1-3-2012

A novel neutrophil derived inflammatory biomarker of pulmonary exacerbation in cystic fibrosis.

Emer P. Reeves
Royal College of Surgeons in Ireland

David A. Bergin
Royal College of Surgeons in Ireland

Sean Fitzgerald
Royal College of Surgeons in Ireland

Elaine Hayes
Royal College of Surgeons in Ireland

Joanne Keenan
Dublin City University

See next page for additional authors

Authors
Emer P. Reeves, David A. Bergin, Sean Fitzgerald, Elaine Hayes, Joanne Keenan, Michael Henry, Paula Meleady, Isabel Vega-Carrascal, Michelle A. Murray, Teck Boon Low, Cormac McCarthy, Emmet O'Brien, Martin Clynes, Cedric Gunaratnam, and Noel G. McElvaney

A novel neutrophil derived inflammatory biomarker of pulmonary exacerbation in cystic fibrosis.

Emer P. Reeves[a*], David A. Bergin[a*], Sean Fitzgerald[a], Elaine Hayes[a], Joanne Keenan[b], Michael Henry[b], Paula Meleady[b], Isabel Vega-Carrascal[a], Michelle A. Murray[a], Teck Boon Low[a], Cormac McCarthy[a], Emmet O'Brien[a], Martin Clynes[b], Cedric Gunaratnam[a] and Noel G. McElvaney[a].

[a] *Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland.*

[b] *National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin 9, Ireland.*

[*]E.P.R. and D.B. contributed equally to this work.

Address for correspondence: Dr Emer P. Reeves PhD MSc, Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland; e-mail: emerreeves@rcsi.ie

Running title: A neutrophil biomarker of exacerbations.

Key words: Cystic fibrosis, alpha-1 antitrypsin, CD16b, infection, inflammation, pulmonary exacerbation.

Abstract

Background: The focus of this study was to characterize a novel biomarker for cystic fibrosis (CF) that could reflect exacerbations of the disease and could be useful for therapeutic stratification of patients, or for testing of potential drug treatments. This study focused exclusively on a protein complex containing alpha-1 antitrypsin and CD16b (AAT:CD16b) which is released into the bloodstream from membranes of pro-inflammatory primed neutrophils.

Methods: Neutrophil membrane expression and extracellular levels of AAT and CD16b were quantified by flow cytometry, Western blot analysis and by 2D-PAGE. Interleukin-8 (IL-8), tumour necrosis factor-alpha (TNF-alpha) and AAT:CD16b complex were quantified in CF plasma (n=38), samples post antibiotic treatment for 14 days (n=10), chronic obstructive pulmonary disease (n=10), AAT deficient (n=10) and healthy control (n=14) plasma samples by ELISA.

Results: Cell priming with IL-8 and TNF-alpha caused release of the AAT:CD16b complex from the neutrophil cell membrane. Circulating plasma levels of IL-8, TNF-alpha and AAT:CD16b complex were significantly higher in patients with CF than in the other patient groups or healthy controls ($P<0.05$). Antibiotic treatment of pulmonary exacerbation in patients with CF led to decreased plasma protein concentrations of AAT:CD16b complex with a significant correlation with improved FEV1 ($r= 0.84$, $P=0.005$).

Conclusion: The results of this study have shown that levels of AAT:CD16b complex present in plasma correlate to the inflammatory status of patients. The AAT:CD16b biomarker may become a useful addition to the clinical diagnosis of exacerbations in CF.

1. Introduction

Cystic fibrosis is an autosomal recessive condition and although a multi-organ disorder, end-stage lung disease with chronic bacterial infection is the major cause of morbidity and mortality in affected individuals (1). The pathogenesis of lung disease induced by CF is a direct result of decreased chloride secretion and hyper-absorption of sodium, resulting in the retention of dehydrated mucus within the airways (2). This thickened mucus provides an ideal environment for bacterial infection in the respiratory tract. *Staphylococcus aureus* (*S. aureus*) is the major bacterial pathogen in early years but subsequently *Pseudomonas aeruginosa* (*P. aeruginosa*) becomes the prominent pathogen in adult patients (3, 4). Adding to this lung microbiome in CF, obligate anaerobes in CF sputum samples including *Prevotella*, *S. saccharolyticus*, *Peptostreptococcus prevotii* and *Actinomyces* have been detected (5, 6). Intravenous (*i.v.*) antibiotics are normally used for acute infective exacerbations with efficacy assessed by monitoring respiratory function, body weight and circulating markers of systemic inflammation (7).

In addition to ineffective mucociliary and cough clearance, persistent expression of inflammatory cytokines and chemokines plays a major role in the pathogenesis of chronic lung disease (8), leading to recruitment and activation of neutrophils in the CF airways (9). Longitudinal and prospective studies of infants with CF and also within an animal model with mutated CFTR genes, demonstrates that airway inflammation follows respiratory infection (10). Thus an increasing volume of clinical data supports the use of early antimicrobial treatment to inhibit (11) or delay (12) bacterial infection and to achieve decreased hospitalization and improved patient survival. For this reason there has been increased interest in the use of infective exacerbations (frequency and resolution) as

an outcome measure in clinical trials and sensitivity to treatment. However, whilst there are a number of criteria put forward to define an exacerbation (13-16) variability among parameters exists (17). Moreover, as new agents are investigated in clinical trials and new therapeutic targets are identified, it is becoming increasingly important to accurately define the clinical signs and symptoms of pulmonary exacerbations and to define objective markers of infection (18).

