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Proteinase 3 Activity in Sputum from Subjects with Alpha-1-Antitrypsin Deficiency and COPD

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Abstract

Chronic obstructive pulmonary disease (COPD) is associated with tissue damage believed to result from an imbalance between serine proteinases and their inhibitors. Although the role of neutrophil elastase (NE) has been studied, it is likely that other proteinases play a role. The importance of proteinase 3 (PR3) has not been established, as specific substrates have only recently been available.

We studied clinically stable subjects with either alpha-1-antitrypsin (A1AT) deficiency or usual COPD with chronic bronchitis. Sol-phase sputum was analysed for PR3 activity and concentration, NE activity and concentration, concentrations of airway inhibitors (A1AT, secretory leukoproteinase inhibitor, elafin) and markers of neutrophilic inflammation. Twelve patients were also studied during exacerbations.

PR3 activity was present in most sputum samples and greater than NE activity (which was largely undetectable) in both subject groups (A1AT deficiency median PR3 128nM, IQR 33-558nM; NE 0nM, IQR 0-0nM; p=0.0043: COPD PR3 22nM, IQR 0-103nM; NE 0nM, IQR 0-0nM; p= 0.015). PR3 activity was greater during exacerbations than the stable state (p=0.037) and correlated with markers of neutrophilic inflammation.

The regular identification of PR3 activity in sputum from stable subjects with A1AT deficiency or usual COPD suggests it may play a greater role in the pathophysiology than previously thought.

Keywords

Chronic obstructive pulmonary disease; Proteinases; Inflammatory mediators

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide [1]. Apart from smoking cessation and use of long term oxygen therapy (LTOT) in hypoxic patients, current therapies only provide symptomatic benefit, and there is little evidence that they alter disease progression or mortality. Novel therapies are therefore urgently needed to improve quality of life and prognosis.

The pathophysiology of the disease remains unclear although inflammation and tissue damage are thought to be central to the development of COPD. In particular, an imbalance between neutrophil serine proteinases (NSPs) and their inhibitors has been thought to play a key role [2]. This belief is based on the association of alpha-1-antitrypsin (A1AT) deficiency (the major inhibitor of these proteinases) with early onset COPD [3], and the fact that neutrophil elastase (NE) and proteinase 3 (PR3) are able to degrade many critical components of the extracellular matrix, including elastin, type IV collagen, fibronectin and laminin [4, 5]. In addition, these proteinases also produce emphysematous lesions when administered by intra-tracheal instillation in animal models [6, 7] and can produce other features of COPD including mucus hypersecretion [8]. The activities of the NSPs in the lungs are usually controlled by endogenous inhibitors such as A1AT which is mainly derived from the serum and inhibits NE and PR3, secretory leukoproteinase inhibitor (SLPI) which is mainly produced locally and inhibits NE but not PR3, and elafin and its precursor trappin-2 (also produced locally) which inhibit NE and PR3 [9].

The role of NE in the pathogenesis of COPD and particularly emphysema has been studied extensively [10]. However, selective inhibitors of NE have not been fully effective in controlling neutrophil-mediated damage in the airways [11], supporting the likelihood that other proteinases play a role. Further, Korkmaz et al [12] has suggested that A1AT preferentially inhibits NE, and that

PR3 is only inhibited after NE is completely neutralised suggesting that PR3 may play a more important role when both are released. To date there is little direct evidence of the role of PR3, and research has been hindered by a lack of availability of specific substrates and inhibitors.

Proteinase 3 is a multifunctional serine proteinase mainly located in the azurophilic granules and on the cell surface of neutrophils [13]. It is a 29KDa glycoprotein alternatively named myeloblastin, AGP7 and p29b [14, 15]. Both PR3 and NE are released simultaneously upon neutrophil activation and degranulation. PR3 is however, the most abundant serine proteinase in the neutrophil with each cell being estimated to store approximately 3 times more PR3 than NE [16]. A pro-inflammatory role for PR3 has been suggested by its ability to activate TNFα and IL-1β [17] and provoke apoptosis [18]. The amino acid sequence and crystal structure of PR3 is similar to that of NE [19], and until recently no substrate was available that could discriminate between the two proteinases [20]. However, highly sensitive Abz-peptidyl-EDDnp fluorescence resonance energy transfer (FRET) substrates are now available that can differentiate these human NSPs and are capable of measuring subnanomolar concentrations in biological fluids [21].

The current study was designed to investigate the presence and activity of PR3 compared to NE in sol-phase sputum from subjects with A1AT deficiency (A1ATD) and non-deficient COPD, together with the concentrations of their cognate inhibitors. The results provide insight into the contribution of PR3 to the proteolytic activity in the lungs, and hence potential role with implications as a therapeutic target.

Methods

Subject selection

Twenty eight clinically stable subjects (at least 8 weeks after any acute infection) with A1ATD (PiZZ confirmed by genotyping) with a history of chronic bronchitis were identified from the UK national registry for A1ATD, and spontaneously produced sputum was collected as described previously [22]. None of the A1ATD subjects were receiving augmentation therapy. Chronic bronchitis was a feature in 35% of subjects on the UK national registry.

