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Alpha-1-antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients

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ABSTRACT

Rationale

Airways of cystic fibrosis (CF) patients are characterized by neutrophils that release high amounts of elastase, overwhelming the local antiprotease shield. Inhalation of α_1-antitrypsin (AAT) may restore the protease-antiprotease dysbalance and attenuate airway inflammation in CF airways.

Objectives

To assess the best deposition region for inhaled AAT by two different inhalation strategies. To examine the effect of four weeks AAT inhalation on lung function, protease-antiprotease balance and airway inflammation in CF patients.

Methods

In a prospective, randomized study, 52 CF patients received a daily deposition of 25 mg AAT by inhalation for four weeks targeting their peripheral or bronchial compartment. Levels of elastase activity, AAT, pro-inflammatory cytokines, neutrophils, IgG fragments and *Pseudomonas* numbers were assessed in induced sputum before and after the inhalation period.

Results

Inhalation of AAT increased AAT levels and decreased elastase activity levels, neutrophils, pro-inflammatory cytokines and numbers of *Pseudomonas*, but had no effect on lung function. No difference was found between the peripheral and bronchial inhalation mode.

Conclusions

Although no effect on lung function was observed, the clear reduction of airway inflammation after AAT treatment may precede pulmonary structural changes. The AAT deposition region may play a minor role for AAT inhalation in CF patients.

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ABBREVIATIONS

AAT	α_1-antitrypsin
BAL(F)	Bronchoalveolar lavage (fluid)
AKITA	Anlage zur kontrollierten Inhalation von therapeutischen Aerosolen (*engl*: Electronically regulated device for the controlled inhalation of therapeutic aerosols; manufactured by Inamed Gmünden, Germany)
CF	Cystic fibrosis
CFU	Colony forming unit
DTT	Dithiothreitol
$F(ab)_2$	Two antigen-binding fragments (of immunoglobulin)
Fc	Cristallizable fragment (of immunglobulin)
FEV_1	Forced expiratory volume in one second
FVC	Functional vital capacity
IL-	Interleukin
IgG	Immunoglobulin G
ITT	Intent-to-treat
LTB4	Leukotriene B4
MEF_{25}	Maximum expiratory flow at 25 % of vital capacity
mITT	Modified intent-to-treat
P. aeruginosa	*Pseudomonas aeruginosa*
TNF-α	Tumor necrosis factor α

INTRODUCTION

The major cause of death in patients with cystic fibrosis (CF) is respiratory insufficiency resulting from chronic bacterial infection and progressive destruction of the lung [1]. Due to the genetic defect in CF airway epithelial cells, an excessive inflammatory response is present in CF airways characterized by high amounts of interleukin 8 (IL-8). IL-8 attracts neutrophils to the lung resulting in chronic airway inflammation with excessive release of neutrophil elastase overwhelming the antiprotease shield of the airspaces [2].

Neutrophil elastase is a serine protease with a broad substrate specificity, stored as a fully active enzyme together with cathepsin G and proteinase 3 in the azurophilic granules of neutrophils [3]. Besides invading microorganisms that are the physiological target of elastase, a surplus of the enzyme damages host compounds and pulmonary structures including cilia [4], elastin [5], fibronectin [6], surfactant proteins A and D [7,8], immunoglobulins [9], and cell surface receptors on neutrophils [10,11] and lymphocytes [12]. Such damage results in reduced mucociliary clearance [4] and an impaired opsonophagocytosis [11]. Furthermore, elastase stimulates the production of pro-inflammatory cytokines as IL-8 [13] and leukotriene B4 (LTB4) [14].

In the healthy lung, α_1-antitrypsin (AAT) is present at high concentrations and acts as an antiprotease screen to prevent the deleterious effects of free elastase [15]. In CF airways, AAT is complexed [16] and proteolytically inactivated [17], resulting in a dysbalance of proteases to antiproteases. To attenuate the deleterious effects of free elastase on pulmonary structure and host defense mechanisms, the inhalation of AAT has been proposed as a therapeutic strategy in CF patients [18,16].

For effective treatment with inhaled AAT it is essential that the highest possible fraction of the aerosolized drug reaches the target region within the lungs and that the inhalation is performed within a time period which is convenient for the subject. The first aim of this study was to compare the peripheral and the bronchial AAT deposition mode. The second aim was to

examine whether four weeks of AAT inhalation have an effect on the protease-antiprotease balance, percentages of neutrophils, mRNA and protein levels of pro-inflammatory cytokines, numbers of *Pseudomonas aeruginosa* (*P. aeruginosa*) and percentages of immunoglobulin G (IgG) fragments in sputa of CF patients.

MATERIAL AND METHODS

The study was performed as a multicenter, randomized, open-label, parallel group trial conducted at the following 8 study centers in Germany: University of Munich (Co-ordinating investigator M Griese), the St. Elisabethen-Hospital, Department of Internal Medicine, Frankfurt (J Bargon), Department of Internal Medicine (C von Mallinckrodt) and the Department of Pediatrics (H-G Posselt), University of Frankfurt, Department of Internal Medicine (J Hohlfeld) and Department of Pediatrics (M Ballmann), University of Hannover, Medical School, the Department of Pediatrics, University of Giessen (H Lindemann), and the Department of Pediatrics, University of Cologne (E Rietschel). The study was monitored by Acromion GmbH, Frechen, Germany. Documented approval from appropriate ethics committees was obtained for all participating centers prior to study begin, according to Good Clinical Practice (GCP) guidelines, local laws, regulations and organization. Written informed consent was obtained from all subjects before any study procedure took place.

Study population

Inclusion criteria were the diagnosis of CF by clinical symptoms and positive sweat tests or disease inducing mutations, age ≥ 8 years, forced expiratory volume in one second (FEV_1) > 25 % of predicted value, free elastase activity levels detected in the sputum sample at visit 1 (free elastase activity levels two standard deviations above the negative blank sample), at least three times positive tested for *P. aeruginosa* in the last two years and positive testing in induced sputum at visit 1, stable concomitant therapy at least two weeks prior to visit 1 and during the study, and written informed consent.

Exclusion criteria were history of lung transplant, any lung surgery within the past two years, on any thoracic surgery waiting list, severe concomitant disease (serious malignant disease, congestive heart failure NYHA III/IV, cor pulmonale with the need of oxygen therapy), severe liver cirrhosis with ascites, hypersplenism or grade III/IV esophageal varices, selective IgA deficiency with anti-IgA antibodies, active pulmonary exacerbation within the

four weeks prior to screening, current smoker, pregnancy or lactation, and women of child-bearing age without adequate contraception.

Study outline

At the first visit (visit 1), eligible subjects received a random number. Accordingly, each subject received an individually programmed SMART CARD, which was to be used with the AKITA® device (Inamed GmbH, Headquarters and Logistic, Gmünden, Germany) and which determined the respective inhalation pattern with bronchial or peripheral deposition [20]. During the following 2-week run-in period the subjects inhaled isotonic saline once daily in order to get used to the inhalation pattern. At visit 2, baseline measurements were taken and a four-week treatment period began where AAT was inhaled. After two weeks (visit 3) and four weeks (visit 4) all measurements were repeated. The study treatments were added to the regular therapy of the patients. Sputum induction was done only at the study visits in the hospital, as described in detail below.

