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Aα-Val^{360}: a marker of neutrophil elastase and COPD disease activity

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ABSTRACT: Forced expiratory volume in 1 s is currently the most widely used marker of chronic obstructive pulmonary disease (COPD) severity; however, it is a poor surrogate of the emphysematous component and the underlying pathophysiological mechanism, and therefore new markers are urgently needed. Neutrophil elastase (NE) is likely to play a key pathophysiological role in COPD and the current study explores a marker of NE activity as a potential indicator of COPD disease activity.

Aα-Val^{360} was measured in 81 subjects with a clinical diagnosis of COPD, both in the stable state and at presentation with an acute exacerbation, and comparisons were made using lung function tests and computed tomography imaging. The relationship of Aα-Val^{360} with disease progression was also assessed in 40 of the subjects over a 4-yr period.

Baseline Aα-Val^{360} related to physiological and radiological markers of disease severity, was higher at presentation with an acute exacerbation than in the stable state and (at least partly) related to disease progression over the subsequent 4 yrs.

We demonstrate that Aα-Val^{360} is a marker of cross-sectional COPD disease severity and possibly disease progression, and represents a new concept of specific biomarkers. This study therefore reports the first *in vivo* data to support the pathophysiological role of NE in COPD.

KEYWORDS: Aetiology, chronic obstructive pulmonary disease, disease progression, leukocyte elastase

Chronic obstructive pulmonary disease (COPD) is a slowly progressive chronic disease characterised by airflow obstruction that is predominantly irreversible. Forced expiratory volume in 1 s (FEV1) is a recognised prognostic indicator commonly used as a clinical end-point in pharmaceutical trials. Furthermore, guidelines suggest the diagnosis of COPD is only made in people with symptoms and airflow obstruction as defined by a ratio of FEV1 to forced vital capacity (FVC) either <0.7 or the lower limit of normal (LLN) [1]. However, although short-term improvements in FEV1 are considered beneficial and often relate to improvements in patient symptoms, this physiological measure has significant flaws. First, the relationship to healthcare status is weak. Secondly, FEV1 is effort dependent and the day-to-day variability may be greater than the progressive decline observed in patients with COPD over many years [2], making it a poor surrogate for early phase II studies of potential disease-modifying agents. Finally, and importantly, it is recognised that COPD is a group of distinct pathological processes and the FEV1 relates poorly to the presence of emphysema and its severity as quantified by computed tomography (CT) densitometry [3]; therefore, a diagnosis made on spirometric grounds alone may be inappropriate [4]. There is therefore an urgent need to assess COPD more comprehensively and develop biomarkers relevant to the individual components that are validated as markers of disease prognosis and hence can be used as early read-outs for phase II clinical trials.

It is increasingly accepted that COPD is an inflammatory disease and, therefore, a number of potential biomarkers have been studied in subjects with COPD; however, few are central to the pathophysiological process and none have been effectively validated. For instance, although C-reactive protein (CRP), a nonspecific marker of inflammation, relates to mortality in people with mild-to-moderate (but not severe) COPD [5], elevated levels of this marker fail to predict a more rapid decline of FEV1 in longitudinal studies [6]. Also, densitometric analysis of CT scans are increasingly considered in clinical trials since lung densitometry is now accepted by the US Food and Drug Administration as a clinically meaningful end-point for the assessment of emphysema progression [7]. However, the methodology has yet to be standardised and a normal range of lung density

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defined; therefore, CT densitometry cannot yet be considered a validated biomarker and is unlikely to demonstrate the early response required for phase II proof-of-concept studies.

Further progress may be achieved by developing biomarkers of key pathological processes in COPD. Neutrophil elastase (NE) was implicated in the pathogenesis of COPD shortly after the observation of the presence of early-onset emphysema in people with α_1-antitrypsin (α_1-AT) deficiency [8]. At neutrophil degranulation, the NE concentration greatly exceeds that of its major inhibitor (α_1-AT), even in healthy individuals; however, dilution occurs due to diffusion away from the point of release and NE is inhibited rapidly within the neutrophil microenvironment when an equimolar concentration with its inhibitors is reached. There is therefore an area of obligate proteolytic damage, even in healthy subjects, which is exponentially greater in patients with α_1-AT deficiency, explaining their susceptibility to disease [9]. Although the role of NE in subjects with COPD unrelated to α_1-AT deficiency is less well understood, the quantum proteolytic damage in this group of patients may be enhanced as abnormalities within circulating neutrophils relate to overall mortality [10]. Also, an inverse relationship exists between FEV1 and circulating neutrophil numbers [11], and neutrophils from subjects with COPD exhibit abnormal chemotactic responses [12]: adherence and migration under flow conditions [13], enhanced activity as indicated by the proteolytic destructive potential [14], and production of reactive oxygen species [15] compared to appropriate control subjects. Since NE has the potential to replicate many of the pathological features of COPD, a marker of NE activity may represent an ideal biomarker of COPD and disease activity. However, because of rapid inactivation *in vivo*, studies are yet to link NE activity conclusively with the pathogenesis of COPD and we have therefore developed an assay based on a pre-inhibition NE-specific fibrinogen cleavage product (Aα-Val^{360}) [16]. The aim of the current study was therefore to explore the role of NE in the pathogenesis of COPD using Aα-Val^{360} as a marker of disease activity in a well-characterised cohort of subjects with symptoms and/or physiological evidence of COPD.

