



# A specific proteinase 3 activity footprint in $\alpha_1$ -antitrypsin deficiency

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**ABSTRACT**  $\alpha_1$ -Antitrypsin ( $\alpha_1$ -AT) deficiency is a risk factor for emphysema due to tissue damage by serine proteases. Neutrophil elastase (NE) has long been considered the enzyme responsible. However, proteinase 3 (PR3) also produces the pathological features of chronic obstructive pulmonary disease (COPD), is present in the same granules in the neutrophil and is inhibited after NE. We developed a specific footprint assay for PR3 activity and assessed its relationship to an NE footprint in  $\alpha_1$ -AT deficiency.

An ELISA was developed for the specific PR3 fibrinogen cleavage site  $A\alpha$ -Val<sup>541</sup>. Levels were measured in plasma from 239 PiZZ patients, 94 PiSZ patients, 53 nondeficient healthy smokers and 78 individuals with usual COPD. Subjects underwent extensive demographic characterisation including full lung function and lung computed tomography scanning.

$A\alpha$ -Val<sup>541</sup> was greater than the NE footprint in all cohorts, consistent with differential activity. Values were highest in the PiZZ  $\alpha_1$ -AT-deficient patients and correlated with the NE marker  $A\alpha$ -Val<sup>360</sup>, but were ~17 times higher than for the NE footprint, consistent with a greater potential contribution to lung damage.  $A\alpha$ -Val<sup>541</sup> was related cross-sectionally to the severity of lung disease (forced expiratory volume in 1 s % pred:  $r_s = -0.284$ ;  $p < 0.001$ ) and was sensitive to augmentation therapy, falling from 287.2 to 48.6 nM ( $p < 0.001$ ).

An *in vivo* plasma footprint of PR3 activity is present in greater quantities than an NE footprint in patients with  $\alpha_1$ -AT deficiency, is sensitive to augmentation therapy and represents a likely biomarker for dose-ranging studies.



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**This novel assay provides evidence of proteinase 3 activity in  $\alpha_1$ -antitrypsin deficiency, suggesting it may play a significant role in lung tissue damage and act as a specific biomarker for augmentation therapy** <http://bit.ly/32dnt66>

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## Introduction

Proteolytic enzymes have long been implicated in the pathophysiology of chronic obstructive pulmonary disease (COPD), especially in emphysema-dominant phenotypes. This concept originated with the observation that  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT)-deficient individuals were particularly susceptible to the development of emphysema [1].  $\alpha_1$ -AT is a major inhibitor of neutrophil serine proteinases and animal studies identified the central role of neutrophil elastase (NE) as the mediator of pathological changes typical of COPD [2]. However, two other serine proteinases are co-localised in the azurophil granule with NE, *i.e.* proteinase 3 (PR3) and cathepsin G, and are released into the pericellular environment with NE following neutrophil activation. PR3 in particular can produce the same bronchial and emphysematous changes as NE in animal models [3]. In addition, PR3 has also been implicated in cellular apoptosis, which is thought to be another key process in the development of emphysema [4].

PR3 is stored as a proenzyme in the azurophil granule with NE, although the quantity is uncertain. Direct immunostaining indicates increased total PR3 [5] whereas indirect assays by subtraction indicate reduced activity concentrations [6] compared with NE. However, PR3 has a lower association rate constant with  $\alpha_1$ -AT [7], suggesting it is likely to have more prolonged activity than NE before inactivation leading to  $\alpha_1$ -AT/enzyme complex formation, as demonstrated by mathematical modelling [7]. Indeed, studies of airways secretions in both  $\alpha_1$ -AT-deficient and nondeficient COPD indicate that uninhibited PR3 activity is more likely to be present than uninhibited NE activity [8], supporting a more persistent role for PR3 in the development of COPD and emphysema.

The development of emphysema is believed to relate mainly to connective tissue destruction (predominantly elastin) in the lung interstitium. This provides a major impediment to assessing the focused local interstitial activity of these enzymes because of access to the site of action and rapid inhibition *in situ* that is not necessarily reflected in sputum studies.

As neutrophils migrate into the lung they are accompanied by plasma proteins including fibrinogen [9]; thus, we hypothesised that specific fibrinogen cleavage products would be generated at the point of neutrophil activation in the interstitium, acting as a footprint of local enzyme activity and be detectable (although likely at a lower level) in the plasma through lymph recirculation. Indeed, an NE footprint ( $A\alpha$ -Val<sup>360</sup>) is raised in  $\alpha_1$ -AT deficiency, is reduced by  $\alpha_1$ -AT augmentation therapy and increases during exacerbations, providing validation for this concept [10, 11].

