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## Antimicrobial peptides, disease severity and exacerbations in Bronchiectasis

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**Author's contributions:**

Study design: OS, SA, JDC. Patient recruitment and data collection: OS, LP, AS, GSC, ART, FB, SA, JDC. Performed experiments and sample processing; LP, EC, AS, DC, AS, HRK, MO, SO, SV. Writing the manuscript: OS, LP, JDC. Revising of the manuscript and approval of submission: all authors. Responsible for the overall content as guarantors: OS, JDC.

**Short title:** Antimicrobial peptides in Bronchiectasis

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3 **Key messages**  
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8 **What is the key question?** Is there a dysregulation in pulmonary and systemic  
9 antimicrobial peptides (AMP) associated with the frequent exacerbator phenotype in  
10 bronchiectasis?  
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17 **What is the bottom line?** Frequent exacerbators have dysregulation of AMPs with  
18 particularly high levels of the cathelicidin LL-37 and low levels of secretory leukocyte  
19 protease inhibitor (SLPI) in sputum, which could predict time to next exacerbation and  
20 the frequency of exacerbations during follow-up.  
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28 **Why read on?** This is the first study to describe AMP dysregulation in the frequent  
29 exacerbator phenotype in bronchiectasis.  
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## ABSTRACT

**Rationale:** Recently a frequent exacerbator phenotype has been described in bronchiectasis but the underlying biological mechanisms are unknown. Antimicrobial peptides (AMP) are important in host defense against microbes but can be proinflammatory in chronic lung disease.

**Objectives:** To determine pulmonary and systemic levels of AMP and their relationship with disease severity and future risk of exacerbations in bronchiectasis.

**Methods:** A total of 135 adults with bronchiectasis were prospectively enrolled at three European centers. Levels of cathelicidin LL-37, lactoferrin, lysozyme and Secretory Leukocyte Protease Inhibitor (SLPI) in serum and sputum were determined at baseline by ELISA. Patients were followed-up for 12 months. We examined the ability of sputum AMP to predict future exacerbation risk.

**Measurements and main results:** AMP levels were higher in sputum than in serum, suggesting local AMP release. Patients with more severe disease at baseline had dysregulation of airway AMP. Higher LL-37 and lower SLPI levels were associated with bronchiectasis severity index (BSI), lower FEV<sub>1</sub> and *Pseudomonas aeruginosa* infection. Low SLPI levels were also associated with the exacerbation frequency at baseline. During follow-up, higher LL-37 and lower SLPI levels were associated with a shorter time to the next exacerbation, while LL-37 alone predicted exacerbation frequency over the next 12 months.

**Conclusions:** Patients with bronchiectasis showed dysregulated sputum AMP levels, characterized by elevated LL-37 and reduced SLPI levels in the frequent exacerbator phenotype. Elevated LL-37 and reduced SLPI levels are associated with *Pseudomonas aeruginosa* infection and can predict future risk of exacerbations in bronchiectasis.

## INTRODUCTION

Bronchiectasis is a chronic inflammatory lung disease characterized by permanent dilatation of the bronchi. Most patients suffer daily cough and sputum production and some of them experience frequent exacerbations (1). A frequent exacerbator phenotype has recently been described, in which patients consistently have multiple exacerbations over time and this condition independently predicts worse clinical outcomes including increased mortality (2). The underlying biological mechanisms leading to frequent exacerbations in this group have not yet been identified, but frequent exacerbators appear to have greater neutrophilic inflammation and are more susceptible to infection with bacteria, particularly *Pseudomonas aeruginosa* (3, 4).

Antimicrobial peptides (AMP) are important in host defense against pathogenic microbes in the lung (5). Among the most important and abundant antimicrobial peptides in the airway are lysozyme, lactoferrin and the cathelicidin LL-37 (which are proinflammatory mediators released from activated neutrophils, macrophages and bronchial epithelium) and Secretory Leukocyte Protease Inhibitor (SLPI; produced by respiratory epithelial cells and mostly anti-inflammatory) (6, 7). AMP function through multiple mechanisms including protein degradation (lysozyme), nutrient depletion (lactoferrin), cellular disruption and lysis, and inhibition of virulence factors (LL-37, SLPI) (8, 9). AMP are designed to protect against bacterial infection but in the context of chronic lung inflammation bacteria such as *P. aeruginosa* adapt to resist AMP killing, changing methods of iron acquisition and variations in biofilm-associated polysaccharides (10). The result may be an ineffective and excessive AMP response that stimulates inflammation without achieving bacterial clearance (11).

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3 A consistent finding in bronchiectasis is the persistence of bacterial infection  
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5 despite an excessive inflammatory response which instead of achieving bacterial  
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7 clearance results in host damage (3, 12). The contribution of AMP to the vicious cycle  
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9 of bronchiectasis has not been previously explored. We therefore hypothesized that  
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11 AMP are altered in sputum from patients with bronchiectasis and may correlate with  
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13 disease severity and predict future risk of exacerbations.  
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## 19 **METHODS**

### 20 **Study design and Ethics**

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24 This was an international, multicenter, prospective, observational study which  
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26 included consecutive adults with bronchiectasis. The study protocol was approved by  
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28 local institutional review board and all subjects gave signed informed consent.  
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### 33 **Participants**

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36 Patients were recruited from three regional specialist bronchiectasis clinics at the  
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38 Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), Ospedale Maggiore Policlinico  
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40 (Milano, Italy) and Ninewells Hospital (Dundee, UK).  
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43  
44 Bronchiectasis was defined by the presence of bronchial dilatation on high-  
45  
46 resolution CT scanning with a compatible clinical history of cough, sputum production  
47  
48 and/or recurrent respiratory infections. Patients with cystic fibrosis, primary  
49  
50 immunodeficiency, active malignancy, active allergic bronchopulmonary aspergillosis,  
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52 interstitial lung disease, active mycobacteria disease, HIV infection and long-term oral  
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54 corticosteroid treatment were excluded.  
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### 58 **Clinical assessments**

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3 All patients were clinically stable, defined by the absence of an exacerbation that  
4 required antibiotic treatment within 30 days. Frequent exacerbators were defined as 3 or  
5 more exacerbations per year at baseline. Demographic data, number of exacerbations in  
6 the previous year, relevant comorbid conditions and current treatments were recorded.  
7  
8 Exacerbations were recorded as moderate (treated with antibiotics but not requiring  
9 hospitalization) or severe (requiring hospitalization or intravenous antibiotics). All  
10 patients underwent spirometry (13). The underlying etiology of bronchiectasis was  
11 determined after testing recommended by current guidelines (1, 14). The Bronchiectasis  
12 Severity Index (BSI) and FACED scores were calculated (15, 16). Severe disease was  
13 considered when BSI score was  $\geq 9$  points and FACED score was  $\geq 5$  points.  
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### 28 **Longitudinal outcomes**

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30 Patients were followed-up for one year from the time of sputum and serum  
31 collection. During follow-up, patients were visited every 6 months as part of routine  
32 clinical practice at the study centers. Time to first exacerbation was calculated as the  
33 time from sampling to the first day of antibiotic administration because of an  
34 exacerbation.  
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### 44 **Bacteriology**

45  
46 All bacteriology was performed on spontaneous early-morning sputum samples.  
47 Sputum was separated from saliva and the sample split for bacteriology and assessment  
48 of AMP levels. Samples were processed for bacteriology as previously described (17).  
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### 56 **AMP and inflammatory mediator measurement**



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3 Methods of sputum and blood processing were standardized across the 3 sites.  
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5 Sputum samples were processed by ultracentrifugation at 50,000g for 90 minutes  
6  
7 followed by careful extraction of the supernatant. Samples were processed within 2  
8  
9 hours of expectoration and immediately frozen at -80C.  
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11