Inflammatory biomarkers represent a solution to the variation in the clinical criteria defining exacerbations in CF. Thus far identified biomarkers indicative of increased pulmonary inflammation with *P. aeruginosa* infection include serum levels of G-CSF (19) and matrix metalloproteinase-1, -8 and -9 (20). Alternatively levels of cytokines including IL-8 (21), neutrophil released elastase (22), myeloperoxidase (23) and cathepsin B and S (24) have been evaluated as markers of pulmonary exacerbation in sputum samples. However, one aspect of neutrophil physiology which has largely been overlooked is pre-activation or priming of the circulating cell. Neutrophil priming is a prerequisite for homing to the lung and can be used as read-out for the *in vivo* action of pro- and anti-inflammatory cytokines. Consequently, we hypothesized that markers of neutrophil priming in the systemic compartment would serve as an early read-out for pulmonary exacerbation in CF and should be normalized upon optimal treatment. Recently we have shown that the acute phase protein alpha-1 antitrypsin, is bound to the circulating neutrophil via interaction with the glycosylphosphatidyl-inositol (GPI) linked membrane protein CD16b (FcγRIIIb) (25). Moreover, soluble immune complex (sIC) primed the circulating neutrophil to release AAT from the cell membrane within a protein complex with CD16b. Within the present study we investigated the potential of

neutrophil released AAT:CD16b protein complex to serve as a potential marker of infective inflammation and resolution of pulmonary exacerbation in adult patients with CF. Some of the results of this study have been previously reported in the form of an abstract (26).

2. Materials and methods

2.1 Chemicals and reagents

All chemicals and reagents were endotoxin free and were purchased from Sigma-Aldrich unless indicated otherwise.

2.2 Patient groups

Four patient groups and healthy control volunteers were recruited to this study as follows: **1:** Asymptomatic alpha-1 antitrypsin deficient individuals (AATD) not receiving therapy were recruited from the Irish Alpha-1 Antitrypsin Deficiency Registry (n=10, mean age 43.95 ± 8.53). AATD patients had plasma AAT levels <11 μM and were clinically stable with a forced expiratory volume in one second (FEV1) of 98.2% ± 17.48% predicted. **2:** Adult patients with COPD (n=10) and a history of smoking were recruited as previously described (27). **3:** Patients with CF (n=38, mean age 26.8 ± 5.9) were genotyped for cystic fibrosis transmembrane conductance regulator mutations. Treatment of an acute bacterial exacerbation in 10 patients with CF involved *i.v.* administration of colomycin (2 million units *i.v.* 3 times daily (*t.d.s*)), piperacillin/tazobactam (4.5g *i.v. t.d.s.*), flucloxacillin (2g *i.v.* 4 times daily (*q.d.s*)), meropenem (2g *i.v. t.d.s.*) and/or ciprofloxacin (400mg *i.v.* 2 times daily (*b.d.*)). An exacerbation was defined by previously validated criteria (28). **4:** All non-CF bronchiectasis patients (n= 6, mean age 64 ± 6.7, mean % FEV1 of 59% predicted) were recruited from a specialized non-CF bronchiectasis clinic and had no evidence of an exacerbation at the time of recruitment. **5:** Control volunteers (n=14, mean age 34.72 ± 3.17) had no respiratory symptoms and were not on medication. All participants gave

written informed consent to participate in the study, which was approved by Beaumont Hospital Ethics Committee.

2.3 Neutrophil isolation and membrane fractionation.

Neutrophils were purified from whole blood and membranes were isolated as previously described employing sucrose density ultracentrifugation (25). The methodology for flow cytometry, proteomic analysis, SDS-PAGE and Western blotting of neutrophil plasma membranes can be found within the supplemental materials and methods.

2.4 Enzyme linked immunosorbent assays (ELISAs)

The concentration of IL-8 or TNF-alpha in plasma samples was measured by enzyme linked immunosorbent assay (ELISA), conducted in accordance with the manufacturer's instructions (R&D Systems). For the detection of AAT:CD16b complex within plasma samples of healthy control, AATD or patients with CF, 5μg/ml of specific mouse anti-CD16b (R&D systems) was employed as a capture antibody and 50ng/ml of polyclonal goat anti-AAT specific antibody (Abcam) as a detection antibody, followed by an anti-goat IgG conjugated to HRP. Prior to each assay, wells from Nunc 96-well microtiter plates were coated with capture antibody suspended in Buffer A (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.3) over night at 4°C. The wells were subsequently washed in Buffer A, blocked with bovine serum albumin (BSA; 1% w/v) for 1h and exposed to patient plasma (100μl). As the ELISA was based on quantification of AAT complexed to CD16b that is captured by the anti-CD16b antibody, serial dilutions of

purified human AAT was simultaneously coated in triplicate in the Nunc 96-well microtiter plate to establish a standard curve as previously described (29). Controls included all reagents except capture, detection antibody or biotinylated secondary antibody.

2.5 Statistical analysis

All experiments were performed a minimum of three times and results expressed as means ± standard error of the mean. The data was analyzed with the GraphPad Prism version 4.03 for Windows (GraphPad Software, USA). Continuous data were tested for normality (1 sample Kolmogorov - Smirnoff test) and where normal were compared using an unpaired student t-test. Differences were considered significant at $P \leq 0.05$. Quantification of protein abundance was determined using the biological variation analysis module of Decyder™ using ANOVA-1 analysis for comparing across the different groups (healthy controls, non-CF bronchiectasis and CF samples pre- and post-antibiotic treatment). Differential expression was defined as greater than 1.5-fold change in expression with a P-value ≤ 0.05.

3. Results

3.1 Proinflammatory mediators cause release of AAT and CD16b from neutrophil membranes.

We have previously shown that exposure of neutrophils to sIC causes release of AAT and CD16b from the neutrophil membrane in a complex form (25). To build on this background information the effect of the proinflammatory stimulus IL-8, on release/shedding of AAT and CD16b from the neutrophil membrane was investigated. Neutrophils were primed with IL-8 (10ng/2x10^7 cells) and after 10 min of treatment a significant decrease in cell surface AAT was detected by flow cytometry (control untreated mean fluorescence = 50.98 ± 0.67 and IL-8 treated cells mean = 17.62 ± 0.06, $P<0.05$) (Fig. 1A). Moreover, the observed decrease in membrane AAT corresponded with a reduction in CD16b expression (control untreated mean fluorescence = 38.75 ± 0.11 and IL-8 treated cells mean = 19.22 ± 0.05, $P<0.05$) (Fig. 1B). Furthermore, we have previously shown that release of AAT:CD16b complex from the cell membrane in response to sIC involves surface sheddase activity including ADAM-17 (25). We therefore investigated whether ADAM-17 activity was required for concomitant release of AAT with CD16b in response to TNF-alpha. This was confirmed when cells were primed with TNF-alpha (10ng/2x10^7 cells) and the extracellular supernatants analysed by Western blot for the presence of released CD16b and AAT (Fig. 1C). TNF-alpha induced release of CD16b and AAT at 5 and 10 min post exposure, an effect inhibited by inclusion of the specific ADAM-17 inhibitor, TAPI-1 (10 μM) (30). TAPI-1 prevented release of AAT and CD16b in response to TNF-alpha and significantly reduced the extracellular detectable level of the two proteins by approximately 80% at the 5 min time

point (P<0.05) (Fig. 1D). Collectively, these results demonstrate that neutrophils primed by pro-inflammatory mediators including IL-8 and TNF-alpha, rapidly release AAT and CD16b from the neutrophil membrane.

