all figures, concentrations of interleukin 1β (IL-1β) are given on the right-hand axis and concentrations of tumour necrosis factor α (TNFα) and interleukin 8 (CXCL8) are given on the left-hand axis. (A) Changes in raw data over 1 month for a representative patient (patient 5) for the sputum sol phase mediators IL-1β, TNFα and CXCL8. Data points represent the measured concentration of one mediator for a single patient on a single visit (in units as per figure legend). (B) Changes in mediators over 1 month for patient 5 using a 3-day rolling mean. Data points represent the mean measured concentration of one mediator for 3 consecutive days for a single patient. A 3-day rolling mean was determined for each mediator and cell count in this patient by averaging the results from visits 1, 2 and 3 (rolling mean "3a" on x axis), then from visits 2, 3 and 4 (rolling mean "3b") etc, until the final three visits were incorporated into a rolling mean ("3i"). (C) Changes in mediators over 1 month for patient 5 using a 5-day rolling mean. Data points represent the mean measured concentration of one mediator for 5 consecutive days for a single patient. A 5-day rolling mean was determined for each biomarker in this patient by averaging the results from visits 1–5 (rolling mean "5a" on x axis), then from visits 2–6 (rolling mean "5b"), etc, until the final visit ("rolling mean 5g).

tissue.[20] To date, one study has provided data regarding the reproducibility of induced sputum: three samples taken at 4-weekly intervals showed no significant differences in neutrophil counts or CXCL8 concentrations in 12 patients with mild to moderate COPD.[21] The concentration of mediators in exhaled breath condensate (EBC) is often at levels close to the lower limit of detection (and often below the lower limit of quantification) for most assays, making accurate measurements difficult.[22] There is high variability in repeated measurements of EBC inflammatory indices,[23] which is likely to be dependent upon difficulty both in quantification as well as the dilution caused by water vapour. Bronchoalveolar lavage (BAL) fluid samples are also complicated by dilution from the lavage which is operator-dependent, variable and for which no correction factor has been determined.[8] Furthermore, the variability of biomarkers using BAL fluid is currently unknown because of practicalities of repeated sampling.

Spontaneous sputum collection is non-invasive and has no inflammatory sequelae to limit sequential sampling. Furthermore, spontaneous sputum samples are the only medium where an artificial dilution factor is absent.[8 24] However, there is still an inherent physiological dilution present in all lung secretions which will have an impact on the ability to perform accurate serial measurements.

Sputum is a mixture of lower airway and oropharyngeal secretions. Lower airway secretions are affected by exudation of plasma-derived proteins into the airway lumen[25] which occurs both when the epithelial mucosa is damaged[26] and intact, both structurally and functionally,[27] and presents a dilutional factor inherent in all sampling methodologies.

Oropharyngeal contamination can be reduced by patient education and confirmed by the percentage of squamous cells in the sample, which should typically be less than 10%. In our study, squamous cells accounted for less than 1% of cells seen in the samples, suggesting that oropharyngeal contamination was low. Although we took the precaution of asking patients to rinse their mouths prior to expectoration, the rinse itself or any remaining oropharyngeal secretions could still dilute the sputum samples. Indeed, this could explain some of the intra-patient variability seen day to day, and similar effects could influence the between-patient variability.

If mediator and cell values were overwhelmingly influenced by dilution, it would be predicted that mediator concentrations between patients would correlate well, as dilute samples would have low measured concentrations of mediators and concentrated samples would have high measured concentrations of mediators, even if all patients were in the same inflammatory state and mediator secretion was identical. The data for GROα, however, suggest that this is not the case. In our study GROα failed to correlate with any other mediator except CXCL8. This suggests, firstly, that the remaining correlations reflect different inflammatory states per patient and, secondly, that GROα does not reflect inflammatory differences between patients or the neutrophil content of airway secretions. GROα may be less important in the development or progression of COPD than other mediators. It is possible that GROα has a different spectrum of activity than previously suspected,[28] or that GROα is maximally expressed in COPD patients with chronic bronchitis irrespective of the degree of inflammation.[29] However, the fact that GROα does not correlate with other biomarkers provides sound evidence that the relationships seen between other mediators are not purely dilutional in nature. Furthermore, the correlations with systemic markers of disease (BMI and FEV1) and the use of repeated sampling (when any