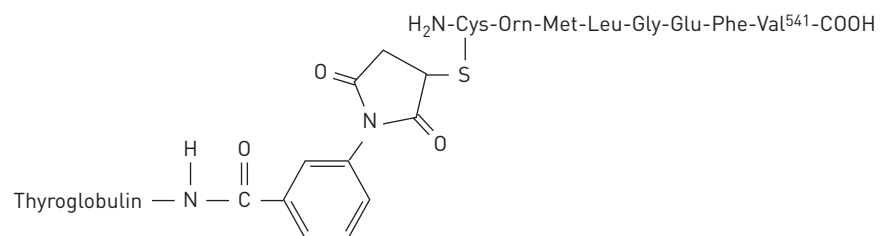
The purpose of the present study was to develop a specific “footprint” assay for PR3 also based on fibrinogen cleavage that would be measurable in plasma, and to assess its relationship to respiratory dysfunction in  $\alpha_1$ -AT deficiency and sensitivity to change during augmentation therapy.

## Materials and methods

### $A\alpha$ -Val<sup>541</sup> assay

The methodology and validation of the assay is covered in the supplementary material. Cleavage of fibrinogen with PR3 produced several fragments, one of which was detected in plasma from  $\alpha_1$ -AT-deficient patients and digested to obtain the carboxyl-terminal amino acid sequence ending at position 541.

Briefly, we generated antiserum that recognised the free carboxyl amino acid of  $A\alpha$ -Val<sup>541</sup> (normally linked with Ser<sup>542</sup>). The peptide COM<sup>536</sup>LGEFV<sup>541</sup> was conjugated to thyroglobulin using the cross-linking reagent Sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester) (figure 1) and rabbits were immunised with the conjugate. The subsequent antiserum was harvested and an indirect ELISA developed as described in the supplementary material.



**FIGURE 1** The peptide conjugate. 6-mer peptide MLGEFV with an additional cysteine (Cys) and unnatural ornithine (Orn) at the amino terminus (COM<sup>536</sup>LGEFV<sup>541</sup>). Coupled to bovine thyroglobulin using Sulfo-MBS.

Once the assay was established, the antiserum was validated in several ways. 1) Carboxyl-terminal extensions (1-mer and 6-mer) were added to COMLGEFV and a further peptide had the terminal valine removed, and these were used to confirm antibody specificity. 2) The NE-specific peptide also with a terminal valine (CJTSESSV from the  $A\alpha$ -Val<sup>360</sup> assay) was tested to ensure no cross-reactivity. 3) Various concentrations of PR3-cleaved, NE-cleaved or uncleaved fibrinogen were measured to determine the degree of recognition of the peptides generated in these preparations (all human plasma samples contain some of the enzyme-specific peptides as a result of physiological activity of neutrophils in health). The results of these validation experiments are shown in supplementary figures E4–E7.

#### *A $\alpha$ -Val<sup>360</sup> assay*

The relationship between  $A\alpha$ -Val<sup>541</sup> and the NE-specific cleavage product  $A\alpha$ -Val<sup>360</sup> was determined using plasma samples from  $\alpha_1$ -AT-deficient patients, COPD patients and healthy smoking controls. The full methodology for the  $A\alpha$ -Val<sup>360</sup> assay has been described previously [10].

#### *Study subjects*

The initial cohort for this study consisted of 180 homozygous Z allele (PiZZ) patients with severe  $\alpha_1$ -AT deficiency enrolled as part of the UK National Institute of Health Research (NIHR) Rare Diseases Translational Research Collaboration. The subjects (all ex-smokers for >12 months or never-smokers) were recruited from six UK sites (Birmingham, Cambridge, Leicester, Nottingham, Royal Brompton (London) and Southampton) and gave written informed consent (South Birmingham Research Ethics Committee LREC 3359).

Subjects underwent extensive demographic characterisation including full, post-bronchodilator lung function testing and densitometric analysis of high-resolution chest computed tomography at full inspiration using an established analysis programme (Pulmo CMS; Medis Specials, Leiden, The Netherlands). The severity of airflow obstruction varied from none to very severe as quantified by Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease (GOLD) staging criteria [12]. Plasma was taken and stored at  $-80^{\circ}\text{C}$  until analysed.

