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Identification of Pseudomonas aeruginosa and airway bacterial colonization by an electronic nose in Bronchiectasis

Guillermo Suarez-Cuartin, MD, PhD[1,2], Jordi Giner, RN, PhD[1,2], José Luis Merino, BSc, PhD[3], Ana Rodrigo-Troyano, MD[1,2], Anna Feliu, RN[1,2], Lidia Perea, BSc[2], Ferran Sanchez-Reus, MD, PhD[2,4], Diego Castillo, MD, PhD[1,2], Vicente Plaza, MD, PhD[1,2], James D. Chalmers, MD, PhD[5], Oriol Sibila, MD, PhD[1,2].

[1]Department of Respiratory Medicine, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona. Barcelona, Spain. [2]Institut d'Investigació Biomédica Sant Pau (IIB Sant Pau). Barcelona, Spain. [3]Electronic Systems Group. Universitat de les Illes Balears. Palma de Mallorca, Spain. [4]Department of Microbiology, Hospital de la Santa Creu i Sant Pau. Universitat Autònoma de Barcelona. Barcelona, Spain. [5]Scottish Centre for Respiratory Research, University of Dundee. Dundee, UK.

Corresponding author: Dr. Oriol Sibila. Servei de Pneumologia, Hospital de la Santa Creu i Sant Pau. C/. Sant Antoni M. Claret 167, 08025 Barcelona (Spain). E-mail: osibila@santpau.cat

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Abstract

Rationale: Airway colonization by Potentially Pathogenic Microorganisms (PPM) in bronchiectasis is associated with worse clinical outcomes. The electronic nose is a non-invasive technology capable of distinguishing volatile organic compounds (VOC) in exhaled breath. We aim to explore if an electronic nose can reliably discriminate airway bacterial colonization in patients with bronchiectasis.

Methods: Seventy-three clinically stable bronchiectasis patients were included. PPM presence was determined using sputum culture. Exhaled breath was collected in Tedlar bags and VOC breath-prints were detected by the electronic nose Cyranose 320®. Raw data was reduced to three factors with principal component analysis. Univariate ANOVA followed by *post-hoc* least significant difference test was performed with these factors. Patients were then classified using linear canonical discriminant analysis. Cross-validation accuracy values were defined by the percentage of correctly classified patients.

Results: Forty-one (56%) patients were colonized with PPM. *Pseudomonas aeruginosa* (n=27, 66%) and *Haemophilus influenzae* (n=7, 17%) were the most common PPM. VC breath-prints from colonized and non-colonized patients were significantly different (accuracy of 72%, AUROC 0.75, p<0.001). VOC breath-prints from *Pseudomonas aeruginosa* colonized patients were significantly different from those of patients colonized with other PPM (accuracy of 89%, AUROC 0.97, p<0.001) and non-colonized patients (accuracy 73%, AUROC 0.83, p=0.007).

Conclusions: An electronic nose can accurately identify VOC breath-prints of clinically stable bronchiectasis patients with airway bacterial colonization, especially in those with *Pseudomonas aeruginosa.*

Abbreviations list

BSI: Bronchiectasis severity index

CF: Cystic fibrosis

E-nose: Electronic nose

PPM: Potentially Pathogenic Microorganisms

VOC: Volatile Organic Compounds

Introduction

Non-cystic fibrosis bronchiectasis (hereafter called "bronchiectasis") is a chronic respiratory condition characterized by irreversible dilation of the bronchi and chronic airway inflammation [1]. Recent studies have observed an increased prevalence of bronchiectasis across Europe and the United States [2-4], and a high annual economic burden that increases with disease severity and the number of exacerbations [5].

Airway colonization by potentially pathogenic microorganisms (PPM) is an important cause of morbidity in bronchiectasis patients, and *Pseudomonas aeruginosa* is one of the pathogens most frequently isolated in those cases [6]. The presence of *P. aeruginosa* airway colonization is associated with more frequent exacerbations and a higher mortality rate compared to patients without *P. aeruginosa* infection [6,7]. Therefore, microbiological assessment determined by sputum culture analysis is one of the key factors in the characterization of bronchiectasis patients. However, sputum culture has limitations such as time delay for results and the difficulty to obtain proper sputum samples [8,9]. Thus, sputum analysis is not used routinely as a standard of care for the management of bronchiectasis patients in many hospitals [10,11]. In these cases, other techniques for microbiological characterization may be helpful.

