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Microfibrillar-associated protein 4: A potential biomarker of chronic obstructive pulmonary disease

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KEYWORDS
Microfibrillar-associated protein 4;
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Summary
Background: Microfibrillar-associated protein 4 (MFAP4) is a matricellular glycoprotein that co-localises with elastic fibres and is highly expressed in the lungs. The aim of this study was to test the hypothesis that plasma MFAP4 (pMFAP4) reflects clinical outcomes in chronic obstructive pulmonary disease (COPD).

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Chronic obstructive pulmonary disease;
Acute exacerbation of COPD;
BODE index;
Modified Medical Research Council score

Methods: pMFAP4 was measured by an AlphaLISA immunoassay in stable COPD ($n = 69$) at baseline and at follow-up until 24 months after inclusion and in acute exacerbations of COPD (AECOPD) ($n = 14$) at baseline and until 6 months after inclusion.
Results: The majority of patients (89%) were in GOLD II and III. Multiple linear regressions showed positive associations between pMFAP4 and the Global initiative for Obstructive Lung Disease (GOLD) grade ($p = 0.01$), modified Medical Research Council score ($p < 0.0001$) and BODE index ($p = 0.04$). Negative associations were found with 6-min walking distance ($p = 0.04$) and bronchodilator-induced reversibility ($p = 0.02$). The pMFAP4 levels varied less than 25% between the baseline and a 3 month follow-up in 83% of the patients. The pMFAP4 levels appeared unaffected in the acute phase of severe AECOPD but rose to an increased stable level within one month after hospitalization.
Conclusion: Increased pMFAP4 was associated to the severity in COPD and has the potential to serve as a stable disease biomarker. This observation warrants confirmation in a larger longitudinal COPD population.

Background

Chronic obstructive pulmonary disease (COPD) is characterised by pulmonary emphysema and a progressive airflow limitation caused by small airway disease [1]. Emphysema results from the degradation of the lung parenchyma, partly due to an inflammation induced protease-antiprotease imbalance [2].

Airflow is quantified using forced expiratory volume in one second (FEV_1) and FEV_1 is currently the only validated biomarker in COPD. However, FEV_1 is poorly correlated with symptom burden and outcomes in COPD, which makes it an inadequate surrogate marker of disease activity, a poor predictor of disease progression and an ineffective tool for the evaluation of treatment responses [3]. Thus, there is a pressing need for new, non-invasive and cost effective biomarkers in COPD [4].

Extracellular matrix turnover products have been suggested as alternative markers of emphysema development and respiratory disease activity [5–7], and microfibrillar-associated protein 4 (MFAP4/MAGP-36) is a matricellular glycoprotein highly expressed in the lung [8]. Immunohistochemistry and immunogold electron microscopy have demonstrated that MFAP4 is colocalised with microfibrils in elastic fibres in pulmonary blood vessels and in the alveolar septae [9,10]. MFAP4 is moreover increased in cirrhotic liver disease [11]. MFAP4 binds to collagen, elastin and the collagen-like regions of pulmonary surfactant proteins A (SP-A) and D (SP-D) [9,10,12] and is suggested with a protective role in photodamaging of the skin through the regulation of metalloproteinase expression [13].

The specific objectives of this pilot-study testing the hypothesis that MFAP4 levels in plasma (pMFAP4) reflect COPD severity were the following: I) to evaluate the association of MFAP4 levels with parameters reflecting disease severity in a stable COPD cohort; II) to evaluate the variability of pMFAP4 expression over time in stable COPD; and, III) to evaluate pMFAP4 variation during and after an acute exacerbation of COPD (AECOPD).

Methods

Ethical considerations

Study protocols were approved by the Regional Scientific Ethics Committee for Southern Denmark, and oral and written informed consent were obtained from the study subjects.

Stable COPD patients

The patients evaluated in this study were from the Danish subpopulation of the ECLIPSE cohort (Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points; Clinicaltrials.gov identifier NCT00292552; GSK study code SCO104960) [14] and were followed up after 3, 6, 9, 12, 18 and 24 months. Briefly, patients with stable COPD, aged 40–75 years and with a smoking history of at least 10 pack-years were enrolled [14]. The measured or estimated clinical outcomes were FEV_1; FVC and FEV_1/FVC; a 6-min walking test; the percentage of low attenuation area (LAA%); exhaled carbon monoxide, *eCO*, with *current smoking* defined as an eCO >12 ppm; the modified Medical Research Council (mMRC) dyspnoea scale; and the BODE index calculated according to standards, using the *B*ody Mass Index, *O*bstruction Index, *D*yspnoea score and *E*xercise capacity. COPD subjects were asked about exacerbations 3, 6 and 12 months after enrolment in the study. In addition, they were contacted by telephone every month by the study staff and asked about details of exacerbations during the previous month. Specifically, subjects were asked whether they had been unwell in the last month, whether they had seen a doctor or been to hospital and whether they had taken any medication for exacerbations (oral corticosteroids or antibiotics). The data were analysed 12 months after enrolment into the study as described previously [15].

Control subjects

EDTA-plasma samples were obtained from 54 smoking and 52 non-smoking control subjects who were recruited from

the blood donor bank at Odense university hospital. The subjects' ages, genders and smoking habits were obtained through the register forms.