Twenty two patients with usual COPD (normal PiMM A1AT phenotype) were recruited from primary care at the start of an acute exacerbation. These subjects had a clinical diagnosis of COPD based on a history of chronic bronchitis and exertional dyspnoea, with or without supportive spirometry at presentation (but subsequently confirmed to meet the diagnostic criteria [23]). Sputum was collected at the start of an acute exacerbation (day 1) and then after resolution of the episode during convalescence (day 56). In total, 12 day 1 samples and 22 day 56 samples of sufficient volume were available for the study.

All subjects had full demographic data collected including smoking history, full pulmonary function tests (PFTs) and a high resolution CT (HRCT) scan performed in the stable clinical state. The A1ATD subjects had their health status assessed using the St George's Respiratory Questionnaire (SGRQ).

The study was approved by the local research ethics committee and all subjects gave informed consent.

<u>Preparation of sputum samples</u>

Spontaneous sputum samples were collected (as free from saliva as possible after a mouthwash) and divided into two aliquots; one aliquot was used to obtain quantitative microbiological culture as described previously [24], and the second was ultracentrifuged (50,000 x g for 90 minutes at 4°C) to obtain the sol-phase which was stored at -70°C until analysis.

Measurement of PR3 and NE activities in sol-phase sputum

The enzymatic activities of PR3 and NE in the samples were evaluated by measuring the hydrolysis of specific substrates using pure proteinases as standards. Pure PR3 (Merck, UK) and pure NE (Athens Research and Technology, USA) were active site titrated against pure A1AT (Athens Research and Technology, USA), which had been previously titrated against porcine pancreatic elastase (PPE, Sigma-Aldrich, UK) of known activity. The activity of PPE was determined using Lineweaver-Burk double reciprocal plot analysis with N-succinyl-ala-ala-ala-p-nitroanilide (SlaaapN, Sigma-Aldrich, UK) as the substrate and published kinetic constants [25].

The PR3 activity assays were performed using the FRET substrate Abz-VAD-norV-ADRQ-EDDnp (Alta Biosciences, UK) which was prepared as described previously [21]. This substrate has a catalytic constant Kcat/Km of 3400$mM^{-1}s^{-1}$ and shows no significant hydrolysis by NE [26]. Pure PR3 was diluted in buffer (50mM Hepes, pH 7.4, 150mM NaCl, 0.05% Igepal CA-630) to an active concentration of 10nM and serially diluted in the same buffer to 0.625nM. Buffer alone was used as a blank for the assay. Sol-phase sputum samples were diluted 1 in 60 and the standards and samples (150µL) were added to a black opaque polypropylene low binding plate (Sigma, UK) in duplicate. A 1 in 60 dilution was initially chosen as most values fell within the range of the standard curve. Subsequently, 3µL of 1mM FRET substrate was added to each well and the fluorescence was measured (excitation 320nm, emission 420nm) at regular intervals for as long as the curve was linear using a Biotek Synergy 2 Multi-Mode Microplate Reader at 37°C. The activities of PR3 in the samples were obtained by interpolation from the standard curve. Any sample with a value above or below that of the standard curve was repeated at a suitable dilution. Using this FRET substrate, reliable measurements were obtained for PR3 in the range of 0.1-10nM [21]. The intra-assay coefficient of variation (CV) was 3.98% and the inter-assay CV was 13.35%. All results were corrected for the initial sample dilution.

The NE activity assays were performed using SlaaapN as the substrate which has a catalytic constant Kcat/Km of 465$M^{-1}s^{-1}$ and shows no significant hydrolysis with PR3 [27]. Pure NE was diluted in buffer to 1µM and then serially diluted to 15.6nM. Buffer alone was used as a blank for the assay. Sol-phase sputum samples were studied undiluted with appropriate controls. The standards, samples and control samples (30µL) were added to a 96 well plate in duplicate and the substrate SlaaapN (150µL of 1mg/ml) was added, except for the control wells where buffer was added instead of substrate. The optical density (OD) at 410nm was read at regular intervals up to 60 minutes using a Biotek Synergy HT plate reader at 37°C. The activities of NE in the samples were obtained by interpolation from the standard curve after subtraction of control values. The lower limit of detection for this substrate was 15nM and the intra- and inter-assay CVs were 3.48% and 4.76% respectively. Any samples with values below this level were re-assayed using the NE specific FRET substrate Abz-APEEIMRRQ-EDDnp providing reliable measurements for NE in the range of 0.1-10nM [21]. The intra-assay CV was 3.42% and the inter-assay CV was 4.76%.

The enzymatic activities of both *Pseudomonas aeruginosa* culture supernatants and pure *Pseudomonas* elastase (Merck, UK) were assessed using the above substrates to confirm no cross reactivity with the assays for human proteinases.