Treatments administered

To determine the optimal region for AAT deposition, two inhalation modes were programmed in a double-blinded fashion on a SMART CARD connected to the inhalation device (AKITA inhalation device with the Pari LC Plus or the LC Star nebulizer). Patients were randomly assigned to either bronchial or peripheral deposition, according to the method described by Brand et al. [20].

Peripheral deposition was achieved by a slow inhalation flow (200 ml/sec) and an individualized inhalation volume calculated from the individual inspiratory capacity determined by spirometry. Therefore, the formula inhalation volume $2 \times e^{(-1.5/\text{inspiratory capacity})} + 0.25$ was used [20]. The nebulizer used was a Pari LC Star delivering an aerosol with a mass median diameter (MMD) of 3.5 µm.

Bronchial deposition was achieved by a very slow inhalation flow of 100 ml/sec and a low inhalation volume of 60 % of the individual inspiratory capacity, but maximal 0.5 l. The nebulizer used was a Pari LC Plus generating an aerosol with a MMD of 5.0 µm. The two nebulisers had an identical outer appearance.

The SMART CARDs were programmed to deposit 25 mg AAT (Prolastin®, purified AAT in a buffer containing 100-210 mM sodium, 60-180 mM chloride and 15-28 mM sodium phosphate) during the treatment phase. Prolastin® is made from human blood plasma and is primarily monomeric in solution (>99.5 %), manufactured by Bayer Corporation (Clayton, NA, USA). During the run-in phase, equal amounts of isotonic saline instead of AAT were inhaled with the respective inhalation pattern. The daily inhalations took place in the evening between 18.00 and 23.00 pm. The time required for the inhalations was between 5 min and 15 min, depending on the lung function of the patient. Treatment compliance (date and time of inhalation, number of breaths, and completeness of each breath) was monitored on the SMART CARD.

Induced sputum

Induced sputum was obtained at the study visits in the hospital between 9 am and 1 pm following the AAT inhalation the evening before. Before sputum was induced, subjects underwent physiotherapy and spirometry, then two puffs of salbutamol were inhaled and 5 ml of 5.85 % sodium chloride were nebulized with the Pari LC Plus for 15 min connected to a Pari Master compressor. A physician was present during sputum induction.

The pooled sputum was divided into 3 different samples, one for bacteriology (0.5-1 ml), one for AAT, elastase, cytokine and neutrophil assessments (3 ml) and one for IgG determination (3 ml). The samples for bacteriology were shipped overnight at 4°C to the Max von Pettenkofer Institute, Munich South-German reference lab for *P. aeruginosa*, University of

Munich. The samples for AAT, elastase, cytokines, neutrophil and IgG analysis were incubated 1:1 with dithiothreitol (DTT) (sputolysin, Calbiochem Biosciences, Beeston, U.K.), filtered through Nitex gaze-filters and washed with Hanks solution. The sputum suspension was centrifuged at 500xg for 10 min at 4°C and the supernatant was centrifuged at 4000xg for 20 min at 4°C. Thereupon, the supernatant was mixed with a protease inhibitor (0.5 mM EDTA, 500 µM Pefabloc (Merck, Darmstadt, Germany), 5 µM E-64, 50 µM Bestatin (both Roche, Basel, Switzerland)) to block free elastase activity and to avoid *in vitro* proteolysis. Aliquots of the supernatant were frozen at -70°C until final analysis. Cytospin slides were prepared with the cell pellet using 200,000 cells per slide. At least 400 cells were differentiated by May-Grünwald-Giemsa. The viability was assessed by the trypan blue dye exclusion test. The remaining cells were suspended in Hanks buffer at 4°C and were immediately processed for flow cytometry.

The levels of AAT were measured by ELISA. Levels of free elastase activity were analyzed by a chromogenic assay according to standard protocols as described by Hilliard et al. [21]. Numbers of *P. aeruginosa* were quantified according to the method described by Hogardt et al. [22].

Flow cytometry

CD45-APC mouse IgG1 (Pharmingen, Heidelberg, Germany) and mouse IgG1-APC (Immunotech, Marseille, France) as isotype control were used. Calculations were performed with Cell Quest analysis software (Cell Quest Pro, Becton-Dickinson, Heidelberg, Germany). Resuspended sputum cells were blocked with human IgG for 20 min to avoid non-specific binding, were incubated with monoclonal antibodies for 40 min, washed two times and were analyzed by flow cytometry (Becton-Dickinson, FACS Calibur, Heidelberg, Germany) as described previously [23]. Prior to the study, the discrimination between neutrophils and alveolar macrophages was optimised. Gating of neutrophils was based on light scatter

properties and positive expression for CD45. In our experimental setting, macrophages were to a lesser extent disturbing, than the considerable amount of apoptotic or dead cells present in CF sputa. Therefore, propidium iodide (5 µg/ml; Sigma, St Louis, MO, USA; 5 µg/ml) and Annexin V-FITC (5 µg/ml; dilution 1/100; Boehringer Mannheim GmbH, Mannheim, Germany) were used to discriminate intact viable leukocytes (Annexin V^-, PI^-) from apoptotic (Annexin V^+, PI^-) and necrotic (Annexin V^+, PI^+) cells. Only viable neutrophils were included in the analysis. These gates were used to analyze 10,000 cells neutrophils/sample.

Immunoglobulin G

For the assessment of IgG fragments, a modified method according to Fick et al. [24] was used. 10 µl of the sputum supernatant pretreated with a proteinase inhibitor (see above), were lyophilized, dissolved in 20 µl sample buffer and reduced for 10 min at 70°C (NuPAGE Reducing Agent, Invitrogen, Madison, WI, USA). The sample together with 500 µl of antioxidant (NuPAGE antioxidant, Invitrogen, Madison, WI, USA) was subjected to electrophoresis (10% Bis-Tris Gele) and 500 ng human IgG was used as standard. *In vitro*, the effect of human leukocyte elastase (HLE) on IgG was assessed by incubation of 500 ng IgG isolated from human serum (Sigma, Missouri, USA) with HLE (40 U/ml; EPC, St. Louis, MO, USA) for 2h at 37°C. The proteins were transferred on nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) for semi-dry Western blotting (Xcell II Blot). IgG fragments were identified by incubation for 12h with 2.5 µl of a specific goat-anti-human IgG antibody. After intensification of the signal by chemiluminescence, the blots were scanned with a computing densitometer (Fluor-S MultiImager, BioRad, Richmond, CA, USA) and were semi-quantitatively analyzed using Quantity One (BioRad, Richmond, CA, USA). To detect corresponding protein-spots between gels, all blots were stacked and spots were matched according to molecular weight.