Plasma was also analysed from two other patient groups in the UK  $\alpha_1$ -AT-deficient registry (ADAPT) programme described elsewhere [13]. A cohort of 94 individuals with the PiSZ genotype ( $\alpha_1$ -AT levels  $\sim 40\%$  of normal), 59 PiZZ  $\alpha_1$ -AT-deficient patients with “early” disease defined as those without spirometric evidence of COPD (forced expiratory volume in 1 s (FEV<sub>1</sub>)/forced vital capacity (FVC)  $>0.7$ ) or with GOLD Stage I (mild) obstruction collected as part of an Alpha 1 Foundation cohort of early/mild disease and 78 usual COPD patients, including both current (n=30) and ex-smokers (n=48), without  $\alpha_1$ -AT deficiency (FEV<sub>1</sub>/FVC  $<0.7$  and FEV<sub>1</sub>  $<80\%$  predicted) recruited through the NIHR-funded Clinical Research Facility (Queen Elizabeth Hospital Birmingham, Birmingham, UK). Finally, 53 healthy smokers (FEV<sub>1</sub>/FVC  $>0.7$ , age 41–79 years) were studied to determine a healthy range for  $A\alpha$ -Val<sup>541</sup>. Samples from 39  $\alpha_1$ -AT-deficient patients were repeated after 12–24 months while still clinically stable to assess consistency of the result. Plasma was also assessed for 21  $\alpha_1$ -AT-deficient patients before (baseline) and at 6 months during augmentation therapy with  $\alpha_1$ -AT, and for 15 patients before and during placebo therapy, as described previously [10] to determine the effect of replenishment. No patient in any group had experienced an acute exacerbation in the 6 weeks prior to assessment.

#### *Statistical analysis*

Statistical analysis was performed using SPSS Statistics version 22.0 (IBM, Armonk, NY, USA). Normality was tested using the Shapiro–Wilk test and statistical significance between groups was taken as  $p < 0.05$ .  $A\alpha$ -Val<sup>541</sup> and  $A\alpha$ -Val<sup>360</sup> were not normally distributed, and therefore expressed as median and interquartile range (IQR) for group data, and correlations were performed using Spearman’s  $r_s$  with log-transformed data. The Wilcoxon signed-rank test was used to determine any differences in paired samples (*i.e.*  $A\alpha$ -Val<sup>541</sup> stability over time or before/after augmentation therapy) and the Mann–Whitney test was used to determine any differences between groups. Where multiple comparisons were made, a Holm–Bonferroni correction was applied.

## **Results**

The results of the assay development and validation are described in detail in the supplementary material. The inter- and intra-assay coefficients of variation were 23.6% and 8.5%, respectively.

The demographic features of the  $\alpha_1$ -AT-deficient patients, nondeficient COPD patients and healthy control subjects are summarised in table 1. Lung function was corrected for age, sex, ethnicity and height using the Global Lung Function Initiative 2012 equations [14]. Lung densitometry was available for 191 of

TABLE 1 Demographic features of healthy individuals, chronic obstructive pulmonary disease (COPD) patients,  $\alpha_1$ -antitrypsin-deficient subjects with the PiSZ genotype and the combined PiZZ cohort

	Healthy	COPD <sup>#</sup>	PiSZ	PiZZ <sup>¶</sup>
<b>Subjects</b>	53	78	94	239
<b>Age years</b>	61.5±10.5	66.0±9.4	52.4±15.1	57.2±11.2
<b>Sex</b>				
Male	28 (52.8)	50 (64.1)	50 (55.6)	117 (49.0)
Female	25 (47.2)	28 (35.9)	40 (44.4)	122 (51.0)
<b>FEV<sub>1</sub> % pred</b>	109.97±15.76	54.49±22.46	90.00±32.66	66.21±32.02
<b>FEV<sub>1</sub>/FVC %</b>	76.93±6.28	47.59±15.68	63.28±21.12	48.44±20.51
<b>Kco % pred</b>	97.60±17.55	64.89±19.87	85.62±27.75	63.52±18.92
<b>Perc15 HU</b>	NA	NA	NA	-950.79±26.73
<b>HU910 %</b>	NA	NA	NA	44.40±18.29
<b>HU950 %</b>	NA	NA	NA	19.90±14.67

Data are presented as n, mean±sd or n (%). FEV<sub>1</sub>: forced expiratory volume in 1 s; FVC: forced vital capacity; Kco: transfer coefficient of the lung for carbon monoxide; Perc15: density below which the lowest 15% of voxels occurs; HU910 and HU950: proportion of voxels with density below these Hounsfield unit thresholds. Full analysis of computed tomography densitometry (used widely in observational and clinical trials) was available for 191 patients and this dataset is summarised for the PiZZ  $\alpha_1$ -AT-deficient patients only. <sup>#</sup>: FEV<sub>1</sub> n=77, FEV<sub>1</sub>/FVC n=76 and Kco n=28; <sup>¶</sup>: FEV<sub>1</sub> n=237, FEV<sub>1</sub>/FVC n=238 and Kco n=233.

the 239 PiZZ subjects and mean results are also shown using accepted parameters to quantify emphysema as described previously [15].

