# Original article

# *Pseudomonas*-induced lung damage in cystic fibrosis correlates to bactericidal-permeability increasing protein (BPI)-autoantibodies

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**ABSTRACT**

**Objective.** *Lung damage is the most common cause of death in cystic fibrosis (CF). It is induced by bacterial colonization and inflammatory activity perpetuates its course. Autoantibodies directed against BPI (bactericidal permeability increasing protein), called BPI-ANCA, have recently been associated with cystic fibrosis. Here we confirm this association and evaluate the relation between ANCA and total IgG level as they relate to bacterial colonization, pulmonary function, and musculoskeletal symptoms.*

**Methods.** *BPI-ANCA, MPO-ANCA, and PR3-ANCA were measured with ELISA in 46 adult patients with CF. Total IgG was determined by immunoturbidimetry. Results were correlated to bacterial colonization, lung function and musculoskeletal symptoms.*

**Results.** *BPI-ANCA was found in 33 patients. In the whole group, both BPI-ANCA and total IgG were inversely correlated to lung function, but in patients chronically colonized with* Pseudomonas aeruginosa (P. aeruginosa), *BPI-ANCA alone was correlated to lung damage (p = 0.01). Median lung function, measured as forced expiratory volume in 1 second, in* P. aeruginosa *colonized patients with high levels of BPI-ANCA was 43% of the predicted value. In BPI-ANCA negative, the corresponding figure was 83%. In patients not colonized with* P. aeruginosa, *this relation was less evident. No correlation between ANCA and musculoskeletal symptoms was seen.*

**Conclusion.** P. aeruginosa *induced lung damage in CF patients is associated with the presence of BPI-ANCA.* P. aeruginosa *colonized patients without BPI-ANCA have almost normal lung function. We suggest that BPI-ANCA discriminate* P. aeruginosa *colonized CF patients with severe lung damage from those whose disease is less destructive. Vasculitis like symptoms in CF are not ANCA associated.*

## Introduction

CF is a genetic, recessive disorder due to mutations on chromosome 7 resulting in defective chloride channel proteins in epithelial cells, causing highly viscous mucus. Organ systems involved are the gut and the respiratory tract, dysfunction of the latter being the cause of more than 90% of the deaths (1). Several different mutations are described and genotype-phenotype relationships have shown a strong correlation for the severity of the pancreatic disease. In lung disease, however, there is much more variability (2, 3).

*Pseudomonas aeruginosa* (*P. aeruginosa*), a gram-negative bacterium, colonizes the respiratory tract of most adult CF patients. An inflammatory process is hereby initiated and neutrophil granulocytes are recruited to the affected tissue. Bacteria, enzymes released from granulocytes and highly viscous mucus in the airways lead to organ damage and aggravated gas exchange (4). The patients rarely have fever or elevated acute phase proteins in serum, rapidly deteriorating lung function being their only clinical evidence of active ongoing inflammatory process. Hypergammaglobulinemia is a marker of unspecific inflammatory activity. Earlier studies have shown correlation between poor lung function and high levels of IgG and some of its subclasses (5-8). The clinical use of total IgG as a marker of destructive disease is however limited as most patients have normal levels. Under-

standing the lung disorder and monitoring its course is an important and challenging task.

Anti-neutrophil cytoplasmatic antibodies, ANCA, are autoantibodies directed against different proteins in the cytoplasm of neutrophil granulocytes (9, 10). In systemic vasculitidies, ANCAis directed against proteinase 3 or myeloperoxidase. The ANCA level corresponds to disease activity, and there is evidence that ANCA is involved in the pathogenesis of these diseases (11). In CF, ANCA is directed against bactericidal-permeability increasing protein (BPI), which is a 55 kD antibacterial peptide mainly located in neutrophil granulocytes (12). Prevalences of IgG-BPI-ANCA between 39% and 91% have been reported in CF with the higher prevalences appearing in adult populations. High levels of BPI-ANCA and reduced lung function has been reported (13-16), as well as association with *P. aeruginosa* colonization (16). It is not known whether BPI-ANCA constitutes only an indicator of chronic *P. aeruginosa* colonization or if it gives additional information concerning disease development.