The electronic nose (e-nose) is a non-invasive diagnostic device that contains an array of electronic chemical sensors capable of identifying volatile organic compounds (VOC) breath-prints [12,13]. The e-nose has demonstrated diagnostic value in identifying different airway respiratory diseases such as Chronic Obstructive Pulmonary Disease (COPD) [14], asthma [15] and cystic fibrosis (CF) [16]. Furthermore, several studies have shown that the e-nose is

also able to detect respiratory infections. In COPD, the e-nose has successfully distinguished patients with and without airway bacterial infection during clinical stability [17] and acute exacerbations [18]. Some studies have suggested that specific bacteria such as *P. aeruginosa*, may produce distinguishable VOC [19-21]. The e-nose has been able to identify the presence of *P. aeruginosa* infection both in CF patients [22] and in swabs of bacteria obtained from *in vitro* cultures [23]. However, data regarding the use of the e-nose in bronchiectasis, and its potential role identifying *P. aeruginosa* is lacking.

We hypothesized that the e-nose could accurately discriminate VOC profiles from bronchiectasis patients with and without airway bacterial colonization, especially in those with *P. aeruginosa*. Therefore, the aim of this study was to explore if an electronic nose can reliably discriminate airway bacterial colonization in clinically stable patients with bronchiectasis.

Methods

Study design and Ethics

This cross-sectional study included clinically stable bronchiectasis patients with and without airway bacterial colonization. The study protocol was approved by the institutional ethics committee (IIBSP-BRO-2013-154) and patients gave their informed consent. This study was registered in www.clinicaltrials.gov on April 2014. ClinicalTrials.gov ID: NCT02163642.

Study population

Patients were consecutively recruited from a specialist clinic at the Hospital de la Santa Creu i Sant Pau in Barcelona, Spain, between June 2014

and May 2016. **Figure 1** shows the study approach for patient enrolment. The diagnosis of bronchiectasis and its etiological assessment were established according to national and international guidelines [1,24]. Post-infective aetiology was defined as patients with a history of previous severe lower respiratory tract infections due to bacterial and viral pneumonia, pertussis or tuberculosis [1]. Exclusions were: patients with age less than 18 years; unable to give informed consent or with other respiratory diseases such as CF, active allergic bronchopulmonary aspergillosis, active non-tuberculous mycobacterial infection or pulmonary fibrosis with traction bronchiectasis, as well as patients receiving immunoglobulin replacement therapy or chronic systemic corticosteroid treatment. Sample size was calculated as described in previous studies [17,18].

Clinical and functional assessment

All subjects were included during clinical stability, defined as the absence of an exacerbation requiring antibiotic or systemic corticosteroid treatment within the previous 30 days. A detailed clinical history was obtained from all participants, including demographic data, smoking status, relevant comorbid conditions, current treatment and the number of previous outpatient and hospitalized exacerbations. Severity of disease was assessed with the Bronchiectasis Severity Index (BSI) and FACED scores [25,26]. Spirometry was performed according to international recommendations [27], using the reference values for Mediterranean population [28].

Bacteriology

Spontaneous sputum samples for bacteriology were obtained from all participants on inclusion, and were processed as we described previously [29]. Quality of sputum was evaluated using the Murray-Washington criteria [30]. Patients were classified according to sputum bacteriology assessment into three groups: non-colonized, colonized by *P. aeruginosa* and colonized by other PPM (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and other Gram-negative bacilli).

Exhaled breath analysis

Breath samples were obtained from all participants to assess VOC profiles with the e-nose as we described previously [17,31]. In summary, exhaled breath samples were collected in 10 litre Tedlar bags after 3 minutes of tidal breathing through a Hans-Rudolph valve with an expiratory silica reservoir exposed to dry air and an inspiratory filter. The e-nose device (Cyranose 320®; Smith Detections, CA, USA), a chemical vapour analyser with 32 organic polymeric Nano-composite sensor arrays, was then connected to the Tedlar bag for 5 minutes. The exposure to exhaled breath generated a breath-print VOC profile for each subject.

All participants stopped their inhaled medications and fasted for at least 12 hours before the breath sampling.

Data analysis

Results are presented as mean and standard deviation (SD) for continuous parametric data, and median and interquartile range (IQR) for

continuous non-parametric data. Categorical data are presented as frequencies and percentages. Statistical analysis was performed using the SPSS 22 software for Windows (SPSS; Illinois, USA). A *p* value of less than 0.05 was considered significant.

