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Original Article

Sputum and serum calprotectin are useful biomarkers during CF exacerbation

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Abstract

Background: Adequate monitoring of cystic fibrosis lung disease is difficult. CF exacerbation offers a unique setting to test the utility of biomarkers in the assessment of changing airways inflammation. We hypothesised that levels of calprotectin in sputum (and serum) would change informatively following treatment of an exacerbation.
Methods: 27 patients with CF were recruited at onset of pulmonary exacerbation. Sputum and serum were collected at the start and end of antibiotic therapy. Sputum calprotectin, interleukin-8 (IL8), and myeloperoxidase (MPO) were measured, as were serum calprotectin, CRP and vascular endothelial growth factor (VEGF).
Results: Sputum calprotectin decreased following treatment of an exacerbation ($p<0.05$), and was superior to other sputum markers. Serum calprotectin, CRP, and VEGF also decreased significantly ($p=0.002$, $p=0.002$, $p=0.013$ respectively). Serum calprotectin level following treatment had predictive value for time to next exacerbation ($p=0.032$).
Conclusions: This study demonstrates the superiority of calprotectin (in sputum and serum) as a biomarker of CF exacerbation over better-established markers.

1. Introduction

Cystic fibrosis (CF) lung disease is characterised by chronic bacterial infection which begins in early childhood, is persistent throughout life and rapidly evolves to evade host defence systems [1]. Patients with CF experience recurrent episodes of increasing pulmonary symptoms, termed exacerbations, which are accompanied by a decrease in lung function [2]. Viral infections, including respiratory syncitial virus, may initiate pulmonary exacerbation [3] but an increase in the density of colonising organisms [4] or the acquisition of new pathogenic organisms [5] are also important and as such antibiotic therapy decreases the bacterial density of respiratory secretions [6,7].

A major difficulty in studying the aetiology and pathophysiology of CF exacerbations is the lack of consensus for diagnostic criteria despite a definite clinical need [8]. Exacerbation has been defined in major CF therapeutic trials from empirical data [9–11]. Nevertheless in routine practice clinical judgement and changes in lung function are most commonly used to dictate the need for therapy [11]. Irrespective of definition, CF exacerbation represents an *in vivo* state of increasing inflammation in CF lung disease.

Sputum obtained from CF subjects contains a mixture of proteins which may serve as objective measures of lung inflammation. Interleukin 8 [IL-8] [11–16], myeloperoxidase [MPO] [17–20], matrix metaloproteinase 9 [MMP-9] [21] and neutrophil elastase [NE] [13,16,22] have all been advocated and studied. NE and IL-8 correlate inversely to lung function, suggesting a relationship of sputum markers to disease severity [23]. We have recently described calprotectin (also known as calgranulin A/B, S100A8/A9, MRP8/14, CF antigen) in BALF [24] and sputum [25] as a biomarker of CF lung disease. Calprotectin was first described in the serum of CF patients in 1975[26], and later became known as CF antigen[27]. In spite of being present in CF lung secretions in high concentrations, the function of calprotectin in the CF lung and its mechanism of action have yet to be explored. Calprotectin is highly abundant in neutrophils, has pro-inflammatory properties via activation of

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TLR-4[28], and has been demonstrated as central to lung inflammation in non-CF models of lung infection[29].

Sputum protein profiles [17] and cytokine levels such as IL-8 have been demonstrated to change following treatment of CF exacerbations with antibiotic therapy [6,7], although this is not a consistent finding in all studies[30]. Altering the level of bacterial burden in the lung with antibiotic therapy may alter the inflammatory milieu. Thus we may use this model to study the clinical significance of new markers of CF lung disease. Other groups have used exacerbation in CF to demonstrate the presence of novel biomarkers, for example prostaglandin E_2 and cys-leukotrienes, mediators of oxidative stress, were elevated in CF exacerbation compared to stable CF [31] as was HMGB1[32], although these studies did not utilise serial samples in the same patients. Serial monitoring has been used for serum vascular endothelial growth factor, which appears to be a marker of inflammatory change following treatment of CF exacerbation with antibiotic therapy [33].

On the basis of our previous work with calprotectin [24,25] we hypothesised that calprotectin (sputum and serum) would change informatively following treatment of CF exacerbation. We wished to compare its utility to previously assessed biomarkers, sputum IL-8 and serum CRP and VEGF.