<u>Measurement of PR3 and NE concentrations in sol-phase sputum</u>

The concentrations of PR3 and NE in the samples were measured by enzyme linked immunosorbent assay (ELISA) using commercially available kits. The PR3 ELISA (Biorbyt, UK) only detects unbound PR3, whereas the NE ELISA (Cambridge Bioscience, UK) detects both free NE and NE bound to inhibitors. The lower limits of detection were 5pM for the PR3 ELISA and 14pM for the NE ELISA. The intra- and inter-assay CVs for these ELISAs were 3.59% and 9.19% respectively for the PR3 ELISA and 5.07% and 13.76% respectively for the NE ELISA.

Measurement of inhibitors in sol-phase sputum

The concentration of A1AT was measured by a locally developed ELISA. In brief, 96-well plates (Nunc Maxisorb, UK) were coated overnight with 200µL of 2µL/ml goat anti-human A1AT antibody (Binding site, Birmingham, UK) in carbonate buffer pH 9.6. After blocking with 1% bovine serum albumin (BSA), sol-phase sputum samples (diluted 1:100 to 1:10,000) were added and a human serum protein calibrator (Dako, UK) containing 1.24g/L A1AT was used to develop a range of standards (1.9-62ng/ml) and the plates were incubated for two hours at room temperature. Following this, the next antibody [200µL of 2.6µL/ml goat anti-human A1AT peroxidase conjugate (Binding site, Birmingham UK) in phosphate buffered saline (PBS), albumin 0.1% (w/v), and Tween®20 0.05% (v/v)] was added and incubated for two hours at room temperature. The substrate [tetramethylbenzidine (TMB) solution (Sigma, UK)] was then added and the reaction was stopped with 0.1M H_2SO_4 when a suitable colour change had developed. The OD was then read at 450nm with a 570nm wavelength correction and sample concentration was determined from the standards by interpolation. The intra- and inter- assay CVs were 4.66% and 4.35% respectively.

The concentrations of SLPI and elafin were measured using commercially available ELISA kits (R&D systems, UK) according to the manufacturer's instructions. The intra- and inter-assay CVs were 6.37% and 10.76% respectively for the SLPI ELISA and 7.67% and 4.46% respectively for the elafin ELISA.

Other measurements in sol-phase sputum

Markers of neutrophilic inflammation were measured when sufficient sample remained, including interleukin (IL)-8, leukotriene (LT)-B4 and myeloperoxidase (MPO). IL-8 and LTB4 were measured using commercially available ELISA kits (R&D systems, UK). The intra- and inter-assay CVs were 5.97% and 7.22% respectively for the IL-8 ELISA and 6.34% and 11.9% respectively for the LTB4

ELISA. MPO activity was measured as described previously [28]. The intra- and inter- assay CVs for the MPO activity assays were 3.85% and 10.07% respectively.

Statistical analysis

Statistical analyses were performed using PASW statistics 18 for Windows. Normality was tested using the Kolmogorov-Smirnov test. PR3 activity, PR3 concentration, NE activity, NE concentration, IL-8, LTB4, MPO, A1AT, SLPI, FEV1 and SGRQ total score were not normally distributed therefore non-parametric tests were used and data are presented as medians and inter-quartile ranges (IQR). Mann-Whitney U tests or Wilcoxon signed rank tests were used for comparisons of independent or related data respectively. Correlations were assessed using Spearman's rank correlation coefficient. Normally distributed data are presented as mean ±standard error of mean (SEM) and independent or paired T-tests were used for comparisons of data for independent or related measures respectively. To compare variables between A1AT deficient and COPD subjects after adjusting for baseline differences, the data were log transformed and linear regression was used with group as one of the independent factors. The unstandardized residuals were tested for normality. For statistical purposes, enzyme activity below the lower limit of detection was taken as 0.1nM. Results were deemed statistically significant if $p \leq 0.05$.

Results

Baseline characteristics

Baseline characteristics of the two subject groups are shown in Table 1. The A1ATD subjects were younger, had a lower pack year smoking history, FEV1 (% predicted), FEV1/FVC ratio and KCO (% predicted) compared to the COPD subjects. Of the 28 A1ATD subjects, 7 were never smokers, 19 were ex-smokers and 2 were current smokers. Of the 22 COPD subjects, 15 were ex-smokers and 7 were current smokers. There were no significant differences in sex distribution or radiological evidence of emphysema between the two groups.

Table 1- Baseline characteristics

	A1ATD	COPD	P-value
Age Mean (SEM)	55 (1.9)	65 (1.9)	0.002
Number of males (%)	22 (79%)	19 (86%)	NS
Smoking history (pack years) Mean (SEM)	21 (3.9)	54 (10.0)	0.004
FEV1 (% predicted) Mean (SEM)	49 (5.9)	72 (5.8)	0.004
FEV1/FVC ratio Mean (SEM)	0.41 (0.04)	0.49 (0.04)	0.031
KCO (% predicted) Mean (SEM)	69 (5.2)	101 (6.1)	0.001
Number with radiological evidence of emphysema on HRCT (%)	22 (79%)	14 (64%)	NS

A1ATD: alpha-1-antitrypsin deficiency, COPD: chronic obstructive pulmonary disease, SEM: standard error of the mean, FEV1: forced expiratory volume in one second, FVC: forced vital capacity, KCO: carbon monoxide transfer coefficient, HRCT: high resolution computed tomography, NS: not significant.

