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ELSEVIER
Journal of Cystic Fibrosis 13 (2014) 63 – 68
Journal of Cystic Fibrosis
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Original Article

Inflammatory and immunological biomarkers are not related to survival in adults with Cystic Fibrosis

K.L. Moffitt [a,1], S.L. Martin [a], A.M. Jones [b], A.K. Webb [b], C. Cardwell [c], M.M. Tunney [a,1], J.S. Elborn [d,*,1]

[a] School of Pharmacy, Queen's University Belfast, United Kingdom
[b] Manchester Adult CF Centre, South Manchester University Hospitals NHS Trust, Manchester, United Kingdom
[c] Centre for Public Health, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, United Kingdom
[d] Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, United Kingdom

Received 28 November 2012; received in revised form 7 June 2013; accepted 8 June 2013
Available online 13 July 2013

Abstract

Background: Chronic *Pseudomonas aeruginosa* pulmonary infection is associated with a decline in lung function and reduced survival in people with Cystic Fibrosis (CF). Damaging inflammatory and immunological mediators released in the lungs can be used as markers of chronic infection, inflammation and lung tissue damage.
Methods: Clinical samples were collected from CF patients and healthy controls. Serum IgG and IgA anti-*Pseudomonas* antibodies, sputum IL-8 and TNFα, plasma IL-6 and urine TNFr1 were measured by ELISA. Sputum neutrophil elastase (NE), cathepsin S and cathepsin B were measured by spectrophotometric and fluorogenic assays. The relationship between IgG and IgA, inflammatory mediators and long-term survival was determined.
Results: IgG and IL-6 positively correlated with mortality. However, multivariate analysis demonstrated that after adjusting for FEV_1, IgG was not independently related to mortality. A relationship was observed between IgG and IL-6, TNFα, TNFr1 and between IgA and IL8, cathepsin S and cathepsin B.
Conclusions: These data indicate that biomarkers of inflammation are not independent predictors of survival in people with CF.
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Keywords: Antibody titre; Inflammation; Proteases; *Pseudomonas aeruginosa*; Cystic Fibrosis; Mortality

1. Introduction

Chronic *Pseudomonas aeruginosa* pulmonary infection is associated with a decline in lung function, increased exacerbations and reduced survival in people with CF. In the lungs, chronic infection is accompanied by airway inflammation, resulting in the release of damaging inflammatory and immunological mediators from a number of sources including the airway epithelium and circulating neutrophils and macrophages [1]. Such mediators, including neutrophil elastase (NE), IL-8 and TNFα, correlate with disease severity in diverse populations of CF patients [2–4] and can be used as markers of chronic infection, inflammation and lung tissue damage.

The initial humoral response to bacterial challenge in the lungs is the production of secretory IgA (sIgA) by plasma cells in the pulmonary mucosa, followed by the systemic release of IgG. Antibody response to *P. aeruginosa* in CF can be used as a biomarker and lung inflammation and tissue damage are related to antibody titres [5–8]. Specific antibodies against *P. aeruginosa* antigens increase when chronic infection is established and a high number of anti-*Pseudomonas* precipitins have been shown to be related to poor prognosis [5,9]. Despite increased anti-pseudomonal antibodies, the humoral response does not confer protective immunity against this pathogen in the CF lung [10].

In some studies, increased antibody titre against *P. aeruginosa* precedes isolation of the pathogen from the respiratory tract and

* Corresponding author at: Centre for Infection and Immunity, Health Sciences Building, 97 Lisburn Road, Queen's University Belfast, BT9 7BL, United Kingdom. Tel.: +44 28 9097 2098; fax: +44 28 9098 2671.
E-mail address: s.elborn@qub.ac.uk (J.S. Elborn).
[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.jcf.2013.06.002

serological studies demonstrate a higher incidence of infection than that of microbial culture [11,12]. However, this pattern has not been consistently demonstrated and this may be due to variation in sample collection, assay methods and microbiological facilities available [13]. Furthermore, study results may be further confounded by not all patients showing an early antibody response [14]. Therefore, serological and molecular techniques may not be particularly helpful in detection of early or intermittent colonisation. To date, there has been little agreement regarding the clinical value of measuring serum antibody response against pathogens such as *P. aeruginosa* [15], particularly in relation to CF children [16]. Anti-pseudomonal IgG titre may have potential diagnostic value and can be used as a prognostic tool to identify CF patients at risk of establishing a chronic infection [17,18]. For many years this method has been used diagnostically in the Danish CF centre in Copenhagen to distinguish between intermittently colonised and chronically infected patients [19].