12 Serum and sputum Lactoferrin, Lysozyme (AssayPro, St. Charles, MO, USA),  
13  
14 LL-37 (Hycult Biotech, Plymouth, PA, USA), SLPI and Matrix metalloproteinase 9  
15  
16 (MMP-9) (R&D Systems, Minneapolis, MN, USA) levels were measured by validated  
17  
18 commercially available ELISA kits. Sputum samples were diluted 1/25000 for  
19  
20 Lactoferrin and Lysozyme, 1/20 for LL-37 and 1/2000 for SLPI assays. The limits of  
21  
22 detection were 0.625 ng/ml for lactoferrin, 0.0781 ng/ml for lysozyme, 0.14 ng/ml for  
23  
24 LL-37 and 0.025 ng/mL for SLPI. Neutrophil elastase activity in sputum supernatants  
25  
26 was measured by activity based immunoassay (ProAxis LTD, Belfast, UK) as  
27  
28 previously described (18).  
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### 35 **Western Blot**

36  
37 To investigate the degradation of SLPI in bronchiectasis airway sputum the  
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39 samples were subjected to denaturing gel electrophoresis on 15% SDS-PAGE gels  
40  
41 before being transferred to nitrocellulose. After blocking with 5% skimmed milk in  
42  
43 PBST the membranes were incubated overnight in anti-SLPI antibody at 0.1µg/ml  
44  
45 (R&D cat no AF1274) at 4°C. After incubation with Anti-goat HRP (BioRad, Watford  
46  
47 (UK) cat no 1721034) at 1/2500 dilution for 1 hour the blot was washed thoroughly in  
48  
49 PBST before SLPI proteins were visualized using Millipore Immobilon Western  
50  
51 Chemiluminescent HRP substrate (cat no WBKLS0100) used as per manufacturers  
52  
53 recommendation. A representative autoradiographic film is shown.  
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### **Air-liquid interface nasal epithelial cell culture**

Nasal basal epithelial cells obtained from healthy and bronchiectasis patients were grown and seeded into 24-well transwell inserts at p2 or p3. Cells were fully differentiated into ciliated air-liquid interface cultures using Pneumocult ALI+ media (Stemcell technologies, Cambridge UK). Cells were apically treated with 100µl/well of PBS (control), Neutrophil Elastase at 3µg/ml (Sigma, Elastase from human leukocyte, E8140-1UN), with or without elastase inhibitor (AstraZeneca, AZD9668) at 10nM (a dose sufficient to completely block elastase activity as determined in preliminary experiments) or inhibitor alone. After 30mins of incubation at 37°C, apical supernatants were collected. An additional 100µl/well PBS used to wash the apical surface of the cells and added to the corresponding supernatants. Baseline characteristics of healthy controls are described in the Methods section of the online data supplement.

### **Statistical analysis**

Statistical analysis was performed using SPSS version 22. Categorical variables are presented as frequencies and percentages and comparisons performed using  $\chi^2$  or Fisher exact test when required. Continuous variables are presented as mean and standard deviation (SD) in parametrical variables and median and interquartile range (IQR) in non-parametrical ones. Differences were analysed using Student t-test, ANOVA test or their corresponding non-parametrical test when required (Kruskal-Wallis and Mann-Whitney tests). The relationship between linear variables was studied using linear regression and Spearman rank correlation (rho and p-value), with the latter included because relationships between inflammatory biomarkers were not assumed to be linear. Data are reported with the rho and p-values derived from the Spearman rank correlation. The contribution of different proteases to SLPI levels was studied with

multiple linear regression. Exacerbation rate was analyzed using a negative binomial model with time in study as an offset. Time to first exacerbation was modeled using Cox's proportional hazard regression. Exacerbation rate and time to first exacerbation were adjusted for BSI, site and fully adjusted for bronchiectasis severity, gender, aetiology, inhaled corticosteroid use and site. Patients lost to follow-up or dying within the 1 year follow-up were censored in the analysis. Discrimination between groups for prediction of hospitalization for severe exacerbations was tested using the area under the receiver operator characteristic curve. A p value <0.05 was considered significant.

## RESULTS

### Patient characteristics

A total of 135 adults with stable bronchiectasis were included in the study. **Table 1** shows the baseline characteristics of the population. Mean age was 69 ( $\pm 10$ ) years and 56% were females. The majority of patients had idiopathic or post-infective bronchiectasis and the mean BSI score was 8.0 ( $\pm 4.3$ ) and FACED score was 2.4 ( $\pm 1.5$ ), indicating moderate to severe bronchiectasis. Most of the patients (65%) were receiving inhaled long-acting bronchodilator treatment, whereas 43% were receiving inhaled corticosteroid treatment and 4% were receiving inhaled antibiotics. 322 exacerbations were recorded in the previous year, including 32 (10%) that require hospitalization. 68 patients (50.4%) were frequent exacerbators defined as 3 or more exacerbations per year at baseline. Frequent exacerbators had worse lung function and more severe disease compared to non-frequent exacerbators (see online table E1).

### AMP levels in sputum and blood

1  
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3 All measured AMP in patients with bronchiectasis were higher in sputum than in  
4  
5 serum. Lactoferrin was the AMP with highest pulmonary expression, with a median  
6  
7 (IQR) of 114.6 (45.7-250.6)  $\mu\text{g/ml}$ . In serum, lactoferrin levels were 4.3 (2.3-6.5)  
8  
9  $\mu\text{g/ml}$ . Lysozyme was the AMP with highest systemic expression with a median (IQR)  
10  
11 of 5.8 (4.4-7.3)  $\mu\text{g/ml}$ . In sputum, lysozyme levels were 68.9 (42.1-105.2)  $\mu\text{g/ml}$ . LL-37  
12  
13 levels were 1444 (28.1-8054) ng/ml in sputum and 328.3 (204.3-533.1) ng/ml in serum  
14  
15 while SLPI levels were 536.4 (161.7-2729) ng/ml in sputum and 120.8 (85.6-152.2)  
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17 ng/ml in serum.  
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21 No correlations between pulmonary and systemic AMP levels were found.  
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23 Spearman rank correlation between sputum and serum lactoferrin was  $\rho=-0.05$   
24  
25 ( $p=0.59$ ), between sputum and serum lysozyme was  $\rho=0.06$  ( $p=0.49$ ), between  
26  
27 sputum and serum LL-37 was  $\rho=0.08$  ( $p=0.36$ ), and between sputum and serum SLPI  
28  
29 was  $\rho=0.03$  ( $p=0.73$ ).  
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### 35 AMP levels and disease severity

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37 Patients with severe disease had elevated LL-37 levels and lower SLPI levels.  
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39 Using the BSI score, the highest sputum LL-37 levels were detected in patients with  
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41 severe BSI score in comparison to patients with moderate and mild disease (3835 (56.9-  
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43 13502) vs 108.2 (21.1-6456) vs 148.7 (17.3-2743) ng/ml,  $p=0.007$ ). Sputum SLPI levels  
44  
45 were the lowest in the most severe group in comparison to moderate and mild patients  
46  
47 (341.6 (88.8-1635) vs 608.5 (275.9-2678) vs 875.5 (444.1-7415) ng/ml,  $p=0.004$ )  
48  
49 (**Figure 1**). Using the FACED score, similar relationships were observed to those seen  
50  
51 with BSI although overall group differences were not statistically significant ( $p=0.05$   
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53 and  $p=0.1$  respectively, see online Figure E1). No correlation among systemic AMP  
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55 levels and disease severity were found.  
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3 A weak inverse significant correlation was also observed between sputum LL-37  
4 (rho=-0.25, p=0.004), lactoferrin (rho=-0.23, p=0.006) and lysozyme (rho=-0.19,  
5 p=0.02) with FEV<sub>1</sub>, whereas sputum levels of SLPI had a weak correlation (rho=0.17,  
6 p=0.05) (see online Figure E2). No correlations between serum determinations and lung  
7 function tests were observed.  
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12 Frequent exacerbators had significant lower airway SLPI compared to infrequent  
13 and non-exacerbators (350.2 (101.7-2583) vs 560.7 (269.2-2424) vs 934.3 (385.1-7206)  
14 ng/ml, p=0.01). Sputum lysozyme, lactoferrin and LL-37 were not associated with  
15 baseline exacerbation frequency (see online Figure E3). Systemic AMP levels were not  
16 associated with exacerbation frequency.  
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### 28 **AMP levels and airway infection**