2. Methods

The Lothian Hospitals Ethics Committee granted approval for this study. Patients were recruited at the time of a pulmonary exacerbation requiring antibiotics, as determined by the patient's physician on the basis of increased breathlessness, increased sputum production and a decrease from baseline FEV_1. Sputum and serum were collected for the assessment of biomarkers within 24 hrs of commencing treatment with antibiotics and again at cessation. This ranged from one to three weeks of therapy. FEV_1 was recorded at these time points.

Sputum was processed within 2 hours of collection as described previously [34]. In brief, sputum plugs were harvested and processed with 4x weight/volume 0.1% dithiothreitol (DTT) after which 4x weight/volume PBS was added. Samples were filtered through 48 μm mesh and centrifuged at 1200 rpm to remove the cells. Supernatant was stored at -80 °C until further analysis. The cell pellet was re-suspended in PBS, cytospins prepared and stained with May-Grunwald-Giemsa for differential cell counting. All counts were expressed as percentage of the population counted. All samples utilised in the study contained <40% squamous cells ensuring samples were from the lower airway.

Table 1
Patient Demographics, colonising organism and antibiotic treatment.

Patient	M/F	Genotype	Age	FEV1% Pred Start	FEV1% Pred End	Colonising Organism	Treatment For Exacerbation
1	f	ΔF508/3659ΔC	20	67	66	BC, SM, HI, SA	TO, CFZ
2	f	ΔF508/ΔF508	30	42	49	PA, SA	TO, CFZ
3	f	ΔF508/ΔF508	20	13	13	PA	CO, MER
4	f	ΔF508/ΔF508	18	55	60	PA	TO, CFZ
5	f	ΔF508/G551D	18	23	39	BC	UnK
6	m	ΔF508/Unk	46	36	38	PA, SM	TO, CFZ
7	f	ΔF508/ΔF508	21	56	59	PA, SA	TO, MER
8	m	ΔF508/ΔF508	18	58	59	MRSA, SA	TO, CFZ
9	f	ΔF508/G551D	31	31	49	PA	CFZ, CIP
10	m	ΔF508/ΔF508	18	33	43	SA, SM	TAZ, MIN
11	f	ΔF508/ΔF508	20	60	73	PA, Asp	TO, MER
12	m	ΔF508/Unk	32	75	88	PA	CIP, AZI
13	f	ΔF508/P67L	27	45	49	SA, BMV	TO, CFZ
14	f	ΔF508/G542X	23	50	82	SA, HI	TO, CFZ
15	f	ΔF508/ΔF508	22	66	75	PA, SA	CO, CFZ
16	f	ΔF508/Unk	17	38	44	SA	FL, COAMOX
17	m	ΔF508/G542X	22	56	52	PA	TO, MER, AZ
18	m	ΔF508/G551D	41	24	31	SM, PA, SA. Asp	TO, MER
19	m	ΔF508/ΔF508	24	41	49	SA, PA, SM	TO, CFZ
20	m	ΔF508/ΔF508	37	21	27	BC, PA	TO, MER
21	f	ΔF508/ΔF508	22	45	52	PA	TO, MER
22	f	ΔF508/ΔF508	18	15	15	PA, SA	AZ, MER
23	m	ΔF508/G551D	20	28	28	PA	TO, CFZ
24	f	ΔF508/ΔF508	26	61	64	PA, SM, Asp	TO, CFZ
25	m	ΔF508/ΔF508	17	23	28	PA	CO, MER
26	m	ΔF508/ΔF508	17	33	45	PA, SA	TO, CFZ
27	m	ΔF508/3849+10 kb C→T	22	34	49	PA	TO, CFZ

Unk = unknown.

Colonising organisms relate to most recent sputum culture prior to exacerbation recorded for each patient. PA=*Pseudomonas aeruginosa*, BC=*Burkholderia cenocepacia*, BMV=*Burkholderia multivorans,* SA=*Staph aureus*, SM=*Stenotrophomonas maltophillia*, HI=*Haemophilus influenzae*, Asp=*Aspergillus fumigatus*. Treatment for exacerbation was with intravenous antibiotics apart from subject 12 who received oral treatment. AZ=aztreonam, AZI=azithromycin, CIP=ciprofloxacin, CFZ=ceftazidime, CO=colomycin, COAMOX=coamoxiclav, FL=flucloxacillin, MER=meropenem, MIN=minocycline, TAZ=tazobactam/pipericillin, TO=Tobramycin.

