Antimicrobial peptide levels are linked to airway inflammation, bacterial colonisation and exacerbations in chronic obstructive pulmonary disease

Louise J.P. Persson[1], Marianne Aanerud[1], Jon A. Hardie[2], Roy Miodini Nilsen[3], Per S. Bakke[2], Tomas M. Eagan[1,2] and Pieter S. Hiemstra[4]

Affiliations: [1]Dept of Thoracic Medicine, Haukeland University Hospital, Bergen, Norway. [2]Dept of Clinical Science, University of Bergen, Bergen, Norway. [3]Centre for Clinical Research, Haukeland University Hospital, Bergen, Norway. [4]Dept of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands.

Correspondence: Louise J.P. Persson, Dept of Thoracic Medicine, Haukeland University Hospital, Jonas Liesvei 65, N-5021 Bergen, Norway. E-mail: louise.persson@med.uib.no

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ABSTRACT Antimicrobial peptides (AMPs) are effectors of host defence against infection, inflammation and wound repair. We aimed to study AMP levels in stable chronic obstructive pulmonary disease (COPD) and during acute exacerbations of COPD (AECOPD), and to examine their relation to clinical parameters and inflammatory markers.

The 3-year Bergen COPD Cohort Study included 433 COPD patients and 325 controls. Induced sputum was obtained and analysed for levels of the AMPs human cathelicidin (hCAP18/LL-37) and secretory leukocyte protease inhibitor (SLPI), and for the inflammatory markers interleukin (IL)-8, IL-6 and tumour necrosis factor-α (TNF-α) using immunoassays. Systemic hCAP18/LL-37 and vitamin D levels were also studied. Treating AMPs as response variables, non-parametric tests were applied for univariate comparison, and linear regression to obtain adjusted estimates. The risk of AECOPD was assessed by Cox proportional-hazard regression.

Sputum AMP levels were higher in patients with stable COPD (n=215) compared to controls (n=45), and further changed during AECOPD (n=56), with increased hCAP18/LL-37 and decreased SLPI levels. Plasma hCAP18/LL-37 levels showed a similar pattern. In stable COPD, high sputum hCAP18/LL-37 levels were associated with increased risk of AECOPD, non-typeable *Haemophilus influenzae* colonisation, higher age, ex-smoking and higher levels of inflammatory markers.

Altered levels of selected AMPs are linked to airway inflammation, infection and AECOPD, suggesting a role for these peptides in airway defence mechanisms in COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide [1], to which acute exacerbation of chronic obstructive pulmonary disease (AECOPD) is an important contributor [2]. The hallmark pathology is chronic small airway inflammation and loss of alveolar walls [3]. Increased oxidative stress, protease/anti-protease imbalance and airway infections can further sustain local inflammation [4]; however, the cause of the chronicity of inflammation is as yet mostly unknown.

Human cathelicidin (hCAP18/LL-37) and secretory leukocyte protease inhibitor (SLPI) are antimicrobial peptides/proteins (AMPs) with central roles in the innate immune defence against infection [5]. AMPs are expressed at the airway mucosal surface both constitutively and upon inflammatory and infectious stimuli; cathelicidin expression especially is also regulated by vitamin D [6, 7]. AMPs display broad-spectrum antimicrobial activity against a variety of pathogens, have immunomodulatory properties, and may contribute to tissue repair reactions in the lung [8, 9]. Importantly, not only the presence but also the activity of both AMPs is regulated by factors present in purulent secretions in the airways [10–14].

Previous studies have reported different sputum AMP levels in COPD patients compared to controls [15–18], and higher cathelicidin and/or lower SLPI levels during infectious COPD exacerbations [19–21] and bacterial colonisation [19, 20]. Thus, altered cathelicidin and SLPI levels have been linked to a deteriorated lung environment. Existing studies on this topic in COPD are, however, limited by small sample sizes and a lack of strong data on the possible determinants of airway AMP levels, including clinical parameters and airway inflammatory markers. Furthermore, it is unknown whether AMP levels in patients with stable disease are predictive of future AECOPD.

The present study uses data from the large and well-characterised Bergen COPD Cohort Study (BCCS) [22] and from the Bergen COPD Exacerbation Study (BCES) [23]. Our aims were to examine 1) cathelicidin and SLPI levels in COPD (both stable and during AECOPD) compared to in controls; 2) associations between AMP levels in stable COPD and colonisation with non-typeable *Haemophilus influenzae* (NTHi), inflammatory markers, vitamin D and COPD characteristics; and 3) the relationship between AMPs and susceptibility to future AECOPD.

Methods

Study population

An overview of the study participants in the BCCS and the BCES and samples included in the analyses is presented in table 1 and further summarised in the supplementary material. Detailed descriptions of the study design, inclusion/exclusion criteria, sampling and data collection for the BCCS and BCES have been published previously [22, 23]. In brief, included patients had a clinical diagnosis of COPD, a smoking history of $\geqslant$10 pack-years, a post-bronchodilator forced expiratory volume in 1 s (FEV_1) to forced vital capacity (FVC) ratio <0.7 and FEV_1 <80% predicted.

Written informed consent was obtained from all participants, and The Regional Committee for Medical Research Ethics, Western Norway, approved the study.