#### *A $\alpha$ -Val<sup>541</sup> concentrations in health, COPD and $\alpha_1$ -AT deficiency*

The A $\alpha$ -Val<sup>541</sup> concentrations showed a wide range for all cohorts. The median (IQR) concentration of A $\alpha$ -Val<sup>541</sup> was 270.0 (158.9–440.6) nM in the PiZZ  $\alpha_1$ -AT-deficient cohort but was lower in PiSZ patients (58.8 (39.4–87.1) nM;  $p < 0.001$ ) and lower still for nondeficient COPD patients (20.0 (13.3–32.1) nM;  $p < 0.001$ ) and healthy smokers (27.6 (15.0–40.0) nM;  $p < 0.001$ ). Individual results are shown for each of these patient groups in figure 2.

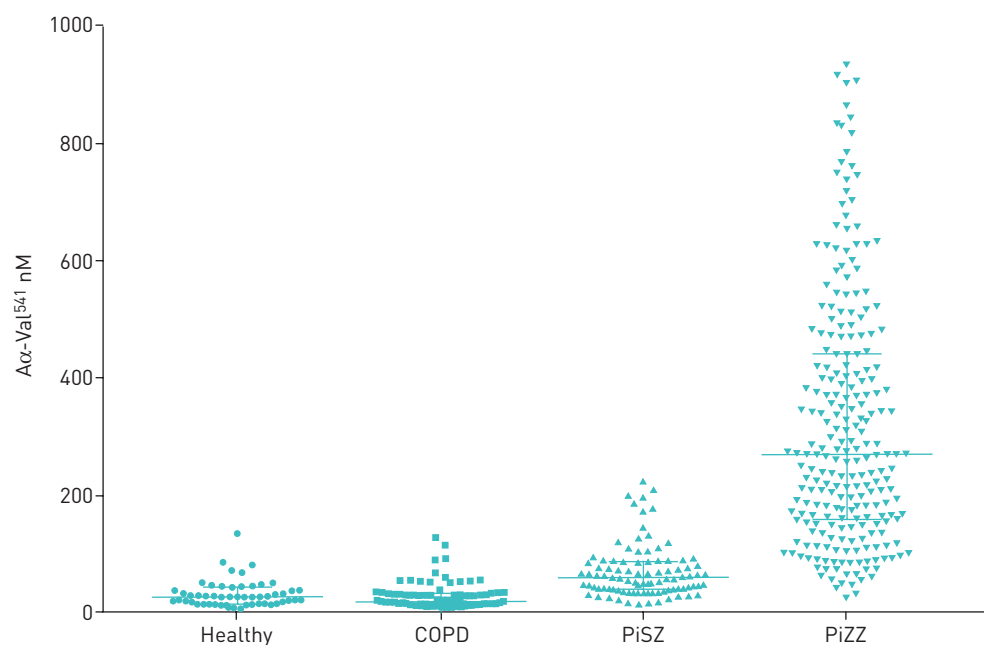


FIGURE 2 Individual A $\alpha$ -Val<sup>541</sup> concentrations for healthy subjects, individuals with chronic obstructive pulmonary disease, and the PiSZ and PiZZ  $\alpha_1$ -antitrypsin-deficient patient cohorts ( $p < 0.001$  between all groups except healthy smokers *versus* COPD ( $p = 0.112$ ), tested by the Mann-Whitney U-test). Each point represents the result for a single patient. The median and interquartile range are shown as horizontal lines.

### Effect of smoking

None of the  $\alpha_1$ -AT-deficient patients were current smokers. However, the usual COPD group consisted of 30 current and 48 ex-smokers. The median (IQR) A $\alpha$ -Val<sup>541</sup> was lower in ex-smokers (17.5 (11.8–30.0) nM) compared with current COPD smokers (22.6 (15.2–35.3) nM;  $p=0.05$ ). In addition, it was also lower than in healthy smokers (27.6 (15.0–40.0) nM;  $p=0.036$ ). There was no difference between current COPD smokers and healthy smokers ( $p=0.765$ ).

### Relationship to baseline demographic data

The relationship of A $\alpha$ -Val<sup>541</sup> to baseline demographics of the PiZZ cohort are summarised in table 2. There were significant correlations between A $\alpha$ -Val<sup>541</sup> and airflow obstruction, gas transfer and lung densitometry (where this was available) with the exception of the percentage of voxels less than –910 HU. No correlations were seen with baseline data from the other three groups. Individual A $\alpha$ -Val<sup>360</sup> values showed no significant correlations with baseline demographics.