3.2 Increased levels of proinflammatory mediators and CD16b:AAT complex in plasma from CF patients.

Quantification of IL-8 by ELISA revealed significantly elevated levels of IL-8 in CF plasma compared to COPD and control subjects (553.8 ± 82.2 versus 249.9 ± 26.2 and 168.3 ± 51.2 pg/ml plasma respectively, P<0.05) (Fig. 2A). In accordance with results obtained for IL-8, significantly elevated levels of TNF-alpha were measured in CF plasma compared to COPD and control subjects (10.43 ± 2.12 versus 1.56 ± 0.75 and 4.16 ± 1.02 pg/ml plasma respectively, P<0.05) (Fig. 2B). Moreover, individuals with CF were more likely to have elevated plasma levels of AAT:CD16b compared to COPD and control groups (Fig. 2C). Significantly higher levels of AAT:CD16b protein complex were detected in CF patient plasma (6.47 ± 0.65 ng/ml) compared with results from the other groups (COPD = 0.73 ± 0.26, AATD = 0.01 ± 0.005 and control = 0.80 ± 0.24; P<0.05) (Fig. 2C). This sandwich ELISA employed a detection antibody to AAT and measured negligible levels of AAT:CD16b complex in plasma of AATD individuals. Accordingly, this sample cohort served as a negative control and a measure of accuracy of the AAT:CD16b ELISA.

Next, we analyzed the correlation between levels of AAT:CD16b and proinflammatory mediators (IL-8 and TNF-alpha) within CF plasma. The combined IL-8 and TNF-alpha levels in plasma showed a significant positive correlation with the

concentration of AAT:CD16b complex (n = 62, r = 0.4223; P = 0.0001; Fig. 3). Taken together these results illustrate a novel correlation between increased pro-inflammatory mediators and AAT:CD16b complex plasma levels.

3.3 Levels of AAT:CD16b complex in CF patients decrease post antibiotic therapy.

To determine if levels of AAT:CD16b complex in plasma could reflect the inflammatory states of individuals with CF, we carried out analysis of patients plasma pre- and post-antibiotic therapy. Patients were treated for an acute microbial exacerbation related to *Pseudomonas aeruginosa* (*P. aeruginosa*) and/or *Staphylococcus aureus* (*S. aureus*) bacterial infection predominately. As illustrated in Table 1, an improvement in spirometry measurements was observed in most cases following treatment (pretreatment FEV1 % predicted mean of 35.20, range 23-48; post-treatment mean 43.60, range 24–65).

CF plasma samples before and after intravenous antibiotic therapy were quantified for AAT:CD16b complex by ELISA. Of the 10 patient samples analyzed, levels of AAT:CD16b decreased after the administration of antibiotic therapy with the exception of 2 (Fig. 4A). These latter two patients were deemed nonresponsive to treatment and clinical symptoms did not improve with therapy, as measured by a decreased increment in FEV1 (Table 1: CF4 & CF6).

Proteomic analysis was carried out to evaluate the expression of the AAT:CD16b complex on isolated neutrophil membranes pre- and post-therapy. The 6 patients with CF selected for proteomic analysis were representative of the whole group (same degree of exacerbation) and controls for this experiment included membrane samples from 6 non-

CF bronchiectasis (inflammatory control) and 6 healthy control donors. Antibiotic treatment modulated expressions of multiple protein spots present on analytical 2-D gel images compared to the samples collected without treatment on day 0. In total, ~750 spots were detected on the 2-D analytical gels by DeCyder software. When comparing the membrane protein spots on the gels from day 0 with that of 14-day post treatment and control membranes, 36 spots showed differential expression. Two of these spots were down-regulated in CF samples on day 0, while they were increased at least 1.5-fold in both the membrane samples from the 14-day-treated CF patients and the samples from the control persons (non-CF bronchiectasis and healthy control membranes, $P<0.05$ by ANOVA-1). Those 2 unknown spots were excised from the gels and identified as AAT and CD16b by LC-MS/MS (accession number gi994572 and gi703025, respectively). Densitometry of Western blots of CF membranes for AAT and CD16b pre- and post-antibiotic therapy, confirmed proteomic results (Fig. 4B&C) and indicated significantly increased expression of AAT and CD16b ($P=0.01$ and $P=0.05$ respectively) on CF neutrophil membranes post antibiotic treatment. Of clinical importance, post antibiotic treatment, a positive correlation was found between % increase in FEV1 and reduced circulating plasma levels of AAT:CD16b ($n = 10$, $r = 0.84$, $p < 0.005$; Fig. 5). Collectively these results indicate that treatment of an exacerbation with antibiotic therapy in CF results in decreasing levels of plasma AAT:CD16b, with a corresponding increase in the level of membrane bound complex and improved FEV1.

4. Discussion

Pulmonary exacerbations are important contributors to morbidity and are associated with lung function decline over 1 year in CF (31). However, a foremost obstacle in investigating the processes and functional changes associated with CF exacerbations is the lack of a clear diagnostic standard. A circulating biomarker of neutrophil priming prior to airway neutrophil migration and activation would serve as a specific biomarker for the early detection of CF exacerbations and the subsequent response to antibiotic treatment. In this study we have combined well-defined clinical groups and demonstrated that plasma levels of neutrophil released AAT:CD16b complex correlate with circulating plasma levels of pro-inflammatory mediators. Post antibiotic treatment of CF pulmonary exacerbation, we observed an increase in FEV1 and a corresponding decrease in plasma protein concentration of AAT:CD16b complex. These results indicate that the AAT:CD16b plasma biomarker effectively reflects exacerbations of the disease.