Blood was collected into serum tubes with pre-added clotting activator (Monovette serum collection tubes, Sarstedt AG and Co, Germany). The tube was then mixed by inverting 5 times. Blood was left to clot at room temperature for 45 minutes. Tubes were centrifuged at 1800×*g* for 15 minutes at room temperature. Separated serum was removed into cryovials (Nunc, Thermo Fisher Scientific, Denmark) as above and stored at -80 °C until further analysis. A separate EDTA blood sample was taken for routine haematology (white cell count).

Calprotectin was measured in sputum and serum by a double antibody sandwich ELISA, using monoclonal and polyclonal antibodies against human calprotectin complex (gift of Erling Sundrehagen, Norway). Interleukin 8 (Biosource, UK); myeloperoxidase (Assay Designs, Michigan, USA); CRP; and VEGF (Quantikine, R and D Systems, Oxford, UK); were measured using commercial kits according to the manufacturers' instructions. All standard curves and dilutions for sputum ELISAs were performed in the presence of 0.05% DTT to ensure accurate measurement of mediators in sputum as samples had been processed with DTT.

2.1. Prediction of future exacerbations

To investigate whether serum calprotectin at the end of exacerbation could predict patient outcome the clinical case notes were reviewed 1 year following completion of the study and the time to next exacerbation calculated in days. A cut off 9.1 μg/ml (median value in stable non-exacerbating CF subjects) was employed and this divided the group into 13 (<9.1 μg/ml) and 12 (>9.1 μg/ml) patients. The same analysis was performed for CRP using a cut off level of 10 mg/ml (upper limit of normal).

2.2. Statistical analyses

Data analyses were performed with GraphPad Prism software (GraphPad, La Jolla, Ca, USA). Normally distributed data were analysed by paired t test and non-normally distributed data by Wilcoxon sign rank test. Kaplan Meier curves were compared by log rank (Mantel Cox) testing.

3. Results

Twenty-seven patients completed the study (demographics in Table 1). FEV_1 improved over the course of an exacerbation, increasing from 41.8 (SEM 3.2) to 49.1 (3.6)% predicted ($p=0.001$). Whole blood white cell count decreased from 11.8 (SEM 0.9) to 9.0 (1.5) ($p=0.004$) and sputum neutrophils from 98.8% to 97.5% ($p=0.04$), see Table 2.

3.1. Sputum results

Due to limitations in sputum sample size not all patients could be assessed for all biomarkers (priority was given to sputum calprotectin which was measured in all 27 paired samples). There was a significant reduction in the level of calprotectin from median 619.4 (IQ range; 484.1- 971.9) μg/ml to 274.4 (184.0-570.9) μg/ml ($p=0.013$; Fig. 1). Sputum IL8 and MPO were measured in 26 paired samples (Table 2). Sputum IL8 showed a trend to decrease following treatment, from median 30.8 (18.8-53.5) ng/ml to 20.6 (10.3-60.6) ng/ml ($p=0.11$). Sputum MPO showed a trend to decrease following treatment, from median 41.3 (18.6-49.8) μg/ml to 24.4 (8.8-45.5) μg/ml ($p=0.07$).

Table 2
Measurements taken at the start and end of exacerbation treatment.

Measurement	Start of Exacerbation	End of Treatment
FEV_1% predicted	41.8(3.2)	49.1(3.6) **
Sputum Calprotectin μg/ml	619.4 (484.1- 971.9)	274.4 (184.0-570.9) *
Sputum IL8 ng/ml	30.8(18.8-53.4)	20.6(10.3-60.5)
Sputum MPO μg/ml	41.3(18.6-49.8)	24.4(8.8-45.5)
Sputum Neutrophil %	98.8(97.2-99.6)	97.5(95.6-98.7) *
WCC 10^9	11.8 (0.9)	9.0(1.5) **
CRP mg/ml	35.6(8.6-75.2)	9.9(3.0-23.5) **
Serum Calprotectin μg/ml	21.5 (13.3-55.5)	9.3 (6.5-18.2) **
Serum VEGF	385 (226- 582)	236 (143- 412) *

Data are displayed as median (IQR) or mean (SEM) depending on normality of distribution. Paired analysis was performed to investigate which markers changed most significantly with treatment, for exact p values please see text.
* $p<0.05$.
** $p<0.01$.