### Relationship to the NE footprint marker A $\alpha$ -Val<sup>360</sup>

The PR3 activity marker showed a positive correlation with the previously validated NE activity footprint marker A $\alpha$ -Val<sup>360</sup> ( $r_s=0.266$ ;  $p<0.001$ ) in the PiZZ cohort (figure 3). The intercept of this relationship using linear regression was log 1.86, suggesting that PR3 activity would remain ~72 nM after the NE had been completely inhibited. A positive correlation was also seen in the COPD cohort ( $r_s=0.232$ ;  $p=0.021$ ) with the intercept using linear regression (log 0.70), suggesting that PR3 activity would be minimal (~5 nM) if NE footprint activity was zero. No significant correlation was observed between the two markers (A $\alpha$ -Val<sup>360</sup> and A $\alpha$ -Val<sup>541</sup>) in the PiSZ ( $r_s=0.074$ ;  $p=0.242$ ) or the healthy smoking ( $r_s=0.079$ ;  $p=0.289$ ) groups.

### Ratio of the two footprints

Values for the A $\alpha$ -Val<sup>360</sup> footprint were lower than for the A $\alpha$ -Val<sup>541</sup> footprint in each patient group. The median (IQR) A $\alpha$ -Val<sup>360</sup> value in healthy smokers was 9.2 (7.3–11.5) nM and this was lower than for nondeficient COPD patients (11.7 (9.1–13.2) nM;  $p<0.001$ ), which in turn was lower than for PiSZ patients (13.5 (11.0–18.2) nM;  $p<0.001$ ). However, the data for PiSZ patients were similar to the data for PiZZ patients (14.5 (11.7–18.8) nM;  $p=0.116$ ).

The A $\alpha$ -Val<sup>541</sup>/A $\alpha$ -Val<sup>360</sup> ratio also showed wide variation with a median (IQR) of 2.6 (1.8–4.4) for healthy smokers and 1.9 (1.4–2.8) for nondeficient COPD patients. In the COPD group the ratio was higher for current smokers than ex-smokers (2.0 (1.7–3.1) and 1.6 (1.1–2.6), respectively;  $p=0.008$ ). Ratios were higher ( $p=0.0067$ ) for PiSZ subjects (4.1 (2.4–6.8)) compared with healthy smokers and more so ( $p<0.001$ ) for PiZZ subjects (17.1 (10.0–29.3)). The data for individual patients are shown in figure 4.

### Stability

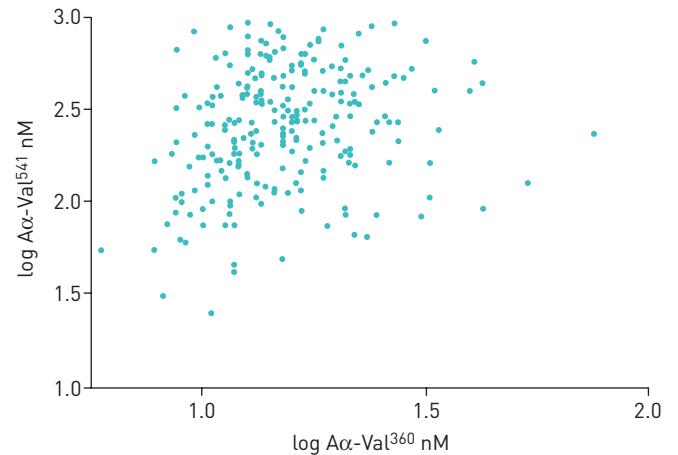
Plasma was collected from 39  $\alpha_1$ -AT-deficient (PiZZ) patients at baseline and 12–24 months later to assess A $\alpha$ -Val<sup>541</sup> stability. The median (IQR) concentration at baseline (281.4 (168.4–332.9) nM) was not significantly different to the second sample (215.2 (135.6–352.9) nM), indicating relative stability over at

TABLE 2 Correlation coefficients and significance values for A $\alpha$ -Val<sup>541</sup> together with the number of patients assessed with complete data for analysis from the combined PiZZ cohort

Clinical	Patients n	Correlation coefficient $r_s$	p-value
Age	239	0.059	0.183
FEV <sub>1</sub> % pred	237	–0.284	<0.001 <sup>#</sup>
FEV <sub>1</sub> /FVC	238	–0.247	<0.001 <sup>#</sup>
Kco % pred	233	–0.124	0.030 <sup>#</sup>
Perc15 HU	191	–0.198	0.003 <sup>#</sup>
HU910 %	191	0.115	0.057
HU950 %	191	0.182	0.006 <sup>#</sup>

FEV<sub>1</sub>: forced expiratory volume in 1 s; FVC: forced vital capacity; Kco: transfer coefficient of the lung for carbon monoxide; Perc15: density below which the lowest 15% of voxels occurs; HU910 and HU950: proportion of voxels with density below these Hounsfield unit thresholds. Lung densitometric analysis assessing emphysema is shown as Perc15, HU910 and HU950. <sup>#</sup>: p-values which remained statistically significant following Holm–Bonferroni correction for multiple comparisons.