PR3 and NE activities in sputum from clinically stable subjects

Detectable PR3 activity was found in all sputum samples from the A1ATD subjects (median 128nM, IQR 33-558nM) and in 64% of samples from the COPD subjects (median 22nM, IQR 0-103nM). PR3 activity was greater in A1ATD subjects compared to those with usual COPD (p= 0.004) after adjusting for baseline differences in age, pack year smoking history, FEV1 (% predicted) and KCO (% predicted). Unbound PR3 concentration measured by ELISA confirmed these differences and values correlated strongly with PR3 activity (Spearman's rho 0.879, p<0.001) indicating that most of the unbound PR3 was enzymatically active.

Detectable NE activity was found in 21% of A1ATD sputum samples (median 0nM, IQR 0-0nM) and 5% of usual COPD samples (median 0nM, IQR 0-0nM). The concentration of NE measured by ELISA (free and bound to inhibitors) was not significantly different (p=0.296) between subjects with A1ATD (median 330nM, IQR 140-813nM) and usual COPD (median 214nM, IQR 84-564nM). In the A1ATD group, 3 subjects had higher NE activity than PR3 activity. These 3 subjects were colonised with *Pseudomonas aeruginosa* whilst none of the subjects with usual COPD were. Neither *P. aeruginosa* culture supernatants nor purified *Pseudomonas* elastase were able to hydrolyse the substrates used for the NE assays (data not shown), suggesting that the NE activity was specific. Of note, *Pseudomonas* elastase activity is not inhibited by A1AT or the other airway serine proteinase inhibitors [29].

The activity results are summarised in Figure 1. PR3 activity was greater than NE activity in subjects with A1ATD (p=0.004) and those with usual COPD (p=0.015).

NSP inhibitory proteins in sputum

As expected, the concentration of A1AT was higher in sputum from subjects with non-deficient COPD compared to those with A1ATD (COPD median 405nM IQR 234-744nM, A1ATD median 51nM

IQR 19-83nM: $p<0.001$). In addition, usual COPD subjects also had higher concentrations of SLPI (COPD median 2.8µM IQR 1.7-4.4 µM, A1ATD median 1.3µM IQR 0.8-2.7 µM: $p=0.002$) and elafin (mean 316±37pM vs 200±39pM: $p=0.039$). SLPI was quantitatively the predominant NSP inhibitor in sputum from both subject groups. The contribution to the total airway inhibitory capacity of elafin was negligible, being found in subnanomolar concentrations in all samples. As shown in Figure 2, airway inhibitors of PR3 were quantitatively less than those of NE.

The median concentration of NE measured by ELISA (free and bound) was less than the median total concentration of its cognate inhibitors both in subjects with A1ATD and usual COPD, as shown in Figure 3 and would explain why the majority of sputum samples from both subject groups showed no detectable NE activity. It was not possible to measure the total PR3 concentrations since the ELISA available was only able to detect unbound PR3. Nevertheless, the presence of PR3 activity in the sputum samples indicates that the amount of PR3 exceeds the functional concentration of its inhibitors.

<u>Relationship between PR3 activity and clinical status</u>

In the 12 subjects with usual COPD who had sputum samples available at the start of an exacerbation and at 8 weeks following the exacerbation when clinically stable, ten day 1 (exacerbation) samples and 6 day 56 (stable) samples had detectable PR3 activity and the group values were significantly greater during exacerbation than in the stable state ($p=0.037$). These data are shown in Figure 4. In this sample of patients, no significant difference was found in NE activity between exacerbation and stable clinical state (data not shown).

Markers of neutrophilic inflammation were measured in the majority (n=21) of usual COPD samples and in a subset of A1ATD samples (n=11) where sufficient sample remained. The demographics of the COPD subjects where the measurements could be made were not significantly different to those where it couldn't. The A1ATD subjects who did not have the measurements available were older (59 years vs 50 years: p=0.026) and had a lower KCO (59% predicted vs 82% predicted: p=0.031) than those where measurements were available. There were no significant differences in sex, pack year smoking history, smoking status, FEV1 % predicted, FEV1/FVC ratio, residual lung volumes or SGRQ total score.

The concentrations of IL-8 (A1ATD median 6.86nM IQR 2.02-12.39nM, COPD median 2.77nM, IQR 1.13-8.24nM: p=0.312), LTB4 (A1ATD median 6.52nM IQR 4.15-20.02nM, COPD median 4.72nM IQR 2.77-17.50nM: p=0.463) and the MPO activities (A1ATD median 0.40 units/ml IQR 0.19-0.57 units/ml, COPD median 0.58 units/ml IQR 0.28-1.65 units/ml: p=0.293) were not significantly different between patient groups.