Table 4 Comparison of the inter-patient coefficient of variation (CV) for each mediator using raw data or data manipulated using a 5-day rolling mean

	IL-1β (pM)	TNFα (pM)	CXCL8 (nM)	MPO (mg/ml)	LTB4 (nM)	GROα (nM)	Sputum macrophages (10⁶/ml)	Sputum neutrophils (10⁶/ml)	Median CV (IQR)
Median	43.3	2.07	3.6	1.8	6.1	12.3	0.29	2.65	
IQR	7.5–413	0.2–55	1.4–14.4	1.1–3.5	1.5–18.6	5–19.5	0.13–0.75	1–6.4	
CV calculated from raw data	53.5	407.8	145.4	102	99.8	60.9	100	115.4	101% (90–123)
CV calculated using 5-day rolling mean	52.15	234.6	131	92.15	78.2	58.5	110	89.4	91% (73–115)

The median biomarker concentration (and IQR) is calculated from all measurements for all patients. In order to calculate the inter-patient CVs, the mean logged concentrations were calculated for each biomarker in each patient (either using daily data or data from the first 5-day rolling mean).
IL-1β, interleukin 1β; TNFα, tumour necrosis factor α; CXCL8, interleukin 8; MPO, myeloperoxidase; LTB4, leucotriene B4; GROα, growth-related oncogene α; IQR, interquartile range.

variable dilutional factors would be minimised) also makes this explanation unlikely.

We reassessed marker variability within patients by the use of a rolling mean. This technique requires multiple data points in order to overcome fluctuations due to variable sample dilution. A 5-day rolling mean had the optimal effect on intra-patient variability. However, a 3-day rolling mean also reduced intra-patient variability and was able to demonstrate most of the key relationships between mediators. This supports its use as a technique for studying real changes in inflammation in interventional studies. Two 3-day collections of spontaneous sputum (pre- and post-intervention) in each patient would provide a sound basis for interpreting inflammatory changes following treatment.

Simple power calculations were undertaken to assess the effect of single or multiple data points on sample size in a crossover or parallel interventional study design. In a putative trial where a single uncorrected data point is taken for each patient both before and after intervention, our calculations suggest that fewer patients would be needed to see a 50% reduction in mediators and cells using spontaneous sputum than would be needed to detect the same reduction in mediator-positive cells in bronchial biopsies.[30] The numbers needed can be reduced dramatically by using three data points, with a small further reduction with five data points. These results were not altered if data from three separate days were used (eg, days 123, 456, 789, etc) compared with overlapping days (eg, days 123, 234, 345) which could be influenced by repeated inclusion of a single day.

The wide inter-patient variability is determined from a relatively small cohort of patients. It is possible that others

may have inflammation vastly outside the range documented here which would affect the power calculations for a parallel study. However, the single-day data are consistent with stable state results in our previous larger studies of over 100 patients with a wide range of FEV_1,[4 7 31 35] suggesting that the current data are representative.

By choosing spontaneous sputum, we limited our recruitment to patients with chronic bronchitis. Although there remains some controversy as to the importance of sputum production in COPD, a body of evidence is emerging that suggests that chronic bronchitis may be a predictor of mortality and morbidity independent of FEV_1. Previous work has concluded that the inflammatory burden is higher in patients with chronic bronchitis than in matched patients without chronic bronchitis.[31] Furthermore, chronic mucus hypersecretion is consistently associated with both an excess decline in FEV_1 and an increased risk of subsequent hospitalisation because of COPD;[32] mucus hypersecretion is associated with death from respiratory infections.[33] Given the importance of this symptom in COPD and its relationship with inflammation, it is important to understand the relationships between inflammatory mediators in this group of patients. Studying spontaneous sputum samples prevents comparison with healthy controls, which is a limitation of the current study. However, it has been established that the mediators and cells measured in the current study are raised in patients with COPD compared with healthy controls in studies using induced sputum.[5–7] Furthermore, the aims of the current study were to document the variability of measured mediators and their inter-relationships in spontaneous sputum samples from patients with COPD and chronic bronchitis. A further limitation is that effective