MATERIAL AND METHODS

Patients aged 40–80 yrs who were smokers or ex-smokers with a diagnosis of COPD (based on symptoms with and without supportive spirometry) who presented in primary care with an acute exacerbation were recruited to the study. All had a history of chronic bronchitis [17] and exertional breathlessness and had the normal PiM α_1-AT phenotype (Heredilab, Salt Lake City, UT, USA). Exacerbations were defined by the presence of increased dyspnoea, cough and sputum production (although the volume of the latter had not always increased) and new or increased sputum purulence was a feature of a proportion of patients [18]. Spirometric confirmation of COPD was not used as an entry requirement, to allow the inclusion of participants with a broad range of phenotypes, and therefore subgroup analyses were performed for patients with chronic bronchitis and dyspnoea but with FEV1 and FEV1/FVC within the normal range, and also for subjects with COPD defined spirometrically. Normality was defined as within 1.64 standardised residuals (SRs), as recommended by American Thoracic Society/European Respiratory Society guidelines, to overcome sex and age differences in lung function, a threshold which is often termed the LLN [19].

Subjects were assessed at the onset of an exacerbation and all provided a spontaneous sample of sputum over a 4-h period after waking. Sputum samples were analysed macroscopically using a standardised colour chart used to classify sputum colour (Bronkotest; Heredilab) and those with mucopurulent or purulent sputum (grade 3–8) were treated with antibiotics (oral cefuroxime), while those with mucoid sputum (grade 0–2) were not. The patients were assessed in detail 8 weeks after the episode (when in the stable clinical state) with full lung function tests and a high-resolution CT (HRCT) scan of the thorax.

Aα-Val^{360} was measured in plasma samples obtained both at presentation with the exacerbation and when clinically stable, using a highly specific assay as described previously [16]. In addition, comparisons were made with physiological parameters and visual assessment of the HRCT scan. All scans were assessed for the presence or absence of visible emphysema by an experienced thoracic radiologist using established criteria [20]. Plasma CRP was measured by ELISA using commercially available pre-prepared plates and standards (Binding Site; Birmingham, UK). Other inflammatory markers relevant to neutrophilic inflammation including myeloperoxidase (MPO), interleukin (IL)-8, and leukotriene B_4 (LTB_4) in sol-phase sputum samples and plasma α_1-AT/NE complexes were also measured (as described previously [16, 21]) and related to the Aα-Val^{360} concentration. Aα-Val^{360} was also measured in plasma samples obtained from 39 healthy controls.

Finally, the patients were reviewed 4 yrs later (where possible) and repeat lung function testing and an HRCT performed at full inspiration using the same General Electric Prospeed Scanner (General Electric Medical Systems, Milwaukee, WI, USA). Densitometric analysis was performed to assess emphysema progression accurately using the voxel index (-950 HU) and the 15th percentile point in both the upper zone (level of aortic arch) and lower zone (level of inferior pulmonary vein).

Post-bronchodilator (salbutamol 400 μg and ipratropium 60 μg *via* a large-volume spacer) spirometry was performed using a wedge bellows spirometer (Vitalograph, Maids Moreton, UK) and gas transfer measurements by the single-breath carbon monoxide method. The European Community for Steel and Coal reference equations [22] were used to derive predicted values for spirometry, while the reference equation of COTES [23] was used for transfer coefficient of the lung for carbon monoxide (*KCO*).

The study was approved by the South Birmingham Research Ethics Committee, Birmingham, UK.

Statistical analysis

Statistical analyses were performed using SPSS 17.0.1 (Chicago, IL, USA) for Windows. Data are presented as mean±SE, normality was tested using the Kolmogorov–Smirnov test and statistical significance was taken as $p<0.05$.