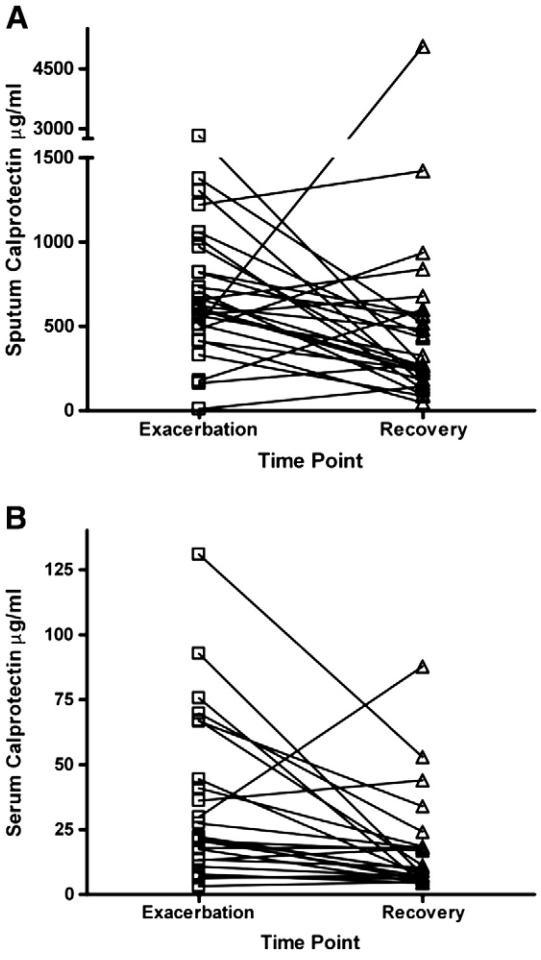

Fig. 1. Panel A. Sputum calprotectin decreases over the course of an exacerbation following treatment with antibiotics; $p=0.013$. Individual data are shown for 27 subjects. Panel B. Serum calprotectin decreases over the course of an exacerbation following treatment with antibiotics; $p=0.002$. Individual data are shown for 25 subjects.

3.2. Serum results

Serum was available in 25 patients from 27 recruited as two declined venepuncture. Serum calprotectin decreased from median 21.5 (13.3-55.5) μg/ml to 9.3 (6.5-18.2) ($p=0.002$; Fig. 1). Serum CRP decreased from median 35.6 (8.7-92.0) mg/ml. to 9.9 (3.0-23.5) ($n=22$ paired samples [3 single samples were above the limit of detection of the assay: 300 mg/ml]; $p=0.002$; Table 2). Serum VEGF decreased from median 385 (226- 582) pg/ml to 236 (143- 412) ($p=0.013$; Fig. 2).

3.3. Significant correlations

Serum calprotectin was negatively correlated with FEV_1 (Spearman r -0.49 [$p<0.012$] pre-treatment vs. -0.38 [$p=0.056$] post-treatment), giving an overall Spearman r of -0.48 ($p=0.0004$) for calprotectin and FEV_1 before and after exacerbation treatment. Serum CRP correlated less well with lung function (Spearman r -0.32 [$p=0.12$] pre-treatment vs. -0.26 [$p=0.21$] post-treatment), giving an overall Spearman r of -0.36 ($p=0.011$) for CRP and FEV_1 before and after exacerbation treatment. Sputum calprotectin did not significantly correlate with lung function.

3.4. Predictive values of serum markers

The median time to exacerbation in patients with calprotectin >9.1 μg/ml was 70 days compared to 112 days in the <9.1 μg/ml group ($p=0.032$; Fig. 4). Three patients in the >9.1 μg/ml group died within 18 months of their final study visit. CRP failed to show a difference in the median time to next exacerbation 81 (<10 mg/ml) vs. 84 (>10 mg/ml) days ($p=0.12$; Fig. 3).

4. Discussion

We have demonstrated that treatment of an exacerbation with antibiotic therapy in CF results in decreasing levels of sputum and serum calprotectin. Serum CRP and VEGF also decreased. We have also demonstrated a predictive value of serum calprotectin at the end of exacerbation treatment for time to next exacerbation.