Data acquisition and measurements

A study physician examined all participants and performed a structured interview regarding smoking habits, comorbidities, general medical history (including history of AECOPD) and medication use at study visits every 6 months. Blood samples and induced sputum samples were collected at regular visits and during exacerbations. Spirometry was performed both pre- and post-inhalation of 0.4 mg salbutamol on a Viasys MasterScope (Viasys Healthcare, Hoechberg, Germany). Data sampling during exacerbations was performed as described previously [23].

Induced sputum

Induction was initiated by inhalation of 0.4 mg salbutamol, followed by inhalation of 5 mL hypertonic NaCl (3%) for 3×7 min from a Devilbiss UltraNeb 99 (DeVilbiss Healthcare, Somerset, PA, USA). Sputum supernatant was obtained after processing according to the whole sample method, aliquoted and kept frozen in −80°C freezers for later analyses of AMPs and inflammatory markers. Sputum culture was performed to assess NTHi colonisation (supplementary material).

AMPs, inflammatory markers and vitamin D

Sputum and plasma cathelicidin were measured by ELISA according to the manufacturer's protocol (Hycult Biotech, Uden, the Netherlands). SLPI was measured by an ELISA system developed at the Laboratory for Respiratory Cell Biology and Immunology, Leiden University Medical Centre, Leiden, the Netherlands [24]. Sputum samples were analysed for levels of the inflammatory markers interleukin (IL)-6, IL-8 and tumour necrosis factor-α (TNF-α) by a multiplex immunoassay (Bio-Plex Precision pro; Bio-Rad

TABLE 1 Overview of study participants and samples

	Bergen COPD Cohort Study		Bergen COPD Exacerbation Study
	Controls	COPD	COPD/AECOPD
Subjects	325	433	356/157
Induced sputum samples[#]	52	472	83
One sample per individual[¶] (% of subjects with ⩾1 sample)	45 (14)	255 (59)	56 (36)
Paired samples[+]	NA	40	40
Unpaired samples[§]	**45**	**215**	**56**
Sputum culture[#]	23	248	93
One sample per individual[¶]	23	163	64
NTHi-positive[¶]	NA	**44**	NA
Culture negative[¶,f]	NA	**112**	NA
Blood samples[##]	324	420	285
One sample per individual[¶]	324	**420**	143
Paired samples[+]	NA	131	131
Unpaired samples[§]	**324**	**289**	**143**

Data are presented as n, unless otherwise indicated. Bold formatting indicates the samples/populations selected for the main analyses. COPD: chronic obstructive pulmonary disease; AECOPD: acute exacerbation of chronic obstructive pulmonary disease; NTHi: non-typeable *Haemophilus influenzae*; NA: not assessed/not used in analyses. [#]: total number of samples, including repeated samples; [¶]: the first sample taken was selected to achieve one sample per individual; [+]: paired samples are from COPD patients who gave samples both in the stable phase and during AECOPD; [§]: the stable samples from COPD patients with paired samples were excluded to avoid paired samples in comparative analyses (stable COPD *versus* AECOPD); [f]: no potential airway pathogen grew in culture; [##]: collected at baseline and during AECOPD.

Laboratories, Inc., Hercules, CA, USA) as described previously [23]. Trained personnel performed a sputum differential cell count, including a neutrophil count (expressed as the percentage of total non-squamous cells). Vitamin D deficiency was defined as 25(OH)D levels <20 ng·mL^{-1}. For further description of blood sampling and vitamin D measurements, see supplementary materials. No samples had been previously thawed.

Statistical analyses

Stata 13.0 was used for analyses (StataCorp LP, College Station, TX, USA). For univariate comparisons of AMP levels across study groups, non-parametric tests were applied (supplementary materials).

Patient characteristics associated with cathelicidin levels in stable COPD with p<0.20 were selected and tested one by one in a linear regression model with log-transformed cathelicidin levels as a dependent variable, and retained in the multivariate model if p<0.20 (model 1). Generated coefficients were anti-logged and expressed as geometric mean (GM) ratios with 95% confidence intervals. Explanatory variables in model 1 (age, FEV_1, smoking habits and number of exacerbations the last year before baseline) were used to assess adjusted effect estimates of NTHi status and inflammatory markers on cathelicidin levels in separate models (model 2 and 3–6 respectively). Additional adjustment was done for the percentage of sputum neutrophils. Regression models were not built for SLPI, because SLPI showed few univariate associations with the variables investigated.

The predictive value of sputum and plasma cathelicidin levels (in quartiles) for risk of later exacerbations was analysed by Cox regression in patients with stable COPD. Models were adjusted for number of exacerbations the last year before baseline, age, sex, smoking habits, use of inhaled steroids and FEV_1.

Strategies used to build the multivariate models are given in more detail in the supplementary material. A significance level of 0.05 was used for all analyses.

Results

Controls were younger, more often current smokers and of normal weight compared to COPD patients (table 2).

Differences in levels of AMPs between controls, patients with stable COPD and those with AECOPD are shown in figure 1a and b. Plasma and sputum cathelicidin levels were higher in COPD compared to in controls, and further increased during AECOPD; no correlation was found between plasma and sputum levels (table 3). An inverse change in the sputum levels of the two AMPs was observed during AECOPD, which was also illustrated by their inverse correlation (supplementary figure S1) and inverse association with the percentage of sputum neutrophils (table 4, supplementary figures S2 and S3). AMP levels in controls are described in supplementary table S3 and the supplementary results.