Aα-Val^{360} and α_1-AT/NE complex concentrations were not normally distributed and, therefore, correlations with other inflammatory markers and lung function were performed using Spearman's rho. Multivariate analysis was performed using linear regression and stepwise entry of independent factors.

Mann–Whitney U-tests were used to compare nonparametric values in subjects with and without visible emphysema on HRCT scan, while unpaired t-tests were used for parametric data. Comparisons were made between values obtained at the onset of an exacerbation and stable state data using paired t-tests (for normally distributed data) and paired Wilcoxon rank tests (for nonparametric data).

RESULTS

Initial stable state assessment

81 subjects (36 female and 45 male) with chronic bronchitis and exertional dyspnoea and a broad range of spirometric results (table 1) were included in the study. Of these patients, 58 achieved the spirometric criteria consistent with COPD (with a FEV1/FVC <LLN), while 61 subjects had a FEV1/FVC <0.7 and therefore met the alternative spirometric threshold for COPD.

The stable-state plasma Aα-Val^{360} concentration related to baseline FEV1 % predicted (r= -0.340, p=0.001) and *K*CO % pred (-0.246, p=0.013). Multivariate analysis accounting for age, sex, smoking history, height and sputum colour demonstrated that the stable-state Aα-Val^{360} was an independent predictor of *K*CO (standardised β coefficient -0.243, R^2 change 0.048; p=0.037); however, the independent relationship with FEV1 fell short of conventional levels of significance (standardised β coefficient -0.231, R^2 change 0.037; p=0.070). Importantly, in subjects with FEV1/FVC below the normal range, similar relationships were also observed between Aα-Val^{360} and FEV1 % pred (r= -0.297, p=0.013) and *K*CO % pred (r= -0.214, p=0.054).

In the full cohort, plasma Aα-Val^{360} showed a reasonable correlation with plasma α_1-AT/NE complex in the stable state (r=0.459, p<0.001) (fig. 1); however, there was no relationship with high-sensitivity CRP (a nonspecific measure of inflammation). Also, the α_1-AT/NE complex did not relate to either FEV1 % pred (r= -0.087, p=0.451) or *K*CO % pred (r= -0.172, p=0.126). Furthermore, the Aα-Val^{360} was significantly higher (p<0.001) in these subjects with chronic bronchitis and dyspnoea (n=80) with a median Aα-Val^{360} value of 20.76 nM (interquartile range (IQR) 13.99–25.44 nM) than healthy controls (n=39) with a median value of 3.50 nM (IQR 2.35–5.14 nM).

TABLE 1 Aα-Val^{360} in 81 stable-state subjects with symptoms of chronic obstructive pulmonary disease and their baseline demographic and lung function data#

Age yrs	65.75±0.92
Height m	1.64±0.01
FEV1 L	1.79±0.08
FEV1 % pred	73.28±2.86
FEV1 SR	-1.44±0.16
FEV1/FVC	0.55±0.02
FEV1/FVC % pred	72.27±2.35
FEV1/FVC SR	-3.01±0.25
RV L	2.20±0.04
RV % pred	126.20±3.78
RV SR	1.52±0.22
TLC L	6.03±0.15
TLC % pred	107.24±1.66
TLC SR	0.60±0.14
***K*CO mmol·min^{-1}·kPa^{-1}·L^{-1}**	1.41±0.48
***K*CO % pred**	96.69±3.21
***K*CO SR**	-0.28±0.20
Plasma Aα-Val^{360} nM	24.39±2.20
Plasma α_1-AT/NE complex nM	2.63±0.18
Plasma hsCRP nM	730.60±146.67
Sputum MPO nM	0.82±0.17
Sputum LTB_4 nM	9.41±2.22
Sputum IL-8 nM	6.79±1.04

Data are presented as mean±SE. Plasma samples refer to stable-state results at baseline. Sputum results refer to the 55 subjects able to produce spontaneous sputum in the stable state at baseline. FEV1: forced expiratory volume in 1 s; % pred: % predicted; SR: standardised residual; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; *K*CO: transfer coefficient of the lung for carbnon monoxide; α_1-AT: α_1-antitrypsin; NE: neutrophil elastase; hsCRP: high-sensitivity C-reactive protein; MPO: myeloperoxidase; LT: leukotriene; IL: interleukin. #: lung function tests were performed on the same day as the plasma samples were obtained.