Severe acute exacerbation of COPD (AECOPD) patients

Fifteen AECOPD patients were recruited from September 2012 through June 2013 at the Medical Emergency Ward, Odense University Hospital. The inclusion criteria were an age of between 40 and 80 years; $FEV_1 < 80\%$ and $FEV_1/FVC < 0.7$; at least 1 exacerbation in the year prior to inclusion; and a smoking history of at least 10 pack-years. The most important exclusion criteria were pneumonia confirmed by chest X-ray, diagnosis with other respiratory disease, congestive heart disease with an ejection fraction of <40%, or recent cancer. A full list of inclusion and exclusion criteria and of therapeutic treatment is shown in the supplementary methods sections. Blood samples were collected at inclusion (day 0), on days 1, 3–5, 6–8, 9–11, and at 4 weeks and 3 and 6 months after inclusion.

Measurement of MFAP4 in plasma (pMFAP4)

pMFAP4 was measured by a modified AlphaLISA® immunoassay from Perkin and Elmer using two monoclonal antibodies, HG-HYB 7-14 and HG-HYB 7-18, as previously described [8]. One unit/mL of MFAP4 corresponds to 38 ng/mL of MFAP4 in serum.

Immunohistochemical staining of transplant lung tissue

The immunohistochemical staining for microfibrillar-associated protein 4 (MFAP4) was performed on lung parenchymal paraffin embedded tissue from non-emphysematous control donor lungs and from transplant recipients with severe emphysema caused by cigarette smoking or α^1-antitrypsin deficiency. The tissue samples were acquired from the transplant biobank at Department of Pathology, Rigshospitalet, Copenhagen University Hospital. Immunohistochemical staining was carried out using an anti-MFAP4 (HG-HYB 7-14) antibody as described previously [8].

Statistical methods

pMFAP4 was normally distributed in all of the subgroups of the COPD patients and controls. The comparisons between two groups were performed using a *t*-test for groups with equal variances, and the comparisons between three or more groups were done using indicator variables in univariate and multivariate linear regression models. A Spearman's correlation analysis and multiple linear regressions were used when analysing the association between the dependent variable (pMFAP4) and the continuous explanatory variables. The covariates and interaction terms in multiple linear regressions were tested by subsequently reducing the model by backward elimination followed by a comparison of Akaikes Information Criteria and by using a threshold of $p < 0.2$. The best reduced model included age, gender and the dichotomous variable exhaled carbon monoxide of >12 ppm as covariates.

The relationship between pMFAP4 and exacerbations was evaluated in 3 different ways: 1) as a continuous variable with numbers of exacerbations; 2) as a categorical variable of "frequent exacerbator" defined by ≥2 exacerbations the year prior to inclusion [14]; and, 3) a categorical variable defined as an exacerbation between baseline and 3 month follow-up.

The variability in pMFAP4 over time was assessed in a mixed effect model adjusted for age, current smoking and gender, with inclusion levels as references. The within-patient and between-patient variance was estimated by allowing the intercept to vary at random. All statistical analyses were performed with STATA version 11.

Results

pMFAP4 in patients with stable COPD at baseline

Of the 91 patients included in the Danish sub-cohort of the ECLIPSE [16], baseline blood samples were available from 76 of the stable COPD patients. Four patients were excluded from this study due to a diagnosis of cancer during the study period, and 3 patients provided insufficient information about smoking during the follow-up. The baseline characteristics of the included cohort divided into smoking and non-smoking COPD patients are shown in Table 1. The current smoking COPD patients had non-significantly lower pMFAP4 relative to the ex-smoking COPD patients ($p = 0.2$). Age was positively associated with pMFAP4 ($\beta = 0.09$, $p = 0.03$) and women had a higher pMFAP4 relative to men, 8.7 ± 2.6 U/mL versus 7.5 ± 2.3 U/mL, respectively ($p = 0.046$).

pMFAP4 in control subjects

pMFAP4 was not significantly different between COPD subjects and control subjects. However, the control population was younger than the COPD population, with a mean age of 46 ± 12 years in the cigarette smoking controls versus 41 ± 14 years in the non-smoking controls. Age was positively associated with pMFAP4 ($\beta = 0.038$, $p = 0.04$), and pMFAP4 was significantly lower in the cigarette smoking controls relative to the non-smoking controls, 6.8 ± 2.2 U/mL versus 8.4 ± 2.5 U/mL, respectively ($p = 0.003$). There was no difference in pMFAP4 between females and males in the control group, 7.5 ± 2.6 versus 7.6 ± 2.5, respectively ($p = 0.8$).

Association between pMFAP4 and severity of disease in patients with stable COPD at baseline

The investigated patients were distributed with 0% GOLD I, 42% GOLD II, 47% GOLD III, and 11% GOLD IV, respectively. Univariate analysis was used to determine the effects of age ($\beta = 0.093$, $p = 0.01$), male gender ($\beta = -1.2$, $p = 0.05$) and eCO > 12 ppm ($\beta = -0.90$, $p = 0.1$), and

Table 1 Basic characteristics stratified by smoking in stable COPD patients.