TABLE 2 Baseline characteristics of participants in the Bergen COPD Cohort Study and the study sample used in analyses for sputum antimicrobial peptides

	Bergen COPD Cohort Study		Study sample used in analyses on sputum AMPs				
	Controls	COPD	Controls	Stable COPD	AECOPD	p-value[+]	p-value[§]
Subjects n	325	433	45	215	56		
Sex						0.97	0.63
Women	46	40	36	36	39		
Men	54	60	64	64	61		
Age years	58.6±9.8	63.5±6.9	54.1±8.4	63.3±7.0	62.6±6.3	<0.001	0.48
Smoking						<0.001	0.23
Ex	32	56	16	53	62		
Current	54	44	80	47	38		
Never	14	0	4	0	0		
BMI kg·m^{-2}						<0.001	0.07
Normal (18.5–24.9)	88	56	87	55	64		
Cachectic (<18.5)	5	28	2	31	16		
Obese (>30)	7	16	11	14	20		
FEV_1 % predicted	102±10	49±14	102±8	51±13	50±14	<0.001	0.52
Vitamin D ng·mL^{-1}	25.0±9.5	25.2±10.0	21.9±9.4	25.7±9.4	25.9±10.9	0.02	0.88
GOLD 2007 stage[#]							0.54
II		47		53	48		
III		42		41	41		
IV		11		7	11		
Exacerbation frequency[¶]							0.52
0–1		83		82	79		
2+		17		18	21		
Used inhaled steroids % yes		69		63	77		0.05
Resting $P_{a}O_2$ kPA		9.3±1.2		9.4±1.1	9.2±1.0		0.38
Hypoxemia % yes		13		11	11		0.88

Data are presented as mean±SD for continuous variables and as a percentage for categorical variables, unless otherwise indicated. COPD: chronic obstructive pulmonary disease; AMP: antimicrobial peptide; AECOPD: acute exacerbation of chronic obstructive pulmonary disease; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; GOLD: Global Initiative for Chronic Obstructive Lung Disease; $P_{a}O_2$: arterial oxygen tension. [#]: stage II: 50%⩽FEV_1<80% predicted, stage III: 30%⩽FEV_1<50% predicted, stage IV: FEV_1<30% predicted; [¶]: number of exacerbations the year before baseline; [+]: differences between stable COPD (n=215) and controls (n=45) were tested by t-test (age, 25(OH)D, FEV_1) or Chi-squared test (all other variables); [§]: differences between patients with stable COPD (n=215) and experiencing AECOPD (n=56) were tested by t-test (age, 25(OH)D, FEV_1, $P_{a}O_2$) or Chi-squared test (all other variables).

Relationship between AMP levels, COPD characteristics and inflammatory markers

The relationships between potential explanatory variables and sputum AMP levels in COPD patients are presented in tables 3 and 4. Similar relationships for the smaller subsample of paired samples of patients from whom samples were available both in the stable phase and during exacerbations are presented in supplementary table S1. Sputum cathelicidin levels were higher in ex-smokers and older patients in both stable COPD and during AECOPD, and in more advanced disease in the stable state. Furthermore, cathelicidin levels positively correlated with sputum inflammatory markers (IL-8, TNF-α, neutrophils) in the univariate analyses (table 4, supplementary figures S2 and S4). In contrast, few associations were found between plasma cathelicidin levels and COPD characteristics or inflammatory markers (supplementary table S2). When evaluating sputum SLPI in COPD patients, we found few associations with examined covariates except for an inverse correlation with the percentage of neutrophils (supplementary figure S3), a positive correlation with IL-6 (supplementary figure S5) and a trend for lower levels in patients hospitalised for AECOPD (table 3). Overall, the same trends for relationships between AMP levels and COPD were observed when restricting the analyses to the smaller group with paired samples (supplementary table S1).

Adjusted estimates of the effect of explanatory variables on sputum cathelicidin levels in stable COPD are presented in table 5. Overall, the results from the regression models were in line with the univariate findings presented in tables 3 and 4, although the effects of age and FEV_1 were attenuated after adjusting for covariates (table 5; models 1–5). The association between FEV_1 and cathelicidin levels was only statistically significant in the unadjusted analyses, thus could be better characterised as a trend. The association between sputum TNF-α and cathelicidin levels was only seen in patients with high levels of TNF-α (TNF-α >5 pg·mL^{-1}).

