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## EXHALED MOLECULAR PROFILES IN THE ASSESSMENT OF CYSTIC FIBROSIS AND PRIMARY CILIARY DYSKINESIA

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## **ABSTRACT**

### **Background**

Early diagnosis and monitoring of disease activity are essential in cystic fibrosis (CF) and primary ciliary dyskinesia (PCD). We aimed to establish exhaled molecular profiles as the first step in assessing the potential of breath analysis.

### **Methods**

Exhaled breath was analyzed by electronic nose in 25 children with CF, 25 with PCD and 23 controls. Principle component reduction and canonical discriminant analysis were used to construct internally cross-validated ROC curves.

### **Results**

CF and PCD patients had significantly different breath profiles when compared to healthy controls (CF: sensitivity 84%, specificity 65%; PCD: sensitivity 88%, specificity 52%) and from each other (sensitivity 84%, specificity 60%). Patients with and without exacerbations had significantly different breath profiles (CF: sensitivity 89%, specificity 56%); PCD: sensitivity 100%, specificity 90%).

### **Conclusion**

Exhaled molecular profiles significantly differ between patients with CF, PCD and controls. The eNose may have potential in disease monitoring based on the influence of exacerbations on the VOC-profile.

## BACKGROUND

Cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) have a major impact on health and quality of life. Early diagnosis, frequent monitoring and vigorous treatment of respiratory infections are key to preserving lung function [1, 2]. For CF adequate screening methodology is available. PCD, however, remains challenging to diagnose as a single gold standard is lacking [1, 3]. Guidelines for the management of mucociliary diseases are based on the monitoring of symptoms [1, 4]. Unfortunately, the correlation between clinical symptoms and the underlying disease activity is generally poor and can only be established by invasive procedures [5]. Infection and inflammation may be present before clinical parameters change. Therefore, early detection and treatment of respiratory pathogens and exacerbations may improve clinical outcome. This creates the need for non-invasive methods in the monitoring of disease activity in CF and PCD.

Analysis of volatile biomarkers in exhaled breath has shown to provide an attractive method to monitor both infection [6] and inflammation [7–9]. These Volatile Organic Compounds (VOCs) are likely to originate from local and systemic metabolic processes reflecting underlying disease processes. Analysis of individual volatiles by Gas Chromatography – Mass Spectrometry (GC-MS) and measurement of nasal nitric oxide illustrated that concentrations of specific volatiles differ between CF patients and/or PCD patients and healthy controls [10, 11]. While GC-MS is an essential technique to link individual components to pathophysiological mechanisms its clinical applicability is hampered by the need for complex laboratory techniques and highly trained personnel. Alternatively, electronic nose (eNose) technology comprises arrays of promiscuous sensors interacting with the exhaled volatile mixture in its entirety, providing a so-called ‘breathprint’ by using pattern-recognition algorithms [12]. Biomedical application of eNoses is emerging [13] and various studies have demonstrated the value of this technology in the discrimination of patients with asthma [14, 15], COPD [8, 14, 15], lung cancer [16, 17] and ventilator-associated pneumonia [18].

For patients with CF and PCD exhaled breath analysis by electronic nose may allow both early screening and frequent monitoring, because the technique is portable, low cost and provides immediate results. According to START guidelines, the essential first step to assess in CF and PCD is to establish exhaled volatile profiles that discriminate a priori defined disease entities [19]. In this study we therefore hypothesized that patients with cystic fibrosis, primary ciliary dyskinesia and healthy controls have significantly different exhaled molecular profiles as determined by electronic nose. Furthermore we aim to study patients without an exacerbation to assess its potential influence on exhaled breath profiles. The secondary goal of this study was to examine whether significantly different breath profiles could be identified in patients with and without pulmonary exacerbations.

## METHODS

### Design

This study was designed as a cross-sectional case-control study. All patients performed a single study visit during which exhaled breath was analyzed and sputum or cough swabs were cultured. Spirometry was performed in patients six years and older by a trained lung function technician according to ATS/ERS guidelines [20].