Aα-Val^{360} in subjects with and without visible emphysema on HRCT

In the overall group, the plasma Aα-Val^{360} concentration was greater (p=0.013) in those with visible emphysema on HRCT (n=43) compared to those without (n=38). Also, subjects with visible emphysema had a significantly lower FEV1 % pred (p=0.014), FEV1/FVC (p<0.001) and *K*CO % pred (p<0.001) than those without (table 2). However, there was no difference in the plasma α_1-AT/NE complex or sputum myeloperoxidase (MPO), leukotriene (LT)B_4 and interleukin (IL)-8 (in the 55 subjects able to produce a spontaneous sputum sample for the stable-state assessment) between those with and without emphysema.

Subgroup analysis was performed for subjects with chronic bronchitis and dyspnoea but FEV1 and FEV1/FVC in the normal range as confirmed by SRs (table 2). This demonstrated that Aα-Val^{360} was also greater in those with visible emphysema (22.88 nM, IQR 14.09–42.17 nM; n=6) on HRCT compared to those without (13.98 nM, IQR 12.31–21.00 nM; n=17), which is similar to that seen in the larger cohort, but the

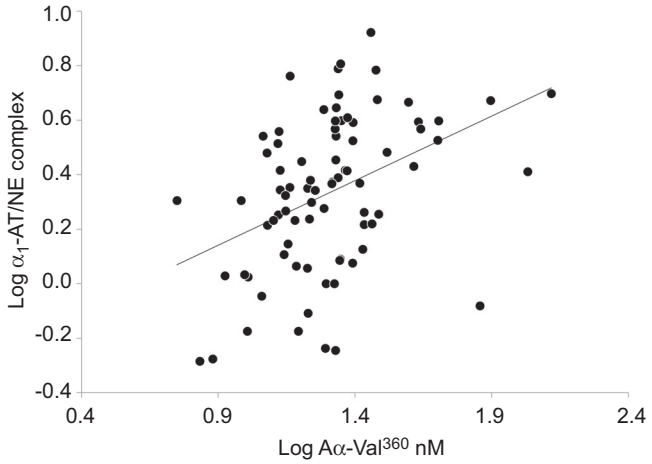

FIGURE 1. The relationship between a marker of neutrophil elastase (NE) activity (Aα-Val^{360}) and elastase release (α_1-antitrypsin (α_1-AT)/NE complex). r=0.459, p<0.001.

TABLE 2 Data for all subjects, subjects with forced expiratory volume in 1 s (FEV_1) and FEV_1/ forced vital capacity (FVC) within the normal range and for subjects with FEV_1/FVC below the lower limit of normal (LLN)

Subset of patients	No visible emphysema on HRCT	Visible emphysema on HRCT	p-value
All			
$A\alpha$-Val^{360} nM	16.98 (13.15–22.29)	21.77 (15.58–27.13)	0.013
FEV_1 % pred	79.75±4.12	67.27±3.80	0.014
K_{CO} % pred	114.76±3.55	80.71±3.76	<0.001
FEV_1/FVC	0.62±0.03	0.48±0.14	<0.001
Subjects with FEV_1 and FEV_1/FVC >LLN			
$A\alpha$-Val^{360} nM	13.98 (12.31–21.00)	22.88 (14.09–42.17)	0.071
FEV_1 % pred	100.77±2.98	100.61±2.38	0.488
K_{CO} % pred	114.88±5.09	93.83±10.43	0.030
FEV_1/FVC	0.77±0.01	0.70±0.02	0.003
Subjects with FEV_1/FVC <LLN			
$A\alpha$-Val^{360} nM	17.96 (15.26–24.13)	21.77 (16.12–27.13)	0.141
FEV_1 % pred	62.73±4.31	61.56±3.63	0.420
K_{CO} % pred	114.67±5.04	78.59±3.98	<0.001
FEV_1/FVC	0.50±0.02	0.45±0.02	0.06

Data are presented as median (interquartile range) or mean±SE, unless otherwise stated. In the whole cohort, subjects with visible emphysema on high-resolution computed tomography (HRCT) had significantly worse lung function tests and a greater $A\alpha$-Val^{360}. Similar differences were seen in subjects with a FEV_1 and FEV_1/FVC within the normal range and those with a FEV_1/FVC <LLN. % pred: % predicted; K_{CO}: transfer coefficient for carbon monoxide.

difference just failed to achieve statistical significance in this subgroup (p=0.071). There was no significant difference in the FEV_1 % pred in those with visible emphysema compared to those without. However, FEV_1/FVC was lower in those with emphysema than those without (0.70±0.01 and 0.77±0.02, respectively; p=0.003). In addition, the K_{CO} % pred was also lower (fig. 2) in those with visible emphysema (93.83±10.43% pred) than those without (114.88±5.09% pred; p=0.030).