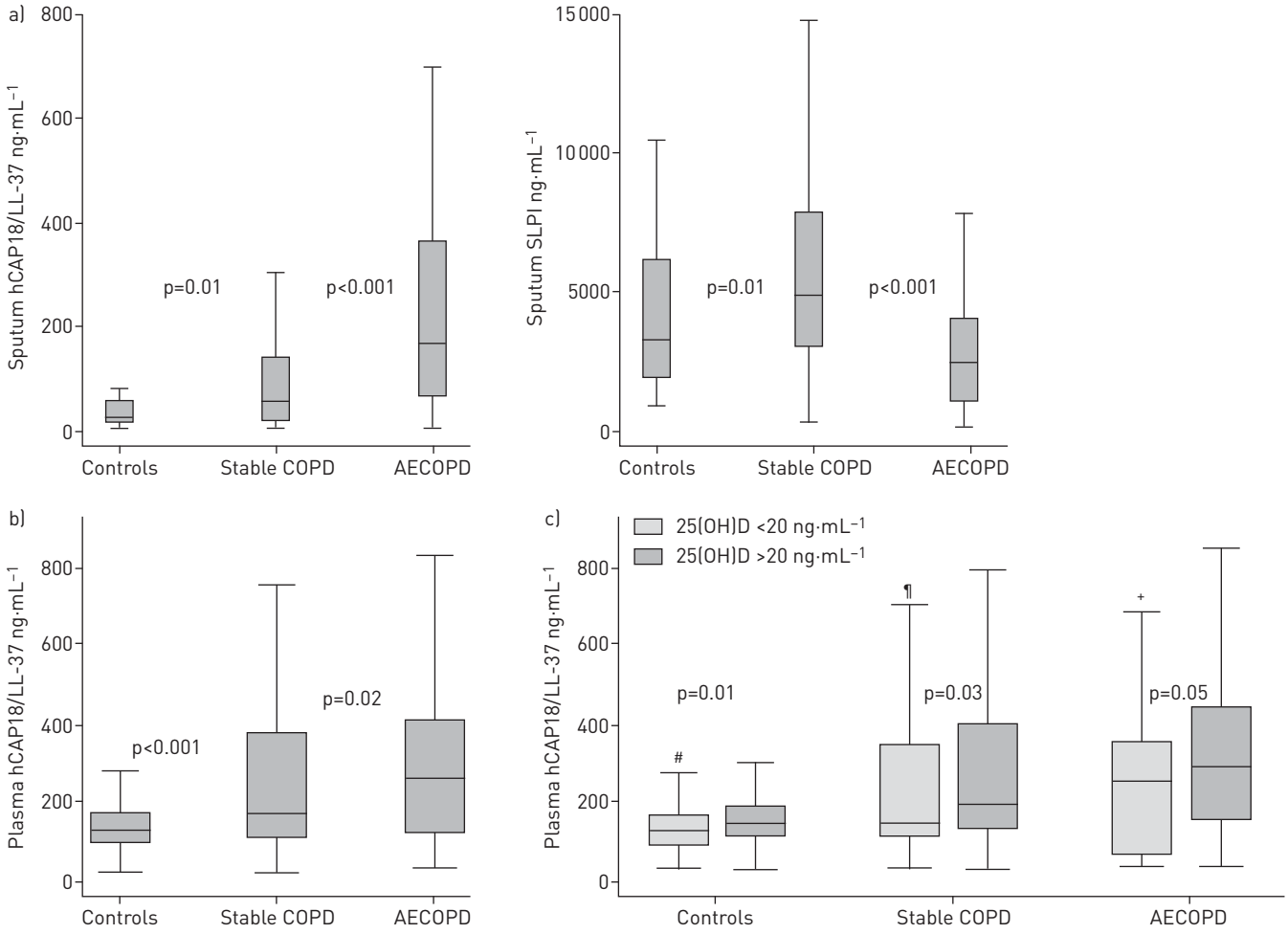

FIGURE 1 a) Sputum antimicrobial peptide levels by study categories: controls, stable chronic obstructive pulmonary disease (COPD) and acute exacerbations of chronic obstructive pulmonary disease (AECOPD) (excludes outside values). b) Plasma hCAP18/LL-37 levels by study categories: controls (n=316), stable COPD (n=274) and AECOPD (n=143) (excludes outside values). c) Plasma hCAP18/LL-37 levels in participants with or without vitamin D (25(OH)D) deficiency by study categories: controls (n=315), stable COPD (n=401) and AECOPD (n=143) (excludes outside values). hCAP18/LL-37: human cathelicidin; SLPI: secretory leukocyte protease inhibitor. #n=107; ¶n=131; +n=51.

Cathelicidin levels and vitamin D status

Correlation analyses showed weak positive correlations between serum 25(OH)D and plasma cathelicidin levels in controls (r=0.10, p=0.06) and in patients with stable COPD (r=0.11, p=0.04), which was an association not found in sputum. Participants with vitamin D deficiency showed significantly lower plasma cathelicidin levels across the three categories of controls, stable COPD and AECOPD (figure 1c). However, when testing vitamin D deficiency in linear regression models, its effect on plasma cathelicidin levels was only significant in AECOPD samples in an unadjusted analysis (GM ratio 0.70, 95% CI 0.51–0.94). This association was lost after adjustment for age, sex, smoking habits and FEV_1.

AMP levels and NTHi status

COPD patients with NTHi-positive sputum cultures did not differ from those with a negative sputum culture with respect to baseline FEV_1, sex, age or smoking habits (data not shown). The univariate comparison showed increased sputum cathelicidin levels in NTHi-positive patients with stable COPD (figure 2). The adjusted effect estimate indicated a more than two-fold increase (GM ratio 2.45, 95% CI 1.41–4.22) (table 5; model 2), although this was influenced by adjustment for the percentage of sputum neutrophils (GM ratio 1.62, 95% CI 0.97–2.70). No association between sputum SLPI levels and NTHi-positivity was observed.