	COPD smokers (21)	COPD ex-smokers (48)	COPD total (69)
MFAP4 (U/mL)	7.7 ± 2.4	8.5 ± 2.5	8.2 ± 2.5
Age (years)	61 ± 7	62 ± 7	62 ± 7
Female (%)	38	60	53
Carbon monoxide (ppm)*	24 (21)	4 (4.5)	7 (19)
Pack-years*	46 (25)	38 (16)	40 (20)
FEV_1 (% pred.)	51 ± 17	49 ± 16	50 ± 16
FVC (% pred.)	89 ± 20	85 ± 20	86 ± 20
FEV_1/FVC	0.45 ± 0.11	0.48 ± 0.13	0.47 ± 0.12
ΔFEV_1 (mL)	178 ± 152	152 ± 111	152 ± 111
6MWD (m)	418 ± 124	422 ± 112	421 ± 117
mMRC*	1 (1)	1 (1)	1 (1)
BMI	25.0 ± 5.2	26.9 ± 4.2	26.2 ± 4.7
Fat free mass (kg/m^2)	17.3 ± 3.1	17.5 ± 2.8	17.4 ± 2.9
BODE index*	2 (4)	3 (3)	3 (3)
LAA%	14 ± 11	19 ± 15	17 ± 14

Values are presented as the mean ± standard deviation, except for *values presented as the median (interquartile range). One pack-year = 20 cigarettes daily, or 50 g of tobacco weekly in one year. MFAP4: Microfibrillar-associated protein 4, FEV_1 (% pred.): post-bronchodilator forced expiratory volume in 1 s in percent of predicted value, FVC (% pred.): post-bronchodilator forced vital capacity in percent of predicted value, FEV_1/FVC: ratio between post-bronchodilator FEV_1 and FVC (obstruction index), ΔFEV_1: FEV_1 reversibility after bronchodilator, 6MWD: 6 min walking distance, mMRC: modified Medical Research Council score, BMI: Body mass index, BODE index: body mass index, obstruction index (FEV_1/FVC), dyspnoea score (mMRC), exercise capacity (MWD), LAA%: percentage of low attenuation area (<−950 Hounsfield units) in computer tomography scan.

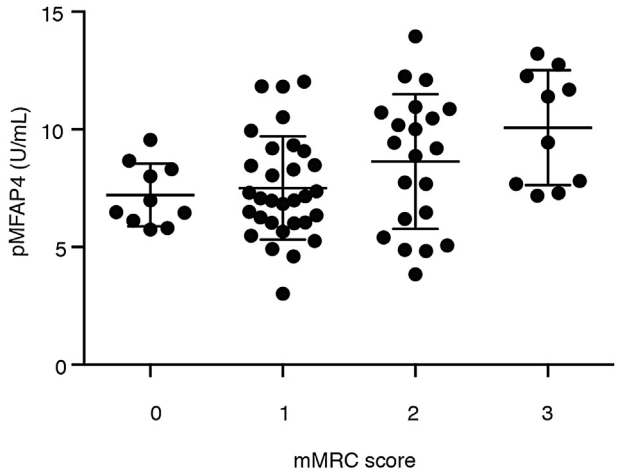

Figure 1 Plasma microfibrillar-associated protein 4 (pMFAP4) stratified by the modified Medical Research Council (mMRC) dyspnoea score.

multiple linear regressions with pMFAP4 as response variable were then corrected for the identified covariates.

Multiple linear regressions showed significant positive associations between pMFAP4 and the GOLD grade, modified Medical Research Council score and BODE index. Negative associations were found with the 6-min walking distance ($p = 0.04$) and bronchodilator-induced reversibility ($p = 0.02$) (Table 2). The Spearman correlation analyses were supported by the outcome of the linear regressions with the exception of the insignificant correlation between pMFAP4 and the GOLD grade (Table 2). The association with the highest level of significance was found between the mMRC score and pMFAP4 (Fig. 1). Neither the tissue density measured as the percentage of low attenuation area (LAA%) nor the presence of exacerbations of disease 1 month before

Table 2 Association between pMFAP4 and clinical outcomes in stable COPD patients.

	ρ	p	β-coeff	CI	p	Effect[a]
GOLD	0.19	0.11	1.09	2.26:1.92	0.01	0.23
mMRC	0.34	0.004	1.08	0.50:1.66	<0.0001	0.40
FEV_1 (L)	−0.37	0.02	−0.89	−2.0:0.17	0.1	
FEV_1/FVC	−0.05	0.7	−0.033	−0.081:0.016	0.2	
ΔFEV_1 (mL)	−0.24	0.04	−0.0048	−0.0088:−0.00082	0.02	−43
6MWD (m)	−0.26	0.03	−0.0051	−0.010:0.0003	0.04	−30
BODE	0.24	0.04	0.42	0.14:0.70	0.004	0.74
LAA%	0.04	0.7	0.02	−0.024:0.065	0.4	
Exacerbation −1	0.46	0.16	−0.43	−2.12:−1.26	0.6	
Exacerbation +1	0.24	0.017	0.11	−0.52:0.75	0.7	
Exacerbation +3	0.06	0.6	−0.04	−1.23:1.16	0.9	

Spearman correlations and multivariate linear regressions with MFAP4 as the dependent variable. The regressions were adjusted for covariates age, gender and exhaled carbon monoxide (eCO) >12 ppm CI: 95% Confidence Interval.
GOLD: Global initiative for Obstructive Lung Disease (GOLD) grade, mMRC: modified Medical Research Council score, FEV_1: forced expiratory volume in 1 s, FVC: forced vital capacity, ΔFEV_1: FEV_1 reversibility after bronchodilator, FEV_1/FVC: ratio between post-bronchodilator FEV_1 and FVC (obstruction index), 6MWD: 6 min walking distance, BODE index: body mass index, obstruction index (FEV_1/FVC), dyspnea score (mMRC), exercise capacity (MWD), LAA%: percentage of low attenuation area (<−950 Hounsfield units) in computer tomography scan. Exacerbation −1: within the month before, +1: within the month after; +3: within three months after.
[a] Effects on clinical outcomes are per increase of 1 standard deviation of pMFAP4 and assessed using the coefficient resulting from multiple regressions.