The aims of this study were to confirm the association between BPI-ANCA of isotypes IgG and IgA and CF in adult patients, as most of the previous studies concern children. We also wanted to study the relationship between BPI-ANCA and bacterial colonization as well as the correlation of these factors with lung function. Secondary vasculitis and vasculitis like manifestations, for example musculoskeletal symptoms, have occasionally been reported in CF patients (17), and therefore we have also investigated ANCA levels to PR3, MPO and BPI in relation to musculoskeletal symptoms.

## Methods

*Study population*

Lund University hospital serves the south of Sweden with centralized care for CF patients. At the time of data collection for this study 54 adult patients were registered as patients at the CF center at Lund University hospital. The clinical CF center program in Lund, as well as in the rest of Sweden, includes annual check up with physiological tests, microbiological diagnosing, and blood sampling. It also includes frequent appointments with physiotherapists for mucus evacuation, physical training, and education in self-treatment. In the case of clinical deterioration, the CF centre is also responsible for the patient care. Patients were asked for informed consent to participate in this study. Forty-six patients agreed to participate in this study. Reasons for not being included were trivial, such as not seeing a nurse assigned to the study during the inclusion period.

*Chart review, scoring for musculoskeletal symptoms, and blood sampling*

Patient charts were reviewed and height, weight, age, and sex were noted. A nurse, experienced in working with CF-patients and with good personal knowledge of each patient, scored the presence of musculoskeletal symptoms on an arbitrary 0-2 scale. Absence of musculoskeletal symptoms was scored 0, moderate symptoms 1, and severe symptoms 2. In connection with blood sampling for medical reasons, serum for ANCA analysis was drawn. Total immunoglobulins in serum were analysed as a part of annual routine check up at the Department of Clinical Chemistry at Lund University Hospital with immunoturbidimetry using reagents from Roche Diagnostics (Mannheim, Germany). CF diagnosis was confirmed genetically in all cases as a clinical routine and the result of mutation analysis was obtained from patient records.

*Lung function test*

Pulmonary function was tested with spirometry following guidelines from the American Thoracic Society (18) at the Department of Clinical Physiology at Lund University Hospital. Forced expiratory volume in 1 second (FEV1) was chosen as a measure of lung function. The results were expressed as proportion of predicted values (FEV1% pred) calculated from the patients' height, age, and sex. In six cases, no spirometry results were obtained on the day of blood sampling due to insufficient patient compliance. In those cases, the most recent result was noted.

*Culture of respiratory secretions*

Samples for respiratory secretion cultures were taken when the patient attended a routine outpatient visit. Sampling, transport, and culturing were performed according to routine procedures. Colonization was defined according to European consensus (19), i.e. three successive positive cultures. All patients provided expectorate. History of bacterial colonization was obtained from patient records as far back in time as possible.

*ELISAfor ANCA*

Serum samples were drawn and analysed for BPI-ANCA of isotypes IgG and IgA, MPO-ANCA and PR3-ANCA. Purified MPO (20), PR3 (21) and BPI (9) was obtained from Wieslab AB (Lund, Sweden), and direct binding ELISA was performed as previously described (22). In short, antigens were coated in microtiter plates at a concentration of 1µg/ml in a bicarbonate buffer. All samples were diluted 1/80 and incubated for one hour. Bound antibodies were detected using alkaline phosphatase conjugated goat anti-human IgG (Sigma, Stockholm, Sweden) and IgA (Eurodiagnostica, Malmö, Sweden). IgG-BPI-ANCA was quantified from a calibrator curve of serum that was serially diluted, and the results expressed as arbitrary units (U). For IgA-BPI-ANCA no calibrator curve was available and therefore the results were expressed as optical density (OD). In each ELISA analysis one positive and one negative control was analysed. BPI-ANCA is not normally found in serum and to decide the positive cut-off level for IgG-BPI-ANCA and IgA-BPI-ANCA, 42 normal sera were analysed. From the result of these normal sera +3SD the level at which BPI-ANCA should be considered positive was determined. In the case of IgG-BPI-ANCAthe level was 20 U, and for IgA-BPI-ANCA it was 0.14 OD. Positive sera were divided into two groups: low level and high level. The high level cut-off was determined arbitrarily, according to cut-offs used in clinical routine analyses, before data was analysed. The upper level cut-off was determined to be 50 U for IgG-BPI-ANCA and 0.40 OD for IgA-BPI-ANCA.