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30 Sputum cultures were positive for bacteria in 86 patients (64%) and negative in  
31 49 (36%). *P. aeruginosa* was the most frequently isolated pathogen in sputum culture  
32 (n=39, 29%), followed by *Haemophilus influenzae* (n=31, 23%), *Staphylococcus aureus*  
33 (n=6, 4%), *Escherichia coli* (n=3, 2%), *Moraxella catharralis* (n=2, 1%),  
34 *Stenotrophomona* (n=2, 1%), *Streptococcus pneumoniae* (n=2, 1%) and *Serratia*  
35 *marcensens* (n=1, 0.7%). Clinical characteristics of patients grouped according to the  
36 presence of *P.aeruginosa*, other pathogens or negative sputum culture (who were  
37 considered non-infected) are showed online (see online Table E2).  
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49 When compared to patients infected by other pathogens and non-infected  
50 patients, bronchiectasis patients with *P.aeruginosa* showed significantly higher sputum  
51 LL-37 levels (3835 (72.2-12435) vs 2425 (36.2-9658) vs 57.8 (17.1-3651) ng/ml,  
52 p=0.002), higher sputum lactoferrin levels (190.7 (68.1-364.3) vs 99.2 (40.4-250.6) vs  
53 79.3 (45.7-201.1) ng/ml, p=0.04), and lower sputum SLPI levels (363.3 (103.7-739.7)  
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3 vs 753.3 (161.7-2624) vs 1066 (309.5-6245) ng/ml,  $p=0.001$ ) (**Figure 2**). No differences  
4  
5 in systemic AMP levels were found between patients infected by *P.aeruginosa*, patients  
6  
7 infected by other pathogens and non-infected patients.  
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10 No differences in sputum AMP levels were found when patients with and  
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12 without chronic macrolide treatment and inhaled corticosteroids were compared In  
13  
14 addition, no differences were found among patients with and without nebulized  
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16 antibiotics, although the small proportion of treated patients ( $n=6$ , 4%) did not exclude a  
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18 potentially effect (see online table E3).  
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### 23 **AMP and Elastase activity**

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26 A significant correlation between sputum LL-37 ( $\rho=0.36$ ,  $p=0.002$ ), sputum  
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28 lactoferrin ( $\rho=0.38$ ,  $p<0.001$ ), sputum lysozyme ( $\rho=0.26$ ,  $p=0.03$ ) and elastase  
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30 activity were found (see online Figure E4). A previous study identified that in cystic  
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32 fibrosis SLPI was degraded by neutrophil elastase into lower molecular weight cleavage  
33  
34 products (19), while a further study found degradation by MMP-9 (20). We  
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36 hypothesized that proteases activity in the bronchiectasis airway would be responsible  
37  
38 for lower levels of SLPI. We found, however, no significant correlation between SLPI  
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40 and elastase activity ( $\rho=-0.09$ ,  $p=0.46$ ) or MMP-9 ( $\rho=-0.13$ ,  $p=0.2$ ) (**Figure 3A and**  
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**3B**). In multiple linear regression incorporating elastase activity and MMP-9, proteases  
accounted for a maximum of 23% of the variance in SLPI levels.

By western blot, we identified a heterogeneous pattern of SLPI cleavage with no  
association between neutrophil elastase activity and SLPI cleavage pattern (**Figure 3C**).

Based on previous work that suggest neutrophil elastase can prevent release of  
SLPI from lung epithelial cells (21) we tested whether exogenous neutrophil elastase  
inhibited SLPI secretion from primary airway epithelial cells. We observed that nasal

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2  
3 epithelial cells from controls and bronchiectasis secreted similar amounts of SLPI  
4 (301.6 (167.4-676.4) vs 163.1 (128.8-340.4),  $p=0.46$ ) (**Figure 3D**). The addition of  
5  
6 exogenous elastase decreased the secretion of SLPI to 21.6 (13.8-37.7)% of the  
7  
8 secretion from cells treated with control buffer ( $p=0.03$ ). The NE inhibitor alone had no  
9  
10 significant impact on SLPI release, while the addition of NE and inhibitor still resulted  
11  
12 in inhibition of SLPI release suggesting that this effect was independent of NE protease  
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14 activity ( $p=0.028$ ) (**Figure 3E**).  
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### 22 **Longitudinal outcomes**

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24 132 out of 135 patients (98%) completed 12 months of follow-up. During this  
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26 time, a total of 310 exacerbations have been recorded in 102 patients, including 36  
27  
28 severe exacerbations. The median number of exacerbations per patient was 2.0. In  
29  
30 univariate analysis, patients with 3 or more exacerbations during follow-up had higher  
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32 levels of LL-37 (2976 (48.8-15472) vs 416.4 (21.8-7251) vs 61.4 (19.8-2865) ng/ml,  
33  
34  $p=0.02$ ) and lower levels of SLPI (316.3 (89.9-944.1) vs 753.3 (343.1-3914) vs 1261  
35  
36 (265.2-6521) ng/ml,  $p<0.001$ ) in comparison to those patients with 1 or 2 exacerbations  
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38 and patients without exacerbations (**Figure 4**).  
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43 In an exploratory analysis we examined outcomes for patients above and below  
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45 a cut-off representing the median of the population (to the nearest whole number).  
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47 Patients with LL-37  $\geq 1500$  ng/ml had a higher frequency of exacerbations (Incidence  
48  
49 Rate Ratio (IRR) 1.57, 95%CI 1.04-2.37,  $p=0.02$ ). Patients also had a shorter time to  
50  
51 next exacerbation with LL-37 levels above the median for the population (Hazard Ratio  
52  
53 (HR) 1.68, 95%CI 1.13-2.50,  $p=0.01$ ). These findings persisted after adjusting for BSI  
54  
55 and site (see table E4 in the online data supplement). LL-37 showed an area under the  
56  
57 curve (AUC) of 0.76 (0.65-0.86),  $p<0.0001$  for predicting hospitalization (see online  
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59  
60

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3 Figure E5).  
4

5 For SLPI, low levels were not significantly associated with a higher frequency of  
6 exacerbations (IRR 0.76, 95%CI 0.50-1.51,  $p=0.1$ ) but did demonstrate a shorter time to  
7 next exacerbation when a cut-off of 1000 ng/ml was used (HR 0.63, 95%CI 0.42-0.94,  
8  $p=0.02$ ) (**Figure 4**). After adjusting for BSI and site, a trend to predict the time to first  
9 exacerbation was observed (see online table E4). SLPI showed an AUC of 0.74 (0.62-  
10 0.86),  $p<0.0001$  for predicting hospitalization for severe exacerbation (see online Figure  
11 E5).  
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## 24 **DISCUSSION**