The primary aim of this study was to determine whether serum IgG and IgA titre against *P. aeruginosa* in clinically stable, chronically infected adult CF patients correlated with mortality and the production of host inflammatory mediators. We hypothesised that IgG antibodies to *P. aeruginosa* would predict long-term survival.

2. Methods

2.1. Patients and controls

Two separate cohorts of clinically stable CF patients were recruited at the Manchester and Belfast Adult CF centres [3,20]. Manchester patients were recruited over a 6-month period between July and December 2001, and Belfast patients during the year 2000. Data and clinical samples were collected from 63 CF patients attending the Belfast Centre (mean age 27.7 ± 8.9 years, 37M, 26F) and from 118 age-matched healthy controls [19]. Patients from Manchester (n = 40, mean age 27.7 ± 8.3 years, 20M, 20F) were subdivided into two groups based on the *P. aeruginosa* strain type causing infection: those infected with sporadic strains and those infected with a transmissible (*P. aeruginosa* strain, MA) strain. Of these 103 CF patients, 36 had no *P. aeruginosa* infection and 67 were chronically infected with *P. aeruginosa* for at least 12 months prior to recruitment. Both studies had ethical approval and patients and controls gave written informed consent. Demographic and clinical data was recorded for all CF patient cohorts at baseline including age, sex, CF genotype, %FEV_1, %FVC, white cell count (WCC), C-reactive protein (CRP) and body mass index (BMI) (Table 1). Mortality data was retrospectively obtained in 2013 and the time in months until death or lung transplantation used for subsequent analysis.

2.2. Biochemical measurements

Biochemical analysis of samples (sputum, urine, serum, plasma) from these patients was performed as part of two previously reported studies [3,4]. Sputum IL-8 and TNF-α, plasma IL-6 and urine TNFr1 were measured by ELISA. Sputum NE, cathepsin S and cathepsin B were measured by spectrophotometric and fluorogenic assays.

Table 1
Demographics of Cystic Fibrosis groups.

	Manchester	Belfast	Combined
Sex, male (female)	20 (20)	37 (26)	57 (46)
CF genotype, no. (%)			
F508/F508	23 (57.5)	20(31.7)	43 (41.7)
F508/other	14 (35)	24 (38.1)	38 (36.9)
Other/other	3 (7.5)	19 (30.2)	22 (21.4)
Age, y, mean (SD)	27.9 (8.5)	27.7 (8.9)	27.8 (8.7)
% FEV_1, mean (SD)	44.1 (18.69)	68.0 (24.99)	58.77 (25.5)
BMI, mean (SD)	20.42 (2.71)	22.47 (4.34)	21.74 (3.94)

2.3. Comparison of anti-pseudomonal immunological response with survival

Serum samples which had been stored at −80 °C since collection were assayed by ELISA for IgG and IgA antibodies against *P. aeruginosa* antigens. Micro-titre plates (96-well) were coated overnight with antigenic preparation from St-Ag: 1–17 (Statens Serum Institute). This antigenic preparation has been shown to have a high sensitivity of 97% [21]. Following blocking, diluted serum was added to each well and incubated for 2 h at room temperature. Horseradish peroxidase conjugated anti-human IgG or IgA (DAKO Co. Golstrup, Denmark) was added to each well. Optical Density was measured at 450 nM and the titre was arbitrarily defined as ELISA units (EU) from a standard curve developed using *P. aeruginosa* positive serum (Statens Serum Institute). Samples were assayed in duplicate and data presented as mean ± SD. The relationship between IgG and IgA and both inflammatory mediators and survival was then determined.

2.3. Statistical analysis

Where necessary, results were log transformed to achieve approximate normal distribution of data. A Pearson's correlation coefficient was used to measure the strength of the linear association between antibody titres, demographic/clinical parameters and sputum inflammatory biomarkers. Immunological response in control subjects, CF patients chronically infected with *P. aeruginosa* and *P. aeruginosa* negative CF patients were analysed using one-way ANOVA with post-hoc testing. The relationship between IgG and long-term survival was determined using Cox proportional hazard modelling to calculate hazard ratios (and 95% confidence intervals) and allowed adjustment for individual level confounders (age, gender, lung function and BMI). Hazard ratios (and 95% confidence intervals) were also calculated for the association between sputum inflammatory biomarkers and survival. Statistical analysis was performed with the SPSS (SPSS Version 19, Chicago, Illinois, USA) software package.