Cytokine protein levels

Levels of IL-8, TNF-α and IL-1β were analyzed in sputum supernatant in duplicates by a sandwich ELISA according to the manufacturer´s instructions (R&D Systems, Minneapolis, MN, USA). Levels of LTB4 were measured in sputum supernatant by a sandwich ELISA according to the manufacturer´s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The detection limits were 3.5 pg/ml for IL-8, 0.12 pg/ml for TNF-α, 1 pg/ml for IL-1β and 1.25 pg/ml for LTB4. Furthermore, cytokine levels in sputum were analyzed with and without DTT pre-treatment at the same dilution used for sputum samples.

Cytokine mRNA levels

Messenger RNA levels of IL-8, TNF-α and IL-1β were analyzed in induced sputum cell pellets in duplicates by a quantitative real-time RT-PCR system (Icycler, Biorad Hercules, CA, USA) according to the manufacturer´s instructions. The following forward (F) and reverse (R) primer pairs were used: IL-8-F: TCTTGGCAGCCTTCCTGATT, IL-8-R: TCCAGACAGAGCTCTCTTCCATC; TNF-α-F: AGGAACAGCACAGGCCTTAGTG, TNF-α-R: AAGACCCCTCCCAGATAGATGG; IL-1β-F: CAGGGACAGGATATGGAGCAA IL-1β-R: ATGTACCAGTTGGGGAACTG. Induced sputum cells were lysed in Trizol LS Reagent (Invitrogen, Life technologies, Karlsruhe, Germany). Total RNA (200-500 ng) was isolated according to the manufacturer´s instructions and reverse transcribed into cDNA. Expression levels of cytokines were determined in duplicates by real time RT-PCR using SYBR green and the iCycler iQ detection system (Biorad, Hercules, CA, USA). Threshold cycle (CT) values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA expression.

Statistics

The primary analysis population was a modified intent-to-treat (mITT) population, including all randomized subjects who received any amount of study medication (AAT) and had at least one evaluation of the primary efficacy variable, i.e. free elastase activity in induced sputum at baseline (visit 2). The primary efficacy comparison for the change in free elastase activity in induced sputum from baseline to endpoint was a two-way ANCOVA with treatment group and center as fixed factors (main effect model) and baseline measurement of free elastase activity in induced sputum as covariate in the mITT population. Based on the standard deviations (SD) of elastase activity levels in sputum as described previously [3,25], a sample size of 24 subjects per treatment group was calculated to detect a change in free elastase activity in induced sputum in the magnitude of the SD at a 5% two-sided alpha and a power of 80%. All further calculations were made in an exploratory way, including the evaluation of the overall treatment effect as pre-post comparisons for the combined deposition groups (Wilcoxon and sign test with Bonferroni correction for multiple comparisons).

RESULTS

Study population, deposition modes and safety

Seventy-two CF subjects were enrolled. Thirty-seven subjects were randomized to the group for peripheral deposition and 35 to the group for bronchial deposition (Figure 1). Thirteen subjects dropped out during the run-in-phase prior to AAT treatment because of the following protocol violations: two patients could not produce sputum anymore, in one subject the diagnosis of IgA deficiency was noticed after inclusion into the study, one patient was not able to perform daily inhalations with the AKITA due to his personal breathing pattern, four subjects withdrew their consent or stopped the study for personal reasons, and five patients made other protocol violations that excluded them from the study. Therefore, 59 subjects received at least one dose of study drug and were valid for safety and intention to treat (ITT) analysis. Seven subjects were excluded because of missing or invalid data for the primary efficacy variable (free elastase activity in induced sputum), because no sputum was produced or the sputum was unavailable for analysis. The group of subjects left (modified ITT group) was defined before the study as the primary analysis population. Thus, the analysis was based on 28 subjects using inhalation for peripheral deposition and 24 subjects using inhalation for bronchial deposition. The baseline characteristics were not statistically different between the two deposition groups (Table 1). Electronically recorded compliance with the study drugs was good.

The number of subjects with adverse events was 10/30 (treatment phase: 7/28) in the peripheral deposition group and 13/29 (treatment phase: 8/24) in the bronchial deposition group. With the exception of one severe adverse event (sinusitis), all other events were of mild or moderate intensity. No serious adverse events occurred. All adverse events had resolved or improved by the end of the study. Three subjects in each group experienced drug-related adverse events. One of these events (fatigue) was attributable to the inhalation maneuver or the saline, as it already occurred during the run-in phase. Two other drug-related adverse events

(fatigue, hemoptysis) arised in the bronchial deposition group and three in the peripheral deposition group (pruritus, influenza-like illness, gastro-intestinal pain).

No significant differences between the peripheral and bronchial deposition group were noted at baseline, after two and four weeks of AAT inhalation with respect to free elastase activity, AAT level, percentage of neutrophils, *P. aeruginosa* counts, cytokine levels, IgG fragments in sputum (Table 2), lung function (FEV_1, FVC, MEF_{25}), number of exacerbations and overall rate of adverse effects (not shown). As no difference of any parameter analyzed between peripheral or bronchial AAT deposition site was found, the overall effect of inhaled AAT was assessed in an explorative analysis by combining the two deposition groups and making before-after comparisons.

Alpha-1-antitrypsin and free elastase activity

Treatment with 25 mg AAT increased the concentration of AAT after two ($p < 0.001$) and four weeks of treatment ($p < 0.001$) (Figure 2A) and slightly reduced the levels of free elastase activity in induced sputum after four weeks of treatment ($p < 0.05$) (Figure 2B). Furthermore, the AAT inhalation decreased percentages of sputum neutrophils after four weeks of treatment ($p < 0.05$) (Figure 3A) and reduced *P. aeruginosa* counts after four weeks of treatment ($p < 0.05$) (Figure 3B), while FEV_1 did not change (Figure 3C).

Pro-inflammatory cytokines

DTT treatment had no significant effect on IL-8, TNF-α, IL-1β or LTB4 levels (data not shown). Sputum protein levels of IL-8 (Figure 4A), IL-1β (Figure 4B), TNF-α (Figure 4C) and LTB4 (Figure 4D) and sputum mRNA levels of IL-8 (Figure 4E), IL-1β (Figure 4F) and TNF-α (Figure 4G) decreased significantly after two and four weeks of AAT inhalation as compared to the baseline. The changes of IL-8 ($r=0.48$, $p<0.01$), IL-1β ($r=0.39$, $p<0.05$), TNF-α ($r=0.4$, $p<0.05$) and LTB4 ($r=0.45$, $p<0.05$) protein levels and the changes of neutrophils ($r=0.52$,

$p<0.01$) correlated positively with the changes of free elastase levels.