Analysis of patients having FEV_1/FVC <LLN demonstrated that the average $A\alpha$-Val^{360} was also greater in those with visible emphysema (n=37) compared to those without (n=21), but this difference was not significant (p=0.141). There was no difference in the FEV_1 % pred or the sputum markers between the two groups of patients, although FEV_1/FVC (p=0.06) and K_{CO} % pred (p<0.001) were lower in subjects with visible emphysema on HRCT (table 2).

$A\alpha$-Val^{360} during an acute exacerbation of COPD

The $A\alpha$-Val^{360} was higher at the onset of the exacerbation than in the stable state even when stratified by sputum colour into visibly purulent or nonpurulent episodes (table 3). Furthermore, $A\alpha$-Val^{360} at the onset of the exacerbation was significantly greater (p=0.030) in subjects who presented with purulent sputum compared with those with mucoid sputum. Interestingly, although $A\alpha$-Val^{360} fell in both groups following resolution, the difference between these two groups persisted (p=0.024) (table 3). In addition, the stable-state sputum IL-8 (p<0.001) and plasma α_1-AT/NE complex (p=0.036) were higher in those who experienced an exacerbation associated with purulent sputum. However, importantly, there was no longer a difference in the sputum colour or sputum MPO and LTB_4 concentrations in the stable state between those who had presented with purulent and those with nonpurulent sputum. In the stable state, no correlation was seen between $A\alpha$-Val^{360} and sputum MPO (r=0.059, p=0.337); however, there remained positive correlations with the key neutrophil chemoattractants LTB_4 (r=0.227, p=0.048) and especially IL-8 (r=0.486, p<0.001).

Longitudinal analysis

40 individuals were alive and consented to assessment with full lung function tests and densitometric analysis of HRCT

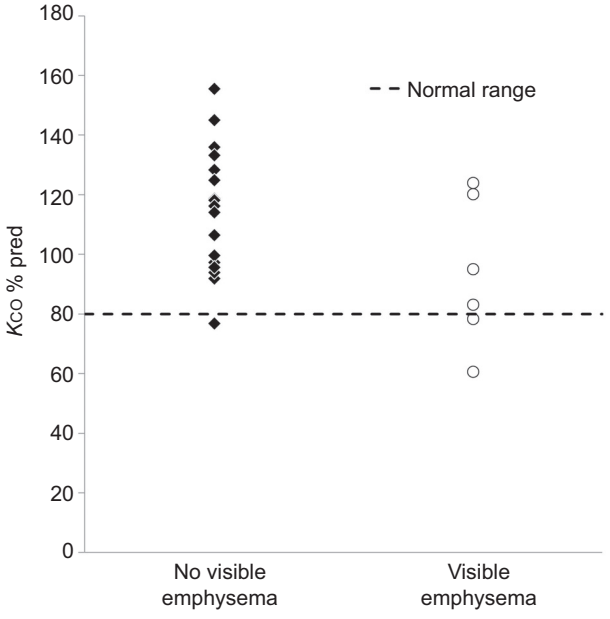

FIGURE 2. Transfer coefficient for carbon monoxide (K_{CO}) in subjects with ''normal spirometry'', demonstrating the significantly lower values (p=0.016) in subjects with visible emphysema on high-resolution computed tomography compared to those without. % pred: % predicted.

TABLE 3 Aα-Val^{360} in subjects with chronic obstructive pulmonary disease at exacerbation onset compared to stable state

	Aα-Val^{360} nM		p-value#
	Exacerbation onset	Stable state	
All	23.72 (18.11–35.41)	21.28 (13.99–24.65)	0.005
Nonpurulent sputum	21.22 (17.42–27.44)	20.00 (12.83–21.69)	0.022
Purulent sputum	26.29 (19.43–39.07)	21.83 (14.91–28.45)	0.043

Data are presented as median (interquartile range), unless otherwise stated. Median Aα-Val^{360} was significantly higher in subjects with chronic obstructive pulmonary disease. #: exacerbation *versus* stable state.

scans at both baseline (stable state) and at follow-up 4 yrs later. Aα-Val^{360} obtained from subjects at baseline related cross-sectionally to both baseline and follow-up physiological and radiological measures (table 4). However, there was no relationship between α_1-AT/NE complex concentration and any physiological or radiological parameter at either baseline or follow-up. Both physiological and radiological markers demonstrated disease progression in the 40 subjects (table 5); however, universal significance was not observed in all parameters. The absence of a significant change may at least partly be explained by the lack of sensitivity of these physiological and radiological tests for detecting disease progression in subjects with COPD and again supports the need for new markers of disease severity and activity.