Breath-print data from all participants was analysed using a pattern-recognition application built in the MATLAB software (v.R2012a) as we described previously [17,31]. In short, raw data was reduced to three principal factors by principal component analysis (PCA). These PCA factors were used to perform a univariate ANOVA, followed by *post-hoc* least significant difference test. Patients were then classified into a categorical division using a linear canonical discriminant analysis, calculated as the one that obtained the better percentage of correctly classified subjects. The discriminant function was trained with all minus one subject samples. Then, the remaining samples were tested. This process known as the “leave-one-out” method was repeated for all subjects, thus building the percentage of correctly classified patients which defined cross-validation accuracy values [14,17,18,31]. A Receiver Operating Characteristic (ROC) was obtained using the discriminant function results. The area under the ROC curve was calculated with multiple logistic regression.

Results

Patient description

Seventy-three clinically stable bronchiectasis patients were included; 47 (64%) were female and median age was 69 years (IQR 60-76.5 years). Mean FEV_1 was 65.9 ± 23.3% of predicted; median BSI score was 7 points (IQR 6-11

points) and median FACED score was 2 points (IQR 1-4 points). The most frequent aetiologies were post-infective (47%) and idiopathic (19%).

Forty-one (56%) patients were classified as colonized. The most frequently isolated PPM were *P. aeruginosa* (n=27; 66%), *Haemophilus influenzae* (n=7; 17%), *Escherichia coli* (n=2; 5%) and *Streptococcus pneumoniae* (n=2; 5%). Other isolated PPM included *Moraxella catarrhalis*, *Achromobacter xylosoxidans* and *Staphylococcus aureus* (n=1 each, 2%).

Baseline characteristics of colonized and non-colonized subjects are summarized in **Table 1**. Patients with airway colonization had lower lung function values (mean FEV1 57.6 ± 20.5 vs. 76.8 ± 22.5, $p<0.001$) and more severe bronchiectasis (BSI score median 10 vs. 6, $p<0.001$; FACED score median 3 vs. 2, $p<0.001$). Colonized patients were subsequently classified into 2 subgroups according to the isolated microorganism in sputum culture; 27 subjects (66%) were colonized with *P. aeruginosa*, and 14 (34%) with other PPM. Demographic and clinical characteristics of these subgroups are shown in **Table 2**. Patients with *P. aeruginosa* airway colonization were more associated with post-infective aetiology, had more severe bronchiectasis (BSI score median 11 vs. 6, $p=0.01$; FACED score median 4 vs. 2, $p=0.001$), and had a higher use of long-acting beta agonists (85% vs. 35%, $p=0.001$) and inhaled corticosteroids (74% vs. 35%, $p=0.01$) compared to those patients colonized with other PPM.

Table 1. Demographics and clinical characteristics of bronchiectasis patients with and without airway colonization.

	Colonized (N=41)	**Non Colonized (N=32)**	**p**
Age (median, IQR)	68 (60.5-77.5)	69.5 (59-75.8)	0.726
Female gender (n,%)	25 (61%)	22 (68.8%)	0.491
Smoking status (n,%) Never Former Current	 31 (75.6%) 10 (24.4%) 0	 24 (75%) 7 (21.9%) 1 (3.1%)	0.514
Cardiovascular disease (n,%)	9 (22%)	5 (15.6%)	0.496
Diabetes mellitus (n,%)	4 (9.8%)	2 (6.3%)	0.588
MRC dyspnoea score (median, IQR)	2 (2-3)	2 (1-2)	*<0.001*
Aetiology (n,%) Post infective Connective tissue disease Primary ciliary dyskinesia Immunodeficiency Inactive ABPA COPD Others Idiopathic	 18 (43.9%) 7 (17.1%) 2 (4.9%) 2 (4.9%) 3 (7.3%) 1 (2.4%) 2 (4.9%) 6 (14.6%)	 16 (50%) 2 (6.3%) 2 (6.3%) 1 (3.1%) 0 2 (6.3%) 1 (3.1%) 8 (25%)	0.521
FVC % of predicted (mean ± SD)	74.7 ± 18.4	87.9 ± 20.6	*0.005*
FEV_1 % of predicted (mean ± SD)	57.6 ± 20.5	76.8 ± 22.5	*<0.001*
Number of exacerbations in the previous year (median, IQR)	3 (2-4)	2 (1-3)	0.392
Bronchiectasis severity index score (median, IQR)	10 (6-13)	6 (4-8)	*<0.001*
FACED score (median, IQR)	3 (2-4)	2 (1-3)	*<0.001*
LABA use (n,%)	28 (68.3%)	18 (56.3%)	0.290
LAMA use (n,%)	19 (46.3%)	8 (25%)	0.061
ICS use (n,%)	25 (61%)	16 (50%)	0.348
Chronic macrolides (n,%)	10 (24.4%)	6 (18.8%)	0.563