Research within this area has identified a number of possible biomarkers of CF exacerbation. For example, by employing a proteomic approach of induced sputum from adult patients with CF, proteolytic degradation and glycosylation of mucins MUC5B and MUC5AC (32) or degradation of IgG and alpha-1 antitrypsin, were proposed as predictors of CF lung exacerbation (23). By immunometric techniques sputum levels of IL-8 have also been shown to significantly alter following treatment of CF exacerbations with antibiotic therapy, suggestive of a noninvasive outcome measure to assess response to therapy in CF patients (33). However, Downey et al (2007) did not find a reduction in sputum IL-8 levels (7), indicating that sputum IL-8 correlates poorly with lung function,

thus questioning the suitability of IL-8 as a reliable biomarker of CF exacerbations. Therefore as an alternative to using sputum, serum samples from patients with CF have also been compared with control sera in order to recognize protein expression profiles specific to CF during an exacerbation. Compared to healthy controls, matrix metalloproteinase (MMP-1, -8 and -9) and G-CSF serum levels were elevated in adult patients with CF and correlated with pulmonary exacerbation and antibiotic treatment (19, 20). Serum levels of S100A12 (34), S100A8/A9, CRP and vascular endothelial growth factor (35) have also been suggested as serum markers of acute infectious exacerbations and to decrease significantly post treatment of an exacerbation. Moreover, whilst CF exacerbations were not shown to modulate neutrophil function (36), neutrophil derived proteins including myeloperoxidase in peripheral blood appeared to reflect inflammatory changes post antibiotic treatment (37). Myeloperoxidase however is a component of neutrophil primary / azurophilic granules and peroxidase exocytosis is a tightly regulated process. Within the present study, rather than identifying markers of neutrophil activation we have focused on a plasma marker of neutrophil pre-activation or priming. Priming can be induced by pro-inflammatory stimuli which are increased in an *in vivo* state of CF exacerbation and which prepare the neutrophil for a state of readiness preceding complete activation. The fundamental mechanism leading to a primed response involves increased levels of cytosolic calcium (38), upregulated tyrosine phosphorylation (39) and cytoskeletal rearrangements (40). *Pseudomonas* alginate (41), TNF-alpha and IL-8 have been shown to be important priming agents for CF neutrophils (42). Recently we have shown that AAT is localized to the neutrophil plasma membrane within lipid rafts bound to CD16b (FcγRIIIb) and is released in complex form (AAT:CD16b) by

priming with sIC (25). CD16b is unique in that it is the only Fc receptor linked to the plasma membrane by a GPI anchor and the metalloprotease ADAM-17 has been implicated in the shedding of CD16b. Within the present study we show that priming of neutrophils *in vitro* with IL-8 and TNF-alpha causes release of CD16b and AAT to the extracellular milieu and *in vivo*, AAT:CD16b complex plasma levels correlate strongly with these pro-inflammatory mediators.

Although antibiotic responsiveness in the absence of quantitative cultures and serotype switching of bacterial species is only suggestive of infective exacerbations, within our study combination antibiotic treatment of individuals for an acute exacerbation involving *P. aeruginosa* and/or *S. aureus* infection resulted in an improvement in FEV1 in 8 of 10 patients tested. In addition, the changes in plasma AAT:CD16b following antibiotic therapy suggested a direct association of plasma levels of AAT:CD16b complex with a changing state of airway inflammation and FEV1. Indeed, a soluble form of CD16b released from neutrophils and bound to IgG (43) has previously been detected in plasma. However, although the soluble CD16b concentration has been shown to increase at inflammatory sites (43, 44), a vigorous examination of its use as a plasma biomarker of inflammation or in the analysis of antibiotic efficiency has not been previously performed. Moreover, the minimum level of soluble CD16b detected by use of a monoclonal antibody in normal serum was 7.3nmol/L (43). In contrast, the lowest detectable level of plasma AAT:CD16b was 0.006nmol/L. These results suggest increased sensitivity of the dual ELISA for detection of the complex biomarker compared to CD16b on its own, possibly due to masking of antibody binding sites within the protein complex. The present study is however not without limitations. For example

correlates of disease states between healthy controls, COPD, AATD and CF individuals are not directly comparable since only patients with CF were in exacerbation and only a proportion of these patients had before and after samples of plasma analysed. Moreover, data indicate that a fraction of the patients with CF would be indistinguishable from the other groups when plasma AAT:CD16b complex is used as a sole marker of exacerbation. However, in support of the use of AAT:CD16b as an indicator of response, proteomic and Western blot analysis of patient neutrophil plasma membranes pre- and post-antibiotic treatment revealed increased membrane bound expression of both AAT and CD16b post treatment. Moreover, in this study we have developed a novel microplate-based protein binding assay employing the use of two antibodies raised against AAT and CD16b which allows for rapid screening of multiple serum samples for AAT:CD16b interactions. This method measures a precise protein-protein interaction, utilizes relatively small amounts of patient sample, is free of protein modification and does not require specialized instrumentation. Thus the clinical benefit of introducing the described AAT:CD16b protein binding assay as an effective tool for evaluating pulmonary exacerbations is apparent. Conversely however, the cost-effectiveness in terms of health quality gained versus cost of implementation requires further evaluation.

In conclusion, primed neutrophils have been found in peripheral blood during an exacerbation of CF, an observation now supported by our study and the identification of circulating levels of AAT:CD16b. Upon treatment of an exacerbation plasma levels of AAT:CD16b complex decrease significantly, correlating with an improvement in FEV1. These observations illustrate that the expression of neutrophil priming-associated biomarkers in peripheral blood can be used as read out for the inflammatory process in

CF. The AAT:CD16b biomarker may be of benefit to clinical trials determining if drugs-in-process are effective against inflammation and may also become an extremely useful addition to the clinical diagnosis of exacerbations and management of CF.