Risk of exacerbations and cathelicidin levels

Among 219 patients for whom cathelicidin was measured in sputum, 156 had at least one later COPD exacerbation during follow-up. In the 377 patients for whom cathelicidin was measured in plasma at

TABLE 3 Sputum antimicrobial peptide levels by patient characteristics and their correlation with inflammatory markers in patients with chronic obstructive pulmonary disease

	Sputum hCAP18/LL-37 ng·mL^{-1}				Sputum SLPI ng·mL^{-1}			
	Stable COPD	p-value[#]	AECOPD	p-value[#]	Stable COPD	p-value[#]	AECOPD	p-value[#]
Subjects n	215		56		215		56	
Non-missing values for AMPs n	205		55		215		56	
Median (IQR)	58 (20–140)		169 (68–364)	<0.001	4915 (3080–7850)		2486 (1098–4059)	<0.001
Sex		0.17		0.50		0.41		0.36
Women	47 (17–135)		143 (56–313)		5302 (3300–8575)		2669 (1377–4573)	
Men	62 (23–148)		188 (70–400)		4858 (2833–7225)		2313 (819–3987)	
Age years		0.05		0.03		0.43		0.1
40–54	49 (13–104)		177 (68–266)		3631 (2310–7360)		3589 (2580–5000)	
55–64	46 (17–129)		97 (47–244)		5302 (3163–7225)		3252 (1141–4573)	
⩾65	67 (30–203)		214 (105–617)		4720 (3215–8730)		1548 (864–3029)	
Smoking		0.001		0.01		0.98		0.42
Ex	87 (24–187)		193 (93–447)		4828 (2790–8850)		1990 (1030–4240)	
Current	43 (17–93)		97 (33–226)		5010 (3175–7015)		3166 (1141–4130)	
GOLD 2007 stage[¶]		0.02		0.16		0.97		0.79
II	44 (16–108)		98 (45–305)		4870 (3400–7170)		2457 (1054–3804)	
III	63 (25–150)		189 (120–364)		5020 (2766–8880)		2814 (1377–5480)	
IV	105 (44–182)		232 (156–400)		4982 (2780–8635)		2313 (741–4420)	
Percentage sputum neutrophils[+]		<0.001		0.01		<0.01		0.07
0–59.99	21 (10–58)		73 (51–113)		4968 (3800–7360)		5398 (2842–5612)	
60–79.99	35 (16–93)		81 (47–156)		6045 (3785–11240)		3620 (2392–5768)	
80–89.99	93 (31–154)		143 (33–447)		4875 (3493–6185)		2580 (1600–3284)	
90–100	105 (37–292)		255 (128–590)		3930 (1849–7225)		1411 (696–3221)	
Percentage sputum eosinophils[+]		0.58		0.14		0.37		0.27
0–1.99	58 (21–154)		192 (68–400)		4858 (2807–7758)		2335 (908–4130)	
⩾2	63 (21–93)		93 (56–186)		5570 (3825–8850)		3220 (2180–5315)	
Exacerbation frequency[§]		0.18		0.09		0.37		0.55
0–1	58 (19–127)		120 (67–291)		4850 (3099–7360)		2703 (1098–4330)	
⩾2	58 (21–266)		295 (156–546)		5380 (2780–9545)		1990 (1137–3403)	
Use of inhaled and/or peroral steroids		0.87		0.47		0.15		0.14
No	58 (21–126)		113 (33–447)		4858 (3077–6025)		1141 (696–3284)	
Yes	58 (19–166)		172 (70–316)		5255 (3105–8990)		2620 (1377–4130)	
Hospitalization for AECOPD				0.17				0.06
No			143 (56–313)				2703 (1377–4240)	
Yes			179 (113–590)				719 (317–3720)	

Data are presented as median (IQR), unless otherwise indicated. hCAP18/LL-37: human cathelicidin; SLPI: secretory leukocyte protease inhibitor; COPD: chronic obstructive pulmonary disease; AECOPD: acute exacerbation of chronic obstructive pulmonary disease; AMP: antimicrobial peptide; IQR: interquartile range; GOLD: Global Initiative for Chronic Obstructive Lung Disease. [#]: differences were tested by non-parametric Mann–Whitney U and Kruskal–Wallis tests; [¶]: stage II: 50%⩽FEV$_1$<80% predicted, stage III: 30%⩽FEV$_1$<50% predicted, stage IV: FEV$_1$<30% predicted; [+]: missing values in 27 patients with stable COPD for analyses on hCAP18/LL-37, in 30 patients for analyses on SLPI, and in two patients during AECOPD for analyses on both AMPs; [§]number of exacerbations the year before inclusion.

TABLE 4 Correlation matrix between sputum AMPs and continuous variables by Spearman's rho

	Sputum hCAP18/LL-37 ng·mL^{-1}				Sputum SLPI ng·mL^{-1}			
	Stable COPD	p-value	AECOPD	p-value	Stable COPD	p-value	AECOPD	p-value
FEV$_1$ % predicted	−0.15	0.03	−0.26	0.07	−0.05	0.48	−0.08	0.60
Sputum hCAP18/LL-37 ng·mL^{-1}	1		1		−0.04	0.55	−0.48	<0.001
Plasma hCAP18/LL-37 ng·mL^{-1}[#]	−0.06	0.55	0.22	0.11	−0.13	0.19	0.22	0.11
Sputum inflammatory markers								
IL-6 pg·mL^{-1}[¶]	−0.03	0.64	−0.17	0.23	0.40	<0.001	0.55	<0.001
IL-8 pg·mL^{-1}[¶]	0.62	<0.001	0.36	<0.01	0.13	0.06	0.04	0.77
TNF-α pg·mL^{-1}[¶]	0.20	<0.01	0.36	<0.01	0.14	0.04	−0.12	0.36
Neutrophils %[+]	0.46	<0.001	0.40	<0.001	−0.24	<0.001	−0.42	<0.01

hCAP18/LL-37: human cathelicidin; SLPI: secretory leukocyte protease inhibitor. COPD: chronic obstructive pulmonary disease; AECOPD: acute exacerbation of chronic obstructive pulmonary disease; FEV$_1$: forced expiratory volume in 1 s; IL: interleukin; TNF-α: tumour necrosis factor-α. [#]: sputum and plasma samples were available at the same time point in 86 patients with stable COPD and 55 with AECOPD; [¶]: missing values in six patients (both stable and during AECOPD) for analyses on both antimicrobial peptides. [+]: missing values in 27 patients with stable COPD for analyses on hCAP18/LL-37, in 30 patients for analyses on SLPI, and in two patients during AECOPD for analyses on both antimicrobial peptides.