25 This study demonstrates that airway AMP, especially high LL-37 and low SLPI  
26 sputum levels are associated with disease severity, disease activity and airway infection  
27 in patients with bronchiectasis. In addition, patients with higher levels of LL-37 and  
28 lower levels of SLPI had a shorter time to the next exacerbation and an increase  
29 frequency of exacerbations during follow-up. The relationships between LL-37/SLPI  
30 and both prior and future exacerbations suggest that dysregulation of AMP may be one  
31 of the mechanisms underlying the frequent exacerbator phenotype in bronchiectasis.  
32 Our findings suggested that AMP may be ineffective as an innate host defense  
33 mechanism, especially in those bronchiectasis patients with severe disease and with  
34 chronic *P. aeruginosa* infection. This fact may contribute to maintaining a vicious cycle  
35 of increased inflammation without eliminating bacterial infection. If further validated in  
36 prospective larger studies, LL-37 and SLPI could represent novel biomarkers to predict  
37 future risk of exacerbations in patients with bronchiectasis.  
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55 AMP are important in host defense against bacteria, viruses and fungi (5). They  
56 are produced by respiratory epithelial cells, neutrophils and macrophages and play an  
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3 important role in innate lung defense (6, 8). Although no previous comprehensive  
4 studies of AMP in bronchiectasis have been conducted, our results complement and  
5 extend previous observations in other chronic airway diseases such as Chronic  
6 Obstructive Pulmonary Disease (COPD) and Cystic Fibrosis (CF). In COPD, increased  
7 sputum levels of LL-37 were associated with airflow limitation, health status and  
8 exercise tolerance (22), and lower sputum lysozyme and SLPI levels were detected in  
9 patients with bacterial colonization and during acute infectious exacerbations (23). In  
10 bronchiectasis, one previous study demonstrated that high levels of sputum LL-37 were  
11 detected in patients chronically infected with *P. aeruginosa* (24). In our study, we  
12 demonstrated that elevated sputum LL-37 and reduced sputum SLPI levels were  
13 associated with disease severity, worse lung function and the presence of airway  
14 infection, especially due to *P. aeruginosa*. In addition, sputum lactoferrin and lysozyme  
15 levels were also associated to worse lung function and sputum SLPI were lower in  
16 frequent exacerbators. Local production of AMP in the lung, probably related to chronic  
17 airway inflammation, is likely to be the key mechanism in bronchiectasis, since we  
18 observed much higher levels in sputum than in serum and no relationship between  
19 serum levels and disease severity.

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LL-37 is a human cathelicidin produced by neutrophils and epithelial cells in response to pro-inflammatory stimuli including cytokines, pathogen-associated molecular patterns and tissue injury (25). LL-37 displays antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and viruses, neutralizes LPS activity, and protects against endotoxic shock (26). In addition, it is involved in the regulation of inflammation, cell proliferation and apoptosis (27). In our study, we demonstrated that high pulmonary levels of LL-37 are associated to disease severity, worse lung function and airway infection. It may seem contradictory that the presence of airway infection

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3 and exacerbation risk is associated with higher levels of an AMP that should reduce  
4 infection. However, previous studies primarily in CF have demonstrated that the  
5 interaction of LL-37 with DNA and with glycosaminoglycan in the inflamed lung  
6 impairs the antibacterial function of LL-37 and its ability to neutralize LPS (28, 29).  
7  
8 LL-37 is therefore able to enhance mucus production and promote inflammation while  
9 losing its antimicrobial activity (30). These previous findings likely explain the high  
10 levels of LL-37 identified in our study and how LL-37 may contribute to poor  
11 outcomes.  
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21 Bacterial clearance is the primary function of AMP. However, some pathogens,  
22 especially *P. aeruginosa*, have developed different process of adaptation to innate  
23 immune clearance mechanisms in the lung, such as creation of biofilms, resistance to  
24 inflammasome-mediated clearance and resistance to AMP (11). A recent study  
25 evaluating *P. aeruginosa* populations using whole-genome sequencing in patients with  
26 bronchiectasis showed that *P. aeruginosa* populations adapt by accumulating loss-of-  
27 functions mutations. This fact leads to changes in phenotypes including different modes  
28 of iron acquisition and variations in biofilm-associated polysaccharides (10). These  
29 findings may explain why, despite an appropriate host response increasing AMP  
30 secretion, *P. aeruginosa* persists in the lungs and the elevated AMP levels could  
31 contribute to perpetuate an excessive inflammatory response, impacting negatively on  
32 clinical outcomes.  
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49 In contrast to the LL-37 data, in our study sputum levels of SLPI were lower in  
50 the most severe patients and in those infected by *P. aeruginosa*. The main function of  
51 SLPI is to protect local tissue from the detrimental consequences of inflammation, as a  
52 result of its anti-protease activity and anti-inflammatory properties (7, 31). Previous  
53 studies have demonstrated decreased levels of SLPI in patients with CF infected with *P.*  
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3 *aeruginosa* due to neutrophil elastase degradation (19). In keeping with the emerging  
4 data that CF and bronchiectasis have highly diverging pathophysiology we found a  
5 different relationship in bronchiectasis. SLPI levels were not related to sputum  
6 neutrophil elastase activity or MMP-9 levels. SLPI degradation was heterogeneous with  
7 multiple cleavage products and was unrelated to elastase activity in sputum. Most  
8 patients, even those with high levels of SLPI by ELISA, had evidence of SLPI  
9 degradation suggesting that functional SLPI deficiency may be present even in patients  
10 with preserved protein levels. Our data suggest that multiple proteases such as matrix  
11 metalloproteinases and cathepsins are likely contributing to SLPI deficiency in the  
12 airway. However, protease levels only accounted for a maximum of 23% of the variance  
13 in sputum SLPI levels, suggesting an important contribution from reduced SLPI  
14 production. In addition, our data demonstrates that elastase reduces release of SLPI  
15 from epithelial cells independent of its protease activity, as previous data suggested  
16 (21). Other studies have generated a variant of SLPI resistant to degradation by  
17 neutrophil elastase (32). However, our data has important therapeutic implications since  
18 neutrophil elastase inhibitors that are in development for the treatment of bronchiectasis  
19 would not be expected to restore normal SLPI function in view of our findings.  
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42 Our study has limitations. First, this study was exploring multiple biomarkers  
43 and so we acknowledge the risk of spurious associations as a result of multiple  
44 statistical comparisons. We addressed this, however, by looking at baseline data with  
45 multiple markers and then focusing our prospective follow-up study on LL-37 and SLPI  
46 only, markers which had shown a clear association with disease severity at baseline.  
47 The finding of consistent results during follow-up makes spurious associations very  
48 unlikely. Our results were consistent, biologically plausible and robust across all studied  
49 AMP.. Second, the absence of a control group prevents the comparison of AMP levels  
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3 of bronchiectasis patients and healthy subjects. And finally, although our study is the  
4 largest of AMP in bronchiectasis, using well characterized patients in multicenter and  
5 international study, it is not possible to clarify if dysregulated AMP levels are markers  
6 of disease severity more than a cause of exacerbation, and further studies are needed to  
7 better clarify this point. Our study did not explore how sputum biomarkers could be  
8 implemented into clinical practice, which may be limited because of the need for  
9 sputum processing.  
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19 In conclusion, we found that patients with bronchiectasis show dysregulated  
20 AMP characterized by elevated pro-inflammatory LL-37, lactoferrin and lysozyme with  
21 reduced anti-inflammatory SLPI. The frequent exacerbator phenotype is associated with  
22 increased levels of LL-37 and degradation of SLPI, providing the first biological  
23 characterization of this key phenotype.  
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Confidential: For Review Only