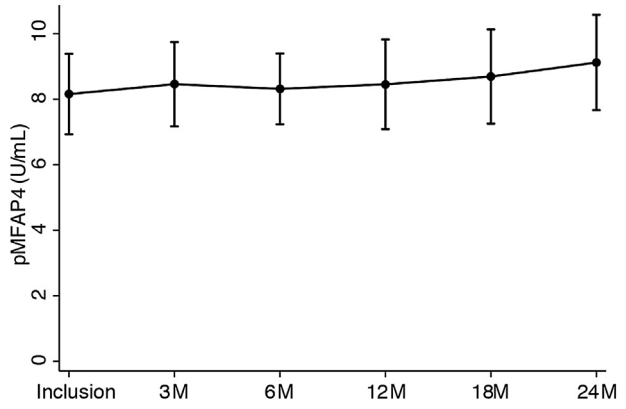

Figure 2 Variability of plasma microfibrillar-associated protein 4 (pMFAP4) in stable COPD. 3M: 3 months, 6M: 6 months, 12M: 12 months, 18M: 18 months, 24M: 24 months follow-up.

nor 1 or 3 months after inclusion were significantly associated to pMFAP4 measured at inclusion.

Variability of pMFAP4 in patients with stable COPD at baseline

The variability of pMFAP4 over time was evaluated in a mixed effects model adjusted for age, gender and current smoking. The within-patient standard deviation of pMFAP4 was 1.4 U/mL, and the between-patient standard deviation was 2.0 U/mL. The within-patient variance made up 32% of the total variance in pMFAP4. The two-year variation in pMFAP4 in patients with stable COPD at baseline is depicted in Fig. 2. An assessment of repeatability between baseline pMFAP4 and the 3 month follow-up showed that pMFAP4 varied less than 25% in 83% of the patients.

pMFAP4 in severe acute exacerbation of COPD (AECOPD) patients

Fifteen patients were enrolled during an admission to the hospital due to AECOPD. These patients were not a subpopulation of the ECLIPSE cohort but comprised a separate population of acute COPD patients. One patient was excluded after 4 weeks due alcohol misuse. Fourteen patients were eligible for the entire 6 months follow-up.

Characteristics of the patients are presented in Table 3. pMFAP4 was found to be significantly higher at 4 weeks ($\beta = 3.6$, $p < 0.00001$), 3 months ($\beta = 3.0$, $p < 0.00001$) and 6 months ($\beta = 3.4$, $p < 0.00001$) relative to inclusion (Fig. 3A). The same pattern was observed for the serum levels of surfactant protein D (sSP-D), where the logarithmically transformed levels (lnSP-D) at 4 weeks ($\beta = 0.28$, $p = 0.008$) and 6 months ($\beta = 0.24$, $p = 0.02$) were significantly higher relative to inclusion. In addition, a decline in sSP-D was seen in the first 10 days after inclusion, with significantly lower sSP-D around day 10 relative to inclusion ($\beta = -0.39$, $p < 0.0001$) (Fig. 3B). The opposite trend was seen in white blood cell counts (WBC), with an increase in the logarithmically transformed levels (lnWBC) peaking around day 10 after inclusion ($\beta = 0.21$, $p = 0.02$) followed by a decrease in WBC with significantly lower values at 4 weeks ($\beta = -0.30$, $p < 0.0001$), 3 months ($\beta = -0.37$, $p < 0.0001$) and 6 months ($\beta = -0.39$, $p < 0.0001$) (Fig. 3C).

Localisation of MFAP4 in the lung

Immunohistochemical staining verified the previous observation that MFAP4 is located in the alveolar septae and in vessels of the lung parenchyma both in the non-diseased lung [9] and likewise in the emphysematous lung tissue (Fig. 4). MFAP4 was further highly expressed in the submucosal layer of the bronchial tissue (not shown). The MFAP4-immunostaining appeared further extended into the adventitia in the emphysematous tissue when compared with non-emphysematous tissue (Fig. 4).

Discussion

The role of MFAP4 in COPD pathogenesis is unknown. MFAP4 is found with relatively high expression in the lung [8] and

Table 3 Basic characteristics stratified by time point in AECOPD patients.

	Inclusion	4 week follow-up	3 month follow-up	6 month follow-up
Age (years)	66 ± 8			
Current smoking (%)	36%			
Pack-years*	60 (34)			
mMRC*	5 (0)	3.5 (1)	3.5 (1.5)	4 (2)
FEV_1 (% pred.)	30 ± 11	36 ± 12	42 ± 21	39 ± 17
FVC (% pred.)	59 ± 9	60 ± 17	70 ± 19	68 ± 13
FEV_1/FVC	0.48 ± 0.18	0.47 ± 0.16	0.48 ± 0.18	0.46 ± 0.16
pMFAP4 (U/mL)	10.6 ± 3.8	14.7 ± 7.3	13.2 ± 6.3	14.1 ± 6.3
sSP-D (ng/mL)*	1047 (847)	1446 (1002)	1277 (1708)	1284 (1170)
WBC (1000/mL)*	10.2 (5.8)	7.6 (2.9)	7 (3.1)	7.7 (2.3)
CRP* mg/L	13 (39)	7.5 (13.5)	1 (12)	4 (6)

Values are presented as the mean ± standard deviation, except for *data presented as the median (interquartile range). One pack-year = 20 cigarettes daily for one year, or 50 g tobacco weekly for one year. mMRC: Modified Medical Research Council dyspnoea scale, FEV_1 (% pred.): forced expiratory volume in one second in percent of predicted value, FVC (% pred.): forced vital capacity in percent of predicted value, FEV_1/FVC: ratio between post-bronchodilator FEV_1 and FVC (obstruction index), pMFAP4: plasma microfibrillar-associated protein 4, sSP-D: serum surfactant protein D, WBC: white blood cell count, CRP: C-reactive protein.