Acknowledgments

This work was supported by The US Alpha-1 Foundation, The Medical Research Charities Group/Health Research Board, and the Program for Research in Third Level Institutes (PRTLI) administered by the Higher Education Authority. We thank all patients who participated within this study.

References

1. Davis PB. Cystic fibrosis since 1938. Am J Respir Crit Care Med. 2006 Mar 1;173(5):475-82.

2. Boucher RC. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. Trends Mol Med. 2007 Jun;13(6):231-40.

3. Hassett DJ, Korfhagen TR, Irvin RT, Schurr MJ, Sauer K, Lau GW, et al. Pseudomonas aeruginosa biofilm infections in cystic fibrosis: insights into pathogenic processes and treatment strategies. Expert Opin Ther Targets. 2010 Feb;14(2):117-30.

4. Kahl BC. Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease. Int J Med Microbiol. 2010 Dec;300(8):514-9.

5. Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med. 2008 May 1;177(9):995-1001.

6. Worlitzsch D, Rintelen C, Bohm K, Wollschlager B, Merkel N, Borneff-Lipp M, et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. Clin Microbiol Infect. 2009 May;15(5):454-60.

7. Downey DG, Brockbank S, Martin SL, Ennis M, Elborn JS. The effect of treatment of cystic fibrosis pulmonary exacerbations on airways and systemic inflammation. Pediatr Pulmonol. 2007 Aug;42(8):729-35.

8. Tabary O, Zahm JM, Hinnrasky J, Couetil JP, Cornillet P, Guenounou M, et al. Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells in vivo and in vitro. Am J Pathol. 1998 Sep;153(3):921-30.

9. Muhlebach MS, Noah TL. Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. Am J Respir Crit Care Med. 2002 Apr 1;165(7):911-5.

10. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Sci Transl Med. 2010 Apr 28;2(29):29ra31.

11. Heinzl B, Eber E, Oberwaldner B, Haas G, Zach MS. Effects of inhaled gentamicin prophylaxis on acquisition of Pseudomonas aeruginosa in children with cystic fibrosis: a pilot study. Pediatr Pulmonol. 2002 Jan;33(1):32-7.

12. Ratjen F, Doring G, Nikolaizik WH. Effect of inhaled tobramycin on early Pseudomonas aeruginosa colonisation in patients with cystic fibrosis. Lancet. 2001 Sep 22;358(9286):983-4.

13. Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med. 1994 Sep 8;331(10):637-42.

14. Rabin HR, Butler SM, Wohl ME, Geller DE, Colin AA, Schidlow DV, et al. Pulmonary exacerbations in cystic fibrosis. Pediatr Pulmonol. 2004 May;37(5):400-6.

15. Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, et al. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. N Engl J Med. 1999 Jan 7;340(1):23-30.

16. Rosenfeld M, Emerson J, Williams-Warren J, Pepe M, Smith A, Montgomery AB, et al. Defining a pulmonary exacerbation in cystic fibrosis. J Pediatr. 2001 Sep;139(3):359-65.

17. Dakin C, Henry RL, Field P, Morton J. Defining an exacerbation of pulmonary disease in cystic fibrosis. Pediatr Pulmonol. 2001 Jun;31(6):436-42.

18. Bilton D, Canny G, Conway S, Dumcius S, Hjelte L, Proesmans M, et al. Pulmonary exacerbation: towards a definition for use in clinical trials. Report from the EuroCareCF Working Group on outcome parameters in clinical trials. J Cyst Fibros. 2011 Jun;10 Suppl 2:S79-81.

19. Jensen PO, Moser C, Kharazmi A, Presler T, Koch C, Hoiby N. Increased serum concentration of G-CSF in cystic fibrosis patients with chronic Pseudomonas aeruginosa pneumonia. J Cyst Fibros. 2006 Aug;5(3):145-51.

20. Roderfeld M, Rath T, Schulz R, Seeger W, Tschuschner A, Graf J, et al. Serum matrix metalloproteinases in adult CF patients: Relation to pulmonary exacerbation. J Cyst Fibros. 2009 Sep;8(5):338-47.

21. Eickmeier O, Huebner M, Herrmann E, Zissler U, Rosewich M, Baer PC, et al. Sputum biomarker profiles in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and association between pulmonary function. Cytokine. 2010 May;50(2):152-7.

22. Sagel SD, Sontag MK, Wagener JS, Kapsner RK, Osberg I, Accurso FJ. Induced sputum inflammatory measures correlate with lung function in children with cystic fibrosis. J Pediatr. 2002 Dec;141(6):811-7.

23. Sloane AJ, Lindner RA, Prasad SS, Sebastian LT, Pedersen SK, Robinson M, et al. Proteomic analysis of sputum from adults and children with cystic fibrosis and from control subjects. Am J Respir Crit Care Med. 2005 Dec 1;172(11):1416-26.

24. Martin SL, Moffitt KL, McDowell A, Greenan C, Bright-Thomas RJ, Jones AM, et al. Association of airway cathepsin B and S with inflammation in cystic fibrosis. Pediatr Pulmonol. 2010 Sep;45(9):860-8.

25. Bergin DA, Reeves EP, Meleady P, Henry M, McElvaney OJ, Carroll TP, et al. alpha-1 Antitrypsin regulates human neutrophil chemotaxis induced by soluble immune complexes and IL-8. J Clin Invest. 2010 Dec 1;120(12):4236-50.

26. Bergin DA RE, Fitzgerald S, Vega-Carrascal I, Hayes E, Keenan J, Clynes M, Low TB, O' Neill SJ, and McElvaney NG. Neutrophil Derived CD16B: Alpha-1 Antitrypsin Complex; A Novel Biomarker Of Inflammation In Cystic Fibrosis. Am J Respir Crit Care Med. 2011;183:A5739. .

27. Greene CM, Low TB, O'Neill SJ, McElvaney NG. Anti-proline-glycine-proline or antielastin autoantibodies are not evident in chronic inflammatory lung disease. Am J Respir Crit Care Med. 2010 Jan 1;181(1):31-5.