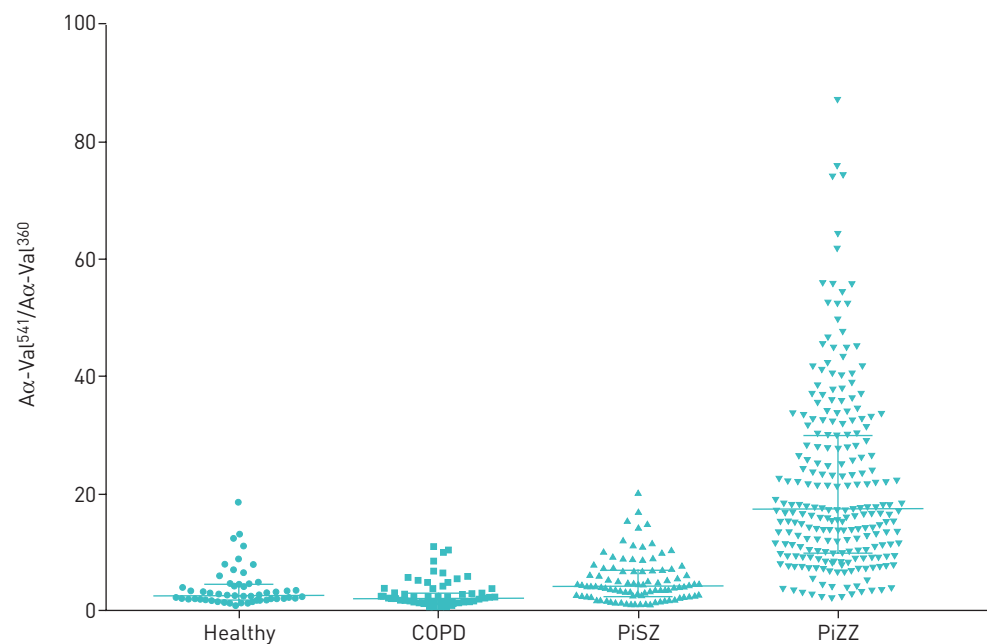
**FIGURE 3** Spearman's correlation ( $r_s=0.266$ ;  $p<0.001$ ) between log values for A $\alpha$ -Val<sup>541</sup> and A $\alpha$ -Val<sup>360</sup> in 239 patients from the PiZZ  $\alpha_1$ -antitrypsin-deficient cohort. Each point represents a sample from an individual patient.



least a year as seen for A $\alpha$ -Val<sup>360</sup> [10]. In addition, unfrozen samples from previous clinical trials stored for longer periods provided comparable group data to that seen with fresh samples, suggesting even long-term storage retains stability.

#### Effect of augmentation in $\alpha_1$ -AT-deficient subjects

Previously we showed that augmentation therapy with  $\alpha_1$ -AT (prolastin 60 mg·kg<sup>-1</sup> weekly) in  $\alpha_1$ -AT deficiency as part of the EXACTLE trial led to a small but significant reduction in the NE footprint, suggesting better local control of NE activity [10]. We assessed the remaining samples from 36 patients in this trial (all measured at baseline: 21 on  $\alpha_1$ -AT treatment and 15 receiving placebo for 6 months) to assess the effect of augmentation on the PR3 footprint. Median (IQR) A $\alpha$ -Val<sup>541</sup> values prior to therapy (287.2 (154.0–375.4) nM) fell on therapy (48.6 (37.7–71.8) nM;  $p<0.001$ ), indicating marked suppression of local PR3 activity, although the data still remained elevated compared with healthy controls ( $p<0.001$ ) and nondeficient COPD patients ( $p<0.001$ ), but comparable to the data for PiSZ subjects. No difference



**FIGURE 4** Comparison of the ratio of A $\alpha$ -Val<sup>541</sup> and A $\alpha$ -Val<sup>360</sup> levels in the PiSZ and PiZZ  $\alpha_1$ -antitrypsin-deficient patients, nondeficient chronic obstructive pulmonary disease (COPD) patients, and healthy smoking controls. The median and interquartile range are shown as solid lines. The median A $\alpha$ -Val<sup>541</sup>/A $\alpha$ -Val<sup>360</sup> ratio was 2.6 for the healthy group, 1.9 for the COPD group, 4.1 for the PiSZ cohort and 17.1 for the PiZZ cohort. The ratio for healthy individuals was significantly lower than for the PiSZ cohort ( $p<0.01$ ) and the PiZZ cohort ( $p<0.001$ ), but significantly higher than the COPD group ( $p<0.01$ ), tested by the Mann-Whitney U-test.

( $p=0.363$ ) was seen in patients receiving placebo (median  $A\alpha\text{-Val}^{541}$  concentration 340.2 (199.0–552.9) nM at baseline and 281.8 (257.5–596.7) nM after 6 months).