3. Results

3.1. Antibody titre and demographic and clinical parameters

There was no significant difference in IgG against *P. aeruginosa* antigens between the two Manchester sub-groups (sporadic, mean 496.0 ± 548.6; transmissible, mean 492.2 ± 440.5; p = 0.64). Therefore, results from both groups (n-40 patients) were combined for further statistical analysis. By univariate analysis (Pearson's correlation coefficient), IgG and all clinical parameters except age were related (Table 2). In contrast, IgA titres were only related to CRP. A significant correlation was also observed between IgG and IgA titre for the 67 patients chronically colonised with *P. aeruginosa* (r = 0.609, p < 0.001; Fig. 1).

3.2. Immunological response to chronic P. aeruginosa colonisation

IgG and IgA titres were compared between control subjects (n = 118), CF patients chronically colonised with *P. aeruginosa* (n = 67) and *P. aeruginosa* negative CF patients (n = 36). There was a significant difference in both IgG (p < 0.001, one-way ANOVA; Fig. 2A) and IgA (p < 0.001; Fig. 2B) between the three groups. Post-hoc testing (Tukey HSD) revealed that, for both IgG and IgA, there was a significant difference (p < 0.001) between control and *P. aeruginosa* positive patients and between *P. aeruginosa* positive and negative patients but no difference between control and *P. aeruginosa* negative patients (Fig. 2).

3.3. Anti-pseudomonal titre as a marker of long-term survival

Cox proportional hazard modelling was used to determine the relationship between IgG titre and long-term survival. Median IgG (110 ELISA units) was used to define two survival groups with patients grouped using a categorical variable above and below 110 (Fig. 3). There was a statistically significant association between IgG titre and mortality (OR = 2.94, 95% CI 1.51–5.76; p = 0.002). However, when analysis was adjusted for all potential confounders (age, gender, lung function [FEV_1] and BMI) this association was no longer apparent (OR = 1.23, 95% CI 0.57–2.66; p = 0.605). Further analysis revealed that adjusting for lung function (OR = 1.19, 95% CI 0.57–2.46; p = 0.642) largely accounted for the observed loss of association. We also examined survival trend across per 10-fold increase in IgG. There was a significant association between IgG titre and mortality (OR = 3.14, 95% CI 1.78–5.54; p < 0.001) which was no longer apparent following adjustment for confounders (OR = 1.75, 95% CI 0.95–3.22; p = 0.072). This loss of association was primarily due to lung function (OR = 1.70, 95% CI 0.95–3.03; p = 0.072), although some residual effect cannot be discounted.

Table 2
Relationship between anti-*P. aeruginosa* IgG and IgA titres and demographic and clinical parameters.

	Age	FEV_1	BMI	WCC	NEUT	CRP[a]
IgG[a]						
r-Value	−0.175	−0.429	−0.336	0.369	0.365	0.671
p-Value	0.252	0.003	0.024	0.013	0.014	<0.001
IgA[a]						
r-Value	−0.065	−0.237	−0.279	0.166	0.236	0.396
p-Value	0.673	0.117	0.063	0.274	0.119	0.007

FEV_1: forced expiratory volume in one second; BMI: body mass index; WCC: white cell count; NEUT: neutrophils; CRP: C-reactive protein.

[a] IgG, IgA and CRP were log transformed to achieve approximate normal distribution of data.

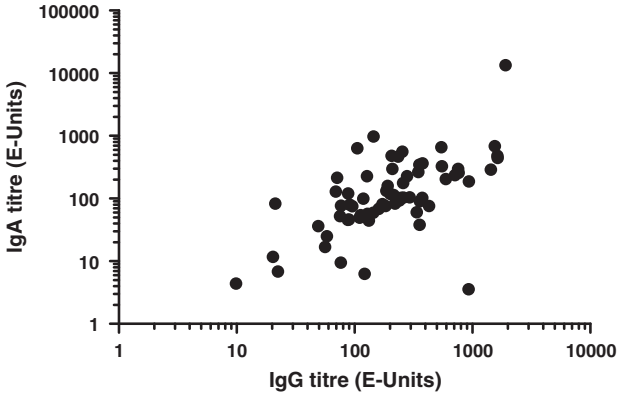

Fig. 1. Relationship between IgG and IgA titre for patients (n = 67) chronically colonised with *P. aeruginosa*. IgA and IgG results were log transformed to achieve normal distribution of data.