Sputum calprotectin decreased following treatment of a CF exacerbation. We have previously demonstrated high levels of calgranulins A and B (the constituent subunits of calprotectin), by mass spectrometry, in CF sputum and BALF[24,25]. Calprotectin may be secreted from stimulated neutrophils [35], or released at cell death [36] and as such is an appropriate marker for inflammation in the CF airway. Faecal calprotectin has been recognised as a marker of organic bowel disease [37] and can differentiate inflammatory bowel disease, which is neutrophil predominant, from irritable bowel syndrome [38]. Calprotectin may play an important mechanistic role in the CF airway and has been previously implicated in early lung disease in animal models [39]. Furthermore functional knock out of calprotectin in a murine model of pneumonia leads to decreases in inflammatory cell recruitment suggesting an integral role in inflammatory cell recruitment [29]. Thus the change in sputum calprotectin following antibiotic therapy implies a direct association of calprotectin with a changing state of airways inflammation. The exploration of a possible role of calprotectin as a pro-inflammatory molecule in the lung requires further work.

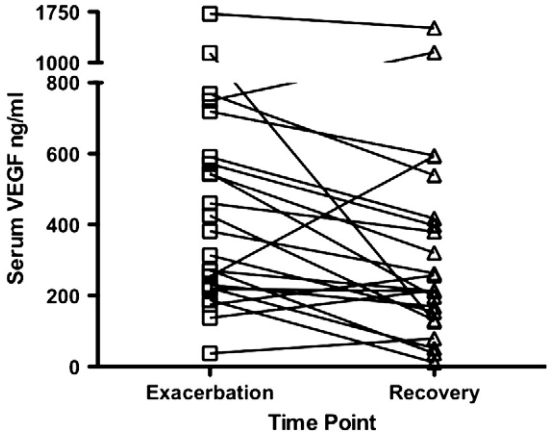

Fig. 2. Serum VEGF decreases over the course of an exacerbation following treatment with antibiotics; $p=0.013$. Individual data are shown for 23 subjects.

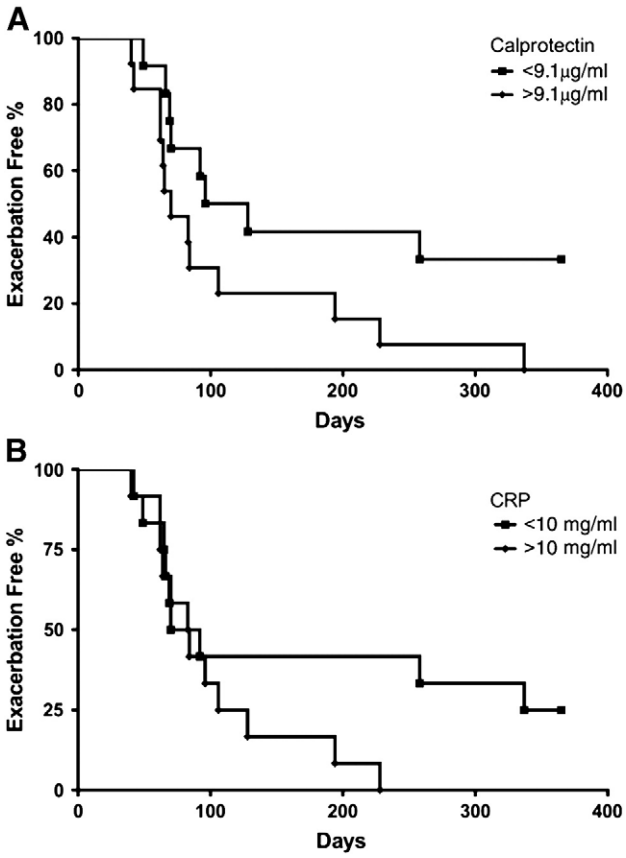

Fig. 3. Panel A. Subjects with serum calprotectin of 9.1 mg/ml at the end of exacerbation treatment have longer time to next exacerbation with median time to exacerbation 112 days vs. 70 days ($p=0.032$). Panel B. Serum CRP of <10 mg/ml did not differentiate time to next exacerbation with median time to exacerbation of 81 vs. 83.5 days ($p=0.125$).