28. Wilmott RW, Amin RS, Colin AA, DeVault A, Dozor AJ, Eigen H, et al. Aerosolized recombinant human DNase in hospitalized cystic fibrosis patients with acute pulmonary exacerbations. Am J Respir Crit Care Med. 1996 Jun;153(6 Pt 1):1914-7.

29. Lovell MA, Lynn BC, Xiong S, Quinn JF, Kaye J, Markesbery WR. An aberrant protein complex in CSF as a biomarker of Alzheimer disease. Neurology. 2008 Jun 3;70(23):2212-8.

30. Shao MX, Ueki IF, Nadel JA. Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. Proc Natl Acad Sci U S A. 2003 Sep 30;100(20):11618-23.

31. Konstan MW, Morgan WJ, Butler SM, Pasta DJ, Craib ML, Silva SJ, et al. Risk factors for rate of decline in forced expiratory volume in one second in children and adolescents with cystic fibrosis. J Pediatr. 2007 Aug;151(2):134-9, 9 e1.

32. Schulz BL, Sloane AJ, Robinson LJ, Prasad SS, Lindner RA, Robinson M, et al. Glycosylation of sputum mucins is altered in cystic fibrosis patients. Glycobiology. 2007 Jul;17(7):698-712.

33. Ordonez CL, Henig NR, Mayer-Hamblett N, Accurso FJ, Burns JL, Chmiel JF, et al. Inflammatory and microbiologic markers in induced sputum after intravenous antibiotics in cystic fibrosis. Am J Respir Crit Care Med. 2003 Dec 15;168(12):1471-5.

34. Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, et al. Expression of S100A12 (EN-RAGE) in cystic fibrosis. Thorax. 2003 Jul;58(7):613-7.

35. Gray RD, Imrie M, Boyd AC, Porteous D, Innes JA, Greening AP. Sputum and serum calprotectin are useful biomarkers during CF exacerbation. J Cyst Fibros. 2010 May;9(3):193-8.

36. Brockbank S, Downey D, Elborn JS, Ennis M. Effect of cystic fibrosis exacerbations on neutrophil function. Int Immunopharmacol. 2005 Mar;5(3):601-8.

37. Niggemann B, Stiller T, Magdorf K, Wahn U. Myeloperoxidase and eosinophil cationic protein in serum and sputum during antibiotic treatment in cystic fibrosis patients with Pseudomonas aeruginosa infection. Mediators Inflamm. 1995;4(4):282-8.

38. Forehand JR, Pabst MJ, Phillips WA, Johnston RB, Jr. Lipopolysaccharide priming of human neutrophils for an enhanced respiratory burst. Role of intracellular free calcium. J Clin Invest. 1989 Jan;83(1):74-83.

39. Hallett MB, Lloyds D. Neutrophil priming: the cellular signals that say 'amber' but not 'green'. Immunol Today. 1995 Jun;16(6):264-8.

40. Mukherjee G, Quinn MT, Linner JG, Jesaitis AJ. Remodeling of the plasma membrane after stimulation of neutrophils with f-Met-Leu-Phe and dihydrocytochalasin B: identification of membrane subdomains containing NADPH oxidase activity. J Leukoc Biol. 1994 Jun;55(6):685-94.

41. Pedersen SS, Kharazmi A, Espersen F, Hoiby N. Pseudomonas aeruginosa alginate in cystic fibrosis sputum and the inflammatory response. Infect Immun. 1990 Oct;58(10):3363-8.

42. Taggart C, Coakley RJ, Greally P, Canny G, O'Neill SJ, McElvaney NG. Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-alpha and interleukin-8. Am J Physiol Lung Cell Mol Physiol. 2000 Jan;278(1):L33-41.

43. Fleit HB, Kobasiuk CD, Daly C, Furie R, Levy PC, Webster RO. A soluble form of Fc gamma RIII is present in human serum and other body fluids and is elevated at sites of inflammation. Blood. 1992 May 15;79(10):2721-8.

44. Hommes DW, Meenan J, de Haas M, ten Kate FJ, von dem Borne AE, Tytgat GN, et al. Soluble Fc gamma receptor III (CD 16) and eicosanoid concentrations in gut lavage fluid from patients with inflammatory bowel disease: reflection of mucosal inflammation. Gut. 1996 Apr;38(4):564-7.