### Subjects

Children aged between 6 months and 18 years were included. By only including children we aimed to investigate CF and PCD patients that had relatively limited concomitant bacteria in their airways compared to adult patients. Patients were recruited during outpatient clinics of the VU University Medical Center Amsterdam, The Netherlands, between August 2011 and November 2011. Diagnosis of CF was based on clinical symptoms in combination with an abnormal sweat test (chloride > 60 mmol/l) and/or identification of mutations in both alleles of the CFTR-gene [21]. PCD diagnosis was based on a combination of clinical symptoms, evaluation of ciliary beat frequency and beat pattern by high-resolution, high-speed video microscopy and by transmission electron microscopy of ciliary ultrastructure as recommended by the European Respiratory Society Task Force consensus statement [1]. Furthermore, ciliary motility was also evaluated after cell culture to exclude secondary ciliary dyskinesia [22]. The presence of a pulmonary exacerbation in CF and PCD patients was determined after patients completed the study visit and was defined as the need to start additional antibiotic treatment as a consequence of a recent change in at least two of the following clinical parameters: change in sputum volume or color, increased cough, increased dyspnea, increased malaise, fatigue or lethargy, temperature over 38°, anorexia or weight loss, change in sinus discharge, change in physical findings on examination, decrease in pulmonary function by 10% or more and radiographic changes. This was done according to national CBO guidelines, based on internationally accepted criteria [5, 23, 24]. Healthy children were recruited during orthopedic outpatient clinics of the VU University Medical Center and the Academic Medical Center Amsterdam, The Netherlands. These two academic centers are situated within 10 km of each other and share the same patient population. Children were excluded in case of any pulmonary, inflammatory or metabolic disease. The study was approved by the Medical Ethical Committee of the participating centers. Written informed consent was obtained from parents and patients between 12 and 18 years. The study was registered in the Netherlands Trial Register, [www.trialregister.nl](http://www.trialregister.nl) under NTR 2847.

## Measurements

### *Breath collection*

Exhaled breath was collected using a modified spacer (Babyhaler, GlaxoSmithKline) with reverse valve system allowing tidal inspiration through a face mask and inspiratory VOC filter (A2, North Safety, Middelburg, The Netherlands) and tidal expiration into the spacer. The VOC filter minimizes the influence of environmental VOCs on the breath profile as a potential source of bias. The spacer was connected to the electronic nose during sampling for direct sample analysis during tidal breathing.

### *Electronic nose*

We used a carbon black polymer based Cyranose 320 electronic nose (Smiths Detection, Pasadena, CA, USA). VOCs interact with the array of 32 polymer nanosensors to induce a fully reversible change in electrical resistance. The changes in resistance of all 32 sensors provide the raw data of the eNose and were combined by pattern recognition analysis into a so-called breathprint. This allows simultaneous analysis of the entire VOC profile instead of analyzing individual sensors which would only represent a limited fraction of the measured volatiles. The settings of the eNose used in this study did not provide immediate results to the investigator and patients. Raw eNose data were digitally imported into a study database.

### **Statistical analysis**

The eNose data were analyzed by pattern-recognition algorithms. SPSS (version 16.0) was used for data analysis. Principal component reduction was used to capture the variance of the original breathprint into a set of orthogonal principle components (PCs), whereby reducing the dimensionality of the dataset to minimize the risk of overfitting [25]. Discriminating PCs were selected by unpaired *t*-test and subsequently used in a canonical discriminant analysis. The discriminant functions were used to construct a receiver operator characteristic (ROC) curve. The area under the curve (AUC) and optimum single spot test sensitivity and specificity were determined. This data was internally cross-validated by a bootstrapping procedure to minimize the risk of false positive findings according to current standards [25]. The current sample size was based on previous studies employing electronic noses in discriminating asthma [14, 15], COPD [15] and lung cancer [16] because no previous studies in CF and PCD were available.

## RESULTS

Seventy-three subjects participated in this study, including 25 patients with CF (median age (yr), IQR, 11.4, 7.7-17.9), 25 patients with PCD (10.7, 7.1-14.5) and 23 healthy subjects (9.3, 5.4-12.6). The subject characteristics of the three groups are described in table 1. Median age and sex did not significantly differ between healthy controls recruited from the two outpatient clinics. Nine CF patients (36%) and 4 PCD patients (17%) had a pulmonary exacerbation. The number of positive sputum and cough swab cultures did not differ between patients with CF (16 out of 23 cultures) and PCD (8 out of 18 cultures). The presence of *Staphylococcus aureus* was significantly higher in cultures of CF patients as compared to cultures of PCD patients ( $p=0.04$ ). *Haemophilus influenzae* occurred more frequently in cultures of PCD patients as compared to cultures of CF patients ( $p=0.02$ ). No other significant differences in subject characteristics were found between CF and PCD patients.