The correlations between PR3 activity and other parameters are summarised in Table 2. PR3 activity correlated positively with NE activity in the A1ATD subjects (Spearman's rho=0.586, p=0.001) even though NE activity was detectable only in 6 subjects. PR3 activity correlated positively with IL-8 quantity in both patient groups (A1ATD Spearman's rho=0.791 p=0.004, COPD Spearman's rho=0.650 p=0.001) and MPO activity (A1ATD Spearman's rho=0.612 p<0.001, COPD Spearman's rho=0.799 p<0.001). In the non-deficient COPD group, PR3 activity also correlated positively with LTB4 (Spearman's rho=0.434, p=0.049).

PR3 activity was found to correlate with the total pathogenic bacterial load in the A1ATD group (Spearman's rho=0.578, p=0.001). Subjects with *Pseudomonas aeruginosa* on quantitative microbiological culture had significantly higher NE activity (p<0.001) and PR3 activity (p=0.025) in their sputum compared to subjects who did not. Higher PR3 activity was also found in samples that grew *Haemophilus influenzae* compared to those that did not (p=0.003) but NE activity was not different. The pathogens isolated in the sputum samples are summarised in Table 3. Some subjects grew more than one pathogen (3 A1ATD subjects), whilst some did not grow any (10 A1ATD subjects and 14 COPD subjects).

A significant correlation was also found between PR3 activity and SGRQ total score in the A1ATD subjects (Spearman's rho=0.621, p=0.001). The SGRQ scores were not available for the subjects with usual COPD. No correlations were found between PR3 activity and FEV1 (% predicted) or KCO (% predicted) in either subject group.

Table 2- Correlations of PR3 activity in sol-phase sputum

Diagnosis	Measurement	Number of Samples with Measurement	Spearman's rho	p-value
A1ATD	MPO	11	0.612	<0.001
	IL-8	11	0.791	0.004
	LTB4	11	0.291	0.385
	SGRQ total	25	0.621	0.001
	Total pathogenic bacterial load	28 (18 positive)	0.578	0.001
COPD	MPO	20	0.799	<0.001
	IL-8	21	0.650	0.001
	LTB4	21	0.434	0.049
	Total pathogenic bacterial load	22 (8 positive)	0.291	0.189

A1ATD: alpha-1-antitrypsin deficiency, COPD: chronic obstructive pulmonary disease, SGRQ: St George's respiratory questionnaire, IL-8: interleukin-8, MPO: myeloperoxidase, LTB4: leukotriene B4.

Table 3- Pathogens isolated in the sputum samples

Pathogen name	Number of A1ATD subjects with positive culture of this pathogen	Number of COPD subjects with positive culture of this pathogen
Haemophilus influenzae	9	5
Moraxella catarrhalis	3	0
Pseudomonas aeruginosa	3	0
Haemophilus parainfluenzae	3	3
Streptococcus pneumoniae	1	0
Staphylococcus aureus	2	0
Proteus mirabilis	1	0

A1ATD: alpha-1-antitrypsin deficiency, COPD: chronic obstructive pulmonary disease

Discussion

This study has provided direct evidence that active PR3 is present in sol-phase sputum from clinically stable subjects with chronic bronchitis associated with A1ATD as well as non-deficient COPD. PR3 activity was greater than NE activity in these subjects, which likely reflects both the lower airway inhibitory capacity for PR3 and the greater amount of PR3 contained within the azurophilic granules of the neutrophil compared to NE. In addition, the association rate constants of A1AT and elafin for PR3 are orders of magnitude lower than for NE [30] and therefore NE is likely to be preferentially inhibited compared to PR3 in the inflammatory environment. PR3 is also not inhibited by SLPI which is present in large amounts in respiratory epithelial lining fluid and is the predominant inhibitor of NE in the upper airways. Furthermore, PR3 is capable of degrading SLPI [31], thus potentially enhancing the biological activities of other NSPs that are usually inhibited by SLPI.

PR3 activity was found to be greater during exacerbations than during periods of clinical stability. Exacerbations are often associated with an increased neutrophilic inflammation [32], and would account for the correlations of PR3 activity with other neutrophilic markers. Of interest, PR3 activity showed a positive correlation with worsening health status as measured by the SGRQ. PR3 activity is thus a marker of increased airway inflammation and exacerbations, which are both associated with impaired health status in subjects with chronic respiratory disease [33, 34] suggesting it is an association rather than cause and effect.

The current study did not however demonstrate any relationship between PR3 activity and lung function parameters. In a previous study where PR3 concentration was measured in sputum from 49 subjects with cystic fibrosis (CF), a negative correlation was observed between PR3 concentration and FEV1 (% predicted) [8]. It is possible therefore that the present study did not involve sufficient numbers to detect a relationship between FEV1 and PR3 activity, although other features such as bacterial colonisation (see below) will also complicate this potential relationship.