Table E3 A comparison of the numbers needed in a cross-over study design when one day's data, the mean of three days' data or the mean of five days' data are used for calculations

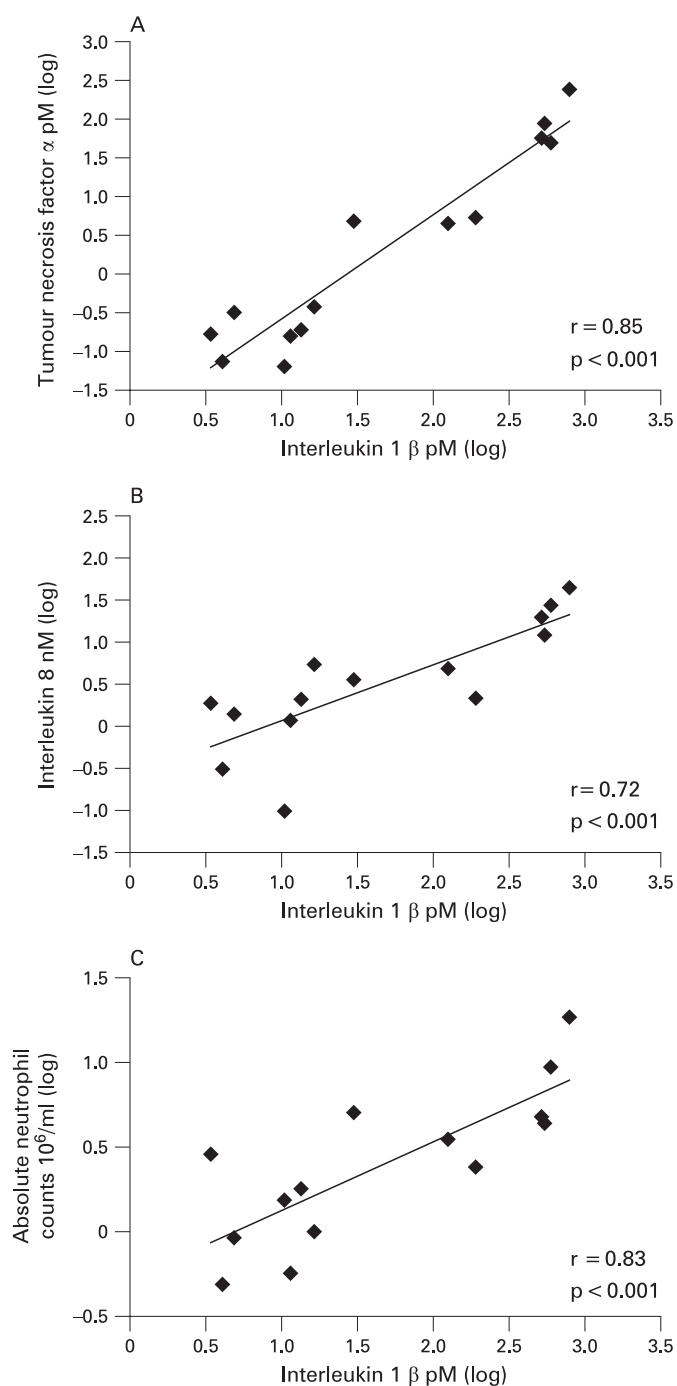|		IL-1β	TNF α	CXCL8	MPO	LTB4	GRO α	Absolute macrophage counts	Absolute neutrophil counts
1 days data	Mean number of patients	10	330	61	62	41	20	52	72
	SEM	0.76	74.72	7.14	15.90	4.79	4.95	11.2	11.11
3 days data	Mean number of patients	2	23	7	16	7	5	12	23
	SEM	0.73	9.08	1.93	4.01	2.13	1.94	1.7	6.69
5 days data	Mean number of patients	2	17	5	13	7	5	6	20
	SEM	0.07	3.12	0.30	1.49	0.45	1.34	0.6	2.25