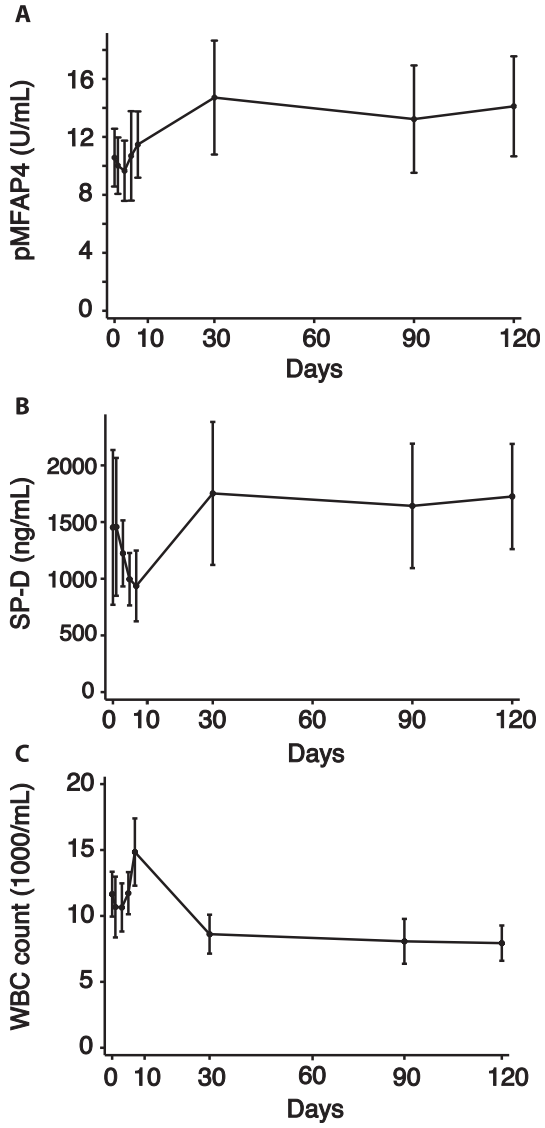

Figure 3 Variability of markers in AECOPD. Variability of A) plasma microfibrillar-associated protein 4 (pMFAP4), B) serum surfactant protein D (sSP-D) and C) white blood cell counts (WBC), from admission (Day 0) for acute exacerbation (AECOPD) and at various intervals during 120 days follow-up.

binding properties to extracellular matrix fibres and smooth muscle cells have previously motivated hypotheses for roles in elastic fibre assembly and in pulmonary emphysema development [10,17]. The present study set out to investigate if MFAP4 variation might reflect COPD severity and the potential utility of plasma MFAP4 (pMFAP4) as a biomarker for clinical outcomes in COPD.

The Danish subgroup of the ECLIPSE cohort of stable COPD patients was examined and we found that pMFAP4 was higher with increasing age and female gender and depressed by current cigarette smoking as demonstrated previously in a population based cohort [18]. We found statistically significant negative associations between pMFAP4 and the 6-min walking distance and FEV_1 bronchodilator reversibility and positive associations were found with GOLD grade, mMRC dyspnoea scale and the composite BODE index. All these associations are previously demonstrated markers of severity of COPD associated with morbidity and mortality [19,20] and the data indicated that increased pMFAP4 reflects disease severity. The pMFAP4 levels varied less than 25% between baseline and 3 month follow-up in 83% of the patients with stable COPD at baseline. In contrast, pMFAP4 was significantly increased at a stable level 1 month after hospitalization although it seemed unaffected during the first days of an acute exacerbation of disease.

The elastin degradation product desmosine has previously been reported to be present at higher levels in GOLD II than in GOLD IV [5], and the rates of lung function decline are higher in GOLD II and III relative to GOLD IV, supporting a higher degree of disease activity in moderate-severe COPD relative to very severe COPD [21]. Such observations for desmosine variation originally prompted a working hypothesis that pMFAP4 would be decreased in severe disease. However, we found the highest levels of pMFAP4 in the stable patients with the most severe clinical outcomes. The observed pMFAP4 variation may however just reflect that there were only 8 subjects with very severe COPD in the present study and that we have predominantly investigated systemic variation of pMFAP4 in GOLD II and III. A limitation the present study is further that we did not compare the pMFAP4 variation to that of desmosine.