**Table 1.** Patient demographics, clinical characteristics and prior treatments.

	Patients ( <i>n</i> = 135)
Age (mean $\pm$ SD)	69.1 (10.5)
Male	59 (43.7)
Smoking status	
Never	82 (60.7)
Ex-smoker	23 (17.0)
Current	30 (22.2)
Comorbidities	
Cardiovascular disease	32 (23.7)
Diabetes mellitus	13 (9.6)
Stroke	12 (8.9)
Treatment	
Inhaled bronchodilators	88 (65.2)
Inhaled corticosteroids	59 (43.7)
Inhaled antibiotics	6 (4.4)
Chronic macrolide therapy	31 (23.0)
FEV <sub>1</sub> (% pred) (mean $\pm$ SD)	78.3 (28.3)
FVC (% pred) (mean $\pm$ SD)	94.3 (27.0)
BMI (kg/m <sup>2</sup> ) (mean $\pm$ SD)	25.9 (5.6)
Aetiology	
Idiopathic	61 (45.2)
Post-infective	22 (16.3)
Post-tuberculosis	12 (8.9)
COPD	12 (8.9)
Rheumatoid arthritis	6 (4.4)
CTD	4 (3.0)
ABPA	4 (3.0)
Primary Ciliary dyskinesia	3 (2.2)
Inflammatory bowel disease	3 (2.2)
Asthma	3 (2.2)
Haematological malignancy associated immunodeficiency	2 (1.5)
Young syndrome	2 (1.5)
Specific antibody deficiency	1 (0.7)
Exacerbations previous 12 months	
0	24 (17.8)
1	28 (20.7)
2	15 (11.1)
3	20 (14.8)
4 or more	48 (35.6)
BSI (mean $\pm$ SD)	8.0 (4.3)
FACED ( mean $\pm$ SD)	2.4 (1.5)

Data is expressed by number (%) as otherwise is indicated. FEV<sub>1</sub>: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity; BMI: Body Mass Index; COPD: Chronic Obstructive Pulmonary Disease. CTD: Connective Tissue Diseases; ABPA: allergic bronchopulmonary aspergillosis BSI: Bronchiectasis Severity Index



## FIGURE LEGENDS

**Figure 1.**-Association of sputum AMP levels and severity of disease. A: Sputum LL-37 levels and BSI score divided in mild (0-4 points), moderate (4-8 points) and severe (9 or more points), B: Sputum SLPI levels and BSI score divided in mild, moderate and severe. *p* values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

**Figure 2.**- Sputum AMP levels (A: LL-37, B: SLPI, C: lactoferrin, and D: lysozyme) and the presence of airway infection. Patients are divided in non-infected, infected by other pathogens different than *P. aeruginosa* and infected by *P. aeruginosa*. *p* values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM

**Figure 3.**-A: Relationship between sputum SLPI and neutrophil elastase activity. B: Relationship between sputum SLPI and sputum MMP-9 levels. Rho and *p*-value were obtained from Spearman rank correlation, while the dotted line is derived from linear regression. C: Western Blot demonstrating degradation pattern of SLPI in sputum. Each column represents an individual patient ordered from left to right in terms of increasing sputum neutrophil elastase activity (measured by immunoassay as described in the methods). Abbreviations (NE: Neutrophil Elastase, FL SLPI= full length secretory leukocyte protease inhibitor, CP1= cleavage product 1, CP2= cleavage product 2, CP3= cleavage product 3). D: SLPI secretion from nasal epithelial cells of control and bronchiectasis patients. E: SLPI secretion from control (CON1 and CON2) and bronchiectasis (BE) nasal epithelial cells untreated (control buffer) or treated with Elastase, Inhibitor or a mixture of Elastase and inhibitor.

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3 **Figure 4.-** A: Sputum SLPI levels and Frequency of exacerbations during 1 year follow  
4 up. B: Time to next exacerbation. Percentage of patients free of exacerbation using LL-  
5 37 1500 ng/ml as a cut-off (Hazard Ratio (HR) 1.68 (95%CI 1.13-2.50, p=0.01). C:  
6 Sputum SLPI levels and Frequency of exacerbations during 1 year follow up. D: Time  
7 to next exacerbation. Percentage of patients free of exacerbation using SLPI 1000 ng/ml  
8 as a cut-off (HR 0.63 95%CI 0.42-0.94, p=0.02). *p* values in panel A and C are obtained  
9 by Kruskal-Wallis test. Graphs are represented as mean and SEM.  
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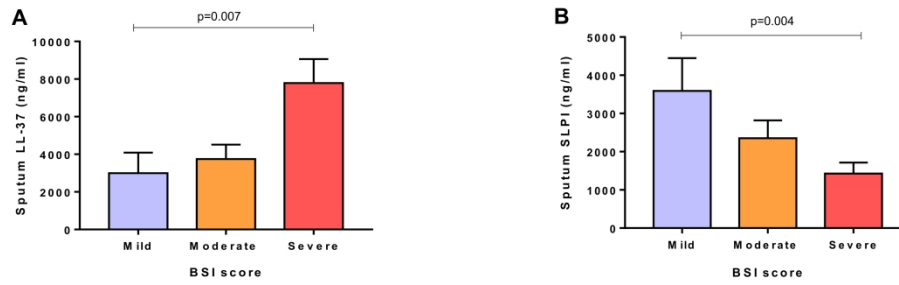


Figure 1.-Association of sputum AMP levels and severity of disease. A: Sputum LL-37 levels and BSI score divided in mild (0-4 points), moderate (4-8 points) and severe (9 or more points), B: Sputum SLPI levels and BSI score divided in mild, moderate and severe. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

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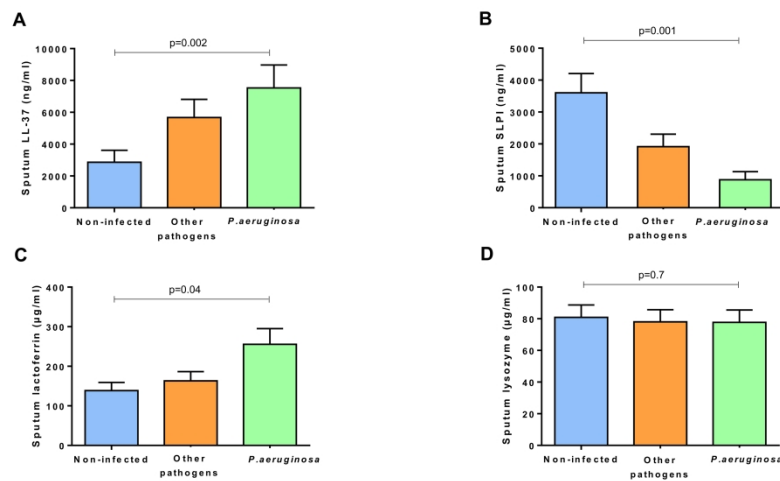


Figure 2.- Sputum AMP levels (A: LL-37, B: SLPI, C: lactoferrin, and D: lysozyme) and the presence of airway infection. Patients are divided in non-infected, infected by other pathogens different than *P. aeruginosa* and infected by *P. aeruginosa*. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM

338x190mm (300 x 300 DPI)

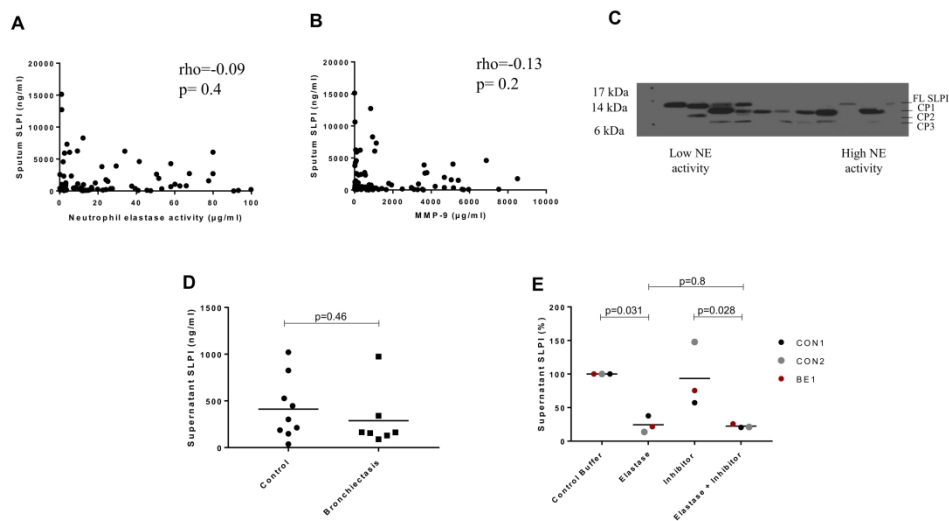


Figure 3.-A: Relationship between sputum SLPI and neutrophil elastase activity. B: Relationship between sputum SLPI and sputum MMP-9 levels. Rho and p-value were obtained from Spearman rank correlation, while the dotted line is derived from linear regression. C: Western Blot demonstrating degradation pattern of SLPI in sputum. Each column represents an individual patient ordered from left to right in terms of increasing sputum neutrophil elastase activity (measured by immunoassay as described in the methods). Abbreviations (NE: Neutrophil Elastase, FL SLPI= full length secretory leukocyte protease inhibitor, CP1= cleavage product 1, CP2= cleavage product 2, CP3= cleavage product 3). D: SLPI secretion from nasal epithelial cells of control and bronchiectasis patients. E: SLPI secretion from control (CON1 and CON2) and bronchiectasis (BE) nasal epithelial cells untreated (control buffer) or treated with Elastase, Inhibitor or a mixture of Elastase and inhibitor.