**Table I.** Mutation of the CFTR gene in relation to age, lung function, and IgG-BPI-ANCAlevel.

| Mutation of CFTR gene | All patients (n=46) | Females (n=20) | Males (n=26) | Median age (years) | Median FEV1% Pred* | Median BPI-ANCA (U) |
|---|---|---|---|---|---|---|
| dF508/dF508 | 24 | 11 | 13 | 22.3 | 70 | 52 |
| dF508/394delTT | 6 | 2 | 4 | 25.7 | 56 | 114 |
| dF508/x** | 12 | 5 | 7 | 26.1 | 62 | 63 |
| 394delTT/x | 2 | 1 | 1 | 30.0 | 39 | 47 |
| x/x | 2 | 1 | 1 | 32.7 | 77 | 19 |

* Lung function is expressed as the proportion of predicted forced expiratory volume in 1 second.
** x represents an allele different from dF508 or 394delTT.

**Table II.** Duration and species of bacterial colonization in 46 adult cystic fibrosis patients (n).

| Bacterial colonization | Duration ≥ 10 years | Duration 5-9 years | Duration 0-5 years | Total |
|---|---|---|---|---|
| *P. aeruginosa* | 18 | 0 | 9 | 27 |
| Non-*P. aeruginosa* | | | | |
| *S. aureus* | 13 | 0 | 1 | 14 |
| *B. cepacia* | 0 | 0 | 1 | 1 |
| *S. maltophilia* | 0 | 0 | 1 | 1 |
| No colonization | - | - | - | 3 |

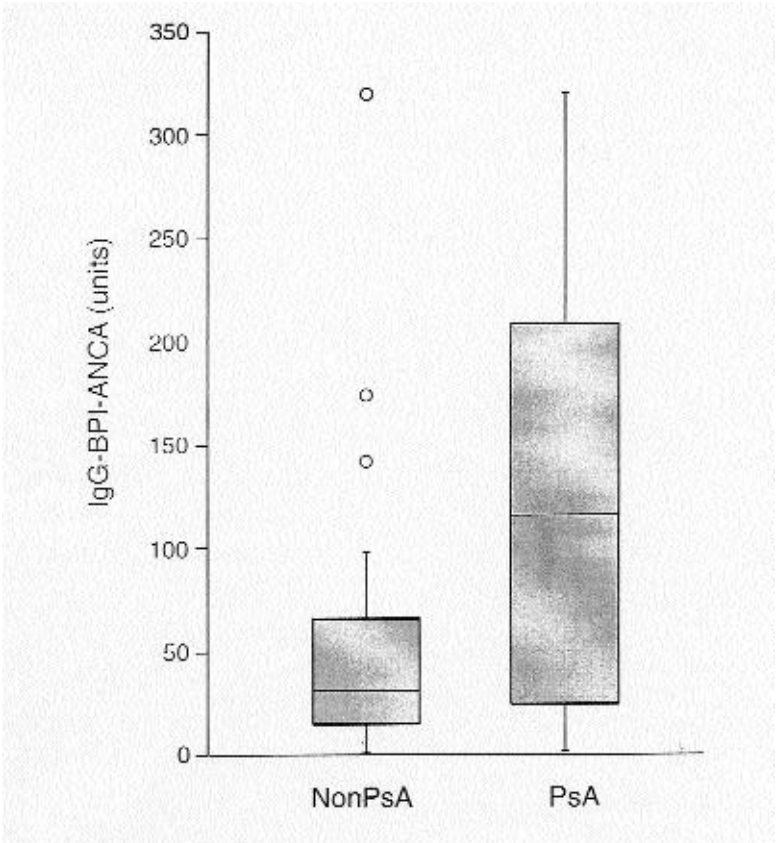


**Fig. 1.** BPI-ANCAin patients colonized with *Pseudomonas aeruginosa* (PsA) (n = 27), and in patients colonized with other bacteria or not colonized at all (non-PsA) (n = 19). Colonization with *P. aeruginosa* in this study was significantly associated with elevated levels of BPI-ANCA(p=0.03).