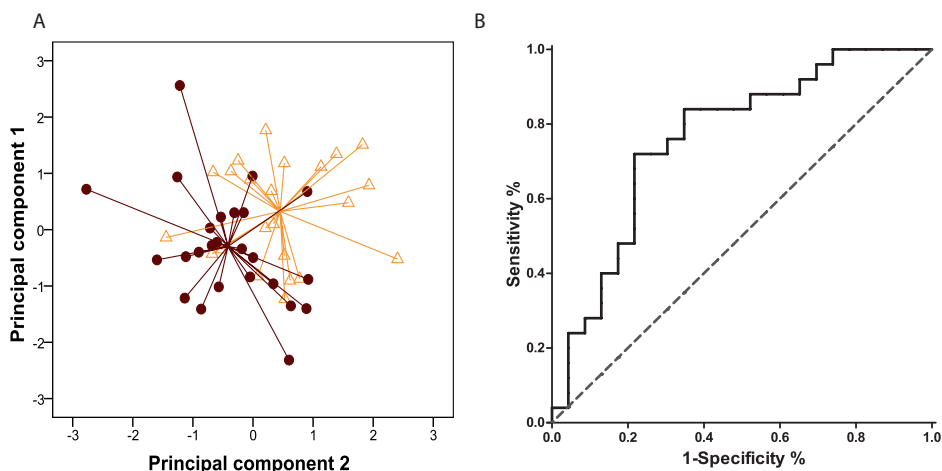
**Table 1.** Clinical characteristics of the study population.

	Cystic Fibrosis	Primary Ciliary Dyskinesia	Healthy Subjects
Subjects (n)	25	25	23
Age (years)*	11.4 [7.7-17.9]	10.7 [7.1-14.5]	9.3 [5.4-12.6]
Male (n/total)	10/25	14/25	14/23
Best FEV <sub>1</sub> in past year (% predicted)*	92.0[81.5-111.0]	104.0 [80.5-109.8]	NA
Best FVC in past year (% predicted)*	99.0 [88.0-116.0]	110.5 [97.0-119.0]	NA
Pulmonary exacerbation (n/total)	9/25	4/23	NA
Positive bacterial cultures (n/total)	15/22	8/18	NA
<i>S. aureus</i> (n)	13 <sup>†</sup>	2	NA
<i>Pseudomonas</i> spp.	5	1	NA
<i>H. influenzae</i>	1 <sup>§</sup>	6	NA
<i>S. maltophilia</i>	1	0	NA
<i>M. avium</i>	1	0	NA
<i>A. denitrificans</i>	1	0	NA
<i>M. catarrhalis</i>	0	2	NA
<i>S. pneumoniae</i>	0	2	NA
Other	1	5	NA

\* Data are presented as median and interquartile range. <sup>†</sup>  $p=0.04$  as compared to PCD. <sup>§</sup>  $p=0.02$  as compared to PCD. N, number; NA, not available; FEV<sub>1</sub>, forced expiratory volume in 1 s pre-bronchodilator; FVC, forced vital capacity, pre-bronchodilator; *S. aureus*, *Staphylococcus aureus*; *Pseudomonas* spp., *Pseudomonas* species; *H. influenzae*, *Haemophilus influenzae*; *S. maltophilia*, *Stenotrophomonas maltophilia*; *M. avium*, *Micobacterium avium*; *A. denitrificans*, *Achromobacter denitrificans*; *M. catarrhalis*, *Moraxella catarrhalis*; *S. Pneumoniae*, *Streptococcus pneumoniae*.

Breathprints from patients with CF ( $p=0.0004$ ) and with PCD ( $p=0.0001$ ) significantly differed from healthy subjects (figures 1A and 2A). The area under the receiver operator characteristic (ROC) curve (AUC) after internal cross-validation reached 0.76 (95% CI 0.62-0.90, sensitivity 84%, specificity 65%) and 0.80 (95%CI 0.67-0.93, sensitivity 88%, specificity 52%), respectively (figures 1B and 2B). Additionally, exhaled breath profiles differed significantly between patients with CF and PCD ( $p=0.001$ ) (figure 3A). ROC analysis resulted in an AUC of 0.77 (95% CI 0.63-0.91, sensitivity 84%, specificity 60%) (figure 3B). CF ( $n=16$ ) and PCD ( $n=19$ ) breath profiles still differed significantly from one another after omitting breath profiles from patients with a pulmonary exacerbation ( $p=0.001$ ). The AUC reached 0.77 (95% CI 0.60-0.95) and sensitivity and specificity increased to 95% and 63%, respectively. Detailed test characteristics for the diagnostic models are presented in table 2.

