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Published in:
Chest

DOI:
10.1016/j.chest.2023.02.047

Publication date:
2023

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Word count: 1109

Short title: Salivary SLPI in bronchiectasis

ACKNOWLEDGMENTS:

The authors thank all the participants in this study for their contribution to the research in bronchiectasis.

Conflict of interest: LP, RF, BS and OS have nothing to disclosure. AS reports grants from Astrazeneca outside the submitted work. SA reports grants from Insmed Incorporated, Chiesi and Fisher & Paykel;

personal fees from McGraw Hill; consulting fees from Insmed Incorporated, Insmed Italy, Insmed Ireland, Zambon, Astrazeneca UK Limited, CSL Behring HmbH, Grifols, Fondazione Charta, Boehringer Ingelheim, Chiesi, Zcube Srl and Menarini; payments from GlaxoSmithKline Spa; and payments for advisory board from Insmed Incorporated, Insmed Italy and Astrazeneca UK Limited, all of them outside the submitted work. JDC reports grants from Astrazeneca, Boehringer Ingelheim, Insmed, Glaxosmithkline, Grifols, Gilead Sciences and Novartis; consulting fees from Astrazeneca, Boehringer Ingelheim, Glaxosmithkline, Grifols, Pfizer, Novartis and Janssen; and payments for expert testimony from Insmed, all of them outside the submitted work.

Funding: This study has been funded by Instituto de Salud Carlos III (ISCIII) through the project "PI21/00419" and co-funded by the European Union. This work has been funded by Contractes Clínic de Recerca "*Emili Letang – Josep Font*" 2022, granted by Hospital Clínic de Barcelona, and by Serra Hunter Program.

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Key words list: saliva, bronchiectasis, antimicrobial peptides, *Pseudomonas*

Abbreviation list: AMP, antimicrobial peptides; AUC, area under the ROC curve; BSI, Bronchiectasis Severity Index; IQR, interquartile range; LABA, long-acting bronchodilator inhalers; LAMA, long-acting muscarinic antagonists; NE, neutrophil elastase; SLPI, secretory leukocyte protease inhibitor.

To the Editor:

Bronchiectasis is a chronic airway disease characterized by permanent bronchial dilatation, mucus production and recurrent airway infections. A research priority in bronchiectasis is to identify biomarkers to address population heterogeneity[1]. We previously showed that sputum antimicrobial peptides (AMP) may have a role in the pathogenesis of airway infection and the prognosis in bronchiectasis. Specifically, we found that low secretory leukocyte protease inhibitor (SLPI) levels and high levels of the active form of the human cathelicidin cationic antimicrobial protein-18, known as LL-37, are associated with *Pseudomonas aeruginosa* infection, disease severity and future exacerbations[2,3]. According to the so-called "oral-lung axis", microaspiration of oral bacteria and salivary proteins may impact lung health[4]. Salivary proteins have been explored as easily available biomarkers related to severity of other respiratory conditions[5,6]. Due to the relevance of airway AMP in bronchiectasis, we hypothesized that salivary AMP levels are altered in these patients. We investigated the relationship between salivary and sputum AMP, and the association between salivary AMP and airway infection, disease severity and future exacerbations in bronchiectasis.

Unstimulated saliva samples were collected as previously described[7] from adult bronchiectasis patients attending a referral single centre in Barcelona (Spain) in 2018-2019. This cohort study was approved by local ethics committee (IIBSP-PRI-2018-105). All patients signed an informed consent. Patients included were clinically stable (defined by 4 weeks without taking antibiotics) and without diagnosed oral diseases. Clinical data, saliva and spontaneous sputum samples were collected in a single visit. Patients were followed for 1-year. Microbiology was performed in sputum as previously described.[8] Saliva was centrifuged at 10000xg for 10 minutes at 4ºC to obtain supernatants. Supernatants from sputum were collected as previously described[2]. Salivary and sputum levels of SLPI

(R&D Systems, Minnesota, USA), LL-37 (Hycult Biotech, Pennsylvania, USA), lactoferrin, lysozyme (AssayPro, Missouri, USA) and neutrophil elastase (NE; ProAxsis, Belfast, UK) were measured by commercial ELISAs. For ELISA measurements, sputum was diluted as previously described[2] and saliva was diluted: lactoferrin 1/2000, lysozyme 1/8000, SLPI 1/4000, LL-37 1/20 and NE 1/5. Total protein content was used to adjust salivary levels (expressed as adjusted ng/ml) using Qubit fluorometer (ThermoFisher, Massachusetts, USA). Statistical analyses were conducted with GraphPad Prism 9 and SPSS version 27. Parametric continuous variables are presented as mean and SD, and median and IQR in non-parametrical ones. The non-parametrical tests Kruskal-Wallis and Mann-Whitney were used to analyse differences. Correlations were studied using Spearman's rank correlation. A $P<0.05$ was considered significant.