baseline, 300 had later exacerbations. Patients within the highest quartile of sputum cathelicidin levels had shorter median time to next exacerbation and had a higher risk of exacerbation compared to patients within the lowest quartile (table 6; hazard ratio 1.64, 95% CI 1.01–2.65). The data indicated a linear relationship across categories of sputum cathelicidin for this outcome. In contrast to sputum, plasma cathelicidin concentration was not related to the risk of later exacerbations.

Discussion

This is the first study to characterise AMP levels in a larger study population together with a wide range of explanatory variables. Associations between demographics, inflammation, infection and AMP levels have not previously been investigated concomitantly. Here, we show that COPD patients have an altered innate immune response as reflected by correlation patterns between sputum AMPs (cathelicidin and SLPI) and sputum inflammatory markers (neutrophils, IL-6, IL-8, TNF-α), and higher AMP levels compared to controls. When compared to stable COPD, AECOPD induced further changes in sputum AMPs, with a significant increase in cathelicidin levels and a correlated decrease in SLPI levels. In adjusted analyses on patients with stable COPD, higher cathelicidin levels were associated with NTHi colonisation, higher age, ex-smoking and an activated airway innate immune response as measured by higher sputum IL-8 and TNF-α levels, and a higher percentage of neutrophils. Interestingly, high sputum cathelicidin levels in the stable phase were predictive of a higher risk of AECOPD during follow-up.

Inflammatory pathways in COPD include activation of both the innate and adaptive immune system, with a prominent role of epithelial cells, macrophages and neutrophils [4]. Neutrophil activation during periods of active inflammation not only induces the release of neutrophil-derived serine proteinases, but also of cathelicidin. Therefore, neutrophils are likely the primary source of cathelicidin levels during acute inflammation, whereas production by airway epithelial cells and macrophages may be more prominent in stable conditions [5]. Moreover, cathelicidin may attract neutrophils directly or indirectly by increased IL-8 expression, and direct macrophage differentiation towards a pro-inflammatory phenotype [25]. Our data support a possible pro-inflammatory role for cathelicidin driven by neutrophilic inflammation, as reflected by higher sputum levels in both COPD compared to controls and during AECOPD compared to stable COPD. These results are consistent with previous studies [15–17, 20, 21]. Cathelicidin levels in the plasma changed in a similar pattern to those in the sputum, but its levels in the two compartments were not correlated. This could indicate that both parallel and independent mechanisms determine cathelicidin airway and plasma levels. The lack of correlation may also result from a higher variability in sputum levels owing to the complex composition of sputum, which is known to interfere with the (immunoassay-based) detection of cathelicidin.

The active vitamin D metabolite 1,25$(OH)_2$D induces cathelicidin expression in various cell types, including macrophages, neutrophils [6] and airway epithelial cells [7]. We found lower plasma cathelicidin levels in participants with vitamin D deficiency, which supports previous *in vitro* studies [6, 7]. Lack of this association in sputum may imply dominance of other pathways/sources of cathelicidin production in an inflammatory state, higher variability of sputum *versus* plasma levels as discussed previously, or that circulating 25(OH)D only poorly predicts local airway vitamin D levels and/or airway responses to vitamin D.

SLPI was initially discovered as an inhibitor of serine proteinases such as neutrophil elastase (NE), and was subsequently found to display broad-spectrum antimicrobial activity [8]; properties that may be highly

TABLE 5 Crude and adjusted geometric mean ratio estimates from linear regression models in patients with stable chronic obstructive pulmonary disease

	Subjects n	Sputum hCAP18/LL-37 ng·mL^{-1}					
		Crude GM ratio	p-value	Adjusted GM ratio [#]	p-value	Adjusted GM ratio [¶]	p-value
MODEL 1							
Age per 10 years	205	1.49 (1.14–1.95)	<0.01	1.03 (1.00–1.06)	0.04	1.24 (0.94–1.63)	0.12
FEV1 % predicted per 10% decrease	205	1.15 (1.0–1.31)	0.05	1.09 (0.95–1.25)	0.20	0.97 (0.84–1.12)	0.68
Smoking							
Ex	116	1		1		1	
Current	89	0.57 (0.39–0.82)	<0.01	0.63 (0.43–0.91)	0.01	0.66 (0.45–0.96)	0.03
Exacerbation frequency[+]							
0–1	168	1		1		1	
⩾2	37	1.49 (0.92–2.41)	0.11	1.40 (0.87–2.25)	0.17	1.58 (0.97–2.57)	0.07
MODEL 2							
Sputum culture							
Negative	94	1		1		1	
NTHi-positive	38	2.25 (1.31–3.88)	<0.01	2.45 (1.42–4.22)	0.001	1.62 (0.97–2.70)	0.07
MODELS 3–5							
Percentage sputum neutrophils per 10%	178	1.41 (1.25–1.59)	<0.001	1.39 (1.22–1.57)	<0.001		
Sputum IL-8 per 100 pg·mL^{-1}	199	1.03 (1.02–1.04)	<0.001	1.03 (1.02–1.03)	<0.001	1.03 (1.02–1.04)	<0.001
Sputum TNF-α pg·mL–1							
0–0.99	127	1		1			
1–4.99	52	0.93 (0.62–1.40)	0.73	0.89 (0.59–1.33)	0.57	0.91 (0.61–1.27)	0.66
⩾5	20	5.26 (2.90–9.55)	<0.001	4.25 (2.32–7.79)	<0.001	3.48 (1.77–6.84)	<0.001