In this study we failed to demonstrate a significant change in sputum IL-8 or MPO following antibiotic therapy, although there were trends to reduction. Decreases in sputum IL-8 following IV antibiotic treatment have been described [6,7], with similar findings reported following nebulised antibiotic therapy [40]. Our study was performed using spontaneously expectorated sputum in line with both Colombo et al [7] and Husson et al [40], but in contrast to Ordonez et al [6], who induced sputum. We do not believe our study was underpowered to demonstrate a change in sputum IL-8 (a secondary outcome), as other groups have demonstrated changes in sputum IL-8 with similar sized patient cohorts [7,40]. The largest study demonstrating changes in sputum IL-8 following antibiotic therapy was performed by Ordonez et al, utilising 42 paired samples and demonstrating a modest decrease in sputum IL-8 (0.5+/- 1.3 log10 pg/ml) [6]. Therefore even if our study was underpowered to detect changes in IL-8 (which we feel unlikely) we have clearly demonstrated the superiority of sputum calprotectin measurement in this population. One possible explanation for our failure to demonstrate a decrease in IL-8, is that our study utilised an adult population with more severe disease compared to Ordonez et al who excluded patients with an FEV_1 of less than 40% [6]. Indeed Downey et al demonstrated no serial change in sputum IL-8 in CF adults following exacerbation treatment, further underlining the possibility that sputum IL-8 is not as powerful a marker in the older patient group[30]. This suggests that IL-8 is a less reliable marker in patients with more advanced lung disease and is consistent with the finding that sputum IL-8 is less well correlated to lung function that other sputum markers such as free elastase [23].

Sputum MPO has been less well studied. As a neutrophil protein we might have expected a change in sputum concentrations following treatment of an exacerbation, and it has been described at high levels in CF sputum compared to control populations [17–20]. The failure to demonstrate a significant change in MPO may be explained by many of the points pertinent to IL-8. And our findings are consistent with a study of oral macrolide antibiotics in CF patients infected with *P. aeruginosa,* which demonstrated no change in sputum MPO following treatment [41]. Also, MPO is a primary granule protein in the neutrophil and as such we could postulate its release from neutrophils may be more tightly controlled than that of calprotectin, a cytoplasmic protein.

Serum calprotectin decreased over the course of an exacerbation. This finding was of higher statistical significance than calprotectin in sputum suggesting less variability in serum sampling than sputum. Calgranulin A (sub-unit of calprotectin) has previously been described in the serum of homozygotes and heterozygotes with CF mutations as *CF antigen* [26]. The serum levels of calprotectin are approximately 4 fold less than those observed in sputum, suggesting that the high concentrations of calprotectin observed in sputum are likely to arise locally in the airways from neutrophils, in particular from necrotic neutrophils, which are more prevalent in the sputum of CF patients with gram negative infection [42]. Changing levels of calprotectin in serum may reflect increased neutrophil recruitment from the bone marrow or leak of calprotectin from the lungs into the systemic circulation due to a breakdown in epithelial barrier integrity although further work is required to investigate this.

Serum CRP and VEGF also decreased significantly. This may have been anticipated as CRP is an acute phase protein previously recognised to change in CF exacerbations [7]. Serum VEGF has also been demonstrated to decrease with treatment of a CF exacerbation, with the main source being postulated as hypoxic lung tissue [33]. In our study both serum CRP and VEGF fell, consistent with previous studies. Serum CRP was demonstrated to show a similar serial change to serum calprotectin following exacerbation treatment but was less well correlated to lung function suggesting a more significant relationship of serum calprotectin to the airway than CRP. However this study does suggest a role for the measurement of CRP in the clinical management of CF exacerbations.

Serum calprotectin concentrations of <9.1 μg/ml at the end of an exacerbation predicted a delayed time to next exacerbation, with the median time being 112 days vs. 70 days for patients with calprotectin >9.1 μg/ml. Indeed three patients in low serum calprotectin group had not exacerbated by 1 year, whereas 3 patients in the high group had died by the time of follow up. CRP was less good in this regard, with no difference in median time to next exacerbation between those patients with normal and those with raised CRP values at the end of exacerbation. Further studies are now required to assess and validate serum calprotectin as a predictor of outcome in CF, but the current data raise the possibility that calprotectin levels may inform whether treatment needs to be prolonged.

We conclude that sputum and serum calprotectin decrease significantly with treatment of an exacerbation and are superior to sputum IL8 and serum CRP and VEGF, all of which have been advocated hitherto, as indicators of response. The additional value of a serum biomarker is recognised because of the greater ease of sample acquisition and processing. Further investigation is required to assess the potential clinical impact of these novel observations.

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Conflict of interest statement

None of the authors have any conflict of interest with regards to this manuscript.

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