*Statistics*

The Mann-Whitney test was used when applicable. To compare means of three or more variables Kruskal-Wallis' test was used (23). To determine the significance of correlation coefficients, Spearman non-parametric rank correlation test was used. Results were considered significant when $p < 0.05$.

## Results

The median age of the 46 patients was 24.6 years (18.4 – 44.6). The median result of forced expiratory volume in 1 second expressed as a proportion of predicted value (FEV1%pred) was 66% (IQR 46-90) in the population. No significant differences in FEV1%pred were seen according to CFTR genotype (Table I).

BPI-ANCAwere analysed for both isotypes IgG and IgAand the results were correlated to the results of musculoskeletal symptoms, bacterial culture and spirometry. We found no major differences in the results of IgG-BPI-ANCA and IgA-BPI-ANCA in their respective correlation to musculoskeletal symptoms, bacterial colonization and lung function. If not indicated otherwise, BPI-ANCA in this text concerns the IgG isotype. BPI-ANCA was present in 33 patients and 24 patients had high levels. Neither age nor sex had any impact on the BPI-ANCA level. Hypergammaglobulinemia, i.e. a level of total IgG above the normal range, was found in 11 cases. Subnormal levels were found in two patients. Colonization of the respiratory tract with *P. aeruginosa* was found in 27 patients. The remaining 19 patients were colonized with other bacteria, in most cases *Staphylococcus aureus* (Table II). This group is referred to as non-*P. aerugi - nosa. P. aeruginosa* colonized patients had lower lung function than non-*P. ae - ruginosa*. Median FEV1%pred was 54 (IQR 38-76) for *P. aeruginosa* and 83 (IQR 65-95) for non-*P. aeruginosa* patients.

In the *P. aeruginosa*-colonized group, the median BPI-ANCA level was 116 U (IQR 25-208) while in the non-*P. ae - ruginosa*-colonized group median BPI-ANCAwas 31 U (IQR 15-64), $p = 0.03$ (Fig. 1).