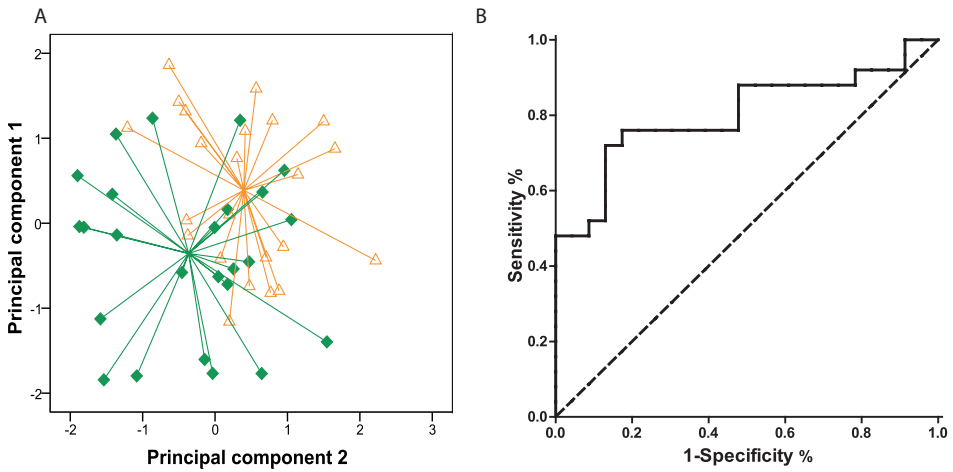
VOC profiles of CF patients ( $p=0.01$ ) as well as PCD patients ( $p=0.01$ ) with and without a pulmonary exacerbation differed significantly. ROC analysis resulted in an AUC of 0.76 (95% CI 0.58-0.95, sensitivity 89%, specificity 56%) and 0.90 (95% CI 0.76-1.00, sensitivity 100%, specificity 90%), respectively.



**Figure 1.** Discrimination of CF patients vs. healthy subjects.

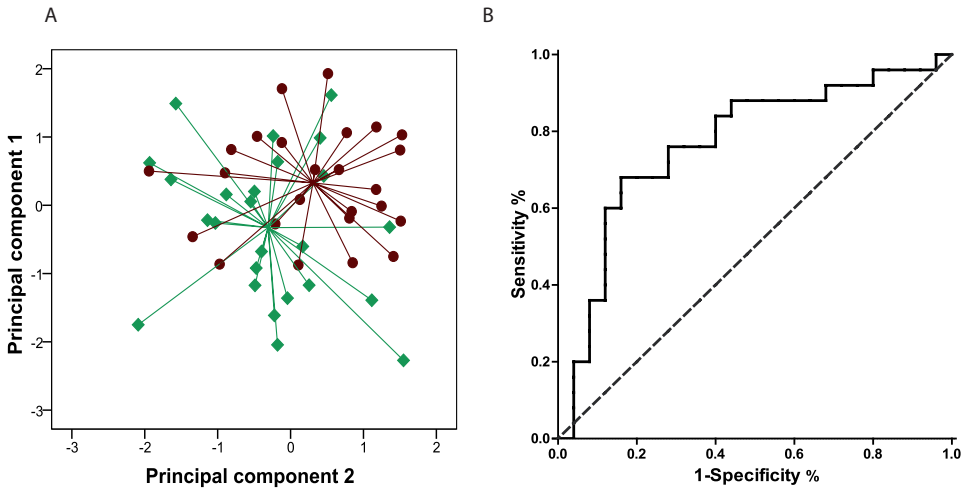
(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with CF (circles) and healthy controls (triangles) along two discriminative principal components.  $P=0.0004$ . (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of CF patients and healthy controls (AUC 0.76).