Immunoglobulin G

CF related proteases were found to cleave IgG into $F(ab)_2$ and Fc fragments [26,24,27]. In sputum samples of the CF patients in this study, IgG bands of 26 kDa, 32 kDa, 36 kDa, 43 kDa and 54 kDa were detected under reducing conditions (Figure 5). In accordance with previous studies analyzing proteolytically cleaved IgG fragments [3,28,29,30,24], the 26 kDa band corresponded to the reduced form of the IgG light chain (LC), the 32 kDa band to the cleaved IgG Fc fragment [28,29] and the 43 kDa band to the cleaved $F(ab)_2$ fragment [29]. The 36 kDa band could not be identified. The 54 kDa band represented the reduced form of the regular IgG heavy chain (HC) of 168 kDa [28,29,30]. The treatment of IgG with HLE cleaved IgG in the two antigen-binding $F(ab)_2$ and the cristallizable Fc fragment. The percentages of 54 kDa IgG increased significantly after four weeks of AAT inhalation (Figure 6A), whereas no significant change was found for the other IgG bands. Percentages of 54 kDa IgG correlated inversely with levels of free elastase activity in sputum before and after AAT treatment (Figure 6B).

DISCUSSION

For effective treatment with inhaled AAT it is essential that the highest possible fraction of the aerosolized drug reaches the target region within the lung. Previously, we have defined the aerosol and breathing characteristics that allow a preferential targeting of inhaled drugs to specific regions within the lungs of CF patients [31,20]. To our knowledge, this is the first study examining possible differences in medication efficacy between the peripheral and bronchial deposition site in the lungs of CF patients. A known and fixed amount of AAT was deposited into the airways of each patient, exactly controlled by the AKITA device, We hypothesized that a more peripheral AAT deposition might be superior to a more bronchial deposition. However, no significant difference between the two deposition modes with regard to the changes in free elastase activity levels and several other parameters were found.

Despite the minor changes in free elastase activity levels after AAT treatment, the inhalation of AAT significantly decreased airway inflammation, as assessed by the analysis of neutrophils and pro-inflammatory cytokines in induced sputum. Recent studies suggest a role for AAT as an anti-inflammatory modulator [33], independent of its antiprotease effect. AAT inhibited LPS-induced TNF-α and IL-1β release dose-dependently from monocytes and IL-8 release from neutrophils *in vitro*. Furthermore, AAT instillation after LPS challenge prevented LPS-induced IL-8 production *in vivo* [33]. Sputum protein and mRNA levels of IL-1β and TNF-α decreased significantly after AAT inhalation in our CF patients. Therefore, we speculate that the AAT inhalation may inhibit the LPS-induced production of TNF-α and IL-1β by epithelial cells and alveolar macrophages [34], thereby attenuating pulmonary inflammation in CF patients. The inhibition of serine proteases other than elastase that are also neutralized by AAT inhalation, as proteinase 3, may contribute to the discrepancy between the small effects of AAT inhalation on elastase activity levels and the clear effects found on inflammatory parameters.

The finding that four weeks of AAT inhalation decreasd neutrophilic inflammation in CF airways is in line with a rat model of chronic *P. aeruginosa* lung infection [35], where aerosolized AAT decreased pulmonary neutrophils and numbers of bacteria [35]. In the study of McElvaney et al. [36], the inhalation of aerosolized AAT in patients with CF increased AAT levels and decreased elastase activity levels in bronchoalveolar lavage (BAL). In contrast to the study of McElvaney et al., the inhalation of AAT in our patients did not decrease elastase activity levels substantially. This discrepancy might be due to the material used, i.e. BAL vs induced sputum. Compared to BAL supernatant where mucus and debris are removed by filtration, induced sputum is a more complex matrix that contains numerous broken and necrotic neutrophils, inclosing high amounts of neutrophil elastase. Therefore, all sputum samples in our study were obtained by defined induction techniques, shipped to a central lab and analyzed under standardized conditions. Nevertheless, the *ex vivo* manipulation of induced sputum samples, that is mixing and centrifugation, probably leads to the release of intracellular elastase into the sputum supernatant. Thus, we speculate that the neutralization of all proteases present in the sputum sample *ex vivo* requires more antiproteases than those dissolved in BAL fluid supernatant. At first glance, it may thus be preferable to use BAL for the assessment of antiprotease treatment effects in CF patients. However in CF patients, elastase and neutrophils are more abundant found in sputum compared to BAL [37,19]. Furthermore, it must be kept in mind that BAL reflects also the alveolar compartment and CF lung disease is primarily confined to the bronchial and bronchiolar compartment.

Martin et al. examined the effect of inhaled recombinant AAT derived from sheep in CF patients and used spontaneous expectorated sputum for the analysis of free elastase levels, AAT levels, myeloperoxidase levels and IL-8 levels [38]. Similar to our observations, the AAT inhalation was not able to neutralize free elastase in sputum completely despite higher doses of AAT used. The deposition mode and compliance were not controlled. In line with

our findings, levels of myeloperoxidase, a marker for neutrophils, were lower in the sputa of the CF patients after the AAT treatment period. Percentages of neutrophils and *P. aeruginosa* counts were not analyzed.

CF proteases are known to cleave IgG into $F(ab)_2$ and Fc fragments resulting in a deficiency of intact IgG proteins and an accumulation of functionally impaired IgG cleavage fragments [3,24]. These IgG fragments influence the function of neutrophils *in vitro* by an impairment of bacteria-induced chemotaxis [29], oxidative burst [28] and enzyme release [30], leading to defective opsonophagocytosis [24]. In our study, high elastase activity levels in sputa were associated with decreased intact (54 kDa) IgG proteins and the inhalation of AAT increased intact IgG proteins in CF airways. Similarly, Fick et al. found in BAL of CF patients an inverse association between intact IgG proteins and free elastase activity [24]. Cleaved IgG fragments (12-43 kDa) in sputum of our CF patients were unchanged after AAT treatment. We speculate that removal of degraded IgG fragments by alveolar phagocytes may account for this observation.

This study has several limitations. As the primary goal was to assess differences between the efficacies of two deposition modes, no placebo control group was included, which substantially limits the conclusions that can be drawn from the data. For future studies examining the effect of AAT inhalation in CF patients, a placebo control group is indispensable. Although very consistent and fitting the current concept of proteolytic airway injury, particular effects of AAT inhalation observed in this study might be non-specific. The two breathing patterns chosen to obtain a more peripheral or a more central deposition still had an estimated overlap of 30%. Thus, with both breathing patterns, the deposition of a substantial fraction of the inhaled drug in the small airways could not be avoided. Thus, we cannot completely exclude that the lack of a difference in the AAT treatment effect between both breathing patterns might be because AAT was deposited in the small airways with both breathing patterns.

Furthermore, the sample size calculation done prior to the study was based on previous observations [3,25], but the elastase activity levels and elastase changes found in our study were markedly lower. Based on the mean and SD for free elastase activity in sputum, as assessed here, a new study intended to detect a drop in free elastase activity from 30 µg/ml to 15 µg/ml with a power of 80% would require about 60 CF subjects per group. Free elastase activity was still detectable in sputum after four weeks of AAT treatment. A lack of adherence to the inhalation could be excluded since electronically monitored compliance was found to be good. Since it is technically feasible to deliver several-fold larger doses of AAT within an adequate inhalation time, AAT amounts higher than 25 mg deposited in the lungs may be reasonable.