The current study has directly measured both PR3 and NE activities in biological samples using highly sensitive and specific substrates. Some previously published studies of NSP activities in airway secretions have used non-specific “elastase” substrates (such as N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide) and potentially measured the combined activities of PR3 and NE [35-37]. Other studies have indirectly measured PR3 activity by measuring the combined activities of PR3 and NE with and without an NE inhibitor [8]. Although SlaaapN has been used to measure NE activity in sputum previously [38], it is less sensitive than the NE specific FRET substrate described here. The direct measurement of individual NSP activities in lung secretions using highly sensitive and specific substrates (as described here) allows the contribution of each NSP to the total proteinase burden to be determined specifically. Although NSPs are released from neutrophils simultaneously upon activation and degranulation, their concentrations can differ due to local factors such as binding to the cell membrane [16], their affinities to endogenous inhibitors at the inflammatory site and their specificities toward peptide or protein substrates [30]. Biological consequences are only likely to occur in the presence (especially persistent presence) of active enzyme.

The results described here are derived from subjects with A1ATD (PiZZ phenotype) and non-deficient COPD with a chronic bronchitis phenotype. Spontaneous sputum production is associated with greater neutrophilic airway inflammation compared to subjects who do not expectorate, which is not related to smoking status [35]. Sputum production may also be associated with an accelerated decline in FEV1 in COPD [39]. Therefore, although it is important to understand the role of NSPs in this subgroup of patients, the results presented here may be less generalizable to subjects who are not spontaneous sputum producers. Spontaneous sputum collection is non-invasive and minimises dilutional errors found with other techniques such as broncho-alveolar lavage [40] and induced sputum collection and may provide critical data in this important phenotype. However, the use of single spontaneously produced sputum samples also has limitations due to the daily variability of sample collection and dilution with nasopharyngeal secretions. This phenomenon has been described in subjects with A1ATD and usual COPD, and could be minimised in future studies by averaging 3 sequential samples [41]. Nevertheless, despite this potential drawback, the differences and correlations described here remain robust.

In the current study, 3 subjects with A1ATD were colonised with *Pseudomonas aeruginosa* and had significantly higher NE and PR3 activities in their sputum compared to subjects who were not. Furthermore, greater PR3 activity was found in samples that grew *Haemophilus influenzae* compared to those that did not. Our group has previously shown that in patients with bronchiectasis, *Pseudomonas aeruginosa* provokes a more intense inflammatory response (with elevated NE and MPO activities) compared to *Haemophilus influenzae*, which in turn is greater than that with *Moraxella catarrhalis* [38]. The 6 subjects with A1ATD who had detectable NE activity in their sputum had significantly lower concentrations of SLPI and elafin compared to those with undetectable NE activity. Previous work has shown that both SLPI and elafin can be cleaved by NE, particularly in the presence of *Pseudomonas aeruginosa* [42, 43] which may therefore represent an effect rather than a cause. There were no other significant differences observed between the subjects with or without detectable NE activity. Correlations of PR3 activity with airway bacterial load and airway inflammation (NE and MPO activity) support the role of colonisation in airway neutrophilic inflammation (as indicated previously [38, 44]), but highlights the association with PR3 activity, which has the potential to drive the pathophysiological processes that influence COPD and its progression. This enzyme may therefore be more important in COPD than has previously been thought.

We have indicated that PR3 activity should be determined when evaluating the proteinase/antiproteinase imbalance in the airways. Whether or not it is central to the process remains to be determined as it remains possible that several proteinases may play a role in the pathogenesis of COPD. In addition to NSPs, both matrix metalloproteinases [45] and cysteine proteinases [46, 47] have been associated with tissue destruction in emphysema both directly and indirectly via their interactions with NSPs and their inhibitors. Strategies to reduce the burden of proteinases in the lungs could potentially offer novel therapies for COPD. Recently, Jegot et al [48] have developed specific PR3 inhibitors. Selective inhibitors of PR3 may thus provide further insight into its role in disease and (based on the studies presented here) could potentially be of therapeutic value in inflammatory lung diseases.

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Conflict of interest

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Figure 1-PR3 and NE activities in sol-phase sputum