**Figure 2.** Discrimination of PCD patients vs. healthy subjects.

(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with PCD (diamonds) and healthy controls (triangles) along two discriminative principal components.  $P=0.0001$ . (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of PCD patients and healthy controls (AUC 0.80).



**Figure 3.** Discrimination of CF patients vs. PCD patients.

(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with CF (circles) and PCD (diamonds) along two discriminative principal components.  $P=0.001$ . (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of CF patients and PCD patients (AUC 0.77).

**Table 2.** Test characteristics of the receiver operator characteristic (ROC) curves for discrimination of CF patients, PCD patients and healthy subjects and for discrimination of patients with and without a pulmonary exacerbation, by exhaled breath analysis.

	N	AUC	95% CI	p-value	Sens (%)	Spec (%)
CF vs. healthy subjects	25 vs. 23	0.76	0.62-0.90	0.002	84	65
PCD vs. healthy subjects	25 vs. 23	0.80	0.67-0.93	<0.001	88	52
CF vs. PCD	25 vs. 25	0.77	0.63-0.91	0.001	84	60
CF pulmonary exacerbation vs. stable disease	9 vs. 16	0.76	0.58-0.95	0.031	89	56
PCD pulmonary exacerbation vs. stable disease	4 vs. 19	0.90	0.76-1.00	0.015	100	90
CF stable disease vs. PCD stable disease	16 vs. 19	0.77	0.60-0.95	0.006	95	63

*Test characteristics of the receiver operator characteristic (ROC) curves for the discrimination of CF patients vs. healthy subjects, PCD patients vs. healthy subjects, PCD patients vs. CF patients and patients with and without a pulmonary exacerbation, by exhaled breath analysis.*

*CF, cystic fibrosis; PCD, primary ciliary dyskinesia; vs., versus; N, number; AUC, area under the curve; 95% CI, 95% confidence interval of the area under the curve; Sens, sensitivity; Spec, specificity.*

## DISCUSSION

Our study shows that CF and PCD patients have significantly different exhaled breath molecular profiles compared to healthy subjects as determined by electronic nose. Furthermore, we observed that CF and PCD have significantly different VOC profiles allowing separation with reasonable sensitivity but rather limited specificity. In addition, we observed that VOC profiles differed significantly depending on the presence of a pulmonary exacerbations. Our results suggest that exhaled breath analysis may have diagnostic and monitoring potential in mucociliary clearance diseases.

To our knowledge this is the first study using eNose technology in CF and PCD. Previously, Robroeks and colleagues identified individual VOCs related to CF by GC-MS analysis of exhaled breath [10]. Our findings extend these results by showing that children with CF have significantly different total VOC profiles compared to healthy subjects. The other novelty of this study was to show that the exhaled marker profile of PCD was significantly different from patients with CF and controls. Compared to nasal nitric oxide measurement in PCD the eNose has lower sensitivity and specificity for detecting PCD [11]. As with the eNose, although to a lower extent, nasal nitric oxide is not specific for PCD and shows overlap with CF, nasal polyps, chronic sinusitis and diffuse pan-bronchiolitis. Even though further studies are necessary eNose technique could be complementary to nasal nitric oxide in the diagnosis of PCD as it may be more easy to perform across all ages and may require less rigorous control of measurement circumstances.

In the present study, all included patients were well characterized according to internationally accepted guidelines, were recruited by the same operator and were derived from the same outpatient clinic [1, 21]. The control subjects were recruited from two outpatient clinics which were located in the same geographical area sharing the same patient population. Subanalysis showed that there was no significant difference between exhaled breath profiles between the healthy controls recruited at the different clinics ( $p=0.16$ ). We aimed to study children in order to minimize the number of respiratory infections as compared to adult patients. However, in 16 out of 23 CF patients and 8 out of 18 PCD patients, pathogens were still detected by culturing. It should be noted that negative culture results do not exclude the presence of airway pathogens, as various recent microbiome studies in CF have demonstrated [26]. Furthermore the number of positive bacterial cultures between CF and PCD patients in this study did not differ although the incidence of *S. aureus* and *H. influenzae* did. The presence of pathogens may therefore be a potential source of bias in the discrimination of CF/PCD from healthy controls. In a post-hoc analysis there was no significant difference between breath profiles in the presence or absence of an infection ( $p=0.33$ ). The exact influence of pathogens on the VOC profile is however difficult to determine because the VOCs resulting from these infections are a combination of VOCs produced by the pathogen itself and the host response to that pathogen. A way of determining VOCs produced by individual pathogens is by studying the production of VOCs from *in vitro* cultures by GC-MS [13]. This however reaches beyond the scope of the current paper.