Figure 7 summarizes the effects of free elastase in the airways of CF patients. Physiologically, elastase is complexed and neutralized by AAT. Due to the elastase/AAT dysbalance in CF airways, free elastase activity damages the pulmonary environment in several ways. Elastase impairs the innate immune response by cleaving the complement receptor 1 (CR1) on neutrophils and the phosphatidylserine receptor on macrophages (not shown) thereby decreasing bacterial killing and clearance of apoptotic cells. Elastase triggers the IL-8 production by bronchial epithelial cells [13]. The released IL-8, in turn, induces elastase secretion by neutrophils and attracts neutrophils to the lung. Elastase impairs the adaptive immunity by cleaving IgG into $F(ab)_2$ and Fc fragments [24]. The impaired pulmonary host defense leads to increased numbers of bacteria in CF airways. Finally, chronic proteolytic damage of pulmonary structures leads to bronchiectasis and lung destruction in CF patients.

In conclusion, these data suggest that four weeks of AAT inhalation reduce airway inflammation in CF patients. Although no effect on lung function was observed, reduced airway inflammation may precede pulmonary structural changes. Longer, placebo-controlled

studies aiming to deposit optimal amounts of AAT into the lungs of CF patients are worthwhile to undertake.

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FIGURE LEGENDS

Fig 1. Study population

The figure shows the algorithm of the patient selection.

Fig 1

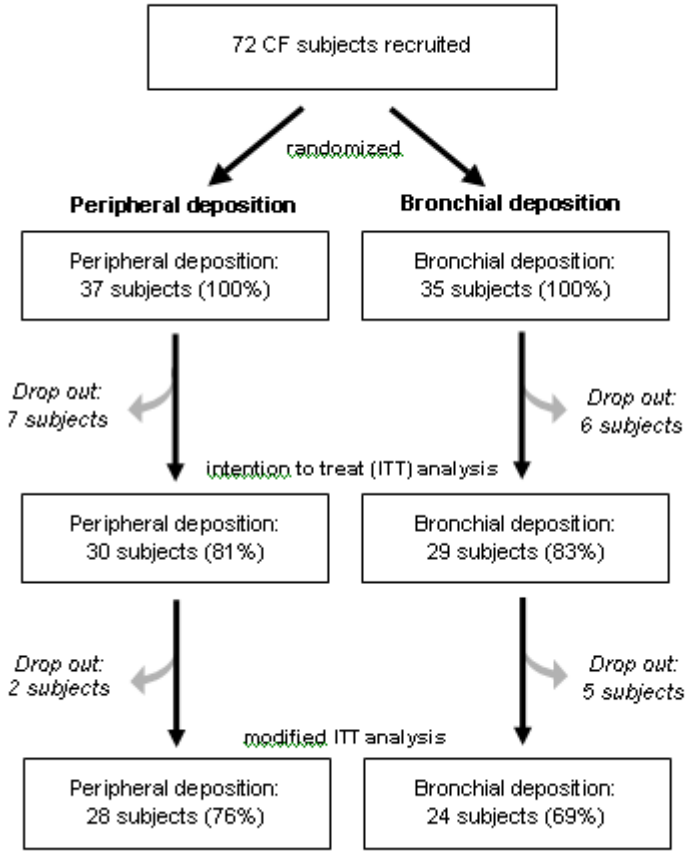


Fig. 2. Levels of α_1-antitrypsin and free elastase activity

Sputum levels of α_1-antitrypsin (Figure 2A) and free elastase activity levels (Figure 2B) at the end of the run in period (baseline) and after two and four weeks of α_1-antitrypsin inhalation. P-values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values ± SEM.

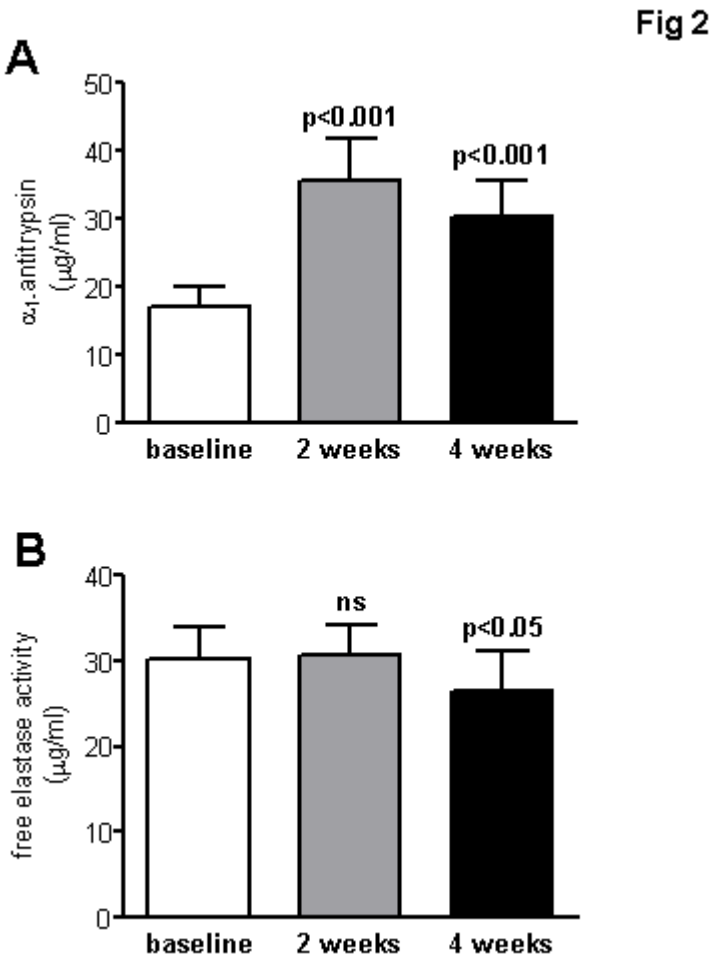


Fig 3. Neutrophils, *P. aeruginosa* and lung function

Percentages of neutrophils (Figure 3A) and *P. aeruginosa* counts (Figure 3B) in induced sputa and forced expiratory volume in one second (FEV_1) (Figure 3C) at the end of the run in period (baseline) and after two and four weeks of α_1-antitrypsin inhalation. CFU: colony forming unit. P-values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values ± SEM.