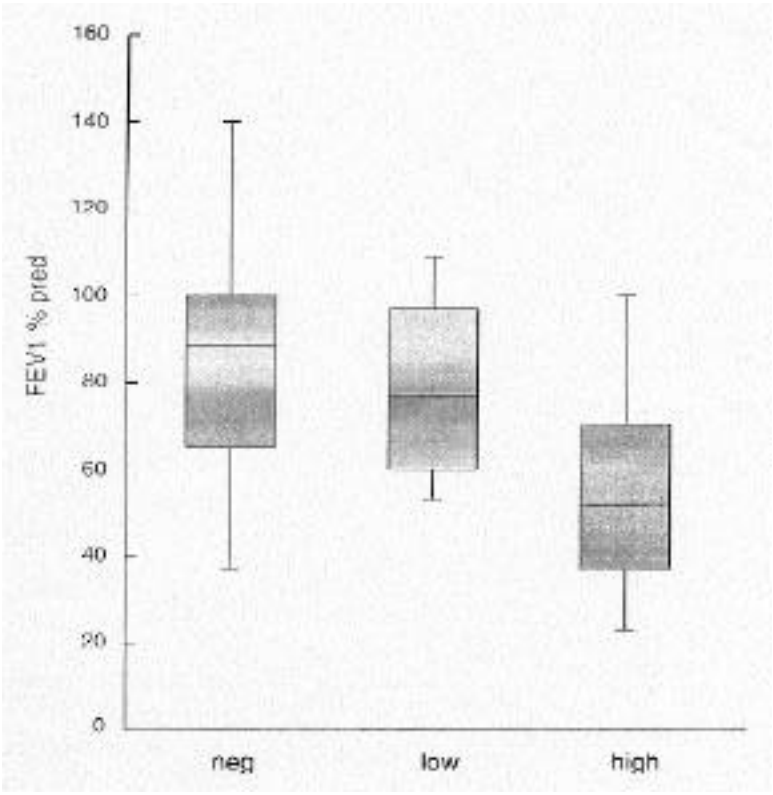


**Fig. 2.** Lung function expressed as a proportion of the predicted forced expiratory volume in 1 second (FEV1%pred) in relation to levels of IgG-BPI-ANCA. There was a strong negative correlation between lung function and the BPI-ANCAlevels. BPI-ANCAnegative patients (n=13) performed 89% of predicted FEV1, patients with low levels (n=9) 77%, and patients with high levels of IgG-BPI-ANCAperformed only 52% (p=0.004).

**Table III.** Lung function in relation to ANCA level and colonization with *Pseudomonas aeruginosa.*

| | | PsA-colonized patients | | Non PsA-colonized patients | |
|---|---|---|---|---|---|
| | | FEV1%pred | n | FEV1%pred | n |
| IgG-BPI-ANCA | | | | | |
| | High | 48 | 18 | 76 | 6 |
| | Low | 82 | 3 | 78 | 6 |
| | Negative | 84 | 6 | 83 | 7 |
| IgA-BPI-ANCA | | | | | |
| | High | 39 | 9 | 59 | 1 |
| | Low | 47 | 11 | 80 | 6 |
| | Negative | 97 | 7 | 88 | 12 |
| All patients | 54 | 27 | 83 | 19 | |

Median results of spirometry, expressed as a proportion of the predicted value of FEV1 in *P. aeruginosa* colonized and non-*P. aeruginosa* colonized patients in relation to levels of BPI-ANCAof isotypes IgG and IgA. In *P. aeruginosa* colonized patients (n = 27) FEV1%pred was significantly inversely associated with the IgG-BPI-ANCAlevel, p = 0.01.

*Musculoskeletal symptoms in relation to ANCA*

Moderate or severe musculoskeletal symptoms were found in seven patients altogether. These patients were all negative for both PR3-ANCA and MPO-ANCA. Their levels of BPI-ANCA did not differ significantly from those who had no musculoskeletal symptoms. Mean BPI-ANCA in patients with no musculoskeletal symptoms was 105 U, and in patients suffering from musculoskeletal symptoms it was 119 U, p= 0.74.

*ANCAcorrelates inversely to lung function*

A negative association between lung function and BPI-ANCA levels in CF patients was demonstrated, p = 0.0008, r = –0.47, confidence interval –0.68 to –0.21. The patients with high levels of BPI-ANCA had a median FEV1%pred of 52% (IQR 38 – 69) The patients who were BPI-ANCAnegative performed a median FEV1%pred of 89% (IQR 65 – 100, Fig. 2).

Pulmonary function was also significantly inversely correlated to the level of total IgG, (p= 0.004, r = -0.41, confidence interval -0.63 – -0.13). Correlation was also strong within the group of patients with normal IgG levels.

*Strong correlation between lung damage and ANCA in* P. aeruginosa *colonized patients*

Chronic colonization with *P. aeruginosa* usually leads to severe lung damage in CF patients, as shown above. Some *P. aeruginosa* colonized patients, however, have almost normal results in physiological tests, namely the BPI-ANCA negative patients. In *P. aeruginosa*-colonized patients, a significant inverse correlation between FEV1% pred and BPI-ANCA (p=0.01, r = -0.48, confidence interval –0.73 – -0.11) was seen. Total IgG did not correlate significantly with lung function in *P. aeruginosa* colonized patients (p = 0.09, r = -0.33, confidence interval -0.64 – 0.07). Spirometry gave a median result of 83% of predicted FEV1 value in the six BPI-ANCA negative, *P. aeruginosa*-colonized individuals. For the *P. aeruginosa* colonized patients with high levels of BPI-ANCA (18 individuals), the corresponding figure was 42%, indicating a severe reduction of lung function.