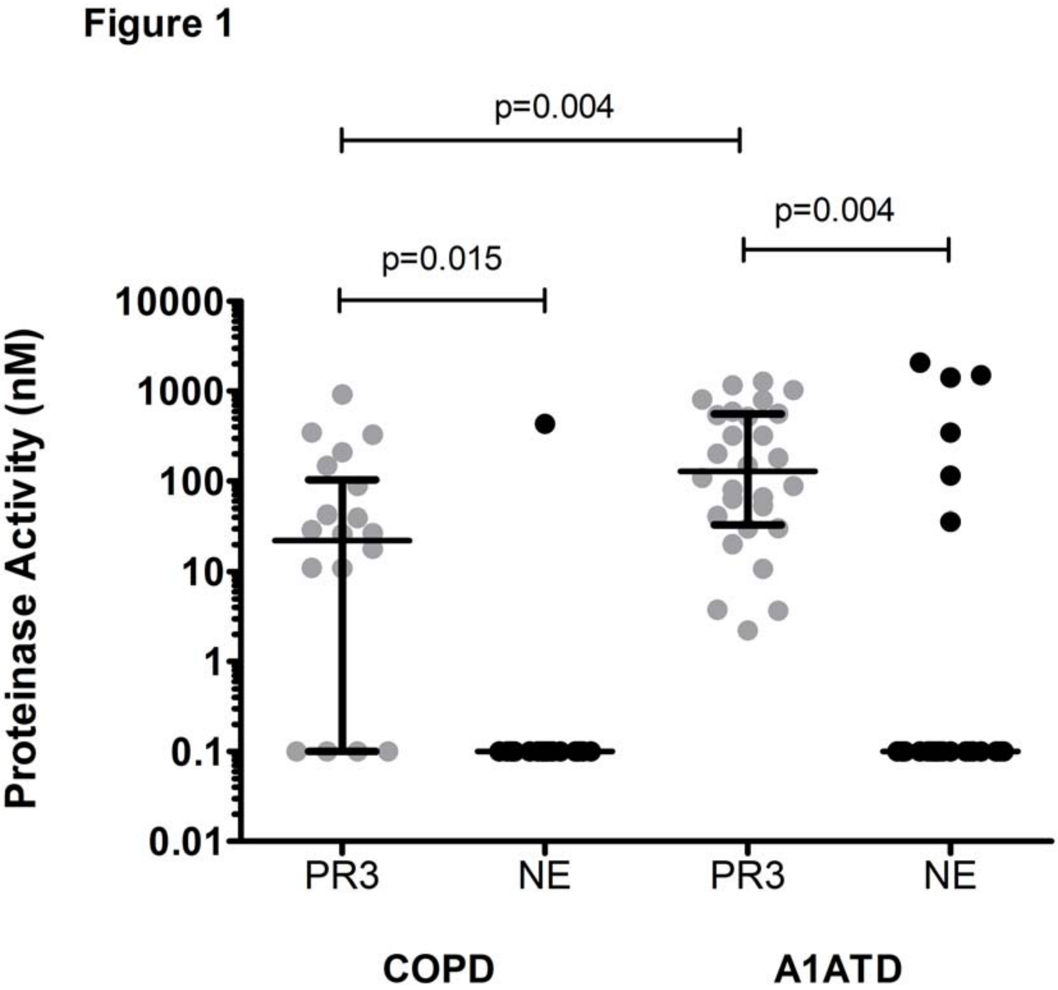

Figure 1: Proteinase 3 (PR3) and neutrophil elastase (NE) activities in sol-phase sputum spontaneously produced by clinically stable subjects with alpha-1-antitrypsin deficiency (A1ATD, n=28) and chronic obstructive pulmonary disease (COPD, n=22). Each point represents data from an individual subject, and some undetectable values are superimposed. Horizontal lines represent the median with inter-quartile range (IQR). PR3 activity was greater than NE activity in both subject groups. Those with A1ATD had greater PR3 activity than subjects with usual COPD. There was no significant difference in NE activity between the subject groups although detectable NE activity was only found in 6 A1ATD subjects and 1 COPD subject. The 3 A1ATD subjects with high NE activity were colonised with *Pseudomonas aeruginosa*. The greater PR3 activity in sputum from

A1ATD subjects compared to COPD subjects remained even when subjects colonised with *P. aeruginosa* were excluded (p=0.006).

Figure 2- NSP inhibitory capacity in sputum from clinically stable subjects with A1ATD and COPD