There was a significant decrease in the FEV1 ($p<0.001$) and *K*CO ($p<0.001$) over the 4-yr period. There was also significant emphysema progression as measured by absolute change in the voxel index (-950 HU) and 15th percentile point in both the upper ($p<0.001$ and $p=0.002$, respectively) and lower ($p<0.001$ and $p=0.021$) zones. Baseline Aα-Val^{360} related to both the subsequent decline in *K*CO % pred ($r= -0.406$, $p=0.008$) and progression in lower zone emphysema expressed as change in the voxel index at -950 HU ($r=0.306$, $p=0.027$); however the relationship of the baseline Aα-Val^{360} with change in the lower zone 15th percentile point did not achieve statistical significance ($r= -0.212$, $p=0.095$). There was no significant association between Aα-Val^{360} and decline in FEV1 % pred ($r= -0.196$, $p=0.113$), or the upper zone voxel index ($r=0.103$, $p=0.264$) and 15th percentile point ($r= -0.049$, $p=0.381$). There was no relationship between the α_1-AT/NE complex and any measure of decline. Receiver operating characteristic analysis of Aα-Val^{360} in subjects who demonstrated a decline in *K*CO % pred over the 4-yr period compared to nondecliners gave an area under the curve of 0.711 ($p=0.037$). An Aα-Val^{360} threshold of 11 nM would have a sensitivity and specificity of 91% and 43%, respectively, for the identification of subjects who will demonstrate a decline in *K*CO % pred over a 4-yr period, while a threshold of 22 nM would have a sensitivity of 33% and specificity of 93%.

In the subgroup analyses of patients with chronic bronchitis and dyspnoea but a baseline FEV1/FVC SR and FEV1 SR within the normal range (n=14), the baseline Aα-Val^{360} also correlated with subsequent decline in *K*CO % pred ($r= -0.534$, $p=0.025$), but not to spirometric or radiological progression. In those with a baseline FEV1/FVC SR <LLN (n=26), the Aα-Val^{360} related to radiological progression (change in lower zone voxel index $r=0.348$, $p=0.041$), but there were no significant relationships with other measurements.

DISCUSSION

We have developed a unique assay based on an NE-specific fibrinogen degradation product (Aα-Val^{360}), which measures the damaging potential of NE at the point of release from the neutrophil prior to its inhibition by the surrounding protease inhibitors [16]. Previous authors have investigated the use of an alternative fibrinogen degradation product (formed by the cleavage of the fibrinogen α-chain at Aα-21); however, this was not pursued further because of its low specificity for NE and the very short half-life of this smaller fibrinogen fragment [24]. In contrast, the Aα-Val^{360} is highly specific and shows stability over time, both of which are important features of any biomarker [16].

In the absence of more suitable gold-standard markers, we opted to relate Aα-Val^{360} to physiological and radiological measures of

TABLE 4 Plasma Aα-Val^{360} was measured in 40 subjects at baseline (stable state) and related to physiology and computed tomography densitometry performed at baseline and at follow-up 4 yrs later

	Correlation with baseline plasma Aα-Val^{360}			
	Baseline		Follow-up	
	r	p-value	r	p-value
FEV1 % pred	-0.319	0.022	-0.357	0.012
***K*CO % pred**	-0.401	0.005	-0.403	0.008
UZ VI -950 HU	0.416	0.004	0.434	0.003
LZ VI -950 HU	0.279	0.041	0.382	0.008
UZ 15th percentile point HU	-0.338	0.017	-0.350	0.013
LZ 15th percentile point HU	-0.224	0.083	-0.299	0.031

Physiological parameters were expressed as % predicted (% pred) corrected for age, height and sex. FEV1: forced expiratory volume in 1 s; *K*CO: transfer coefficient of the lung for carbon monoxide; UZ: upper zone; VI: voxel index; LZ: lower zone.