Fig 3

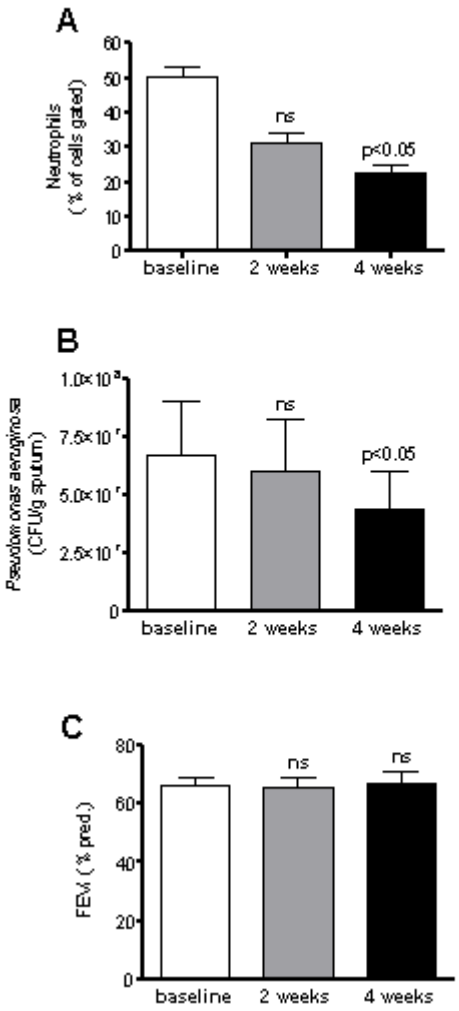
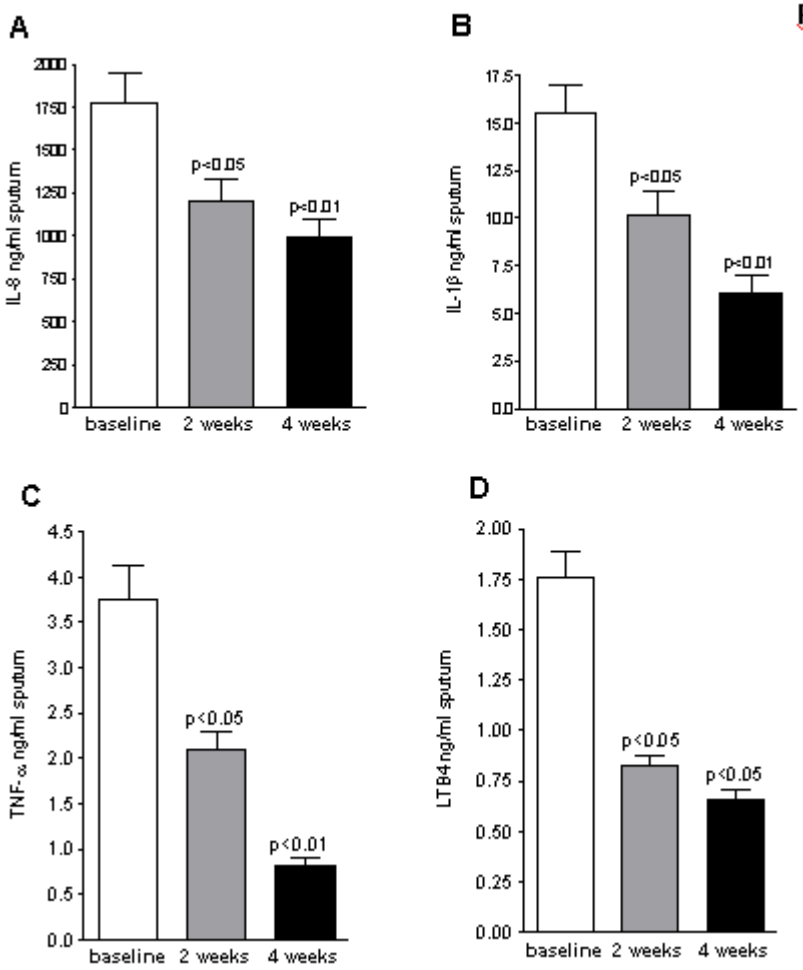


Fig 4. Pro-inflammatory cytokines

A-D: Protein levels of IL-8 (Figure 4A), IL-1β (Figure 4B), TNF-α (Figure 4C) and LTB4 (Figure 4D) in sputum supernatant at the end of the run in period (baseline) and after two and four weeks of α_1-antitrypsin inhalation.

E-G: mRNA levels of IL-8 (Figure 4E), IL-1β (Figure 4F) and TNF-α (Figure 4G) in sputum cell pellets at the end of the run in period (baseline) and after two and four weeks of α_1-antitrypsin inhalation. Threshold cycle (CT) values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA expression. P-values are shown as comparisons to baseline (sign test and corrected by Bonferroni). Bars represent mean values ± SEM.

Fig 4


A
IL-8 ng/ml sputum
2000
1750
1500
1250
1000
750
500
250
0
p<0.05
p<0.01
baseline
2 weeks
4 weeks
B
IL-1β ng/ml sputum
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
p<0.05
p<0.01
baseline
2 weeks
4 weeks
C
TNF-α ng/ml sputum
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p<0.05
p<0.01
baseline
2 weeks
4 weeks
D
LTB4 ng/ml sputum
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
p<0.05
p<0.05
baseline
2 weeks
4 weeks

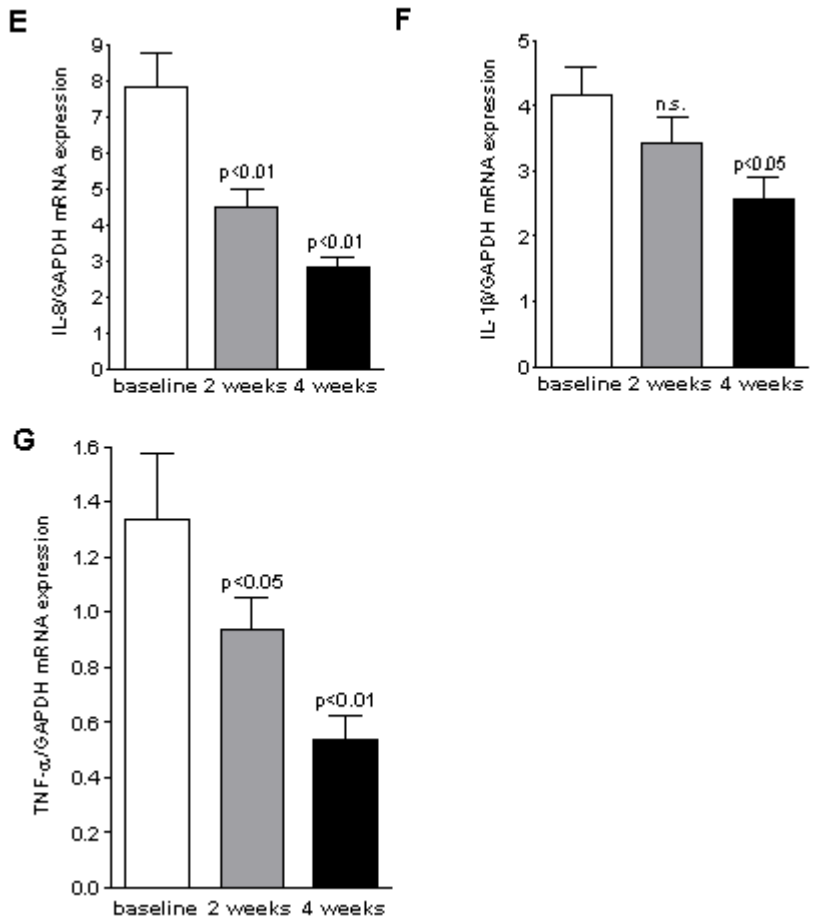


Fig 5. Immunoglobulin G in sputum of CF patients

Reduced IgG fragments in sputum of a representative cystic fibrosis patient before (1) and after (2) four weeks of α_1-antitrypsin inhalation compared to 500 ng human IgG (3) and 500 ng human IgG treated *in vitro* with 40 U of human leukocyte elastase (HLE) for 2h at 37°C (4). The treatment of IgG with HLE cleaved IgG in the two antigen-binding $F(ab)_2$ and the cristallizable Fc fragment.