160 bronchiectasis patients were included in the study. Patients had a mean ± SD age of 69 ± 13 years, 71% females and 46% idiopathic bronchiectasis. 21 patients (13%) had chronic airway *P. aeruginosa* infection and mean Bronchiectasis Severity Index (BSI) was 6±4. 43% of patients had taken inhaled corticosteroids, 46% long-acting bronchodilator inhalers (LABA), 22% long-acting muscarinic antagonists (LAMA), 6% inhaled antibiotics and 5% nasal steroids.

The highest concentration of salivary AMP was the neutrophil biomarker of lactoferrin (median (IQR) 86.3 (45.1-136.1) ng/ml), followed by lysozyme, SLPI and LL-37 (data not shown). A subset of patients (N=67, 42%) also provided sputum samples at the same time. All AMP measured and NE were higher in sputum than in saliva ($P<0.0001$). In these patients with paired samples, sputum SLPI levels were 1462 (488.4-5961) ng/ml. A weak but statistically significant correlation between salivary and sputum SLPI levels was found (rho=0.28, $P=0.03$). No correlations between salivary and sputum LL-37, lactoferrin and lysozyme were found (data not shown).

Patients with *P. aeruginosa* had lower salivary SLPI levels compared with those with a negative result ($P=0.02$) (Fig.1A). Salivary SLPI showed an area under the ROC curve (AUC) of 0.67 (0.55-0.79; $P=0.01$) for discriminating patients with *P. aeruginosa* (Fig.1B). No associations between airway infection and salivary LL-37, lactoferrin, lysozyme and NE were found (data not shown). No significant correlation was observed between salivary SLPI and NE (rho=-0.09, $P=0.2$).

According to BSI[9], severe patients had the lowest salivary SLPI levels compared with moderate and mild patients (Fig.1C). A weak, inverse, but statistically significant correlation was observed between salivary SLPI levels and BSI (rho=-0.21, $P=0.008$). No associations between disease severity and salivary LL-37, lactoferrin and lysozyme were found (data not shown).

The mean number of exacerbations during 1-year follow-up per patient was 1.32 ± 1.31. No association between the frequency of exacerbations and salivary SLPI levels was found (*P*=0.9). Using the median of SLPI as cut-off (0.79 ng/ml), patients with low salivary SLPI levels did not show a significantly increased risk of exacerbation (*P*=0.3, Fig.1D).

Finally, we explored the association between smoking status, inhaled treatments, and salivary SLPI levels. We found similar salivary SLPI levels between current, former and non-smokers. We did not find an association between inhaled corticosteroids, LABA, LAMA, inhaled antibiotics, nasal steroids and salivary SLPI levels (data not shown).

In conclusion, we show that low salivary SLPI levels are associated with disease severity and *P. aeruginosa* airway infection in bronchiectasis. These results are highly consistent with our previous findings of low sputum SLPI levels in bronchiectasis[2,3], suggesting that a disrupted protease/anti-protease balance is extended to the oral cavity. Further studies are needed to better explore the implications on infection and microbiome. Currently, the only validated biomarkers for studying bronchiectasis, depend on the availability of high-quality sputum samples[10]. Since up to 50% of patients with mild-moderate bronchiectasis may be unable to expectorate spontaneously, our potential associations between oral inflammation and clinical status could be equally applied to patients who do and do not spontaneously expectorate. It is important to note, however, that salivary SLPI shows lower discriminatory value than sputum SLPI in identifying severe disease and future exacerbations. This is supported by the weak correlation between salivary and sputum SLPI, suggesting the presence of gradients of antiproteases and proteases across the airways[11]. However, we cannot infer yet what is the cause and what is the consequence[2].

We acknowledge the study limitations. First, not all patients provided paired sputum samples. As reported, not all bronchiectasis patients can easily produce daily spontaneous sputum, emphasising the potential value of alternative biomarker sources such as saliva. Second, we did not culture saliva to investigate infection in the oral cavity. Third, extensive data about oral health was not available in these patients, although none of them had any diagnosed oral disease. Fourth, we did not include a control population to know AMP levels. Finally, the lack of follow-up samples does not allow us to know the stability over the time of salivary AMP.

Overall, our data show that salivary SLPI reflects airway *P. aeruginosa* infection, one of the treatable traits in bronchiectasis. Due to the feasibility of collecting saliva, further work would be helpful to clearly define the AMP oral-lung axis in bronchiectasis and its impact on clinical outcomes.

Acknowledgments

The authors thank all the participants in this study for their contribution to the research in bronchiectasis.

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Figure legend

Figure 1. Salivary SLPI levels in patients with bronchiectasis. Association between adjusted salivary SLPI levels and **(a)** positive microbiological result for *P. aeruginosa,* **(b)** ability for discriminating patients with a positive result for *P. aeruginosa* represented by the area under the ROC curve (AUC), **(c)** disease severity according to Bronchiectasis Severity Index (BSI) and **(d)** time to first exacerbation during 1-year follow-up represented by the percentage of patients free of exacerbation using the median SLPI (0.79 adjusted ng/ml) as cut-off (Hazard Ratio (HR) 1.2 (95% CI 0.8 to 1.9), *P*=0.3). Panels a) and c) show the mean and standard error mean.