3.4. Antibody titre and sputum host inflammatory mediators

Sputum inflammatory biomarkers were available for the Manchester patient group only (n = 40 patients). All results were log transformed to achieve normal distribution of data. By univariate analysis (Pearson's correlation coefficient), IgG and IL-6, TNFα and TNFr1 were related (Table 3). In contrast, IgA titres were related to IL-8, cathepsin S and cathepsin B (Table 3). Cox proportional hazard modelling (Table 4) revealed evidence of an association between IL-6 and survival (p = 0.035) but no evidence of association between other sputum inflammatory biomarkers and survival.

4. Discussion

Our long-term study investigated the relationship between anti-pseudomonal titre and mortality in CF patients. Whilst univariate data analysis showed a significant difference in IgG titre between *P. aeruginosa* patients still surviving and those who had died, or had undergone lung transplantation, multivariate analysis demonstrated that the relationship observed between IgG and mortality was explained by decreased lung function (FEV_1) alone. In a 30-year cohort study, it was reported that CF patients who are treated intensively have lower antibody responses and longer survival after acquisition of chronic *P. aeruginosa* lung infection [17]. Based on our findings, the longer survival observed in this 30 year study may be due to a subsequent improvement in lung function following intensive treatments rather than a reduction in antibody production alone. Plasma IL-6 was

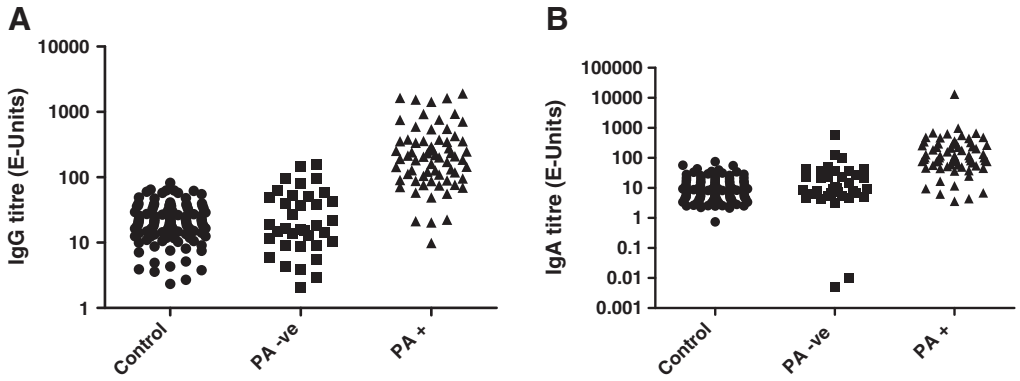

Fig. 2. Comparison of (A) serum IgG and (B) serum IgA titre in healthy control subjects (n = 118), *P. aeruginosa* negative CF patients (PA − ve, n = 36) and patients chronically colonised with *P. aeruginosa* (PA + ve, n = 67). Mean and (95% CI) values for IgG are: control: 24.29 (21.49–27.09); PA − ve: 35.26 (22.35–48.16); PA + ve: 349.7 (246.2–453.2) and for IgA: control: 12.20 (10.01–14.39); PA − ve: 37.03 (4.12–69.94); PA + ve: 381.2 (−13.74–776.2).

the only biomarker in our study to have a positive association with survival.