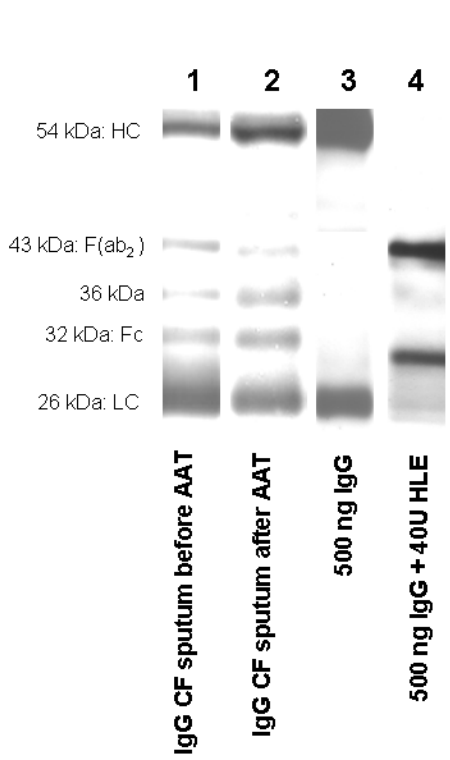


Fig 6. Immunoglobulin G 54 kDa fragments and free elastase activity levels

Figure 6A. Percentages of 54 kDa IgG in induced sputa of CF patients at the end of the run in period (baseline), after two and four weeks of inhalation of once daily 25 mg α_1-antitrypsin. Bars represent mean values ± SEM. P-values are shown as comparisons to baseline (sign test and corrected by Bonferroni).

Figure 6B. Correlation between percentages of 54 kDa IgG and free elastase activity levels in induced sputa of CF patients before inhalation, i.e. at the end of the run in period (white circles), and 4 weeks after the α_1-antitrypsin inhalation (black circles). 54 kDa IgG is expressed as percentage of total IgG detected. Correlations were calculated using Spearman *rho* test.

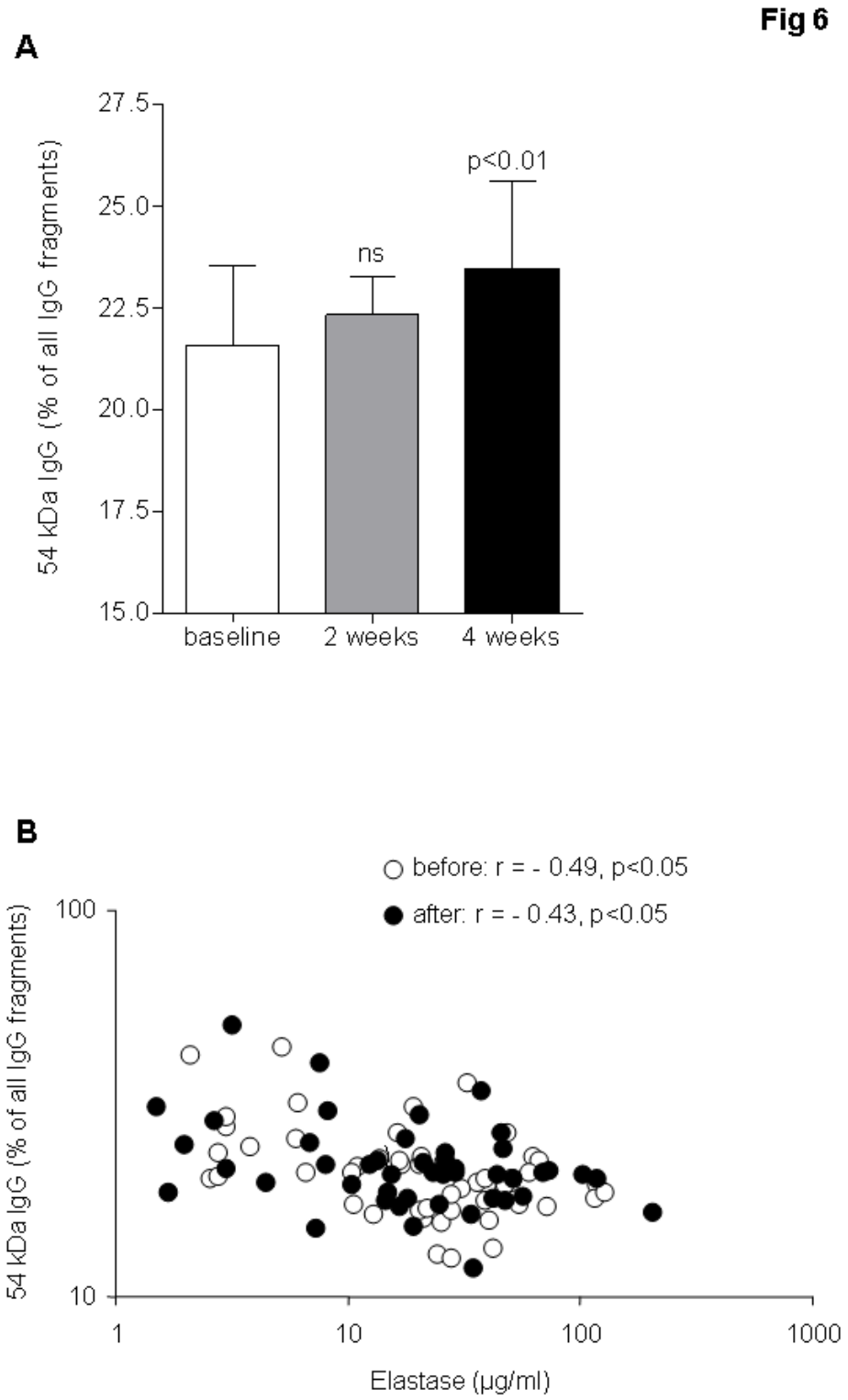
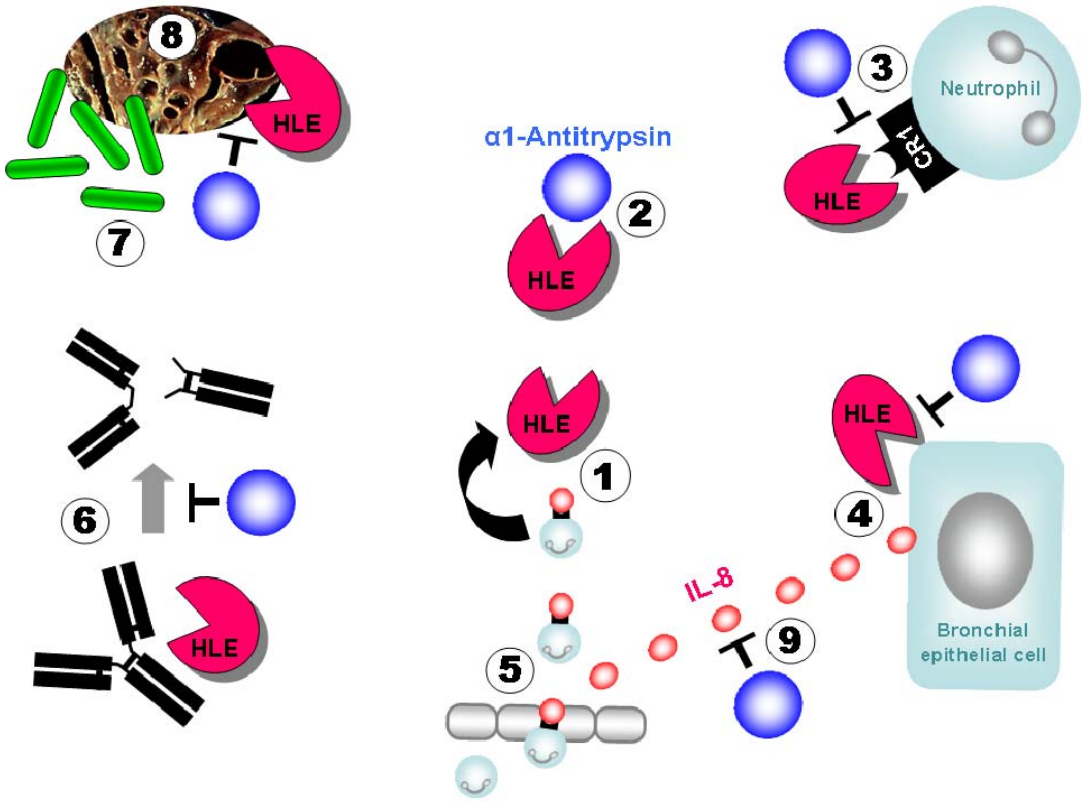