Data are presented as mean GM ratio (95% CI) unless otherwise indicated. hCAP18/LL-37: human cathelicidin; GM: geometric mean; FEV1: forced expiratory volume in 1 s; NTHi: non-typeable *Haemophilus influenzae*; IL: interleukin; TNF-α: tumour necrosis factor-α. [#]: models adjusted for age, smoking habits, FEV1 and number of exacerbations the year before inclusion (⩾2; yes/no); [¶]: models adjusted for age, smoking habits, FEV1, number of exacerbations the year before inclusion (⩾2; yes/no) and sputum neutrophils (%); [+]: number of exacerbations the year before inclusion.

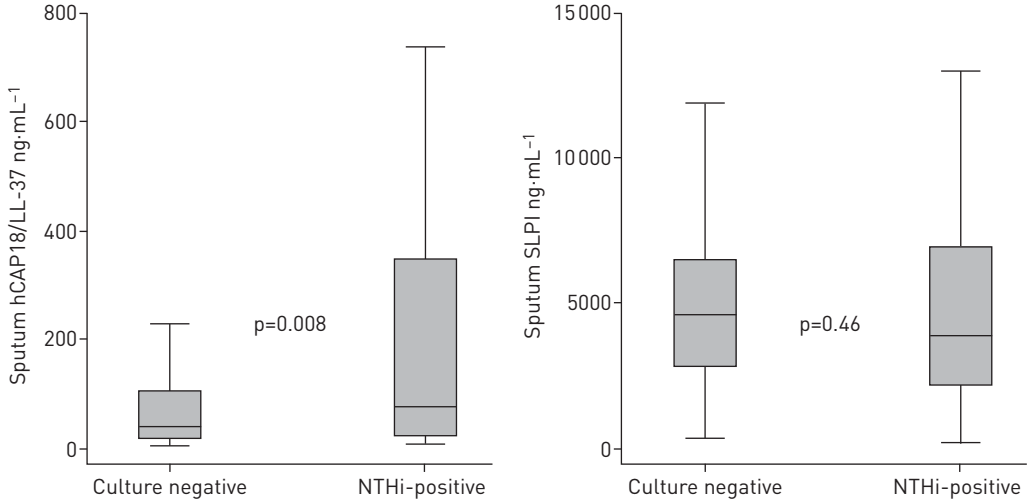

FIGURE 2 Sputum antimicrobial peptide levels in patients with stable chronic obstructive pulmonary disease with non-typeable *Haemophilus influenzae* (NTHi)-positive sputum culture *versus* patients with negative culture (excludes outside values). hCAP18/LL-37: human cathelicidin; SLPI: secretory leukocyte protease inhibitor.

relevant *in vivo* in view of the fact that SLPI is present in high concentrations in mucosal secretions compared to other AMPs [21]. Interestingly, the SLPI target NE was found to increase SLPI gene expression in airway epithelial cell cultures [26, 27], and elevated SLPI levels in bronchoalveolar lavage have been reported in emphysema [28]. Similar to a previous study [18], we report higher sputum SLPI levels in COPD patients compared to in controls, which indicates an amplified host anti-protease defence to inflamed COPD airways. However, several studies describe SLPI deficiency or inactivation of its anti-protease activity during neutrophilic inflammation with concurrent high levels of NE [13, 14, 29]. Mechanisms proposed to explain this finding include extracellular cleavage of SLPI by proteases [13, 14], and extracellular adherence of SLPI–NE complexes to negatively charged cell membranes [26]. In line with this, we observed reduced sputum SLPI levels during AECOPD, and a negative correlation with both the percentage of sputum neutrophils and cathelicidin levels. Also, the system may be overwhelmed during AECOPD so that there can be no further increase in SLPI, which could make the epithelial surface of the lung even more vulnerable to

TABLE 6 Risk of exacerbations by sputum and plasma hCAP18/LL-37 levels, estimated by median time to next exacerbation and Cox regression analyses in patients with chronic obstructive pulmonary disease

	Subjects n[¶]	Median time	Crude estimate			Fully adjusted model[#]		
		days	HR	95% CI	p-value	HR	95% CI	p-value
Sputum hCAP18/LL-37								
Per 100 (ng·mL^{-1})	219		1.03	1.00, 1.07	0.06	1.04	1.00, 1.07	0.04
Quartiles								
Q1 (lowest)	55	399	1			1		
Q2	55	330	0.99	0.62, 1.58	0.98	1.1	0.67, 1.8	0.7
Q3	55	229	1.27	0.81, 2.00	0.29	1.42	0.88, 2.31	0.15
Q4 (highest)	54	224	1.58	1.02, 2.46	0.04	1.64	1.01, 2.65	0.04
p trend[+]					0.02			<0.01
Plasma hCAP18/LL-37								
Per 100 (ng·mL^{-1})	377		1.03	0.98, 1.09	0.21			
Quartiles								
Q1 (lowest)	95	432	1			1		
Q2	95	474	0.86	0.62, 1.20	0.37	0.98	0.70, 1.37	0.89
Q3	93	532	0.77	0.54, 1.06	0.11	0.89	0.63, 1.27	0.53
Q4 (highest)	94	284	1.07	0.77, 1.49	0.68	1.25	0.89, 1.75	0.19
p trend[+]					0.90			0.30