TABLE 5 Baseline and follow-up data for 40 subjects[#]

Parameter	Baseline	4-yr follow-up	p-value
Age yrs	64.07±1.20	68.36±1.20	<0.001
FEV1 L	1.93±0.11	1.79±0.11	<0.001
FEV1 % pred	78.23±4.16	75.79±4.49	0.071
FEV1 SR	-1.24±0.22	-1.25±0.23	0.453
FEV1/FVC	0.57±0.03	0.55±0.03	0.005
FEV1/FVC SR	-2.84±3.78	-3.11±0.36	0.024
***K*CO mmol·min^{-1}·kPa^{-1}·L^{-1}**	1.42±0.07	1.36±0.08	0.006
***K*CO % pred**	95.35±4.73	93.14±5.48	0.273
***K*CO SR**	-0.35±0.30	-0.55±0.40	0.273
UZ 15th percentile point	-920.34±5.70	-929.07±5.03	0.002
UZ VI -950 HU	9.49±1.92	11.59±2.08	<0.001
UZ VI -910 HU	24.70±2.85	27.44±2.69	0.004
LZ 15th percentile point	-907.51±5.44	-913.05±4.75	0.021
LZ VI -950 HU	7.96±1.21	9.53±1.36	<0.001
LZ VI -910 HU	22.96±2.63	25.73±2.58	0.007
Aα-Val^{360} nM	25.73±4.32	NA	NA
α_1-AT/NE complex nM	2.37±0.24	NA	NA

Data are presented as mean± SE, unless otherwise stated. FEV1: forced expiratory volume in 1 s; % pred: % predicted; SR: standardised residual; FVC: forced vital capacity; *K*CO: transfer coefficient of the lung for carbon monoxide; UZ: upper zone; VI: voxel index; LZ: lower zone; α_1-AT: α_1-antitrypsin; NE: neutrophil elastase; NA: not applicable. #: 19 females and 21 males.

COPD disease severity since (despite their flaws) they are widely used and reasonably well validated. We did not compare Aα-Val^{360} with other potential biomarkers since (to date) none have been effectively validated and therefore interpretation of relationships (if any) would be difficult. For example, while urinary desmosine may differ between healthy individuals, smokers with normal lung function and subjects with COPD, it does not correlate with FEV1 and is not influenced by augmentation therapy in α_1-AT deficiency [25] (perhaps because it is neither organ- nor disease-specific).

However, we have previously demonstrated that Aα-Val^{360} relates to FEV1 and demonstrates a response to augmentation therapy in subjects with α_1-AT deficiency [16], and in the current study we demonstrate that Aα-Val^{360} also relates to several specific features of COPD in subjects without α_1-AT deficiency. First, it relates cross-sectionally to physiological and radiological markers of current COPD severity. However, this relationship is likely to be complex (and, therefore, consistent with the strength of the observed associations) since a marker of activity may also relate to the process leading to the current disease state (or severity) or to future disease progression. The strongest relationship was observed between Aα-Val^{360} and severity markers of the emphysematous process (gas transfer and the voxel index) and, in particular, Aα-Val^{360} was the best independent predictor of gas transfer. These data suggest NE activity is central to the pathogenesis of COPD but is likely to be of greatest relevance in the development of the emphysematous component.

Further confirmation of the relationship between NE activity and the emphysematous process was provided by our observation that Aα-Val^{360} is greater in subjects with visible emphysema on HRCT compared to those without. Although the difference was less marked in subjects who also had obstructive spirometry, this may be explained by the smaller number and discordance between the severity of the airway obstruction and alveolar destruction within individuals, since the average observed differences were similar to that seen for the whole cohort.

It is increasingly recognised that COPD is a heterogeneous disease, and the current study demonstrated that visible emphysema was present even in six of the 23 subjects with spirometry within the normal range, and those with visible emphysema also had a greater Aα-Val^{360} and significantly lower gas transfer and lower FEV1/FVC than those without visible emphysema, even though these physiological tests remained either largely or entirely within the normal range. This observation supports the use of a symptomatic diagnosis of COPD (chronic bronchitis and exertional dyspnoea) for inclusion of patients in the current study, since spirometric criteria would have excluded a number of subjects with either ''early'' disease or an emphysema-predominant phenotype. In those with normal spirometry, the difference in Aα-Val^{360} between these two subgroups (with and without visible emphysema on HRCT) fell just short of conventional levels of statistical significance; however the absolute difference in Aα-Val^{360} concentrations mirrored that observed in the entire cohort, suggesting that this is not due to chance alone but rather reflects the smaller number of subjects identified. There is therefore likely to be a subset of patients with an active NE-related disease process yet relatively mild physiological changes that do not meet current spirometric criteria for the diagnosis of COPD, who may be identified by Aα-Val^{360} and benefit from targeted therapeutic intervention to prevent deterioration to the more classical stages of COPD. Clearly, further studies are required to investigate this possibility in depth.