In non-*P. aeruginosa* colonized patients, no significant differences in the median results of FEV1%pred corresponding to ANCA levels were found. Thus, in ANCA negative patients, *P. aeruginosa* colonized or not, the spirometry results indicate that no severe impairment of lung function is present (Table III). In non-*P. aeruginosa* patients the inverse correlation between total IgG and lung function was signifi-

cant, ($p = 0.02$, $r = -0.51$, confidence interval -0.79 – -0.06).

## Discussion

This study confirms the association between BPI-ANCA of both IgG and IgA class with CF in adult patients. Furthermore, we demonstrate that BPI-ANCAis not only a marker of *P. aeruginosa* colonization, but also an indicator of reduced lung function in *P. aeruginosa* colonized CF patients.

Studies presenting survival data from Scandinavia show that centralized, preventative and aggressive treatment, result in a relatively healthy cystic fibrosis population. In this population only 59% were *P. aeruginosa* colonized, a figure corresponding well to earlier Scandinavian data (2, 24, 25).

There is no evident correlation between the chloride channel mutation and clinical course regarding lung function (2, 3, 26). Bacterial colonization, in particular with *P. aeruginosa*, is an obvious risk factor for developing lung damage. However, there are patients who seem not to have a destructive inflammation although chronically colonized with *P. aeruginosa* for many years.

Presence of vasculitis-like symptoms in CF patients has been reported (17, 27). In this study MPO-ANCA and PR3-ANCA was present in low levels in 7 patients altogether (15%). There was no correlation between musculoskeletal symptoms and the presence of MPO- or PR3-ANCA, indicating that musculo-skeletal symptoms in CF patients are not related to ANCA-associated vasculitis.

An elevated level of immunoglobulins (hypergammaglobulinemia) is a non-specific inflammatory marker. In CF, lung damage is caused by an inflammatory process and could thereby be reflected by the level of immunoglobulins. This population demonstrates a correlation between the total IgG level and the degree of lung damage, even though most patients have IgG within the normal range. However, the correlation was not significant in *P. aeruginosa* colonized patients. In non-*P. aeruginosa* patients, nevertheless, total IgG may according to our results have clinical relevance.

Earlier studies on BPI-ANCA in CF (13,15,16,28) show that BPI-ANCAis associated with *P. aeruginosa* colonization as well as with poor lung function. It has been suggested that BPI-ANCA is only an indicator of *P. aeruginosa* colonization. In that case, the test would contribute very little to the diagnostic arsenal, as specific and sensitive serological and bacteriological tests already exist (29). In this study, BPI-ANCAis negative or low in 33% of the *P. aeruginosa* colonized patients, and in this group lung function is relatively preserved compared to the group of patients with high levels of BPI-ANCA. We believe that BPI-ANCA can become a useful tool, as it will discriminate a group of patients within the *P. aeruginosa* colonized group, who seem less affected by their bacterial colonization, namely the BPI-ANCA negative patients. Such discrimination is not possible to make from existing serological and bacteriological tests or, as is seen in this material, from total IgG data.

The biological role of ANCA in CF lung damage is not known. BPI is, as mentioned above, an antibacterial peptide that originates from the neutrophil granulocytes (12). It is possible that ANCA are pathogenic due to the fact that they block BPI and its antibiotic effect. It is also possible that they are only markers indicating the presence of frustrated neutrophils in the tissue. These neutrophils are then releasing the BPI that becomes an antigen.

Patients with *P. aeruginosa* in their airways are considered to have a higher degree of inflammation than non-*P. aeruginosa* patients. In the current study, one group of patients showed no signs of impaired lung function in spite of many years of *Pseudomonas* colonization. This group of patients was characterised by low or negative BPI-ANCA results, indicating that BPI-ANCA may be a marker of ongoing inflammation. Prospective studies are necessary to determine whether BPI-ANCA precedes the development of lung damage. Presence of BPI-ANCA at the time of CF diagnosis in small children (28) indicates that BPI-ANCA may be an early event that could be governed by constitutional factors.

Based on our findings, we suggest that BPI-ANCAmay be a tool for selecting *P. aeruginosa* colonized patients for intense treatment and follow-up.

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