We found that cigarette smoking is associated with reduced levels of pMFAP4. A higher pMFAP4 was also found in ex-smoking COPD subjects compared with current smokers, but the difference was not significant. The variable "exhaled CO $>$ 12 ppm" had a larger effect on pMFAP4 than "current smoking", and there was no significant association between pack-years and pMFAP4. This indicates an acute effect of cigarette smoking on pMFAP4 levels contradicting the working conclusion that increased levels of pMFAP4 reflect disease severity. Active cigarette smoking may thus spur putative associations between pMFAP4 and COPD outcomes. This aspect of pMFAP4 variation warrants investigation in larger cohorts stratified into smoking and non-smoking subjects. Although it is now documented in two independent studies that systemic MFAP4 is depressed by tobacco smoking it is currently unknown how this depression is inferred. We can only speculate this to be caused by cellular dysfunction as a result of toxins within cigarette smoke as previously suggested for the pulmonary marker CC-16 [22].

We estimated associations between pMFAP4 and clinical outcomes in stable COPD patients and found that one 1 standard deviation increase in pMFAP4 corresponded to a fall in bronchodilator FEV_1 of 43 mL, a fall in *6 min walking distance* of 30 m, an increase in the BODE index of 0.74 units, a 0.23 unit increase in GOLD grade and a 0.40 unit increase in the mMRC score. The calculations of the magnitudes of the relations, however, warrant confirmation in larger cohorts with more disease heterogeneity and in particular with more subjects with very severe disease.

There was no association to lung tissue density and pMFAP4 therefore may be suggested to reflect bronchitis related changes in the lung rather than emphysema status in this study of patients with stable COPD at baseline. Yet, data describing the change in LAA% with time were not

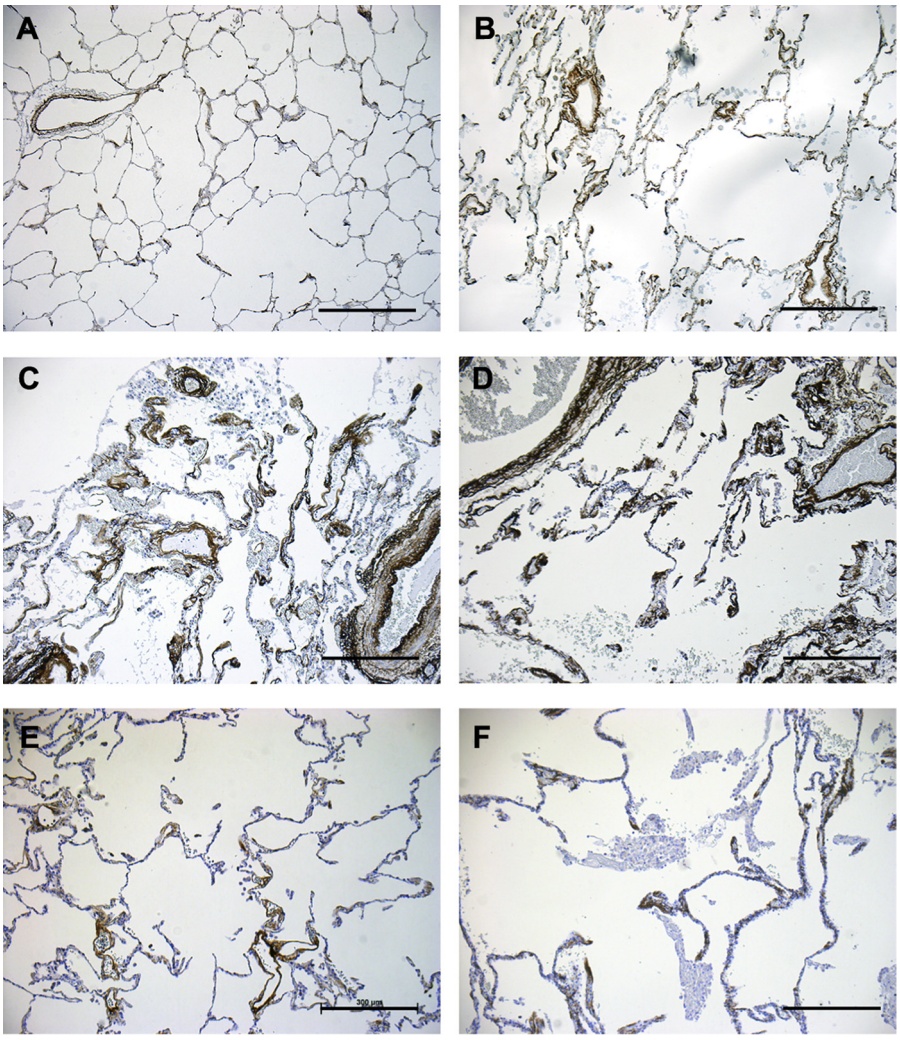

Figure 4 Localisation of microfibrillar-associated protein 4 (MFAP4) in lung tissue. Anti-MFAP4 antibody (HG-HYB 7-14) staining in A and B) parenchymal tissue from two anonymous healthy donor lungs, C and D) parenchymal tissue from two explanted lungs with cigarette smoke-induced emphysema, E and F). parenchymal tissue from two explanted lungs with emphysema due to α^1-anti-trypsin deficiency, non-smoker and ex-smoker, respectively. Original magnification 100×. Bars are 300 μm.

available and we cannot exclude that pMFAP4 variation reflects elastic fibre degradation in the lung.