Secondly, Aα-Val^{360} is a specific marker of pre-inhibition NE activity, while the α_1-AT/NE complex is a marker of total NE release as a result of neutrophil degranulation. We showed that, in general, the Aα-Val^{360} was related to the plasma α_1-AT/NE complex, and that subjects with COPD had higher levels of both Aα-Val^{360} and α_1-AT/NE complex than healthy controls, indicating both greater NE activity and neutrophil enzyme release. However, the differences between Aα-Val^{360} and other markers of neutrophil activation were emphasised by the absence of any correlation between either MPO (a marker of neutrophil degranulation) or α_1-AT/NE complex and the physiological and radiological markers of COPD disease severity (either cross-sectionally or longitudinally), demonstrating that a measure of elastase release alone is a poor surrogate of the enzyme's proteolytic activity and potential influence on disease progression.

Thirdly, Aα-Val^{360} also related to exacerbations of COPD, which are episodes known to relate to physiological progression [26]. Aα-Val^{360} was higher at the onset of an exacerbation than in the stable state 8 weeks later, reflecting greater NE activity which may at least partly impact on subsequent evidence of disease progression. Importantly, the Aα-Val^{360} was higher not only at the onset of an exacerbation in subjects who experienced an exacerbation associated with purulent (neutrophilic) sputum compared to those with mucoid sputum, but also remained

higher in the stable state, supporting the concept that these episodes may mark subjects with a greater likelihood of progression. Furthermore, the higher Aα-Val^{360} in this group of patients was associated with a higher stable state sputum IL-8 and plasma α_1-AT/NE complex concentration, demonstrating a greater ongoing inflammatory process leading to neutrophil recruitment, enzyme release and hence potential tissue damage. Although these data could represent a slower recovery, it is unlikely, since all subjects were seen 2 months after the exacerbation onset, when they were confirmed to be clinically stable. Additionally, the Aα-Val^{360} related both to the stable state and 4-yr follow-up physiological and radiological measures, and these relationships would be less likely if patients had not been in the stable state at the time of assessment (with further resolution after the study). In particular, there was no evidence of an ongoing bacterial trigger since there was no difference in subjective assessment of the stable-state sputum colour or objective measurement of MPO between the two groups of patients.

Although further studies are required, it is probable that patients who experience an exacerbation associated with purulent sputum have greater elastase activity in general (leading to tissue damage which enhances the subsequent risk of a bacterial infection) and, therefore, experience further exacerbations associated with purulent rather than mucoid sputum. It is also possible that subjects who experience a more severe exacerbation have a greater Aα-Val^{360} signal even following recovery and hence decline at a greater rate. However, there is currently no accepted inflammatory marker of exacerbation severity in subjects with COPD to confirm this concept, although it is possible that Aα-Val^{360} itself may fill this role in specific targeted studies.

Finally, there appears to be a relationship between NE activity (measured by Aα-Val^{360}) and COPD disease progression. Current pathophysiological activity is likely to reflect not only preceding but also future disease progression and in the current study we demonstrated baseline Aα-Val^{360} related to deterioration and subsequent disease severity measured by gas transfer. The data also indicates a relationship with Aα-Val^{360} and disease progression measured by CT densitometry, consistent with cause and effect. Nevertheless, the exact contribution to the pathophysiology of COPD in general and emphysema in particular will require a much larger prospective trial in highly characterised patients. However, Aα-Val^{360} did not relate to spirometric decline, suggesting that tissue damage reflected by Aα-Val^{360} is more indicative of the emphysematous process, not only in the presence of established COPD but importantly even in those with emphysema but without airflow obstruction. Clearly larger studies, especially in this latter group, are now indicated, including data on longitudinal progression.

In summary, the current study reports the first *in vivo* data in human subjects which supports the role of NE in the pathophysiology of COPD without α_1-AT deficiency. Furthermore, when considered in combination with previous circumstantial data, the current study suggests that NE may represent (at least part of) a final common pathway leading to tissue destruction in this disease process. Aα-Val^{360} is the first specific biomarker of pre-inhibition NE activity, which relates to cross-sectional measures of disease severity and exacerbations and appears to relate to disease progression in subjects with COPD. Although further work in a larger cohort of patients is required, particularly to explore the relationship with longitudinal physiological markers of disease progression in subjects at risk, Aα-Val^{360} thus represents a new concept of specific biomarkers that may be central to the pathophysiology of COPD.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for the study itself can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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