## Discussion

This article describes a unique epitope of fibrinogen cleavage providing a footprint to identify and track neutrophil PR3 activity *in vivo*. In healthy individuals this footprint is almost three times greater than the equivalent NE footprint and would be consistent with the greater amount of PR3 stored in the azurophil granule reported previously [5]. Both enzymes would be released with neutrophil degranulation, leading to an area of obligate enzyme activity until diffusion dilutes each enzyme to equal that of the surrounding  $\alpha_1\text{-AT}$  [16]. Our data support this concept. The data are also consistent with the greater likelihood of detecting PR3 activity compared with NE activity in the airways of patients with nondeficient COPD [8] where the prevalent lung  $\alpha_1\text{-AT}$  level is further reduced due to restricted diffusion from plasma by the tight epithelial cell junctions [17] and the lack of a significant local inhibitor of PR3 [7].

There are several factors that likely have a greater influence on the *in vivo* activity of PR3 in patients with  $\alpha_1\text{-AT}$  deficiency. These include the low concentration of  $\alpha_1\text{-AT}$ , the higher PR3 concentration in the neutrophil, the reduced association rate constant compared with NE [7] as well as the lack of local lung inhibitors [8]. The net result is reflected in a  $\sim 17$ -fold greater PR3 footprint than that of NE. In addition, although the PR3 and NE footprints correlate, the data do not extrapolate to the origin, suggesting significant PR3 activity would persist even after NE had been inhibited in  $\alpha_1\text{-AT}$  deficiency due to the reasons outlined earlier.

Although both proteinase footprints correlate, only the PR3 footprint related cross-sectionally to lung function and lung densitometry in the PiZZ cohort. The data suggest, at least superficially, that the PR3 footprint would be a better biomarker for the study of lung disease in  $\alpha_1\text{-AT}$  deficiency. However, the relationship between lung disease and biomarkers is complex [18]. Correlations raise the issue of cause and effect, and in our previous study in PiZZ patients the NE footprint (although stable) did not relate to subsequent progression in those with established COPD (*i.e.* the activity of the disease), supporting the concept that it reflected, at least in part, the effect of COPD rather than the cause [19]. This was consolidated further by association of the NE footprint with subsequent progression only in  $\alpha_1\text{-AT}$ -deficient patients without COPD, suggesting that other factors play a role in established COPD [10].

In the nondeficient COPD cohort studied here, the PR3 footprint was lower than for both the PiZZ and PiSZ subgroups. This would be expected due to the normal  $\alpha_1\text{-AT}$  levels in nondeficient patients and was not different to that in healthy smokers. However, only a proportion of the COPD patients remained currently smoking. The ex-smokers had a lower median PR3 footprint, suggesting it is, at least in part, a smoking-related signal in nondeficient COPD.

The PR3 footprint (like the NE footprint) in  $\alpha_1\text{-AT}$  deficiency (although highly variable between individuals) is stable, suggesting it has reached equilibrium and therefore it remains possible that pre-inhibition PR3 activity is a major mediator of damage in established COPD related to  $\alpha_1\text{-AT}$  deficiency. Interpretation of this baseline relationship is, however, further complicated by the fact that many  $\alpha_1\text{-AT}$ -deficient patients studied here were ex-smokers, which would likely have had an amplifying effect on current lung status prior to PR3 footprint assessment. Further studies will therefore be required to determine future disease progression in  $\alpha_1\text{-AT}$ -deficient never- or ex-smokers and any relationship to baseline PR3 footprint activity.

Augmentation therapy with human  $\alpha_1\text{-AT}$  leads to a major reduction in the PR3 footprint (as seen previously for the lower NE footprint) [10] almost, although not quite, back to normal, indicating that this treatment limits the detectable *in vivo* activity of both enzymes (prior to inhibition and complex formation with  $\alpha_1\text{-AT}$ ), but suggests that a higher dose may be required to normalise PR3 activity. The data support, at least in part, the benefits of such therapy on emphysema progression in  $\alpha_1\text{-AT}$  deficiency [20–22] and a role for  $A\alpha\text{-Val}^{541}$  as a biomarker for this treatment effect.

The results for the PiSZ patients are worthy of further comment. There remains uncertainty about the susceptibility of this  $\alpha_1\text{-AT}$  genotype to developing COPD and hence any role for augmentation therapy. The NE footprint in these patients was slightly raised compared with the healthy controls, as was the PR3 footprint and the NE/PR3 footprint ratio. However, the NE footprint in PiSZ patients was similar to that seen in PiZZ patients where lung disease severity and progression are greater. This also suggests that NE may be less important in lung elastin destruction than PR3 in this patient group, and that the lower than normal  $\alpha_1\text{-AT}$  level and decreased association rate constant of PiZZ  $\alpha_1\text{-AT}$  for PR3 [7] result in a mild imbalance of PR3 activity compared with usual nondeficient COPD (indicated by the ratio of the two footprints) and may be a physiological mediator of increased tissue damage, and hence a target for treatment in PiSZ subjects. Clearly, further exploration of this issue is indicated.