Variability is a highly important aspect in the evaluation of biomarkers for chronic disease. Immunodetection of MFAP4 in blood samples has previously been demonstrated to be highly resistant to pre-analysis handling and storage conditions [23]. Here we show that the time-dependent within-patient variability in MFAP4 over 2 years made up approximately one third of the total variation of pMFAP4 in COPD patients. There was a small but significant continuous increase in pMFAP4 over 2 years, which may indicate an increase in pMFAP4 in association with disease progression. However, 83% of the COPD patients with stable disease at baseline had a pMFAP4 variation of <25% within 3 months, and thus the marker appeared relatively stable over time. Mean levels of pMFAP4 in the control subjects did not appear to differ from the mean levels in stable COPD. In contrast, the mean levels of pMFAP4 appeared to be higher in the AECOPD cohort relative to the patients with stable COPD at baseline although only preliminary conclusions may be drawn from the comparison as the independent cohorts were not matched.

The functional role of MFAP4 has yet to be elucidated in detail, although a role in elastic fibre assembly was recently suggested [13]. MFAP4 was found to be increased in pulmonary hypertension [24], and systemic levels were decreased in stable atherosclerosis [8]. These observations suggest that MFAP4 is regulated by cardiovascular disease. The present study thus cannot rule out that the associations found between pMFAP4 and clinical outcomes in stable COPD may be secondary to cardiovascular pathology. The separated analyses of MFAP4 associations to pulmonary or cardiovascular outcomes warrant confirmation in larger cohorts with detailed information about cardiovascular disease.

pMFAP4 increased only slightly during the first 11 days of acute exacerbations. However, the pMFAP4 levels subsequently rose after 4 weeks and remained stable until 6 month follow-up. Systemic SP-D was also lower at inclusion relative to the stable phase but continued to decrease until

days 9—11 after inclusion. After 4 weeks, SP-D also remained stable until 6 month follow-up. Previous studies showed increased levels of SP-D at exacerbation [5,25] and in bacterial pneumonia [26]. In this study, we excluded patients with pneumonia, which may explain the discrepancy between these observations. The observed decline in serum SP-D and the increase in white blood cells during the exacerbation are likely to be caused by glucocorticoid treatment [27,28].

Limitations of this sub-study include the limited number of patients and the lack of information about stable pMFAP4 levels before inclusion and we cannot definitively conclude that pMFAP4 was undisturbed at baseline in AECOPD.

The present study supported that MFAP4 is localised to elastic fibres in the lungs, in alveolar septae, in pulmonary vessels and in the submucosal layer of the bronchi in COPD, as shown previously in non-diseased lung tissue [9]. The fractional area of elastic fibres in the lungs is decreased in COPD [29]. In contrast, there may be increased fibrosis and arterial hyperplasia. These structural changes may all influence the levels of MFAP4 in the lungs. The present study was not designed to enable measurement of the MFAP4 expression in the lung tissue and no attempts were made to quantify the immunostaining.

Conclusions

Plasma levels of MFAP4 represent a novel blood marker that is increased with the severity of stable COPD, and in the months following an acute exacerbation. The pMFAP4 levels appeared stable in stable COPD and varied <25% between baseline and 3 month follow-up in 83% of the patients. Larger longitudinal studies are warranted to confirm these findings and the strength of association.

Conflict of interests

RT-S and BM are employees and shareholders of GlaxoSmithKline, the sponsor of ECLIPSE.

The study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2014.06.003.

References

[1] Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2013;187:347—65.

[2] Tam A, Sin DD. Pathobiologic mechanisms of chronic obstructive pulmonary disease. Med Clin North Am 2012;96:681—98.

[3] Cazzola M, MacNee W, Martinez FJ, Rabe KF, Franciosi LG, Barnes PJ, Brusasco V, Burge PS, Calverley PM, Celli BR, et al. Outcomes for COPD pharmacological trials: from lung function to biomarkers. Eur Respir J 2008;31:416—69.

[4] Vestbo J, Rennard S. Chronic obstructive pulmonary disease biomarker(s) for disease activity needed-urgently. Am J Respir Crit Care Med 2010;182:863—4.

[5] Huang JT, Chaudhuri R, Albarbarawi O, Barton A, Grierson C, Rauchhaus P, Weir CJ, Messow M, Stevens N, McSharry C, et al. Clinical validity of plasma and urinary desmosine as biomarkers for chronic obstructive pulmonary disease. Thorax 2012;67:502—8.

[6] Lindberg CA, Engstrom G, de Verdier MG, Nihlen U, Anderson M, Forsman-Semb K, Svartengren M. Total desmosines in plasma and urine correlate with lung function. Eur Respir J 2012;39:839—45.

[7] Carter RI, Ungurs MJ, Mumford RA, Stockley RA. Aalpha-Val360: a marker of neutrophil elastase and COPD disease activity. Eur Respir J 2013;41:31—8.

[8] Wulf-Johansson H, Lock Johansson S, Schlosser A, Trommelholt Holm A, Melholt Rasmussen L, Mickley H, Diederichsen AC, Munkholm H, Poulsen TS, Tornoe I, et al. Localization of microfibrillar-associated protein 4 (MFAP4) in human tissues: clinical evaluation of serum MFAP4 and its association with various cardiovascular conditions. PLoS One 2013;8:e82243.

[9] Schlosser A, Thomsen T, Shipley JM, Hein PW, Brasch F, Tornoe I, Nielsen O, Skjodt K, Palaniyar N, Steinhilber W, et al. Microfibril-associated protein 4 binds to surfactant protein A (SP-A) and colocalizes with SP-A in the extracellular matrix of the lung. Scand J Immunol 2006;64:104—16.