Even though CF and PCD are both characterized by chronic airway infection and neutrophilic inflammation, they originate from different pathophysiological mechanisms [5, 27]. It is likely that distinct inflammatory and metabolic processes generate partly different metabolites, explaining the different VOC mixtures in exhaled breath for both CF, PCD and healthy subjects [8].

As the determination of distinct breathprints in CF and PCD patients with a pulmonary exacerbation was our secondary objective, we reached a smaller sample size than for our primary objective. Therefore, the latter results are merely hypothesis-generating, requiring further validation in a larger cohort. Previous studies have shown that sputum of patients with PCD exhibit a more than 3-fold increase in interleukin (IL)-8 concentration, a trend towards a lower DNA concentration and lower levels of proteolytic enzymes as compared to subjects with CF, possibly reflecting differences in pathophysiological pathways [28, 29]. The significant differences between VOC profiles of these two mucociliary clearance diseases may thus very well originate from partially distinct inflammatory processes. It will require detailed characterization of individual VOCs by GC-MS to establish the predominant molecular compounds driving the signal. The latter is complementary to probabilistic diagnostic assessment by eNose.

Our study supports the notion that some of the discriminating VOCs are related to the host response by showing that CF and PCD breath profiles could be differentiated with slightly improved test sensitivity and specificity when patients with a pulmonary exacerbation were omitted. This suggests that disease specific VOCs for PCD and CF may exist. Furthermore this suggests that inflammatory changes can be detected by exhaled breath analysis potentially allowing monitoring of disease activity. It is important to investigate this in detail in a study specifically designed for this purpose as the current study was not sufficiently powered to address these questions. Therefore, the latter results are merely hypothesis-generating, requiring further study in a larger cohort.

Comparing gold standard diagnostic groups represents the first step of implementing a novel diagnostic technique into clinical practice according to the STARD guidelines [19]. Even though the currently observed sensitivities are promising, the specificities of the present analysis were still limited. We used bootstrapping procedures to minimize the possibility of false positive results which may occur when analyzing multivariate data [25].

Given the relatively high sensitivity for PCD in the present study, it can be inferred that breath analysis may have additional value in the initial diagnostic work-up of PCD. Particularly, since PCD is likely to be under diagnosed due to the lack of a single gold standard, ambiguous interpretation of diagnostic tests and the partial overlap of symptoms from healthy children suffering from recurrent respiratory tract infections [1, 30].

The observation that exhaled breath profiles differ significantly depending on the absence or presence of a pulmonary exacerbation suggests that the eNose may be a tool for non-invasive monitoring of disease activity in both CF and PCD. As recurrent respiratory infections and inflammation result in progressive lung damage, early detection and treatment are of major importance to improve clinical outcome in these patients. Future longitudinal studies should clarify whether VOC profiling of exhaled breath in CF and PCD patients may add to earlier identification and treatment of respiratory infections as established by quantitative PCR and microbiome technologies.

Furthermore the technological advances of breath analysis techniques are likely to increase its value for CF and PCD. The eNose device used in this study is based on the interaction of polymers with the VOCs inducing a resistance change. Many other techniques exist such as quartz microbalance sensors, metal oxide sensors and optical sensors. It's important to study the value of these sensors in the discrimination of CF and PCD in more detail.

New studies employing chemical analytical techniques such as GC-MS allow the identification of individual VOCs and may aid to connect VOCs to specific disease processes.

Importantly this can also facilitate the selection of the most suitable eNose sensors for the desired application and novel development of disease tailored sensors. This may greatly improve the clinical applicability of exhaled breath analysis by eNose.

In conclusion, our study showed that CF, PCD, healthy children and patients with and without pulmonary exacerbations have significantly different exhaled breath profiles as determined by an electronic nose. Therefore, after optimization and validation, exhaled breath analysis by eNose may eventually qualify as a non-invasive and easy to use tool in the monitoring of CF and PCD in clinical practice.

### **Conflict of interest statement**

None of the authors have conflicts of interest to disclose.

### **ACKNOWLEDGEMENTS**

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