All data is presented in median (quartiles 1-3) unless otherwise indicated.
MRC: Medical Research Council; ABPA: Allergic bronchopulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease; FVC: Forced vital capacity; FEV_1: Forced expiratory volume in 1 second; SABA: Short-acting beta agonists; LABA: Long-acting beta agonists; LAMA: Long-acting muscarinic receptor antagonists; ICS: Inhaled corticosteroids.

Table 2. Demographics and clinical characteristics of bronchiectasis patients with airway colonization by *Pseudomonas aeruginosa* and other potentially pathogenic microorganisms.

	Colonized with *P. aeruginosa* (N=27)	**Colonized with other PPM (N=14)**	**p**
Age (median, IQR)	68 (63-77)	67 (58-78)	0.591
Female gender (n,%)	18 (66.7%)	7 (50%)	0.300
Smoking status (n,%)			
Never	19 (70.4%)	12 (85.7%)	
Former	8 (29.6%)	2 (14.3%)	0.278
Current	0	0	
MRC dyspnoea score (median, IQR)	3 (2-3)	2 (2-3)	0.166
Aetiology (n,%)			
Post infective	16 (59.3%)	2 (14.3%)	
Connective tissue disease	2 (7.4%)	5 (35.7%)	
Primary ciliary dyskinesia	0	2 (14.3%)	
Immunodeficiency	0	2 (14.3%)	
Inactive ABPA	2 (7.4%)	1 (7.1%)	*0.013*
COPD	1 (3.7%)	0	
Others	1 (3.7%)	1 (7.1%)	
Idiopathic	5 (18.5%)	1 (7.1%)	
FVC % of predicted (mean ± SD)	72.3 ± 19.1	79.4 ± 16.6	0.248
FEV_1 % of predicted (mean ± SD)	54.9 ± 22.2	62.7 ± 16.2	0.251
Number of exacerbations in the previous year (median, IQR)	3 (2-4)	2.5 (1-3.3)	0.370
Bronchiectasis severity index score (median, IQR)	11 (9-14)	6 (5.8-12.5)	*0.016*
FACED score (median, IQR)	4 (2-5)	2 (1-3)	*0.001*
LABA use (n,%)	23 (85.2%)	5 (35.7%)	*0.001*
LAMA use (n,%)	14 (51.9%)	5 (35.7%)	0.326
ICS use (n,%)	20 (74.1%)	5 (35.7%)	*0.017*
Chronic macrolides (n,%)	7 (25.9%)	3 (21.4%)	0.750

All data is presented in median (quartiles 1-3) unless otherwise indicated.
PPM: Potentially pathogenic microorganisms; MRC: Medical Research Council; ABPA: Allergic bronchopulmonary aspergillosis; COPD: Chronic obstructive pulmonary disease; FVC: Forced vital capacity; FEV_1: Forced expiratory volume in 1 second; SABA: Short-acting beta agonists; LABA: Long-acting beta agonists; LAMA: Long-acting muscarinic receptor antagonists; ICS: Inhaled corticosteroids.

Breath-print analysis

Bronchiectasis patients with and without airway bacterial colonization had significantly different breath profiles (**Figure 2**). Cross-validation accuracy was 72.1%, and Area under ROC curve (AUROC) was 0.75 (p=0.01) (**Table 3)**.

Subjects with airway bacterial colonization were then analysed. VOC breath-print profiles from subjects colonized by *P. aeruginosa* and by other PPM were markedly different (**Figure 3**), with a cross-validation accuracy of 89.2% and AUROC of 0.96 (p<0.001) (**Table 3**).

In addition, patients with *P. aeruginosa* airway colonization were compared with non-colonized subjects. This analysis also showed significant differences in breath-print profiles (**Figure 4**), with a cross-validation accuracy of 72.7% and AUROC of 0.82 (p=0.007) (**Table 3**).