Numbers required to provide an 80% chance of detecting a 50% decrease in mediators or cells at the 5% level of significance. For 1 day's data, power calculations were performed for each mediator on each visit, and the mean of this is presented (with the standard error of the mean (SEM). For three days' data; power calculations were performed on the first three days (visit 1 to 3), then the next three days (visit 2 to 4) until visit 9–11. These were used to calculate the mean number of patients required. For five days' data; power calculations were performed on the first five days (visit 1 to 5), then the second five days (visit 2 to 6) until visit 7 to 11. These were used to calculate the mean number of patients required.
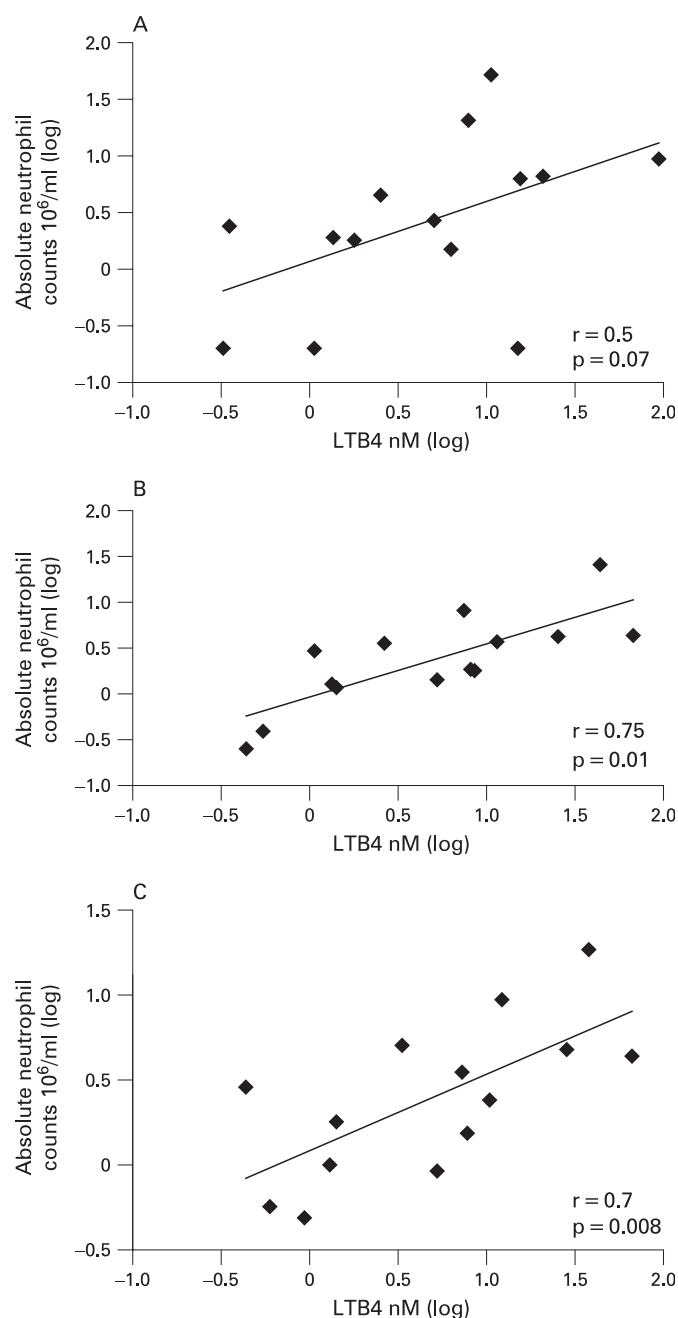
Figure 3 Relationship between sputum sol leucotriene B4 (LTB4) and absolute sputum neutrophil counts using (A) the first day's data for each patient, (B) the mean of the first 3 days' data or (C) the mean of 11 sequential days. In all graphs the data have been log-transformed and the correlation is described using Pearson's correlation coefficient (r) together with the significance of the relationships (p). (A) Data points represent the sputum neutrophil count and sol phase LTB4 concentration for each patient on the first visit. (B) Data are shown for individuals using the mean of the first three samples. (C) Data are shown for individuals using the mean of all 11 samples.