[10] Toyoshima T, Nishi N, Kusama H, Kobayashi R, Itano T. 36-kDa microfibril-associated glycoprotein (MAGP-36) is an elastin-binding protein increased in chick aortae during development and growth. Exp Cell Res 2005;307:224—30.

[11] Molleken C, Sitek B, Henkel C, Poschmann G, Sipos B, Wiese S, Warscheid B, Broelsch C, Reiser M, Friedman SL, et al. Detection of novel biomarkers of liver cirrhosis by proteomic analysis. Hepatology 2009;49:1257—66.

[12] Lausen M, Lynch N, Schlosser A, Tornoe I, Saekmose SG, Teisner B, Willis AC, Crouch E, Schwaeble W, Holmskov U. Microfibril-associated protein 4 is present in lung washings and binds to the collagen region of lung surfactant protein D. J Biol Chem 1999;274:32234—40.

[13] Kasamatsu S, Hachiya A, Fujimura T, Sriwiriyanont P, Haketa K, Visscher MO, Kitzmiller WJ, Bello A, Kitahara T, Kobinger GP, Takema Y. Essential role of microfibrillar-associated protein 4 in human cutaneous homeostasis and in its photoprotection. Sci Rep 2011;1:164.

[14] Vestbo J, Anderson W, Coxson HO, Crim C, Dawber F, Edwards L, Hagan G, Knobil K, Lomas DA, MacNee W, et al. Evaluation of COPD longitudinally to identify predictive surrogate end-points (ECLIPSE). Eur Respir J 2008;31:869—73.
[15] Lomas DA, Silverman EK, Edwards LD, Locantore NW, Miller BE, Horstman DH, Tal-Singer R. Serum surfactant protein D is steroid sensitive and associated with exacerbations of COPD. Eur Respir J 2009;34:95—102.
[16] Moberg M, Vestbo J, Martinez G, Lange P, Ringbaek T. Scientific World Journal 2014 Jan 21;2014:140736. http://dx.doi.org/10.1155/2014/140736. eCollection 2014.
[17] Kasamatsu S, Hachiya A, Fujimura T, Sriwiyanont P, Haketa K, Visscher MO, Kitzmiller WJ, Bello A, Kitahara T, Kobinger GP, Takema Y. Essential role of microfibrillar-associated protein 4 in human cutaneous homeostasis and in its photoprotection. Sci Reports 2011;1:1—10.
[18] Sækmose SG, Schlosser A, Holst R, Johansson SL, Wulf-Johansson H, Tornøe I, Vestbo J, Kyvik KO, Barington T, Holmskov U, Sørensen GL. PLoS One 2013 Dec 4;8(12):e82383. http://dx.doi.org/10.1371/journal.pone.0082383. eCollection 2013.
[19] Celli BR, Cote CG, Marin JM, Casanova C, Montes de Oca M, Mendez RA, Pinto Plata V, Cabral HJ. The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. N Engl J Med 2004; 350:1005—12.
[20] Cote CG, Pinto-Plata V, Kasprzyk K, Dordelly LJ, Celli BR. The 6-min walk distance, peak oxygen uptake, and mortality in COPD. Chest 2007;132:1778—85.
[21] Vestbo J, Edwards LD, Scanlon PD, Yates JC, Agusti A, Bakke P, Calverley PM, Celli B, Coxson HO, Crim C, et al. Changes in forced expiratory volume in 1 second over time in COPD. N Engl J Med 2011;365:1184—92.
[22] Lomas DA, Silverman EK, Edwards LD, Miller BE, Coxson HO, Tal-Singer R. Evaluation of CLtIPSEi: evaluation of serum CC-16 as a biomarker for COPD in the ECLIPSE cohort. Thorax 2008;63:1058—63.
[23] Saekmose SG, Schlosser A, Holst R, Johansson SL, Wulf-Johansson H, Tornoe I, Vestbo J, Kyvik KO, Barington T, Holmskov U, Sorensen GL. Enzyme-linked immunosorbent assay characterization of basal variation and heritability of systemic microfibrillar-associated protein 4. PLoS One 2013;8:e82383.
[24] Abdul-Salam VB, Wharton J, Cupitt J, Berryman M, Edwards RJ, Wilkins MR. Proteomic analysis of lung tissues from patients with pulmonary arterial hypertension. Circulation 2010;122:2058—67.
[25] Shakoori TA, Sin DD, Ghafoor F, Bashir S, Bokhari SN. Serum surfactant protein D during acute exacerbations of chronic obstructive pulmonary disease. Dis Markers 2009;27:287—94.
[26] Leth-Larsen R, Nordenbaek C, Tornoe I, Moeller V, Schlosser A, Koch C, Teisner B, Junker P, Holmskov U. Surfactant protein D (SP-D) serum levels in patients with community-acquired pneumonia small star, filled. Clin Immunol 2003;108:29—37.
[27] Sin DD, Lacy P, York E, Man SF. Effects of fluticasone on systemic markers of inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2004;170:760—5.
[28] Clarke JR, Gagnon RF, Gotch FM, Heyworth MR, Maclennan IC, Truelove SC, Waller CA. The effects of prednisolone in leucocyte function in man. A double blind controlled study. Clin Exp Immunol 1977;28:292—301.
[29] Annoni, et al. Eur Respir J 2012 Dec;40(6):1362—73. http://dx.doi.org/10.1183/09031936.00192611 [Epub 2012 Apr 10].