Table 3. Receiver operating characteristics analyses of breath-prints between bronchiectasis patients with *Pseudomonas aeruginosa* colonization, other PPM colonization and non-colonized.

	Colonized vs. non-colonized bronchiectasis patients	***P. aeruginosa* colonized vs. other PPM colonized bronchiectasis patients**	***P. aeruginosa* colonized vs. non-colonized bronchiectasis patients**
Cross-validation accuracy	72.1%	89.2%	72.7%
Sensitivity	0.84	0.92	0.83
Specificity	0.58	0.85	0.65
AUROC	0.754	0.968	0.829
Positive predictive value	0.70	0.92	0.65
Negative predictive value	0.75	0.85	0.83
p value	0.01	<0.001	0.007

PPM: Potentially pathogenic microorganisms; AUROC: Area under receiver operating characteristic.

Discussion

This study showed that an electronic nose is a non-invasive technology capable of identifying VOC breath-prints from bronchiectasis patients with and without airway bacterial colonization. Moreover, it can accurately distinguish breath-prints of bronchiectasis subjects with *P. aeruginosa* airway colonization

from those colonized with other PPM and non-colonized. These findings suggest that the e-nose could be a useful tool to identify airway bacterial colonization in clinically stable bronchiectasis patients.

Airway bacterial colonization by PPM is one of the most frequent and important causes of morbidity in bronchiectasis patients. Different studies have observed that patients with airway bacterial colonization, either by *P. aeruginosa* or other PPM, have worse clinical outcomes and more severity of disease than non-colonized patients [9,25]. Our study showed a 56% prevalence of colonized patients during clinical stability, and overall these patients also had a history of more frequent exacerbations, lower FEV_1 values and more severe disease. Identifying airway bacterial colonization is important because of its prognostic significance and also because most therapies for bronchiectasis such as inhaled antibiotics, target airway infection. We previously demonstrated an accurate discrimination of COPD patients with and without airway bacterial colonization using the e-nose (sensitivity of 82%; specificity of 96%) [17]. In this study, bronchiectasis patients with airway colonization also had different VOC breath-prints compared to non-colonized ones using the same e-nose technology (sensitivity of 84%; specificity of 58%).

Our study showed a 56% prevalence of PPM airway colonization. This prevalence varies widely between cohorts and countries. Martinez-Garcia et al. observed a PPM airway colonization prevalence of approximately 32% for *P. aeruginosa*, 15% for *Haemophilus influenzae* and 5% for other Gram-negative bacteria [26]. In a different cohort, King and collaborators found a higher colonization rate but predominantly by *Haemophilus influenzae* (47%), *P. aeruginosa* (12%), *Moraxella catarrhalis* (8%) and *Streptococcus pneumoniae*

(7%) [32]. Meanwhile, another study from Chalmers et al. showed a chronic colonization prevalence of approximately 72%, with predominance of *Haemophilus influenzae* (40%), *P. aeruginosa* (16%) and *Moraxella catarrhalis* (14%) [25]. This shows there is a wide range of PPM colonization rates among bronchiectasis patients.

P. aeruginosa is a pathogen frequently associated with airway colonization in bronchiectasis [6]. It has been demonstrated that bronchiectasis patients colonized by *P. aeruginosa* have more frequent exacerbations, a steeper decline in FEV_1 and a higher mortality rate than other patients [6,7,9,25,26]. In addition, patients with airway *P. aeruginosa* colonization have shown higher levels of sputum mucins and airway cytokines, chemokines and neutrophil elastase when compared to other bronchiectasis subjects [29,33]. Neutrophil elastase is also related to worse clinical outcomes and disease severity [34]. In our study, *P. aeruginosa* was the most frequently isolated PPM in 66% of the colonized patients, and as described in previous studies, these subjects had worse lung function values and higher severity scores. Therefore, it is important not only to identify airway bacterial colonization but also to discriminate the presence of specific PPM such as *P. aeruginosa*.