Figure 4 Relationship between sputum sol phase interleukin 1β (IL-1β) and (A) tumour necrosis factor α (TNFα) (B) interleukin 8 (CXCL8) and (C) absolute sputum neutrophil counts. In all graphs the data have been log-transformed. Each point represents the mean of 11 sequential samples for each patient. The correlation is described using Pearson's correlation coefficient (r) together with the significance of the relationships (p).

anti-inflammatory interventions may reduce the ability of the subject to produce spontaneous samples, but this in itself would be proof of efficacy and would be likely to reflect a major reduction in inflammation.[31]

The current study demonstrated clear inter-relationships between most of the measured inflammatory mediators which were enhanced by using the mean mediator concentration for

each patient. Previous studies have reported indirect evidence that some biomarkers may be involved in the pathogenesis of COPD, including the presence of higher concentrations of mediators in disease compared with matched controls and the increases seen during exacerbations.[5 17 28 30 34 35] This study is the first to describe the relationships seen between several biomarkers and the strength of these correlations in lung secretions. IL-1β and TNFα, in particular, correlated strongly

Table E4 Correlations between mediators

Mediator or cell line	Data used for correlations PCC (p)	LTB4	CXCL8	IL-1ß	TNFα	MPO	GROα
LTB4	1 day		0.57 (0.051)	0.78 (0.002)	0.87 (<0.001)	0.65 (0.006)	0.12 (0.7)
	3 days		0.66 (0.01)	0.84 (<0.001)	0.84 (<0.001)	(0.6 (0.012)	0.16 (0.6)
	5 days		0.5 (0.07)	0.8 (<0.001)	0.77 (0.002)	0.62 (0.011)	0.01 (0.99)
	11 days		0.54 (0.03)	0.8 (<0.001)	0.82 (<0.001)	0.58 (0.02)	0.03 (0.9)
CXCL8	1 day	0.57 (0.051)		0.85 (<0.001)	0.84 (<0.001)	0.61 (0.01)	0.22 (0.5)
	3 days	0.66 (0.01)		0.84 (<0.001)	0.83 (<0.001)	0.5 (0.07)	0.23 (0.5)
	5 days	0.5 (0.07)		0.75 (0.001)	0.8 (<0.001)	0.51 (0.04)	0.6 (0.03)
	11 days	0.54 (0.03)		0.72 (<0.001)	0.83 (<0.001)	0.4 (0.12)	0.56 (0.02)
IL-1ß	1 day	0.78 (0.002)	0.85 (<0.001)		0.84 (<0.001)	0.34 (0.2)	0.1 (0.8)
	3 days	0.84 (<0.001)	0.84 (<0.001)		0.96 (<0.001)	0.4 (0.1)	0.06 (0.9)
	5 days	0.8 (<0.001)	0.75 (0.001)		0.87 (<0.001)	0.4 (0.1)	0.15 (0.6)
	11 days	0.8 (<0.001)	0.72 (<0.001)		0.85 (<0.001)	0.31 (0.24)	0.13 (0.6)

Data describes the relationships between mediators and cell counts when one days data is used (here, the first day), or the mean of the first 3, 5 or all 11 days' data. Relationships are expressed as Pearsons Correlation Co-efficient (PCC) and p value.

with all the other measured biomarkers (with the exception of GROα) and neutrophils, indicating an intimate relationship with inflammation.