Figure 2

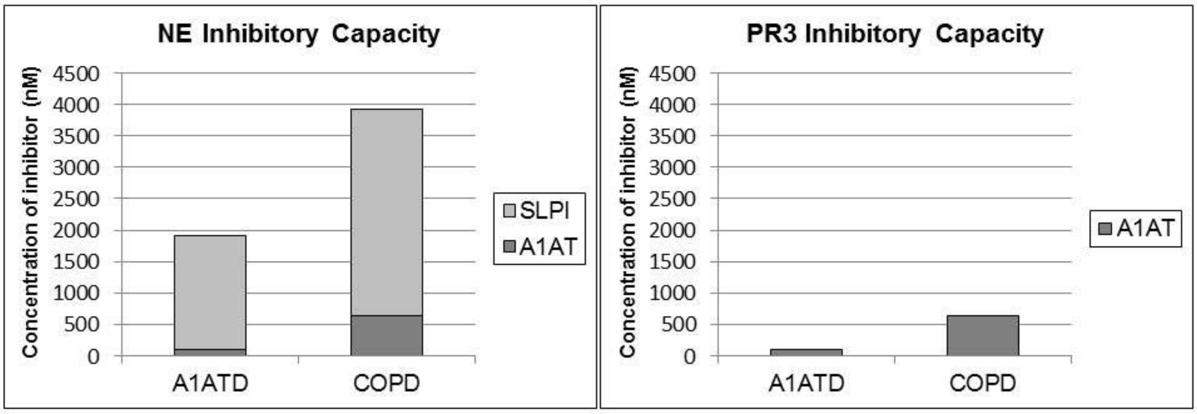

Figure 2: Mean concentrations (nM) of airway inhibitors in sol-phase sputum from subjects with alpha-1-antitrypsin deficiency (A1ATD) and usual chronic obstructive pulmonary disease (COPD). The predominant airway inhibitor of neutrophil elastase (NE) in both subject groups was secretory leukoproteinase inhibitor (SLPI). The contribution of elafin was negligible, being found in subnanomolar concentrations in both subject groups and is therefore not shown on the graph. Proteinase 3 (PR3) thus has fewer and significantly reduced quantities of its airway inhibitors than NE. The PR3 inhibitory capacity is especially low in A1ATD subjects, and indicates why PR3 activity was detected in all samples from this subject group even in the stable clinical state.

Figure 3-Concentration of NE and its inhibitors in sol-phase sputum

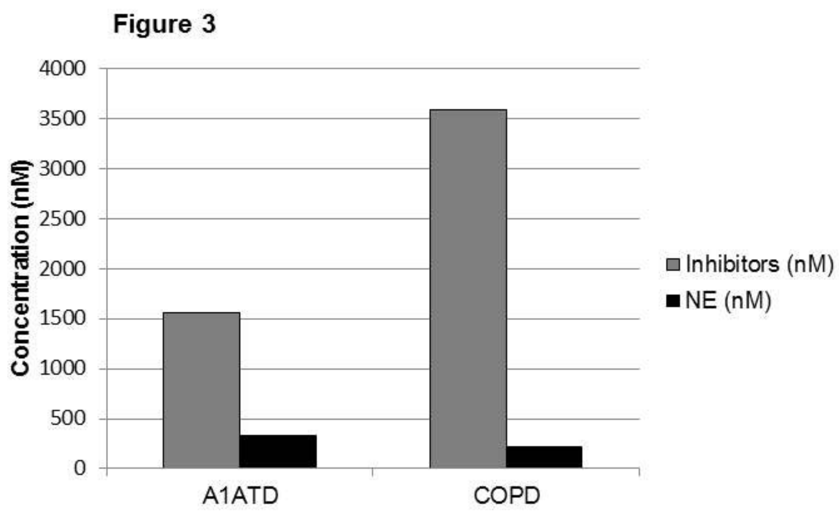

Figure 3: Median concentrations (nM) of neutrophil elastase (NE, free and bound) and the median sum of its inhibitors alpha-1-antitrypsin (A1AT), secretory leukoproteinase inhibitor (SLPI) and elafin in sol-phase sputum from stable subjects with alpha-1-antitrypsin deficiency (A1ATD) and chronic obstructive pulmonary disease (COPD) are shown. In both subject groups, the total concentration of NE is well below that of its inhibitors, particularly in subjects with usual COPD. The lack of NE activity in the majority of sputum samples is thus likely due to the dominance of its inhibitors. The presence of NE activity in some samples therefore likely reflects significant loss of function of the inhibitors.

Figure 4-PR3 activity in COPD patients during an exacerbation and at 8 weeks following the exacerbation when clinically stable

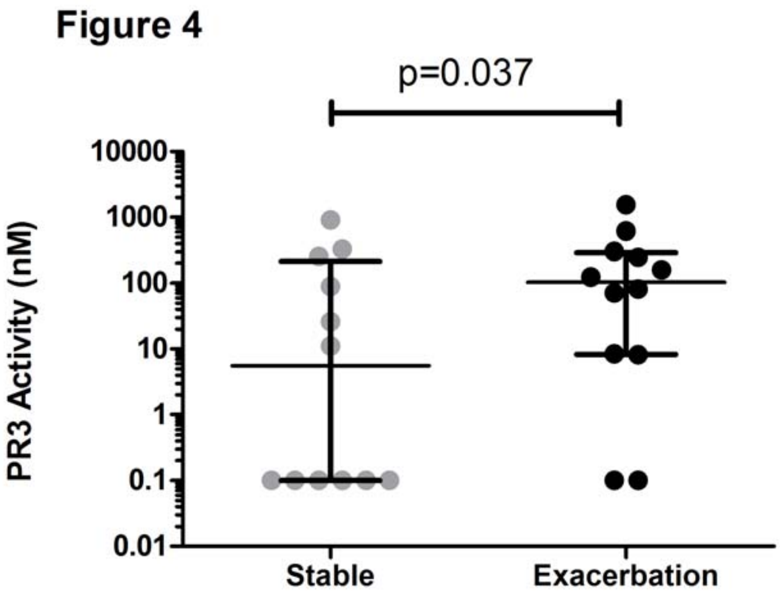

Figure 4: Proteinase 3 (PR3) activity (nM) in sol-phase sputum from 12 subjects with chronic obstructive pulmonary disease (COPD) taken on day 1 of an exacerbation and day 56 when clinically stable. Horizontal lines represent the median and inter-quartile range. PR3 activity was significantly greater during an exacerbation than in the stable clinical state.