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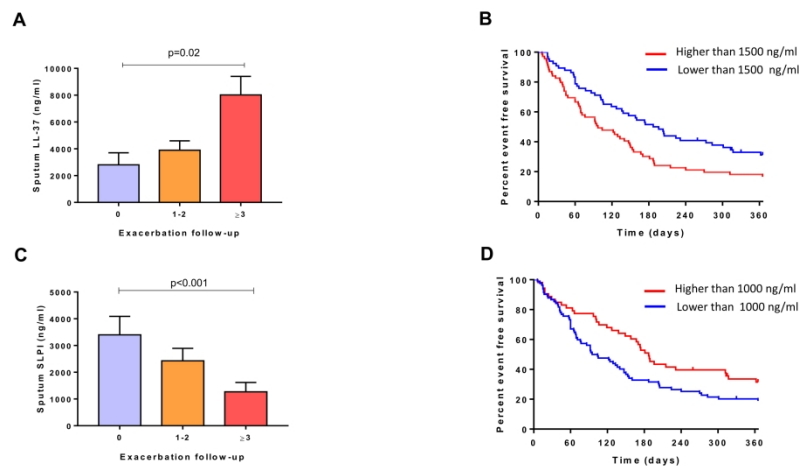


Figure 4.- A: Sputum SLPI levels and Frequency of exacerbations during 1 year follow up. B: Time to next exacerbation. Percentage of patients free of exacerbation using LL-37 1500 ng/ml as a cut-off (Hazard Ratio (HR) 1.68 (95%CI 1.13-2.50,  $p=0.01$ )). C: Sputum SLPI levels and Frequency of exacerbations during 1 year follow up. D: Time to next exacerbation. Percentage of patients free of exacerbation using SLPI 1000 ng/ml as a cut-off (HR 0.63 95%CI 0.42-0.94,  $p=0.02$ ).  $p$  values in panel A and C are obtained by Kruskal-Wallis test. Graphs are represented as mean and SEM.

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6 **Antimicrobial peptides, disease severity and exacerbations in Bronchiectasis**  
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11 Oriol Sibila, Lidia Perea, Elisabet Cantó, Amelia Shoemark, Diane Cassidy, Alexandra Smith,  
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13 Guillermo Suarez-Cuartin, Ana Rodrigo-Troyano, Holly R Keir, Martina Oriano, Samantha Ong,  
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16 Silvia Vidal, Francesco Blasi, Stefano Aliberti, James D Chalmers.  
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25 **ONLINE DATA SUPPLEMENT**  
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## METHODS

### Air-liquid interface nasal epithelial cell culture

For the nasal cilia stuff, healthy controls were recruited from NHS Tayside and University of Dundee staff aged 25 to 60 years old. All controls had no history of rhinitis, asthma or other respiratory disease and were required to be free of upper respiratory tract infection for at least 6 weeks prior to nasal sampling.



## RESULTS

### AMP levels and FACED score

High sputum LL-37 levels were detected in patient with severe FACED score (7633 (719.1-21135) vs 2678 (30.1-9056) vs 329.6 (21.8-6412) ng/ml,  $p=0.05$ ) while sputum SLPI levels were the lowest in the most severe group (236.2 (84.7-901.5) vs 769.7 (125.7-3592) vs 637.4 (296.0-3812) ng/ml,  $p=0.1$ ) (**Figure E1**).

**Table E1.** Patient demographics, clinical characteristics and prior treatments among non-frequent exacerbators and frequent exacerbators.

	Non-frequent exacerbators (n = 67)	Frequent exacerbators (n = 68)	P value
Age (mean ± SD)	69.3 (±10.6)	68.7 (±10.5)	0.3
Male	32 (54.2)	27 (45.8)	0.3
Smoking status			
Never	44 (65.7)	38 (55.9)	0.1
Ex-smoker	13 (19.4)	10 (14.7)	
Current	10 (14.9)	20 (29.4)	
Comorbidities			
Cardiovascular disease	14 (20.9)	18 (26.5)	0.4
Diabetes mellitus	6 (9.0)	7 (10.3)	0.8
Stroke	5 (7.5)	7 (10.3)	0.6
Treatment			
Inhaled long-acting bronchodilator	35 (52.2)	53 (77.9)	0.002
Inhaled corticosteroids	24 (35.8)	35 (59.3)	0.07
Inhaled antibiotics	2 (4.0)	4 (7.0)	0.5
Chronic macrolide therapy	17 (25.4)	14 (20.6)	0.5
FEV <sub>1</sub> (% pred) (mean ± SD)	87.5 (±27.7)	69.3 (±26.2)	<0.0001
FVC (% pred) (mean ± SD)	101.6 (±26.1)	87.1 (±26.2)	0.004
BMI (kg/m <sup>2</sup> ) (mean ± SD)	26.4 (±4.4)	25.4 (±6.5)	0.06
Aetiology			
Idiopathic	33 (49.3)	28 (41.2)	0.1
Post-infective	12 (17.9)	10 (14.7)	
Post-TBC	8 (11.9)	4 (5.9)	
Others	14 (20.9)	26 (38.2)	
Exacerbations previous 12 months			
0	24 (35.8)	0 (0.0)	<0.0001
1	28 (41.8)	0 (0.0)	
2	15 (22.4)	0 (0.0)	
3	0 (0.0)	20 (29.4)	
4 or more	0 (0.0)	48 (70.6)	
BSI (mean ± SD)	5.8 (±3.0)	10.3 (±4.3)	<0.0001
FACED ( mean ± SD)	2.1 (±1.4)	2.6 (±1.7)	0.1

Data are presented as n(%) unless otherwise indicated.

BMI, body mass index; BSI, Bronchiectasis Severity Index; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; PPB, potentially pathogenic bacteria; TBC, tuberculosis; SD, standard deviation.