The current study has both strengths and some weaknesses. The validation of the A $\alpha$ -Val<sup>541</sup> assay confirms its specificity for the cleavage peptide and suggests the predominance of a PR3 signal in all cohorts consistent with its greater concentration than NE in the azurophil granule. In addition, the even greater PR3 footprint signal in  $\alpha_1$ -AT deficiency confirms the potential importance of this enzyme with low concentrations of  $\alpha_1$ -AT compounded by its lower association rate constant and the lack of another local lung PR3 inhibitor [7].

However, there is some controversy concerning the amount of PR3 in the azurophil granule (see Introduction); if the absolute amount of PR3 in the neutrophil is similar to or lower than NE, the difference seen in the footprint assays requires consideration for other possibilities. Both enzymes are promiscuous and can potentially have multiple cleavage sites. For this reason our initial digest and plasma analyses identified a fibrinogen cleavage product produced by PR3 present in the plasma of  $\alpha_1$ -AT-deficient subjects and not generated by NE. Carboxyl-terminal sequencing was consistent with a PR3 cleavage site exposing a neo-epitope, and our subsequent analyses indicated no or only very weak binding by NE-cleaved fibrinogen and no cross-reactivity with our NE-specific cleavage sequence (the A $\alpha$ -Val<sup>360</sup> peptide).

It remains possible that the respective half-lives of these two fibrinogen cleavage products differ, producing some cross-sectional differences in the quantity of both, although this should not vary with  $\alpha_1$ -AT genotype producing the major differences observed here. In addition, augmentation brings these two signals together, suggesting the neo-epitopes generated are predominantly related to activity rather than half-life. Finally, compared with NE, PR3 activity may be preferentially located at the neutrophil membrane where it is less susceptible to inhibition by  $\alpha_1$ -AT [5], facilitating its local pericellular activity and hence fibrinogen cleavage. These issues can only be addressed by complex *in vitro* competition assays, but would require simultaneous pharmacokinetics analysis of patient samples from those receiving both  $\alpha_1$ -AT augmentation and the inclusion of future specific and effective anti-NE or -PR3 therapies.

However, whatever the reason, we have studied several independent cohorts including both a severe  $\alpha_1$ -AT-deficient group (PiZZ), a mild  $\alpha_1$ -AT-deficient group (PiSZ) and a normal  $\alpha_1$ -AT COPD group to consolidate the relationship of the marker to  $\alpha_1$ -AT deficiency and its severity in the stable state, indicating clear differences in the ratio of the two footprints. Whether the PR3 marker predicts future progression of disease in  $\alpha_1$ -AT deficiency is important, and this now requires longitudinal monitoring of disease progression in both the presence and absence of  $\alpha_1$ -AT augmentation therapy. This has important implications for the management of  $\alpha_1$ -AT deficiency as, although  $\alpha_1$ -AT will inhibit both PR3 and NE, the pursuit and development of specific small-molecule inhibitors of NE that do not have broad activity to control both serine proteinases may be inappropriate or only provide partial protection, especially in  $\alpha_1$ -AT deficiency. However, inhibiting NE by an alternative route may paradoxically release enough endogenous  $\alpha_1$ -AT to provide extra PR3 cover. Clearly, such studies need to be undertaken and will provide important insights into neutrophil proteinase biology. The PR3 signal is high and certainly sensitive to augmentation, and may therefore prove essential as a treatment biomarker in future proteinase therapeutic strategies, including dose-ranging studies.

The fibrinogen footprint of PR3 activity is also likely to be an indirect marker of elastin degradation considered central to the pathophysiology of emphysema. Since our results were generated, a recent article using a similar methodology to measure a PR3-specific elastin cleavage product in healthy controls and a COPD cohort indicates similar increased PR3 compared with an NE cleavage product to that seen here [23]. Direct comparison of both assays should, however, be undertaken in a single  $\alpha_1$ -AT-deficient cohort for final confirmation.

We describe a unique *in vivo* footprint of PR3 activity that is present in greater quantities than an NE footprint in subjects with normal  $\alpha_1$ -AT, but especially in the presence of  $\alpha_1$ -AT deficiency. The data suggest that PR3 may play a greater role in lung damage than previously thought and is sensitive to antiprotease therapy. As such, it may provide a critical role as a biomarker for dose ranging of antiprotease therapy. The specificity of the PR3 assay suggests it may also be useful in the assessment of other PR3-dependent diseases such as autoimmune vasculitis.

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