Reduction in neutrophil elastase concentration by recombinant α1-antitrypsin (recAAT) does not alter bacterial loading in the sputum of cystic fibrosis patients

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Table 1. Dose response effect of recombinant α1-antitrypsin (recAAT) on quantitative microbiological counts of CF patients' sputa.

	Mean $\log_{10}$ colony forming units (cfu) per gram of sputum				
recAAT dose	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
Placebo	8.24	8.33	8.07	8.76	9.29
125mg	8.24	8.22	8.38	8.35	8.30
250mg	8.44	8.62	8.46	8.76	8.71
500mg	8.14	8.13	8.33	7.99	9.15

Each value represents the grand mean of 34 male patients' sputum quantitative count, where three serial dilutions were plated in triplicate and replicated twice.

Cystic fibrosis (CF) is the most commonly inherited disease in those of a Caucasian and European background, and has a genetic carriage rate of 1 in 20 persons and an incidence of 1 in 2500 live births. It is an autosomal recessive condition whereby two alleles carrying a polymorphism in the CF transmembrane conductance regulator (*CFTR*) gene phenotypically manifest the disease state through a variety of multi-organ problems associated with a pharmacological dysfunction to regulate chloride ion secretion across cell membranes.

The most common complication of CF is the recurrence of chronic chest infection, usually caused by bacterial pathogens.[1] Patients continue to suffer from recurrent and chronic respiratory tract infections, and morbidity and mortality are due largely to such infections throughout their life.[2,3]

These infections are usually dominated by Gram-negative organisms, especially the pseudomonads, including *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia*. Damage is inflicted on the respiratory epithelium by chronic bacterial colonisation and neutrophils are attracted by chemotactic stimuli. These activated neutrophils release oxygen radicals and proteolytic enzymes, primarily to destroy bacteria but this results in further epithelial damage and destruction of the airways and lung parenchyma. Neutrophil elastase is the most active protease in the airways and is significantly raised in the sputa of CF patients.

The commercial development of recombinant α1-antitrypsin (recAAT) as a treatment in CF to reduce/minimise the tissue-damaging effects of neutrophil elastase has been described recently.[4] In the phase IIa study, 39 patients participated in a prospective, double-blinded, randomised, placebo-controlled trial to examine the effect of recAAT (500, 250 and 125 mg) on sputum neutrophil elastase, other markers of disease and safety parameters.

Subjects from centres across the UK (Belfast, Cambridge, Cardiff, Edinburgh and Sheffield) were randomised to receive nebulised treatment once daily for four weeks, followed by no study treatment for two to four weeks, followed by a two-week rechallenge phase. Although recAAT was not employed as an antibiotic agent in these studies, it was considered important to examine the effects of nebulised recAAT treatment on CF patients in relation to the quantitative bacterial loading of patients' sputa.

Duplicate sputa specimens (1 mL, minimum) were collected from 39 adult male patients (age range: 16.6–69.2 years; mean: 27.5 years) in sterile plastic disposable containers (100 mL) on five visits to the clinic in relation to the study. Patients were randomised to one of the four treatment groups according to a predetermined randomisation list. Randomisation was stratified by centre and for colonisation with *B. cepacia*, which is known to predict a poor prognosis in patients with CF.

The active study medication comprised recAAT (PPL Therapeutics, Roslin, Scotland, UK) at 500 mg, 250 mg or 125 mg doses in a histidine and sodium chloride buffer (reconstituted volumes were 10, 5 and 2.5 mL, respectively). The placebo medication comprised histidine and sodium chloride buffer alone. After an initial screening visit (visit 1), eligible patients returned to the clinic for a supervised first dose and then subsequently once-daily self-administered the study medication as a nebulised solution, using the Pari LC-Star nebuliser with the Turbo Boy compressor (Pari GmbH, Starnberg, Germany), for a period of four weeks. This was followed by a period of two to four weeks with no treatment and then by a two-week rechallenge phase for safety analysis. For the duration of the study, patients were required to attend the clinic once a week to allow assessment (visits 2–5). Patients then had a safety follow-up visit approximately two weeks after the last dosing.