HR: hazard ratio; hCAP18/LL-37: human cathelicidin. [#]: estimate of Cox regression model adjusted for number of exacerbations the year before inclusion, age, sex, smoking habits, use of inhaled steroids and forced expiratory volume in 1 s; [¶]: analyses on plasma included a total of 377 participants, with 219 participants included for the analyses on sputum (crude and adjusted analyses); [+]: P trend was calculated by incorporating the categorised variable as a linear predictor in Cox regression models.

damage. Similarly, SLPI deficiency is previously described in COPD patients colonised with *Moraxella catarrhalis* [19], and in AECOPD induced by a viral or bacterial infection [19–21, 29].

The multivariable analyses portray a situation in which higher cathelicidin levels are related to more severe disease, frequent exacerbations, neutrophilic inflammation and increased levels of the cytokines IL-8 and TNF-α. Golec *et al.* reported similar sputum cathelicidin levels across smoking habits in controls and COPD patients (n=30, early stage COPD) [15], whereas we found higher levels in ex-smoking COPD patients. This discrepancy may be due to the increased power in our study, to the fact that smoking cessation is more frequent in advanced-stage disease, or to the fact that smoking suppresses AMP production, as shown for the AMP human β-defensin-2 [30]. Even though findings held up after adjustment for the percentage of sputum neutrophils, and ex- and current smokers showed similar disease severity, another explanation of cathelicidin release is sustained inflammation in ex-smokers.

In COPD, microbial stimulation of innate immunity is likely an important contributor to inflammation, accompanied by increased neutrophil-derived cathelicidin levels, which may lead to more severe disease. *H. influenzae* (NTHi) is frequently isolated from the airways in both stable COPD and during infection [31], and may contribute to this stimulation. In accordance, it has been shown that bacterial eradication following treatment of an exacerbation is accompanied by resolution of inflammation [32]. Parameswaran *et al.* showed that the acquisition of airway pathogens (NTHi and *M. catarrhalis*) in the event of AECOPD, but not in colonisation, correlated with increased cathelicidin levels compared to a culture-negative state [20]. Another study reported an increase in cathelicidin levels after experimental inoculation of rhinovirus in 20 COPD patients [21]. In contrast to Parameswaran *et al.*, NTHi colonisation in our COPD patients was associated with a more than two-fold increase in cathelicidin levels compared to those in a culture-negative state. We observed a higher percentage of sputum neutrophils and higher IL-8 levels in NTHi-positive patients (data not shown), which support the notion that microbe stimulus is a powerful inducer of inflammation and subsequent cathelicidin release.

Previous studies have linked higher sputum IL-6 and IL-8 [33] and lower SLPI [34] levels in stable COPD to increased risk of exacerbations, while a potential predictive value of high cathelicidin has not previously been investigated.

In the present study, having high sputum cathelicidin levels in the stable state increased the risk of AECOPD during follow-up, and these levels further increased during AECOPD. This may imply that altered lung microbiota, immune and/or inflammatory responses contribute to increased susceptibility to infection in COPD [35, 36]. Also, local factors in COPD airways could inhibit cathelicidin antimicrobial activity, thus explaining the occurrence of pathogen-induced AECOPD despite increased levels of this AMP. Such factors could be products from cells and microbes [10], mucus [12], modification of cathelicidin by citrullination [11] and reduced pH [37].

Strengths of the present study are the larger sample size compared to the few previous studies, a standardised collection of data, and a well-characterised study population. In addition, the included covariates and the risk of later exacerbations in relation to AMP levels have not previously been investigated. However, we acknowledge methodological shortcomings of using sputum culture to assess airway microorganisms compared to new culture-independent techniques that are more sensitive [35], and the lack of sputum culture for all samples. We acknowledge discrepancies in absolute sputum AMP levels in this study compared to previous studies; these may be related to the complex composition of sputum, which can interfere with the detection of AMPs as mentioned previously. Despite attempts to include some never-smoked controls, the number is less than would be expected from the general population, reflecting that our control group was not a random sample from the general population. In addition, more sputum samples of adequate quality were obtained from current smoking controls compared to non-smokers, which may reflect that non-smokers do not as easily produce sputum. However, this reduces the generalisability of our results to controls in a general population and limits comparative analyses between controls and COPD. Also, compared to the COPD group, our control group was smaller, and did not completely match COPD patients with respect to age and body composition.

Furthermore, we cannot exclude a selection bias between participants with or without available sputum samples; patients that agreed to sputum induction may represent a group with lower symptom burden (refusal of induction was the main reason that not all patients were tested). Finally, despite the strength of paired samples in COPD patients (stable phase and during AECOPD), one single measurement of each AMP does not allow us to study the reproducibility of AMP patterns or longitudinal changes.

In summary, our data suggest a role for cathelicidin in the crosstalk between host microorganisms, immune responses and inflammation in COPD. Monitoring cathelicidin levels in COPD may be helpful to distinguish deranged lung homeostasis, and thus possibly serve as a prognostic marker. We confirm

previous findings of an AECOPD-associated decrease in SLPI levels, and add new knowledge on how alterations in AMP levels are related to demographics, inflammation and failure to protect the host from infection.

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