Fig 7. Effects of elastase in CF airways

Upon stimulation with IL-8, human leukocyte elastase (HLE) is released by neutrophils into the airways (1) where HLE is complexed and neutralized by α_1-antitrypsin (AAT) (2). Due to a

HLE/AAT dysbalance in CF airways, free HLE damages the pulmonary environment in several ways (3-8). HLE impairs the innate immune response by cleaving the complement receptor 1 (CR1) on neutrophils (3) thereby reducing bacterial killing. In addition, HLE triggers the IL-8 production by bronchial epithelial cells (4). The released IL-8 attracts large numbers of neutrophils from the circulation through endothelial cells to the alveolar space (5). HLE impairs the adaptive immunity by cleaving immunoglobulin G into $F(ab)_2$ and Fc fragments (6). The impaired host defense leads to increased numbers of bacteria harming the lung tissue (7). Finally, multiple proteolytic damage of pulmonary structures leads to bronchiectasis and lung destruction in CF patients (8). Exogenous supplementation with AAT may attenuate these deleterious processes. Furthermore, AAT may act anti-inflammatory, independent of its antiprotease activity, by reducing free IL-8 levels in CF airways (9).



TABLES

Table 1: Patient characteristics and compliance of the primary analysis population (modified intention to treat population)

Parameter	Peripheral deposition n = 28	Bronchial deposition n = 24	Total n = 52
Race (Caucasian)	28 (100 %)	24 (100 %)	52 (100 %)
Sex (female)	15 (54 %)	11 (46 %)	26 (50 %)
Age (years)	24 ± 8*	27 ± 10	25 ± 9
Weight (kg)	58 ± 10	60 ± 9	59 ± 9
FEV_1 (% of predicted)	65 ± 27	68 ± 25	67 ± 25
Planned inhalation volume per day (l)	50 ± 2	37 ± 0	
Compliance with the study medication (%)			
Run-in (saline)	96 ± 10	86 ± 22	
Treatment (AAT)	95 ± 11	93 ± 16	

*Data are shown as means ± SD

AAT: α_1-antitrypsin; FEV_1: Forced expiratory volume in one second

Table 2: Variables measured in the peripheral and bronchial deposition group and their changes from baseline to endpoint after 4 weeks of α_1-antitrypsin inhalation

	Peripheral deposition n = 28	Bronchial deposition n = 24	Difference between peripheral and bronchial deposition*
Free elastase activity baseline levels [µg/ml]	32.7 ± 31.7	24.9 ± 20.1	
Change after 4 weeks of AAT inhalation [µg/ml]	-7.4 ± 30.0	6.1 ± 31.3	p = 0.19
AAT baseline levels [µg/ml]	20.6 ± 29.3	14.3 ± 13.8	
Change after 4 weeks of AAT inhalation [µg/ml]	6.5 ± 18.9	16.7 ± 33.6	p = 0.19
Neutrophils baseline levels [%]	39.5 ± 23.4	41.8 ± 24.1	
Change after 4 weeks of AAT inhalation [%]	-8.2 ± 20.9	-20.8 ± 30.3	p = 0.11
P. aeruginosa load baseline levels [x10^7 CFU/g]	2.9 ± 5.6	10.6 ± 24.6	
Change after 4 weeks of AAT inhalation [x10^7 CFU/g]	-0.52 ± 7.7	-1.31 ± 9	p = 0.17
IL-8 baseline levels [ng/ml]	1765 ± 867	1682 ± 988	
Change after 4 weeks of AAT inhalation [ng/ml]	- 723 ± 469	- 638 ± 336	p = 0.92
TNF-α baseline levels [ng/ml]	3.6 ± 2.1	3.8 ± 2.8	
Change after 4 weeks of AAT inhalation [pg/ml]	- 1.7 ± 1.3	- 1.2 ± 0.8	p = 0.13
IL-1β baseline levels [ng/ml]	16.2 ± 9.3	13.12 ± 8.3	
Change after 4 weeks of AAT inhalation [pg/ml]	- 8.9 ± 5.9	- 6.7 ± 4.1	p = 0.55

LTB4 baseline levels [ng/ml]	1.72 ± 0.72	1.54 ± 1.1	
Change after 4 weeks of AAT inhalation [pg/ml]	- 1.03 ± 0.42	- 0.92 ± 0.55	p = 0.87
54 kDa IgG fragments baseline levels [% of all IgG fragments]	23.8 ± 7.6	21.4 ± 5.7	
Change after 4 weeks of AAT inhalation [% of all IgG fragments]	+ 1.9 ± 0.4	+ 1.72 ± 1.2	p = 0.62

Data are shown as means ± SD; AAT: α_1-antitrypsin; CFU: colony forming unit; LTB4 Leukotriene B4

*ANCOVA