**Table E2.** Patient demographics, clinical characteristics and prior treatments among non-infected, infected by *P. aeruginosa* and infected by other pathogens

	Non-infected (n = 49)	Infected by other pathogens (n = 47)	Infected by <i>P. aeruginosa</i> (n = 39)	P value
Age (mean ± SD)	67.8 (10.6)	70.9 (10.6)	68.7 (10.3)	0.4
Male	17 (34.7)	24 (51.1)	18 (46.2)	0.3
Smoking status				
Never	31 (63.3)	24 (51.1)	27 (69.2)	0.2
Ex-smoker	9 (18.4)	7 (14.9)	7 (17.9)	
Current	9 (18.4)	16 (34.0)	5 (12.8)	
Comorbidities				
Cardiovascular disease	10 (20.4)	11 (23.4)	11 (28.2)	0.7
Diabetes mellitus	6 (12.2)	6 (12.8)	1 (2.6)	0.2
Stroke	4 (8.2)	4 (8.5)	4 (10.3)	0.9
Treatment				
Long-acting bronchodilator	28 (57.1)	27 (57.4)	34 (85)	0.01
Inhaled corticosteroids	15 (30.6)	24 (51.1)	20 (51.3)	0.07
Inhaled antibiotics	0 (0)	4 (8.5)	2 (5)	0.1
Chronic macrolide therapy	12 (24.5)	9 (19.1)	10 (25.6)	0.7
FEV <sub>1</sub> (% pred) (mean ± SD)	85.5 (23.5)	83.6 (29.3)	62.8 (27.3)	<0.001
FVC (% pred) (mean ± SD)	98.1 (23.7)	99.7 (26.2)	83.1 (29.2)	0.008
BMI (kg/m <sup>2</sup> ) (mean ± SD)	26.3 (5.6)	27.6 (5.7)	23.3 (4.2)	0.001
Bronchiectasis etiology				
Idiopathic	16 (32.7)	29 (61.7)	16 (41.0)	0.1
Post-infective	9 (18.4)	6 (12.8)	7 (17.9)	
Post-tuberculosis	6 (12.2)	1 (2.1)	5 (12.8)	
Others	18 (36.7)	11 (23.4)	11 (28.2)	
Exacerbations previous 12 months				
0	13 (26.5)	9 (19.1)	2 (5.1)	0.1
1	13 (26.5)	8 (17.0)	7 (17.9)	
2	5 (10.2)	4 (8.5)	6 (15.4)	
3	8 (16.3)	6 (12.8)	6 (15.4)	
4 or more	10 (20.4)	20 (42.6)	18 (46.2)	
BSI (mean ± SD)	5.7 (3.2)	7.7 (3.8)	11.5 (4.0)	<0.001
FACED (mean ± SD)	1.7 (1.2)	1.9 (1.1)	3.73 (1.4)	<0.001

Data are presented as n(%) unless otherwise indicated.

BMI, body mass index; BSI, Bronchiectasis Severity Index; FEV<sub>1</sub>, forced expiratory volume in 1 s; FVC, forced vital capacity; PPB, potentially pathogenic bacteria; SD, standard deviation.

**Table E3.-** Sputum LL-37, SLPI, lactoferrin and lysozyme levels in bronchiectasis patients with and without inhaled antibiotic treatment, chronic macrolide therapy and inhaled corticosteroids treatment.

	<b>No chronic macrolides</b> n=104 (77%)	<b>Chronic macrolides</b> n= 31 (27%)	<b>p-value</b>
Sputum LL-37 (ng/ml)	2440.4 (18.4-7422.1)	26.0 (11.4-10667.6)	0.3
Sputum SLPI (ng/ml)	535.2 (191.6-2610.3)	637.4 (89.9-4042.3)	0.6
Sputum Lactoferrin (µg/ml)	118.5 (50.2-248.3)	55.7 (37.0-300.4)	0.2
Sputum Lysozyme (µg/ml)	69.2 (42.1-106.0)	63.7 (37.2-95.2)	0.7

	<b>No ICS</b> n=76 (56%)	<b>ICS</b> n= 59 (44%)	<b>p-value</b>
Sputum LL-37 (ng/ml)	1986.3 (17.6-6074.7)	361.9 (12.1-12334.0)	0.6
Sputum SLPI (ng/ml)	659.0 (173.4-4220.4)	509.9 (129.0-2569.0)	0.5
Sputum Lactoferrin (µg/ml)	117.5 (49.5-239.8)	113.5 (40.39-285.0)	0.7
Sputum Lysozyme (µg/ml)	68.4 (37.5-103.0)	75.6 (42.5-106.2)	0.9

	<b>No inhaled antibiotic</b> n= 129 (96%)	<b>Inhaled antibiotic</b> n=6 (4%)	<b>p-value</b>
Sputum LL-37 (ng/ml)	1443.7 (17.0-8108.1)	2654.7 (29.2-9348.6)	0.3
Sputum SLPI (ng/ml)	560.7 (178.5-3534.5)	246.1 (94.7-861.2)	0.8
Sputum Lactoferrin (µg/ml)	114.6 (46.1-246.0)	136.9 (43.3-385.5)	0.6
Sputum Lysozyme (µg/ml)	69.5 (42.3-105.6)	41.6 (19.1-114.4)	0.5

Data are presented as median (Interquartile range). ICS: Inhaled corticosteroids

**Table E4.-** Analysis of negative binomial model with adjustments.

		<b>Effect estimate</b>	<b>95% CI</b>	<b>p-value</b>
<b>LL-37 <math>\geq</math>1500 ng/ml</b>		<b>IRR</b>		
Exacerbation Rate	Unadjusted	1.57	1.04-2.37	0.02
	Adjusted for BSI	1.53	1.01-2.31	0.04
	Adjusted for site	1.52	1.01-2.28	0.04
	Fully adjusted	1.51	0.97-2.34	0.068
		<b>HR</b>		
Time to first exacerbation	Unadjusted	1.68	1.13-2.50	0.01
	Adjusted for BSI	1.58	1.05-2.36	0.02
	Adjusted for site	1.68	1.11-2.56	0.01
	Fully adjusted	1.72	1.12-2.64	0.01
<b>SLPI &lt;1000 ng/ml</b>		<b>IRR</b>		
Exacerbation Rate	Unadjusted	0.76	0.50-1.15	0.1
	Adjusted for BSI	0.77	0.50- 1.17	0.2
	Adjusted for site	0.73	0.48- 1.11	0.1
	Fully adjusted	0.63	0.39-0.98	0.04
		<b>HR</b>		
Time to first exacerbation	Unadjusted	0.63	0.42-0.94	0.02
	Adjusted for BSI	0.68	0.45-1.03	0.06
	Adjusted for site	0.70	0.46-1.05	0.08
	Fully adjusted	0.65	0.41-1.02	0.059

Fully adjusted includes adjustment for bronchiectasis severity, gender, aetiology, inhaled corticosteroid use and site.

IRR= incidence rate ratio, HR= hazard ratio, BSI=Bronchiectasis Severity Index

**FIGURE LEGENDS**

**Figure E1.** A: Sputum LL-37 levels of FACED score, divided as mild (0-2 points), moderate (2-4 points) and severe (5-7 points), and B: Sputum SLPI levels of FACED score. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean with SEM.

**Figure E2.** Relationship between sputum AMP levels (A: LL-37, B: lysozyme, C: lactoferrin and D: SLPI) and FEV<sub>1</sub>% predicted. Rho and p-value were obtained from Spearman rank correlation.

**Figure E3.** A: Sputum LL-37 and B: SLPI levels and frequency of exacerbations in the previous year. p values are obtained by Kruskal-Wallis test. Graphs are represented as mean with SEM.

**Figure E4.** Relationship between sputum AMP levels (A: LL-37, B: lysozyme, C: lactoferrin) and neutrophil elastase activity (Ns=73). Rho and p-value were obtained from Spearman rank correlation.

**Figure E5.** A: ROC curve of sputum LL-37 for predicting severe exacerbation (Area under the curve (AUC) 0.76, 95% confidence interval (CI) 0.65-0.86, p<0.0001). B: ROC curve of sputum SLPI levels for predicting severe exacerbation (AUC 0.72, 95% CI 0.62-0.86, p<0.0001).