Several studies have used the e-nose technology to discriminate between airway colonization by different PPM, based on the fact that VOC may be produced as a part of bacterial metabolism [35,36]. Lai et al. successfully used the e-nose on swabs from *in vitro* samples to discriminate VOC patterns of common respiratory bacterial pathogens such as *H. influenzae*, *S. pneumoniae*, *S. aureus* and *P. aeruginosa* from control samples [23]. Using the same e-nose as in our study, Shafiek et al [18] demonstrated that COPD patients expressed

different VOC profiles during infectious exacerbations depending on the causative bacteria, especially when comparing *P. aeruginosa* versus *H. influenzae*, although only 8 patients were included in this comparison. In our study, using a higher sample size, the e-nose showed a high accuracy to discriminate VOC breath-prints between airway colonization by different microorganisms, especially when comparing *P. aeruginosa* with other PPM (sensitivity of 92%, specificity of 85%). This high accuracy value may be related to the presence of a specific VOC pattern for different PPM [23,37]. In this order, Goeminne et al. used gas chromatography-mass spectrometry on sputa from bronchiectasis patients to assess *P. aeruginosa* airway colonization status, and observed that not a single but a pattern of VOC are related to the presence of this microorganism [37]. To our knowledge, this is the first study in stable bronchiectasis patients to explore the utility of the e-nose technology not only for discriminating colonized from non-colonized subjects, but also for identifying the presence of *P. aeruginosa* airway colonization.

A key limitation of this study is the relatively small sample size obtained from a single centre and the lack of external validation; however we have previously validated the utility of the e-nose in a smaller COPD cohort [17] and several studies using the same device have lower sample sizes [15,16,31]. In addition, the characteristics of patients in our centre are very similar to those reported from across Europe in terms of demographics, aetiology and bacteriology. This greatly strengthens the generalisability of this study [38,39]. Another limitation is the use of sputum analysis for bacteriology assessment, due to the difficulty of some patients to obtain a good sputum sample and the possibility of contamination with bacteria from the upper respiratory tract.

Nevertheless, the quality of all samples included was evaluated using the Murray-Washington criteria as mentioned above. We consciously decided not to perform molecular diagnostics such as PCR or microbiome characterization since their clinical and prognostic relevance need further evaluation, and sputum sampling is still the standard of care for microbiological assessment in bronchiectasis patients and has a good correlation with results from bronchoscopic procedures in these subjects [40]. In this study it is not possible to affirm whether we are identifying *P. aeruginosa* airway presence, disease severity or medication use. However, bacterial colonization is associated with disease severity and stratifying colonized patients by their severity of disease was not possible due to the low number of non-severe colonized subjects. Also, breath samples were obtained after 12 hours of stopping any inhaled medication in order to avoid this potential confounding factor. Finally, we did not use gas chromatography or mass spectrometry to study the molecular correspondence of the different VOC patterns. A study aiming to identify the different compounds that characterize each group would be of great interest.

In conclusion, the electronic nose is a non-invasive technology that shows promising results in the identification of VOC breath-prints related to airway bacterial colonization in bronchiectasis patients during clinical stability, especially in those colonized by *P. aeruginosa*. Therefore, it may become a useful tool alongside sputum microbiology to improve the proper management of bronchiectasis patients.

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Authorship: Conception and design: OS, GSC, JG. Data acquisition and interpretation: OS, GSC, JG, JLM, ART, AF, LP, FSR, DC, VP, JDC. Data analysis: OS, GSC, JG, JLM, JDC. Preparation of the manuscript: OS, GSC, JG, JLM, JDC. All authors have critically revised and given their final approval of the manuscript.

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Figure Legends

Figure 1. Flow diagram of study approach for patient enrolment

Figure 2. Electronic nose discrimination of colonized vs. non-colonized bronchiectasis patients.

A: Two-dimensional principal component (PC) analyses plot showing the breath-print discrimination.
B: Area Under Roc Curve (AUROC) of 0.75

C: Colonized; NC: Non-colonized.

Figure 3. Electronic nose discrimination of airway colonization with *Pseudomonas aeruginosa* vs. airway colonization with other potentially pathogenic bacteria in bronchiectasis patients.

A: Two-dimensional principal component (PC) analyses plot showing the breath-print discrimination.
B: Area Under Roc Curve (AUROC) of 0.96

C-PA: Colonization with Pseudomonas aeruginosa; C-O: Colonization with other potentially pathogenic microorganisms.

Figure 4. Electronic nose discrimination of bronchiectasis patients with *Pseudomonas aeruginosa* airway colonization vs. non-colonized subjects.

A: Two-dimensional principal component (PC) analyses plot showing the breath-print discrimination.
B: Area Under Roc Curve (AUROC) of 0.82.

C-PA: Colonization with Pseudomonas aeruginosa; NC: Non-colonized.