Figure 1

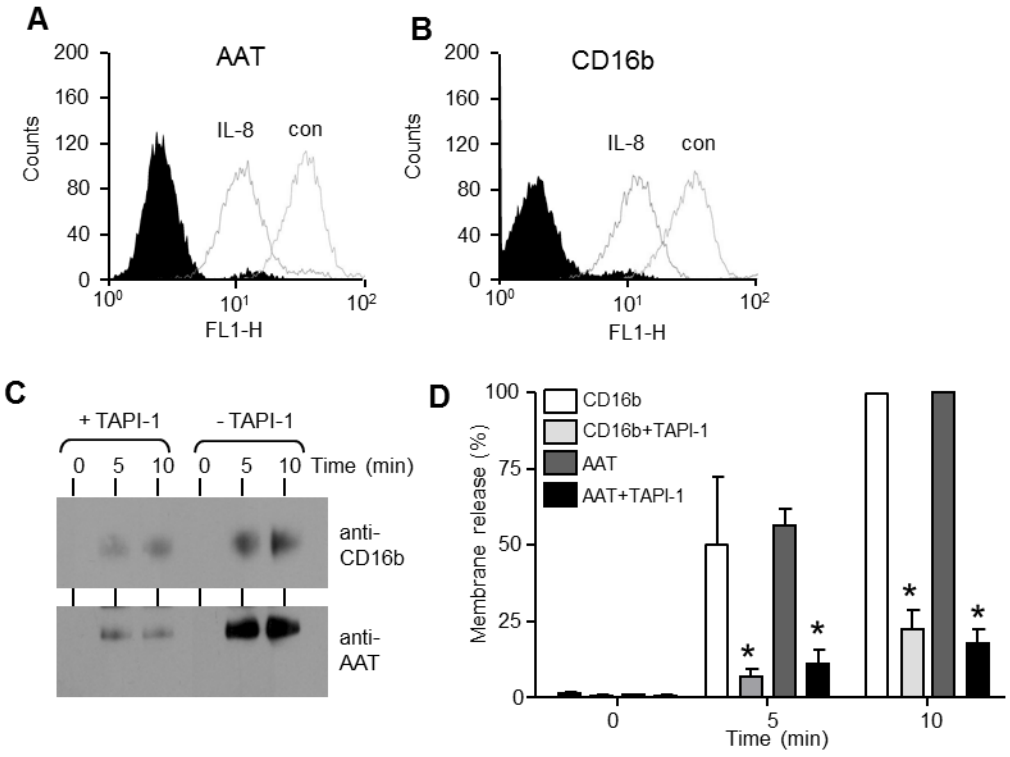

Fig. 1. Pro-inflammatory mediators cause release of AAT and CD16b from the neutrophil membrane.

FACS analysis showing membrane expression of **(A),** AAT or **(B),** CD16b on membranes of un-stimulated control neutrophils (con) or in response to IL-8 (10 ng/2x10^7 cells). The isotype control antibody is illustrated in black (filled). Experiments presented were performed in triplicate on three consecutive days. **C:** Representative immuno-blots (one of 3 separate experiments) showing time course of TNF-alpha (10 ng/2x10^7 cells) induced extracellular release of AAT and CD16b from neutrophils with or without TAPI-1 (10μM). Experiments employed rabbit and goat polyclonal antibody against AAT and CD16b respectively. **D;** Quantification of AAT and CD16b release from neutrophils treated with TNF-alpha in the presence or absence of TAPI-1. TAPI-1 significantly reduced release of AAT and CD16b (*P<0.05 compared to respective untreated sample).

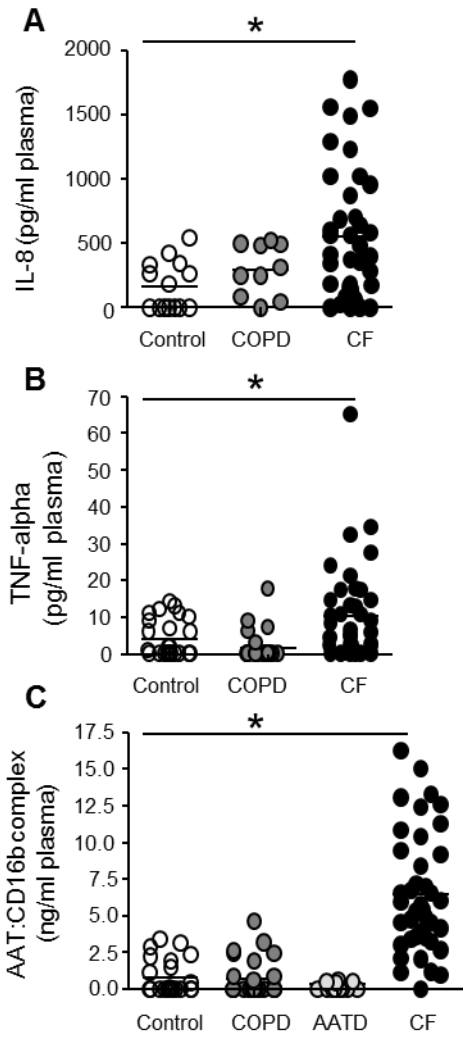

Fig. 2. Protein levels of inflammatory molecules and AAT:CD16b complex in plasma from COPD, AATD, CF and healthy controls.
ELISA analyses of IL-8 (pg/ml) **(A),** TNF-alpha (pg/ml) **(B)** or AAT:CD16b complex (ng/ml) **(C)** in plasma from healthy (control; n=14), COPD (n=10), AATD (n=10) and CF (n=38) individuals. Statistical significance was analysed by ANOVA, *P<0.05.

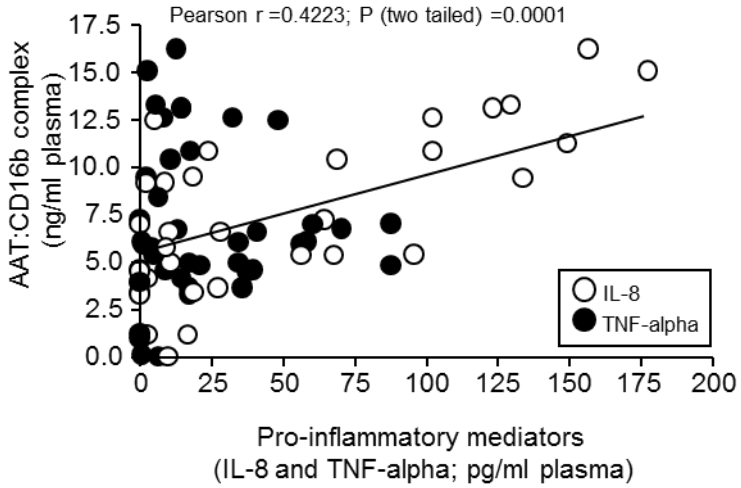

Fig. 3. Positive correlation between AAT:CD16b protein complex and proinflammatory mediators in plasma.

The correlation between IL-8 (○) and TNF-alpha (●) and plasma AAT:CD16b protein complex levels was deemed significant when $\alpha = 0.05$ (Pearson r = 0.42, P<0.0001, R^2 = 0.17).