Using mean results for each patient, this study also demonstrated clear relationships between inflammatory biomarkers and other markers of disease including the FEV_1 (in litres), smoking history and BMI. Many mediators have been found to be raised in COPD, but it is unlikely that all will be central to the pathogenesis of the disease. FEV_1 is the most consistent single predictor of survival and quality of life in COPD,[36] and the relationships described between TNFα, absolute sputum neutrophil and macrophage counts and both FEV_1 and smoking history is highly suggestive that these cells and TNFα are of paramount importance in the pathogenesis of airflow obstruction. Indeed, recent studies suggest that TNFα is associated with the development of emphysema,[37] and there is a wealth of data supporting the central role of macrophage and neutrophil proteinases in COPD.[38] It is recognised that weight loss and low body weight are common in COPD, and that low body weight is associated with increased mortality independent of lung function.[39 40] Further studies have suggested that loss of muscle mass is the most important component of weight loss in COPD, and it is this which affects patients functionally.[41] In the current study, IL-1β, TNFα and LTB4 correlated negatively with BMI, supporting the hypothesis that an increase in lung inflammation is associated with a lower body weight. Previous studies have linked a low BMI to systemic inflammation,[42] but this is the first study to describe the relationship between multiple biomarkers measured in sputum and BMI.

This again provides evidence that these biomarkers may be of importance in COPD and its associated systemic morbidity and supports the concept that wide inter-patient variability is a true finding of inflammatory differences between patients.

Serial measurement of biomarkers enabled us to study their relationships using either 1 day's data or the mean of data from 3, 5, 7 or 11 days for each patient. Given the variability of the raw data, it is perhaps unsurprising that correlations were not always present when 1 day's data were examined, although there was still a significant correlation between some mediators (see tables E4 and E5 in the online supplement). However, in general, using the mean of 3 days' data demonstrated the same relationships and provided the same strength of correlations as using the mean of 11 days' data. This is of importance as it has implications for studies designed to assess the relationships between biomarkers and, perhaps more importantly, biomarkers and clinical measures or outcomes. We have shown that potentially important relationships can be missed if only one data point is used, and that the use of at least three data points increases the likelihood of identifying these relationships while the addition of further data points continues to reduce within-patient variability.

In conclusion, this study documents the variability in mediator concentration both between and within patients with stable severe COPD. Most of this variability is likely to be due to dilution effects, and intra-patient variability is reduced significantly by sequential biomarker measurement which minimises this variation irrespective of alterations in the inflammation signal. This technique would therefore be ideal

Table E5 Correlations between cells and mediators

Mediator or cell line	Data used for correlations PCC (p)	Sputum neutrophils	LTB4	CXCL8	TNFα	MPO	IL-1ß	GROα
Sputum neutrophils	1 day		0.5 (0.07)	0.6 (0.02)	0.46 (0.09)	0.18 (0.53)	0.60 (0.02)	0.13 (0.7)
	3 days		0.75 (0.01)	0.8 (<0.001)	0.84 (<0.001)	0.2 (0.5)	0.77 (0.001)	0.16 (0.6)
	5 days		0.61 (0.01)	0.66 (0.005)	0.71 (0.003)	0.2 (0.5)	0.77 (0.001)	0.3 (0.36)
	11 days		0.7 (0.008)	0.67 (0.004)	0.81 (<0.001)	0.13 (0.64)	0.83 (<0.001)	0.25 (0.4)
Sputum macrophages	1 day	0.38 (0.17)	0.006 (0.9)	0.1 (0.7)	0.08 (0.8)	−0.35 (0.22)	−0.08 (0.8)	0.08 (0.8)
	3 days	0.45 (0.1)	0.05 (0.89)	0.22 (0.44)	0.09 (0.73)	−0.37 (0.2)	0.09 (0.73)	0.22 (0.44)
	5 days	0.29 (0.3)	−0.15 (0.6)	0.11 (0.7)	0.05 (0.8)	−0.14 (0.6)	0.07 (0.8)	0.16 (0.6)
	11 days	0.4 (0.12)	−0.19 (0.5)	0.19 (0.51)	0.09 (0.8)	−0.39 (0.16)	0.03 (0.9)	0 (0.9)