In addition to mortality, we investigated the relationship between antibody titre and inflammatory mediators. Airway inflammation is a major factor in the pathogenesis of CF lung disease and inflammatory mediators as markers of the host response to infection may reflect the intensity of lung injury and relate to changes in clinical status. Our findings report for the first time a significant positive correlation between antipseudomonal IgG and IL-6, TNFα, and TNFr1, whilst antipseudomonal IgA demonstrated a positive correlation with IL8 and the lysosomal cysteine proteases cathepsin S and cathepsin B. Cathepsin S has an important role in antigen presentation but, in addition to cathepsin B, it also utilises a number of extracellular matrix components as substrates thus contributing to the destruction of the lung milieu. This is of particular note as recently cathepsin S and B have been shown to play important roles in the pathophysiology of CF lung disease [4,22]. Correlations observed between IgG and IL-6, TNFα and TNFr1 further highlight interactions between immunological and inflammatory mediators following bacterial challenge within the airways. Microbial secretions from *P. aeruginosa*, in addition to its adherence to the epithelial lining of the respiratory tracts, induce IL-6 release from pulmonary epithelial cells. This is further augmented by the release of TNFα from macrophages. IL-6 acts as an inducer of the acute phase immune response to infection by driving the proliferation and differentiation of beta cells and subsequent immunoglobulin production. Other studies demonstrate that IL-6 strongly enhances IgG and IgA secretion by human lymphocytes following stimulation with bacteria [23]. Surprisingly, we did not observe any correlations between IgG and IL-8 or NE. This may be due to the fact that IL-8 and NE are secreted at the beginning of a pro-inflammatory response to bacterial invasion with a systemic release of IgG occurring downstream at a later time-point. A strong correlation was observed between IgG and IgA in all samples suggesting the inclusion of IgA in preliminary host defence during *P. aeruginosa* invasion and colonisation. Bacterial challenge induces the secretion of IgA within the pulmonary mucosa where it prevents bacterial adhesion to surface epithelium, reduces bacterial growth rate and neutralises bacterial exotoxins [24–26]. Should this response be inadequate, IgG antibodies are subsequently produced which pass into respiratory fluids or serum [24,27]. CF patients with IgA deficiency can be rendered particularly susceptible to infection [28].

We found significant differences between IgG and IgA titres in both our control and CF patients. The residual level observed within the control subjects can be attributed to 'normal acquired

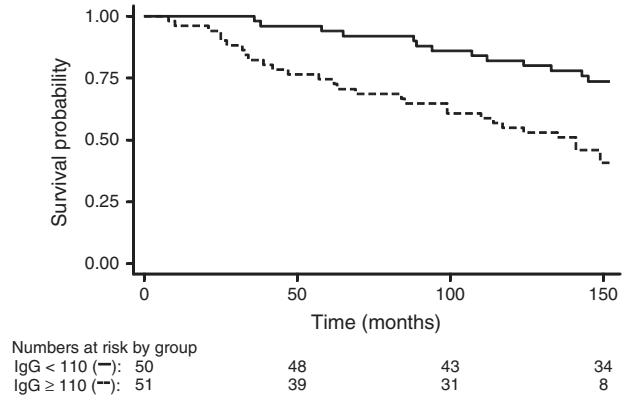

Fig. 3. Kaplan–Meier plot illustrating survival in two groups defined by the median IgG (110 ELISA units) with patients grouped using a categorical variable above and below 110.

Table 3
Relationship between anti-*P. aeruginosa* IgG and IgA titres and sputum inflammatory biomarkers.

	Il-6	IL-8	TNFα	TNFR1	HNE	CAT S	CAT B
IgG							
r-Value	0.646	0.214	0.564	0.448	0.273	0.136	0.208
p-Value	<0.001	0.180	0.001	0.004	0.084	0.397	0.397
IgA							
r-Value	0.219	0.397	0.344	0.278	0.280	0.556	0.590
p-Value	0.168	0.010	0.054	0.087	0.076	<0.001	<0.001

HNE: Human Neutrophil Elastase; CAT S: Cathepsin S; CAT B: Cathepsin B. IgG, IgA and all biomarker results were log transformed to achieve approximate normal distribution of data.

Table 4
Relationship between sputum inflammatory biomarkers and survival.

Biomarker[a]	Hazard ratio (95% CI)	p-Value
IL-6 (per 10 fold increase)	5.80 (1.13, 29.61)	0.035
IL-8 (per 10 fold increase)	1.84 (0.52, 6.55)	0.348
TNFα (per 10 fold increase)	1.87 (0.67, 5.25)	0.234
TNFR1 (per 10 fold increase)	2.74 (0.60, 12.62)	0.195
HNE (per 10 fold increase)	4. 95 (0.46, 52.89)	0.186
CAT S (per 10 fold increase)	4.56 (0.61, 34.28)	0.140
CAT B (per 10 fold increase)	1.56 (0.47, 5.16)	0.465

HNE: Human Neutrophil Elastase; CAT S: Cathepsin S; CAT B: Cathepsin B.
[a] IgG and all biomarker results were log transformed (to base 10) to achieve approximate normal distribution of data and consequently hazard ratios presented are for a 10 fold increase in each variable.

immunity' [29]. Our CF patients chronically infected with *P. aeruginosa* had higher IgG titres compared to those without colonisation which is in agreement with previous studies [30]. Following *P. aeruginosa* infection, the humoral response leads to an increase in the formation and accumulation of immune complexes. These may contribute to severe pulmonary disease in the CF lung and further formation of chronic *P. aeruginosa* infections [31,32].