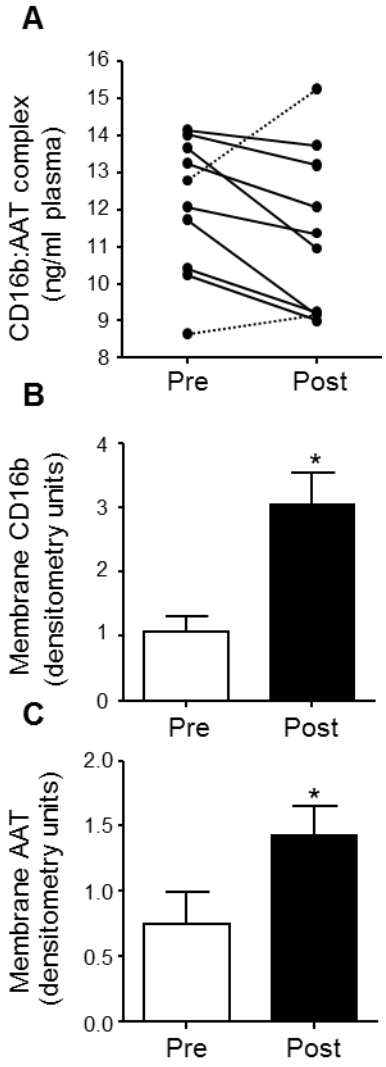

Fig. 4. Levels of AAT:CD16b complex decrease in plasma samples post antibiotic therapy.

A; ELISA quantification of AAT:CD16b complex (ng/ml) plasma levels before (Pre) and 14 days after (Post) antibiotic treatment of patients with CF (n=10). Two nonresponsive patients had higher levels of AAT:CD16b complex (dashed line) **B&C;** Membrane expression of CD16b **(B)** or AAT **(C)** on CF neutrophils before (Pre) and after (Post) antibiotic therapy. The data presented were calculated using constants obtained of Western blot densitometry values. Each measurement is the mean ± S.E (*P<0.05).

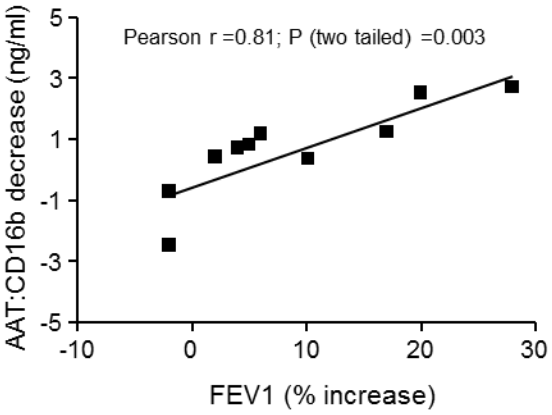

Fig. 5. Positive correlation between reduced plasma levels of AAT:CD16b protein complex and improved FEV1 post antibiotic treatment.

Correlation between FEV1 (% increase) after two weeks antibiotic treatment of individuals with CF (n=10) and changes in plasma levels of AAT:CD16b (ng/ml plasma).

Supplemental Materials and Methods.

Proteomic analysis of neutrophil plasma membranes.

Neutrophil membrane proteins (25 μg) were minimally labelled with 400 pmol Cy2 (CF patients when stable, CF patients during an exacerbation, non-CF bronchiectasis and healthy control membranes: internal control), Cy3 (CF patients when stable or CF patients during an exacerbation) and Cy5 (healthy control or non-CF bronchiectasis) according to manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Six biological repeats of each comparison were used with reverse labelling on three repeats. IEF was performed using immobilised pH gradient (IPG) strips (pH 3-10, 13cm; GE Healthcare) and run for a total of 40k V/h at 22^{o}C. Prior to electrophoresis in the second dimension, IPG strips were equilibrated twice in 10 ml equilibration buffer [30 % (v/v) glycerol, 2 % (w/v) SDS, 6 M urea, 50 mM Tris/HCl, pH 6.8]. The first equilibration was in 10 ml equilibration buffer containing 2 % (w/v) DTT and the second contained 2.5 % (w/v) iodoacetamide. After second dimension SDS-PAGE (10% w/v) gels were scanned using the Typhoon 9400 variable mode imager (GE Healthcare) with image analysis performed using the DeCyder^{TM} Software version 6.5 (GE Healthcare). Statistical analysis and quantification of protein abundance was as previously described using the biological variation analysis module (BVA) of DeCyder^{TM} (32). Protein identification by LC-MS/MS was performed on an Ultimate 3000 nanoLC system (Dionex), interfaced to an LTQ Orbitrap XL (Thermo Fisher Scientific) as previously described (30). Database searches were performed using TurboSEQUEST software (Bioworks Browser version 3.3.1) (Thermo Fisher Scientific). The following filters were applied: for charge state 1, X_{Corr}>1.5; for charge state 2, X_{Corr}>2.0; for charge state 3, X_{Corr}>2.5.

SDS-PAGE and Western blot analyses.

Samples were subjected to SDS-PAGE under denaturing conditions in 12% NuPAGE® gels (Invitrogen™, Carlsbad, CA, USA) following the manufacturer's instructions. After electrophoresis gels were transferred onto 0.2 μm nitrocellulose or PVDF membrane (Sigma-Aldrich, St. Louis, MO, USA) by Western blotting using a semi dry blotter for 1 h at 100mA. Membranes were blocked for 1 h in 3% dry milk (w/v) and 1% bovine serum albumin (BSA) in PBS containing 0.25% (v/v) Tween 20 (PBST). Blots were incubated with 1.0 μg/ml polyclonal rabbit anti-AAT specific antibody (Abcam, UK) or polyclonal goat anti-CD16b specific antibody (R&D Systems, UK). The secondary antibodies were HRP-linked anti-rabbit, or –goat IgG (Cell Signalling Technology, Danvers, MA, USA). Immuno-reactive protein bands were visualized employing SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) after exposure to Kodak® X-Omat LS Film.

Flow cytometry

Cells remained untreated or treated with IL-8 (10ng/2x10^7 cells) for 10 min at 37^{o}C. Neutrophils were fixed (4 % (w/v) paraformaldehyde) and blocked (2% (w/v) BSA) for 1h and incubated with FITC labeled goat polyclonal anti-AAT (1μg/10^6 cells) (Abcam, UK) or mouse anti-CD16b (Santa Cruz) followed by FITC labeled bovine anti-mouse secondary antibody (Santa Cruz). Control samples were exposed to relevant non-specific isotype control IgG or secondary labeled antibody alone and fluorescence counted by flow cytometry. A total of 10,000 events were collected. The data were analyzed using BD FACSDiva software (Franklin Lakes, NJ).