Data describes the relationships between mediators and cell counts when one days data is used (here, the first day), or the mean of the first 3, 5 or all 11 days' data. Relationships are expressed as Pearsons Correlation Co-efficient (PCC) and p value.
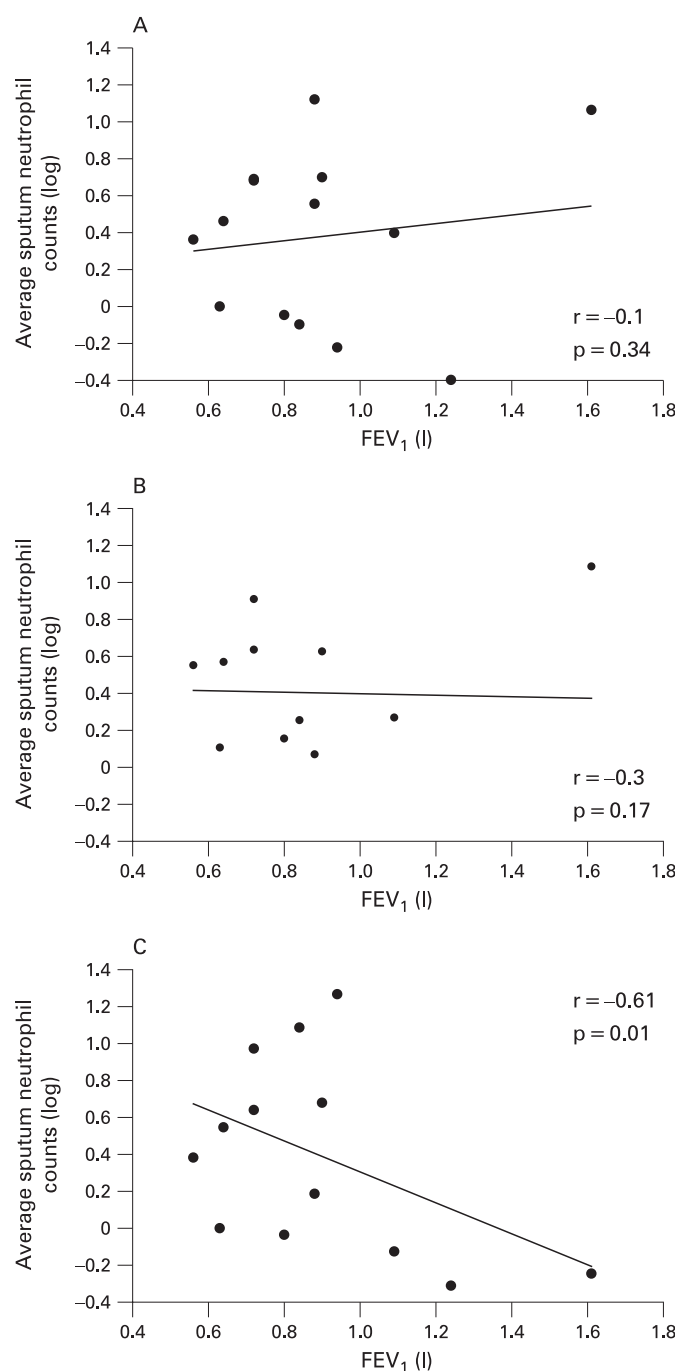
Figure 5 Relationship between forced expiratory volume in 1 s (FEV$_1$) and (A) absolute sputum neutrophil counts using the first day's data for each patient, (B) the mean of the first 3 days' data or (C) the mean of 11 sequential days. In all graphs the sputum neutrophil counts have been log-transformed and the correlation is described using Pearson's correlation coefficient (r) together with the significance of the relationships (p). (A) Data points represent the FEV$_1$ and sputum neutrophil count for each patient on the first visit. (B) Data are shown for individuals using the mean of the first three samples. (C) Data are shown for individuals using the mean of all 11 samples.

for studying inflammatory change in COPD and can be used to power short-term proof of concept studies.

The study demonstrates the positive correlations that exist between sputum inflammatory mediators, neutrophil and macrophage counts, and these biomarkers and other markers of disease severity. By the use of sequential measurements, we have shown that one measurement alone may be insufficient to identify all relationships between mediators. Further sequential sampling reduces the within-patient variability, and using three samples is both reasonable and dramatically reduces the numbers required for both parallel and crossover interventional studies.

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