Sputum was collected immediately after a standardised session of physiotherapy, stored at ambient temperature and processed within 4 h of collection. Fresh sputum (1 mL, minimum) was mixed with an equal mass of Sputasol (Oxoid SR089A, Oxoid, Poole, England) and was incubated in a water bath at 37°C for 15 min, before further processing and enumeration. Serial dilutions of sputum were prepared in quarter-strength Ringer's solution diluent (Oxoid BR52). From the 10^{-4}, 10^{-5} and 10^{-6} dilutions in triplicate, 100 µL inoculum was accurately spread on to the surface of Columbia agar base (Oxoid CM331) supplemented with 5% (v/v) defibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland) and incubated at 37°C for 48 h in a 5% (v/v) CO_2 atmosphere prior to counting. All cultured

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flora, regardless of colonial morphology and appearance were enumerated and the total viable count (TVC) was expressed as $\log_{10}$ colony forming units (cfu) per gram (cfu/g) of original sputum.

Results demonstrated that there was no statistical difference ($P>0.05$; 5%) between sputum counts within a dosing regimen over time, with the exception of the placebo control at visit 1 and visit 6 ($P=0.0104$), or between dosing regimens at any visit (Table 1). The overall sputum loading associated with all patients and all dosing regimens was $\log_{10}$ 8.45 cfu/g sputum.

Recruitment of large numbers of neutrophils to the lungs of CF patients in response to the chronic presence of mainly Gram-negative organisms, specifically *P. aeruginosa*, establishes a chronic cycle of infection/inflammation that leads eventually to irreversible matrix tissue damage as a result of the presence of neutrophil elastase. Hence, any therapeutic intervention that can minimise the effect of elastase on lung tissue would appear to be a prudent approach to maintaining adequate lung function. However, neutrophil elastase is an important agent of non-oxidative killing of bacteria, along with lysozyme, defensins, bacterial/permeability-increasing (BPI) protein, cathepsin G and proteinase 3 activities.[5] Thus, any reduction in the efficacy of bactericidal activity due to therapeutically altered neutrophil elastase should be examined carefully.

In the present study, treatment of the patient population with up to 500 mg recAAT did not change the quantitative loading of bacteria in the lung, suggesting that the bactericidal function of elastase was not significantly altered.

Overall, this study serves to illustrate the importance of monitoring potential changes in the existing quantitative microbial ecology whenever the stringency of a microbiological hurdle is lowered, particularly if an antimicrobial agent is involved. In conclusion, treatment of adult male CF patients with recAAT in this study did not alter the bacterial loading of sputum in this patient population. □

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Molecular (PCR) detection of *Pseudomonas* spp. other than *P. aeruginosa* directly from the sputum of adults and children with cystic fibrosis

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Cystic fibrosis (CF) is the most common genetic disease in Caucasians, with an incidence of 1 in 2500 live births and a carriage rate of 1 in 20 individuals. Patients with CF suffer from recurrent and chronic respiratory tract infections and the majority of the associated morbidity and mortality is due to such infections.

These infections are usually dominated by Gram-negative organisms, especially the pseudomonads, particularly *Pseudomonas aeruginosa*. However, although various other species within the genus *Pseudomonas* have been identified as clinically significant in such patients,[2–4] relatively few studies have examined prevalence, either by conventional culture or by molecular techniques, of such species in patients' sputa.

The aim of this study is to use a multiplex polymerase chain reaction (PCR) assay to determine the presence of genomic DNA from *Pseudomonas* spp. and *P. aeruginosa* directly from the sputa of CF patients.

Sputum (1 mL, minimum) specimens were collected from 116 children and 57 adult patients who had a well-characterised history of CF and placed in sterile (100 mL) plastic disposable containers. Sputum was collected immediately after a standardised session of physiotherapy, stored at ambient temperature and processed within 4 h of collection. Fresh sputum (1 mL, minimum) was mixed with an equal mass of Sputasol (Oxoid SR089A, Oxoid, Poole, England) and incubated in a water bath at 37°C for 15 min, before further qualitative processing for the molecular detection of *P. aeruginosa* and *Pseudomonas* spp.

All DNA isolation procedures were carried out in a class II biological safety cabinet (MicroFlow, England) in a room physically separate from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in accordance with the good molecular diagnostic procedures (GMDP) guidelines proposed by Millar *et al.*,[5] in order to minimise contamination and hence the possibility of false-positive results.

Bacterial genomic DNA was extracted directly from patients' sputa, as well as from the *P. aeruginosa* reference strain ATCC 27853 (Schroeter; Migula), using the Roche High Purity PCR Template Preparation Kit (Roche, England), following the manufacturer's instructions. Extracted DNA was stored at –80°C prior to PCR amplification. With each batch of extractions, a negative extraction control containing all reagents but no sputum was performed, as well as an