Over several decades there has been much research and speculation into the role of antibody titres to *P. aeruginosa* and clinical status. Measurement of serum IgG antibodies by ELISA provides a specific, quantitative and highly sensitive measure of immunological changes associated with all stages of pulmonary infection by *P. aeruginosa*. These changes correlate with a decline in clinical state and lung function [33]. Our findings demonstrate positive correlations between IgG and several clinical and immunological parameters which further support this view. However, the results should be interpreted with caution as we have established that the association between IgG and mortality can be explained by a decline in lung function. Furthermore, our results are based on cross-sectional data and patients who were *P. aeruginosa* negative at the time of sample collection may change in due course to *P. aeruginosa* positive.

Detection of anti-pseudomonal IgG or IgA antibodies in serum has been reported to precede isolation of *P. aeruginos*a by routine microbial culture [14,17]. Brett et al. reported that 90% of CF patients produced specific IgA antibodies before the organism was present in sufficient numbers to be isolated from the respiratory tract [28]. However, the reliability and usefulness of titres are questionable as antibody variance amongst infected patients occurs; some patients show an increase in *P. aeruginosa* specific antibodies shortly after these organisms are cultured whereas other patients can be colonised for 1–2 years without eliciting any systemic immune response [29]. In addition, discrepancies may occur due to the choice of antigenic preparation used in ELISA systems and variability in microbiological culture facilities. The associated host immunological response includes the release of antibodies against a variety of pseudomonal antigens. Whilst many of these are detected in CF patients, no single antigen has been found to detect an antibody response in all CF patients. Nevertheless, it is vital to establish as soon as possible when *P. aeruginosa* colonisation elicits a systemic immune response and subsequent tissue damage. In patients chronically infected with *P. aeruginosa*, a large and sudden increase in antibody titre is a poor prognostic factor with an increase in IgG titre correlating with a decrease in lung function and poor clinical condition [5,8,33]. Our study highlights the association of anti-pseudomonal immunological titres with both clinical parameters and inflammatory biomarkers.

We have demonstrated that IgG is particularly relevant in chronic *P. aeruginosa* infection. Furthermore, in addition to a correlation with IgG, anti-pseudomonal IgA also correlated with the pro-inflammatory biomarkers CRP, IL-8 and cathepsins S and B. Although important in host defence against respiratory viruses, IgA may be less important in bacterial infections; however, measuring specific IgA titres from *P. aeruginosa* may be useful for differentiating between active infection and colonisation [28,34].

Some transmissible strains of *P. aeruginosa* are more virulent that non-transmissible strains, especially within the CF population. Previous studies have shown no difference in inflammatory markers in patients chronically infected with both strains or indeed in their clinical outcome [3,35]. Our findings, which also show no difference between anti-pseudomonal titre in patients chronically infected with either transmissible or sporadic strains of *P. aeruginosa*, further supports this view.

5. Conclusion

We have demonstrated a relationship between anti-pseudomonal IgG and IgA titres and other systemic markers of inflammation. During periods of clinical stability, CF patients infected with transmissible *P. aeruginosa* did not show a heightened IgG or IgA response against specific *P. aeruginosa* antigens, when compared with patients colonised with non-transmissible strains. This is in agreement with previous findings that demonstrate no difference in inflammatory response between these two groups. The univariate correlation between IgG and 10 year survival was explained by a decrease in FEV_1 rather than an increase in anti-pseudomonal antibodies. In addition, pro-inflammatory mediators correlated with IgG response suggest that the IgG response to *P. aeruginosa* can be an immunological marker of chronic infection and declining lung function but cannot be used to predict survival outcome.

Funding

This work was funded through a US–Ireland Project Partnership Grant by the Health and Social Care Research and Development, Public Health Agency, Northern Ireland. M.M.T. was supported by a Health and Social Care Research and Development, Public Health Agency, Northern Ireland, funded by the UK National Institute for Health Research Career Scientist Award.

Conflict of interest

The authors declare no conflict of interest.

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