Figure E1:

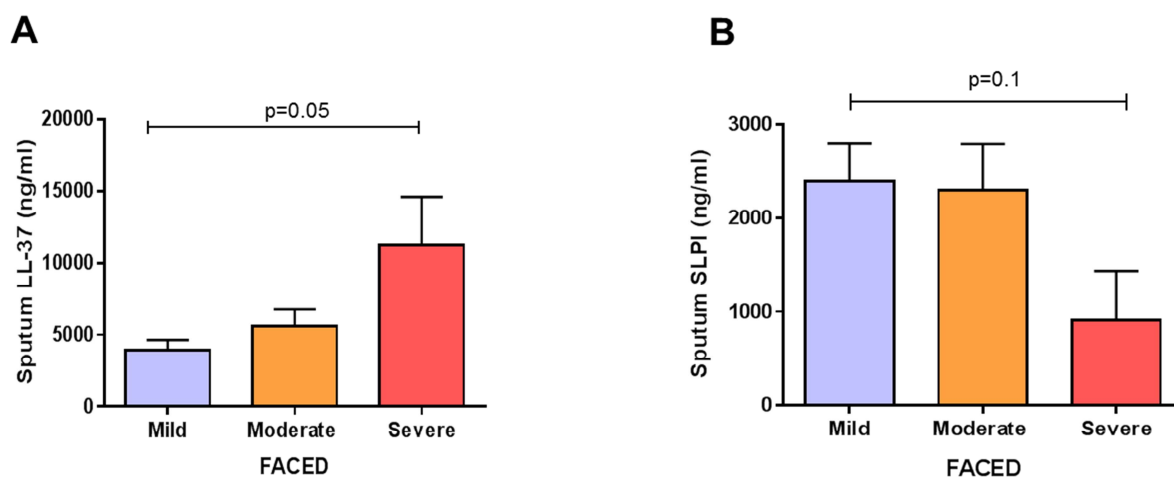
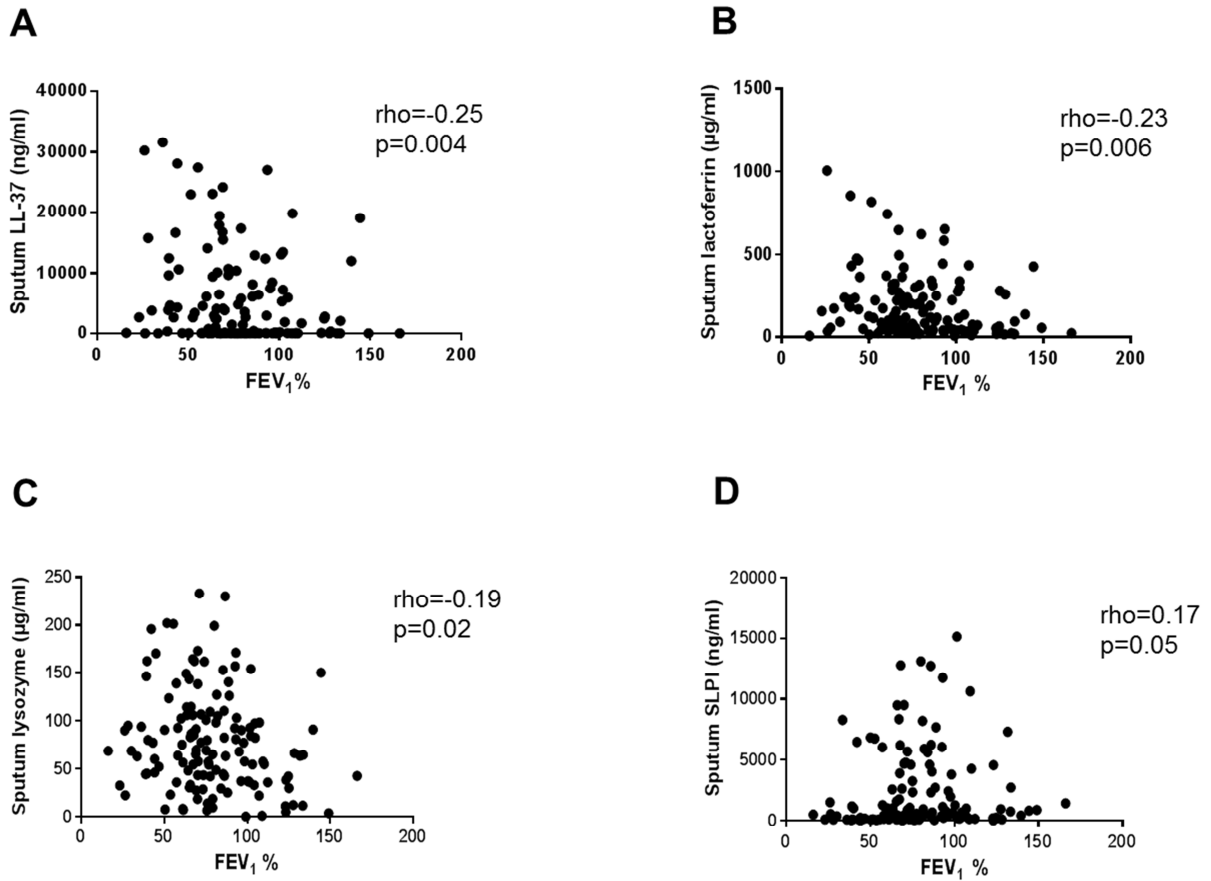


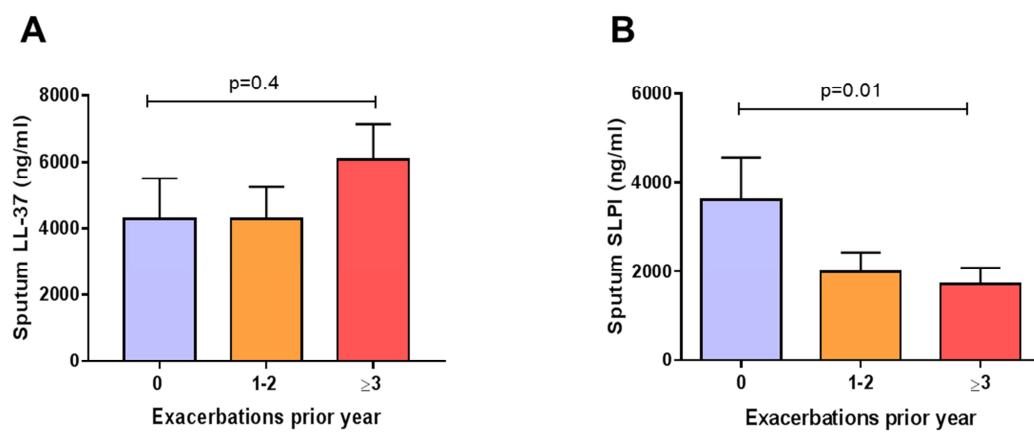
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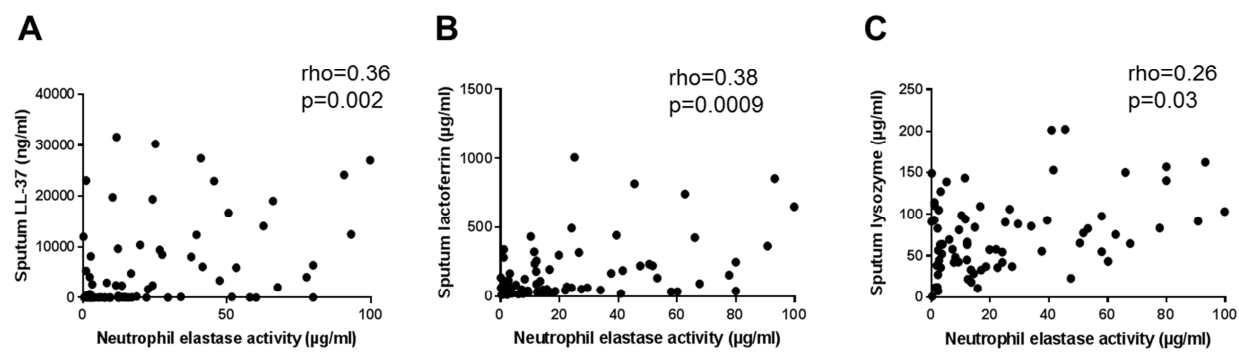


Figure E3:



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Figure E4:



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Figure E5:

