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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Human volatilome analysis using eNose to assess uncontrolled asthma in a clinical setting

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Abstract

Background: Analyses of exhaled volatile organic compounds (VOCs) have shown promising results when distinguishing individuals with asthma. Currently, there are no biomarkers for uncontrolled asthma. Therefore, we aimed to assess, in a real-life clinical setting, the ability of the exhaled VOC analysis, using an electronic nose (eNose), to identify individuals with uncontrolled asthma.

Methods: A cross-sectional study was conducted, and breath samples from 199 participants (130 females, aged 6-78, 66% with asthma) were analysed using an eNose. A multivariate unsupervised cluster analysis, using the resistance data from 32 sensors, could distinguish three clusters of VOC patterns in the training and testing groups. Comparisons between the clusters were performed using the one-way ANOVA, Kruskal-Wallis and chi-squared tests.

Results: In the training set (n = 121), three different clusters covering asthma, lung function, symptoms in the previous 4 weeks and age were identified. The pairwise comparisons showed significant differences with respect to chest tightness during exercise, dyspnoea and gender. These findings were confirmed in the testing set (n = 78) where the training model identified three clusters. The participants who reported fewer respiratory symptoms (dyspnoea and night-time awakenings) were grouped into one cluster, while the others comprised participants who showed similar poor control over symptoms with the distribution of the individuals with asthma being significantly different between them.

Conclusions: In a clinical setting, the analysis of the exhaled VOC profiles using an eNose could be used as a fast and noninvasive complementary assessment tool for the detection of uncontrolled asthma symptoms.

KEYWORDS

asthma, electronic nose, exhaled breath, volatile organic compounds, volatilome

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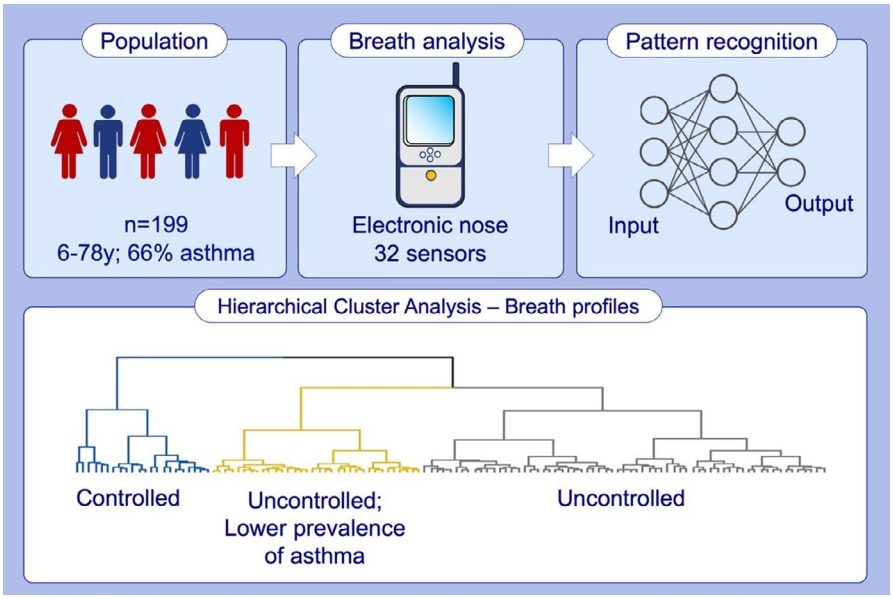

GRAPHICAL ABSTRACT

In a population with asthma and suspicious of asthma (recruited from an outpatient allergy clinic), eNose-driven breath profiles distinguished three clusters, blindly to reference. Participants with less respiratory symptoms are grouped in one cluster while, in the others, participants show poor symptoms control but the distribution of subjects with asthma is different. eNose can screen individuals with uncontrolled asthma symptoms in a clinical setting.

1 | INTRODUCTION

Asthma affects more than 300 million people worldwide and is expected to increase by another 100 million by 2025.[1] This is a chronic inflammatory disorder of the airways leading to hyperresponsiveness, reversible airway obstruction, mucus hyper-production and airway wall remodelling.[2] Although the term asthma is used to describe a set of symptoms and variable airflow limitation mainly due to inflammation, the underlying mechanisms vary heterogeneously among patients leading to difficulties in its management and treatment.[3] Thus, it is critical to ensure the effective management of asthma based on an accurate assessment of the patient characteristics and risk for serious outcomes.

Although the underlying biology remains poorly understood, a patient can be described in terms of the disease phenotypes. Generally, there is no correlation between these phenotypes and treatment responses, and different inflammatory pathways can explain why the therapies are only effective in a subset of patients, such as new biologics in severe eosinophilic asthma.[4] Diagnostic biomarkers are required to appropriately endotype patients and enable more personalised therapy. Furthermore, there is a need to improve the biomarkers to enable their use in clinical practice, particularly for diagnosis and monitoring. Recently, exhaled breath analysis using an electronic nose (eNose) has been suggested as a useful tool for identifying children in need of inhaled corticosteroid therapy [5] and predicting steroid responsiveness.[6] Electronic noses might be a promising tool for assessing asthma control and tailoring personalised treatment.

Breathomics can potentially provide additional information about a patient's condition, as gas chromatography coupled with mass spectrometry (GC-MS) and two-dimensional gas chromatography time-of-flight mass spectrometry (GC × GC-ToFMS), and

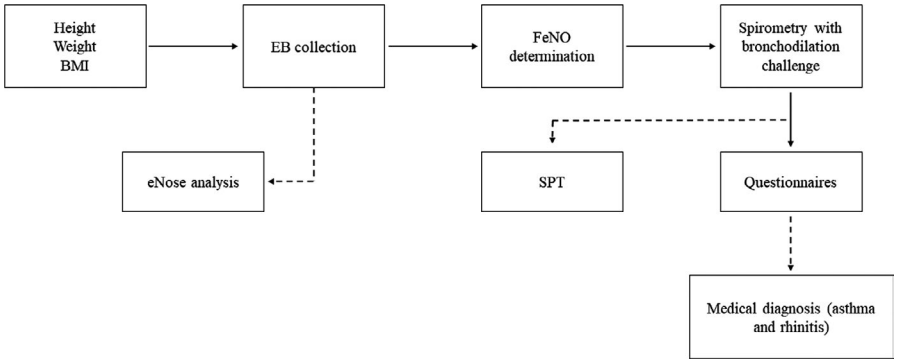

FIGURE 1 Flow diagram of clinical assessment of the participants. Full arrows: Procedures were performed during recruitment at clinical appointments. Dashed arrows: Procedures were performed after the clinical appointment, and specifically, eNose analysis was performed within 5 h after sampling. BMI, body mass index; EB, exhaled breath; FeNO, fractional exhaled nitric oxide; SPT, skin prick tests

TABLE 1 Participants' characteristics

	Training set	Testing set	*P* value
Participants (n)	121	78	
Characteristics			
Gender (male, n)	47	22	.12
Age (years)[a]	31.33 (6-78)	35.22 (7-63)	.09
<12 y old (%)	13.22	14.10	.86
<18 y old (%)	28.10	21.79	.32
Weight (kg)	64.74 (±16.83)	67.38 (±18.03)	.16
Height (m)	1.62 (±0.11)	1.61 (±0.11)	.36
BMI (kg/m^2)	24.45 (±5.26)	25.95 (±6.28)	.06
z-score[b]	0.56 (±1.00)	0.38 (±1.16)	.60
SPT (Positive, %)	83.04	79.41	.54
FeNO (ppb)	43.09 (±45.40)	39.93 (±42.58)	.74
Lung function			
FEV1 (%)	102.21 (±15.60)	98.74 (±16.27)	.13
FVC (%)	109.95 (±13.31)	108.44 (±14.41)	.50
FEV1/FVC (%)	79.67 (±9.17)	78.35 (±11.02)	.36
FEF 25-75 (%)	78.08 (±29.69)	71.01 (±29.59)	.15
PEF (%)	99.07 (±18.29)	97.21 (±18.15)	.48
Positive BD (%)	21.49	25.64	.50
Medical diagnosis of			
Asthma (%)	68.64	70.13	.83
Uncontrolled (%)	33.33	38.46	.55
Rhinitis (%)	87.93	81.82	.24
CS therapy (oral and inhaled)	64.66	64.47	.98
Smoker (%)	7.44	7.69	.95
Intake of food/drinks 2 h prior test (%)	62.81	58.97	.59
Exercise 2 h prior test (%)	0.00	0.00	-
Inhaler 2 h prior test (%)	4.13	5.13	.74

Note: Data are presented as mean ± SD unless otherwise stated. Medical diagnosis of uncontrolled asthma was defined according to the prescription of higher corticosteroid doses.

Abbreviations: BD, bronchodilatation; BMI, body mass index; FEF, forced expiratory flow; FeNO, fractional exhaled of nitric oxide; FEV1, forced exhaled volume in the first second; FVC, forced vital capacity; PEF, peak expiratory flow.

[a]Age is presented as mean (minimum – maximum).

[b]z-score was calculated for girls and boys with ages between 6 and 19 y.

eNose have been previously used to distinguish between the exhaled breath of the asthmatics and healthy subjects.[7,8] Changes in the exhaled volatile organic compounds (VOC) profile of the asthmatics are mainly related to chronic airway inflammation and oxidative stress, which lead to lipid peroxidation of the polyunsaturated

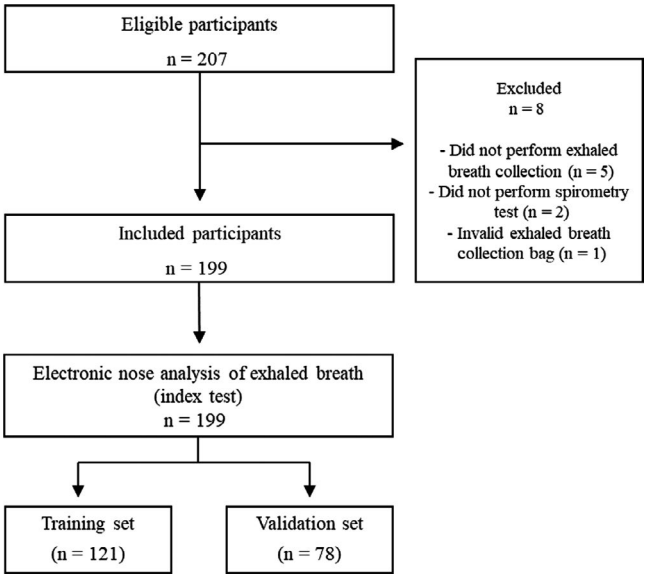

FIGURE 2 Flow of participants through the study

fatty acids present in the cellular membranes.[9] The resultant metabolites are mainly hydrocarbons that are locally released into the airways or bloodstream and are further oxidised by the cytochrome p450 enzymes before being excreted in the exhaled air.[7,10-12] Recently, a study found differences in the breath profiles of eosinophilic and neutrophilic asthma patients with respect to the levels of hexane and 1-propanol (2-10 ppm) and 2-hexanone, undecane, nonanal, 3-tetradecene and 1-pentadecene (0.4-10 ppm).[13] Unlike mass spectrometry, eNoses do not require preprocessing and chemical reference standards due to their ability to quickly analyse and recognise the VOC profiles at a low cost and independent of a skilled operator, once training and calibration have been established.[14] Moreover, the Cyranose 320® can detect VOC concentrations ranging from 100 ppb to 100 ppm.[15] Thus, eNoses are likely to be used in clinical practice. However, there remain major challenges in breathomics, such as the lack of accuracy studies in the larger, representative and intent-to-treat populations.

Therefore, we aimed to blindly explore the differences in the exhaled VOC profiles using an eNose, in subjects with respiratory symptoms (with a medical diagnosis of asthma or suspicious of asthma) recruited from an intent-to-treat population during their appointments, either in a training or testing set, for the assessment of uncontrolled asthma.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

A cross-sectional study of the exhaled breath analysis using an eNose was performed in a training and testing set. We recruited nonpregnant subjects above 6 years of age who had an appointment (monitoring or first visit) at a tertiary care clinic for presenting asthma-like symptoms. We selected a broad age range as we

TABLE 2 Differences between the hierarchical model clusters according to participants' clinical characteristics and lung function parameters (training cohort)

	Cluster T1	Cluster T2	Cluster T3	*P* value
Subjects N	65	22	34	
Characteristics				
Sex (male %)	32.31	59.09	38.24	.08
Age (years)	33.48 (±17.26)	26.23 (±14.10)	30.53 (±16.00)	.18
<12 y old (%)	6.15	27.27	17.65	**.03**
<18 y old (%)	23.08	36.36	32.35	.40
Weight (kg)	65.54 (±14.77)	65.64 (±21.03)	62.62 (±17.87)	.73
Height (m)	1.63 (±0.10)	1.62 (±0.13)	1.60 (±0.12)	.67
BMI (kg/m^2)	24.60 (±4.96)	24.64 (±6.35)	24.05 (±5.20)	.93
z-score[a]	0.39 (±0.97)	0.99 (±0.98)	0.45 (±1.06)	.38
Medical diagnosis of				
Asthma (%)	75.00	76.19	51.52	**.04**
Uncontrolled (%)	31.25	26.67	46.67	.45
Rhinitis (%)	90.48	85.00	84.85	.66
FeNO (ppb)	48.02 (±51.37)	44.62 (±51.08)	32.66 (±23.61)	.54
SPT (%)	80.33	94.74	81.25	.33
Lung function: Baseline				
FEV1 (%)	103.91 (±15.36)	103.77 (±16.52)	97.97 (±15.08)	.17
FEV1 (L)	3.14 (±0.97)	3.22 (±1.02)	2.88 (±0.92)	.54
FVC (%)	111.06 (±13.05)	112.41 (±14.20)	106.24 (±12.82)	.15
FEV1/FVC (%)	80.77 (±9.58)	78.82 (±9.80)	78.13 (±7.85)	.36
FEF 25-75 (%)	81.62 (±30.61)	79.09 (±31.38)	70.68 (±26.10)	.28
PEF (%)	102.22 (±17.85)	100.64 (±19.58)	92.03 (±16.83)	**.03**
FEV1 reversibility (L)	0.20 (±0.23)	0.14 (±0.13)	0.16 (±0.15)	.56
FEV1 reversibility (%)	6.32 (±6.63)	4.45 (±4.19)	6.41 (±6.82)	53
Positive BD (%)	26.15	9.09	20.59	.24
CS therapy (oral and inhaled)	69.35	71.43	51.52	.17
Smoker (%)	9.23	4.55	5.88	.71
Intake of food/drinks 2 h prior test (%)	61.54	40.91	79.41	**.01**
Inhaler 2 h prior test (%)	7.69	0.00	0.00	.11

Note: Data are presented as mean ± SD. Significant differences are indicated in bold.

Abbreviations: BD, bronchodilation; BMI, body mass index; CS, corticosteroid; FeNO, fractional exhaled of nitric oxide; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; PEF, peak expiratory flow; BD, bronchodilation test.

[a]z-score was calculated for girls and boys with ages between 6 and 19 y old.

aimed to identify a general signal in a symptomatic population. The eligible participants were randomly allocated to the training or testing set, and the clinical assessments included exhaled breath collection and eNose analysis, exhaled nitric oxide measurement, lung function and skin prick tests (SPT). Additionally, all participants completed a questionnaire. The medical diagnosis of asthma and allergic rhinitis was subsequently established by a physician according to the guidelines,[2] and the medical definition of uncontrolled asthma was based on the requirement to increase the dosage of corticosteroid (CS) prescription during the appointment. The medical doctor was aware of unwitting nonadherence, and every patient, with asthma or on asthma medication, had inhalation techniques assessed, and environmental measurements and anti-smoking information were provided. In a real-life clinical setting, the medical decision to step up or step down medication takes into consideration the aforementioned outcomes. Thus, we decided to use the "medical uncontrolled asthma" criterion supported by the doctors' decision, without restricting the analysis only to the symptoms scores. The flow of the clinical assessment is presented in Figure 1.

This study was approved by the University Ethical Committee, and written consent was obtained from all the participants before any procedure.

2.2 | Assessments

The height and weight of all the participants were measured. The body mass index (BMI) was calculated stratified according to the World Health Organization (WHO) guidelines.[16] Additionally, z-scores were calculated for the 6- to 19-year-old participants according to the WHO references, using the AnthroPlus software (WHO, Geneva).[17,18]

Exhaled breath collection was performed as described by Dragonieri et al[19] and the European Respiratory Society.[20] Additionally, we evaluated the breath samples collected in ten healthy subjects after 1, 2 and 5 minutes of tidal breathing through a VOC filter to determine the optimal time required to eliminate the environmental influence, using the Cyranose 320® (Sensigent). To identify the differences between the three sample classes, the breath-prints were analysed using the software on-board the eNose (PC nose® software, Sensigent). The samples collected after 2 minutes exhibited a low Mahalanobis distance (0.474) as compared to the 5 minutes samples. Thus, the participants were asked to provide tidal breathing via a two-way nonrebreathing valve attached to a VOC filter (Honeywell A2, North Safety) for 2 minutes, with the nose clipped, to eliminate the effect of the environmental VOCs. Subsequently, the participants performed a maximum forced expiratory manoeuvre into a 1L Tedlar bag (SKC, Inc) attached to the expiratory port of the nonrebreathing valve. The exhaled breath samples were evaluated using Cyranose 320® within 5 hours.

Airway inflammation was assessed by measuring the fractional exhaled nitric oxide (FeNO) levels using the NObreath analyser (Bedfont Scientific Ltd.). The results were expressed in parts per billion and stratified according to the guidelines.[21] Spirometry and bronchodilation challenge were performed before and 15 minutes after the inhalation of 400 μg of salbutamol, according to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines.[22] Allergic sensitisation was measured using SPT. The patients were asked to complete a questionnaire to report any respiratory symptoms in the last 4 weeks, medication taken in the last 48 hours, smoking habits and other diseases. The reported respiratory symptoms included blocked and/or runny nose, sneezing, dyspnoea, chest wheezing, chest tightness upon physical exercise, tiredness while performing daily tasks due to respiratory diseases, waking up during the night and increased usage of medicines because of respiratory diseases. In adults, the frequency of the symptoms was also stratified (never, up to 2 days per week, more than 2 days per week and almost every day).

In total, 207 patients were invited to participate and 199 (training, n = 121; testing, n = 78) were included in the analysis. The flow of participants and their characteristics are summarised in Figure 2 and Table 1, respectively.

2.3 | Data analysis

Resistance values from 32 sensors (variables) of the Cyranose 320® (Sensigent) corresponding to each of the 199 samples (observations) were imported as a DataFrame object into the RStudio software v3.4.2 (R Foundation). The data were scaled to standardise the unit variance (mean = 0, standard deviation = 1). First, cluster analysis was performed on the training data using Ward's minimum variance method to agglomerate the data values according to the Euclidean distances. Hopkins' index was calculated to assess the clustering tendency, retrieving a value of 0.92. The data were considered as clusterable. Then, to assess the optimal number of clusters, the totals within the sum of squares and average silhouette width methods were calculated to confirm/obtain an optimal cluster score for k = 3. The internal validation and cluster stability methods were applied to the "hierarchic," "PAM" (partitioning around medoids) and "k-means" algorithms, with the best scores achieved for the hierarchical model (Table S1). Thus, the training set population was divided into three clusters (T1, T2 and T3) based on the hierarchical clustering method (threshold for 3-class cut-off: 0.0547). Additionally, we calculated the similarity between the clustering methods using the Rand index. Briefly, in cluster analysis, the objects from the same group (or cluster) are more similar to each other than those in the other groups.[23] In the k-means method, each cluster is represented by the mean of the data points belonging to the cluster, as opposed to PAM clustering, where each cluster is represented by one of the objects in the cluster. Hierarchical clustering is an alternative method as it groups the objects based on their similarity. The training model was used to classify the testing cohort (V1, V2 and V3 clusters). Comparisons between the clusters were performed using the ANOVA, Kruskal-Wallis and chi-square tests in the SPSS® statistical package software v25.0 (IBM). Pairwise comparisons between the clusters were also performed. A P value < .05 was considered statistically significant.

3 | RESULTS

Unsupervised hierarchical clustering in the training set resulted in three different clusters covering asthma (P = .04), lung function (P = .03), age (P = .03) and intake of food or drinks 2 hours prior to breath sampling (P = .01) (Tables 2 and 3) (Figure S1, Table S3). Clustering similarity between the hierarchic and k-means models was 0.80 and 0.54 between the hierarchic and pam models, thus strengthening the clusters obtained (Table S2). Briefly, cluster 3 had less participants with asthma but presented a poorer lung function.

Pairwise comparisons between the clusters showed further significant differences, such as chest tightness during exercise (between clusters T2 and T3, and clusters T2 and T1), dyspnoea (clusters T2 and T3), asthma (clusters T3 and T1) and peak expiratory flow (PEF) (clusters T3 and T1). Thus, cluster T3 was characterised

TABLE 3 Differences between the hierarchical model clusters according to asthma-like symptoms (training cohort)

Symptoms (adults)	Cluster T1	Cluster T2	Cluster T3	*P* value
Young and adults (age: 13-78)				
Stuffy nose (%)	78.69	87.50	82.14	.71
Stuffy nose (frequency, mean: 0-3)	1.54 (±1.10)	1.56 (±1.03)	1.79 (±1.03)	.59
Sneezing (%)	91.80	93.75	96.43	.72
Sneezing (frequency, mean: 0-3)	1.77 (±0.92)	1.69 (±0.95)	2.00 (±0.98)	.49
Runny nose (%)	77.05	81.25	82.14	.84
Runny nose (frequency, mean: 0-3)	1.31 (±1.03)	1.31 (±0.87)	1.54 (±1.07)	.63
Shortness of breath/dyspnoea (%)	50.82	37.50	64.29	.22
Shortness of breath/dyspnoea (frequency, mean: 0-3)	0.80 (±0.96)	0.63 (±1.03)	1.11 (±1.03)	.18
High pitch sound in chest/wheezing (%)	50.82	43.75	53.57	.82
High pitch sound in chest/wheezing (frequency, mean: 0-3)	0.95 (±1.13)	0.88 (±1.15)	0.89 (±1.03)	.95
Chest tightness during exercise (%)	52.46	25.00	57.14	.10
Chest tightness during exercise (frequency, mean: 0-3)	0.98 (±1.14)	0.37 (±0.81)	1.00 (±1.09)	.09
Tiredness/difficulty in doing daily tasks (%)	57.38	56.25	57.14	.99
Tiredness/difficulty in doing daily tasks (frequency, mean: 0-3)	1.13 (±1.18)	0.87 (±1.03)	1.00 (±1.02)	.78
Woke up in the middle of the night (%)	45.90	43.75	64.29	.23
Woke up in the middle of the night (frequency, mean: 0-3)	0.85 (±1.09)	0.75 (±1.07)	1.14 (±1.08)	.30
Increased the use of medicines because allergic respiratory diseases (%)	48.89	46.15	52.17	.94
Increased the use of medicines because allergic respiratory diseases (frequency, mean: 0-2)	0.73 (±0.84)	0.69 (±0.86)	0.70 (±0.77)	.99
Children (age: 6-12)				
Stuffy nose (%)	25.00	33.33	83.33	.12
Sneezing (%)	25.00	66.67	33.33	.35
Runny nose (%)	25.00	66.67	66.67	.35
Shortness of breath/dyspnoea (%)	50.00	16.67	33.33	.53
High pitch sound in chest/wheezing (%)	50.00	16.67	16.67	.41
Cough (%)	75.00	66.67	83.33	.80
Chest tightness during exercise (%)	50.00	50.00	50.00	1.00
Tiredness/difficulty in doing daily tasks (%)	50.00	50.00	33.33	.81
Woke up in the middle of the night (%)	0.00	16.67	33.33	.41
Had to miss school (%)	0.00	0.00	16.67	.41
Increased the use of medicines because allergic respiratory diseases (%)	0.00	16.67	33.33	.41
Children, young and adults (age: 6-78)				
Stuffy nose (%)	75.38	72.73	82.35	.65
Sneezing (%)	87.69	86.36	85.29	.94
Runny nose (%)	73.85	77.27	79.41	.82
Shortness of breath/dyspnoea (%)	50.77	31.82	58.82	.14
High pitch sound in chest/wheezing (%)	50.77	36.36	47.06	.50
Chest tightness during exercise (%)	52.31	31.82	55.88	.17
Tiredness/difficulty in doing daily tasks (%)	56.92	54.55	52.94	.93
Woke up in the middle of the night (%)	43.08	36.36	58.82	.19
Increased the use of medicines because allergic respiratory diseases (%)	44.90	36.84	48.28	.73

Note: Symptoms in last 4 wks were auto-reported in a questionnaire. In young and adults, the frequency of symptoms was additionally questioned (0 = never; 1 = up to 2 d in a week; 2 = more than 2 d in a week; 3 = almost every day). The frequency of symptoms was analysed as a numerical variable. No significant differences were found among children (6-12 y old).

TABLE 4 Pairwise differences (*P* value) between the hierarchical model clusters according to asthma-related symptoms (training cohort)

Respiratory symptoms	T1/T2	T1/T3	T2/T3
Young and adults (age: 13-78)			
Shortness of breath/dyspnoea, %	0.34	0.16	0.09
Shortness of breath/dyspnoea (frequency, mean: 0-3)	0.37	0.16	0.09
High pitch sound in chest/wheezing, %	0.62	0.81	0.53
High pitch sound in chest/wheezing (frequency, mean: 0-3)	0.74	0.96	0.80
Chest tightness during exercise, %	0.050	0.68	**0.04**
Chest tightness during exercise (frequency, mean: 0-3)	**0.04**	0.84	**0.04**
Tiredness/difficulty in doing daily tasks, %	0.94	0.98	0.95
Tiredness/difficulty in doing daily tasks (frequency, mean: 0-3)	0.53	0.70	0.68
Woke up in the middle of the night, %	0.88	0.11	0.19
Woke up in the middle of the night (frequency, mean: 0-3)	0.78	0.17	0.20
Children, young and adults (age: 6-78)			
Shortness of breath/dyspnoea, %	0.12	0.45	**0.05**
High pitch sound in chest/wheezing, %	0.24	0.73	0.43
Chest tightness during exercise, %	0.10	0.74	0.08
Tiredness/difficulty in doing daily tasks, %	0.85	0.71	0.91
Woke up in the middle of the night, %	0.58	0.14	0.10

Note: T1: cluster T1; T2: cluster T2; T3: cluster T3. Significant differences are indicated in bold.

by fewer asthmatics, although this cluster had more participants with asthma-like symptoms, such as dyspnoea and chest tightness during exercise, as compared to cluster T2, but was similar to cluster T1. Cluster T3 participants also presented a poorer lung function. Participants from cluster T2, composed predominantly of males and children under 12 years of age, presented a lower prevalence of respiratory symptoms. Cluster T1 included participants with uncontrolled asthma-like symptoms similar to cluster T3. The characteristics of the three clusters (training set) are presented in Tables 2-4 and in the (Tables S3 and S4).

No differences were found between the training and testing populations (Table 1). The breath-prints from the testing set (V1, V2 and V3) showed no significant differences with respect to asthma, PEF and symptoms. However, the trends that included fewer asthmatics in cluster V3 and participants with less respiratory symptoms in cluster V2 were observed. The pairwise cluster analysis showed that cluster V2 included less participants with dyspnoea as compared to cluster V1 ($P = .03$) and less night-time awakenings due to respiratory diseases ($P = .03$) (Tables S5-S8).

4 | DISCUSSION

Our findings support the use of an eNose for evaluating individuals with asthma-like symptoms. In this study, we showed that in patients with respiratory complaints, employing an eNose to analyse the breath-prints distinguished three clusters of subjects differing in the prevalence of asthma, lung function, symptoms in the previous 4 weeks, age and intake of food/drinks 2 hours prior to breath sampling. The participants with less respiratory symptoms were grouped into one cluster, while in the others, participants showed similar poor control over the symptoms, but with a significant difference in the distribution of the subjects with asthma. The testing cohort showed similar clusters regardless of no significant differences in the distribution of the asthmatics. Considered together, our observations support the ability of an eNose to detect the individuals with poorly controlled asthma-like symptoms. This is an important finding as the identification of a specific exhaled breath pattern using an eNose in a reproducible, cheap and noninvasive manner in a clinic setting might assist in the precise and personalised management of asthma.

Our study has a few limitations. First, its cross-sectional design did not allow us to establish a causal relationship between the symptoms and exhaled VOC profile. Cohort studies are required to understand both the determinants and the changes in the exhaled VOC profiles. We also recognise that a third group of patients (validation group) would be essential to strengthen our results. When we divided our population into three subgroups (Training–70%, Testing–20%, Validation–10%), we observed similar results in the training and testing populations, but not in the validation group. This last group

contained few participants, which made it difficult to observe statistically significant differences between the clusters. Second, we did not have the opportunity to assess other biomarkers, such as blood or sputum eosinophils. We recognise that other variables can contribute to differences in the breath-prints, particularly those related to inflammation, as changes in the exhaled breath of patients with distinct inflammatory profiles have been reported.[24,25] Additionally, we did not collect data on the dosage of CS prescribed to each patient with asthma. As this does not preclude adhesion to treatment, in our study, we reported an increase in the CS dosage following the doctor's examination to characterise patients with "uncontrolled asthma" based on a medical decision. Third, reproducibility using a different device was not assessed. Nevertheless, the reproducibility of the Cyranose 320® has already been tested in healthy controls (Cohen's kapa coefficient ranged from 0.75 to 0.91).[26] Moreover, sampling methods for collecting the exhaled VOCs are currently being researched and, despite ERS recommendations, a standardised methodology does not exist.[20] As specified in the ERS technical standard on breath analysis, despite the effect of the environmental VOCs, there are other important considerations, such as the type of sampling, sampling duration, effect of expiratory flow, types of collecting materials and the effects of humidity, food, medications, exercise, smoking and comorbidities. In this study, it was not possible to control the expiratory flow, medication, smoking, comorbidities and intake of food before sampling, which can affect the breath-prints and contribute to overfitting of the results.[15,26-28] The expiratory flow rate can affect the concentration of the exhaled VOCs and, consequently, the sensor response.[29] We did not find statistically significant differences between the clusters with respect to the medication taken, comorbidities and smoking, although other studies have reported differences indicating that these factors are responsible for metabolic alterations that might be reflected in the composition of the exhaled VOCs.[26-28] As the participants were randomly assigned in this study, we could not restrict the intake of food/drinks 2 hours before sampling. As we observed differences with respect to this variable, we suggest that this requirement be fulfilled in future studies to reduce overfitting of the results. Moreover, pregnant women were excluded due to the accelerated metabolism and immune and hormonal alterations observed during pregnancy, which might influence the exhaled VOCs.[30] The sex and age also affect the breath-prints; however, we did not stratify the data with respect to these variables, as the main aim was to identify differences in the breath-prints according to the demographic data, symptoms and lung function. Furthermore, we standardised the sampling using a VOC filter, fixed volume collection bag composed of an inert and reusable material and single expiratory manoeuvre during collection. Methods that remove the effects of humidity are not available. As suggested in the ERS technical standard on breath analysis, we performed a supplementary analysis that involved eliminating the water-sensitive sensors of the eNose (sensors 5, 6, 23, and 31) to obtain similar clusters with similar characteristics. Finally, the testing population should be larger to confirm the results obtained in the training set. The results of the training and testing populations did not completely match, despite the similarities observed.

Regardless of the aforementioned limitations, this study has important strengths. This is one of the first studies to employ an eNose to analyse breath samples in a population with various respiratory symptoms, recruited during appointments, in a clinical setting. A recent study followed a similar approach to evaluate the exhaled breath of COPD and asthma patients, using a different eNose.[25] Cluster analysis showed five combined clusters of chronic airway disease and asthma that were not determined according to the diagnostic label, but rather by the clinical and inflammatory characteristics. In another study conducted in subjects with severe asthma, differences in the exhaled breath related to inflammation and CS use were found.[31] These findings diminish the importance of a diagnostic label and highlight approaches based on the clinical characteristics and treatable traits to understand the condition of each patient. According to the adopted study design, all participants with respiratory symptoms were selected, showing the generalisation of our results and potential clinical application despite comorbidities, smoking, sex, age and intake of CS or food 2 hours prior to sampling. Additionally, we evaluated the respiratory symptoms reported by the participants. The breath-prints were analysed blindly with respect to the reference to better explore the differences found in the exhaled breath of the participants recruited in a real-life clinical setting. Moreover, the rand index results strengthened the outcomes of our clusters. The use of an independent testing set confirmed the results from the training set despite a few incomplete matches, which could be attributed to the sample size, as we observed the same trend in the cluster characteristics for both populations. Furthermore, the elimination of the environmental VOCs during the acquisition of the exhaled air was extremely important, as previous studies have demonstrated good accuracy results using this methodology.[19,26,32-37]

Previous studies have shown that breathomics can identify asthmatics with uncontrolled symptoms and in need of inhaled corticosteroid treatment.[6,37,38] The exhaled breath of patients with controlled and uncontrolled asthma was well discriminated using a model of thirteen compounds (CVV = 80%).[38] Later, the Cyranose 320® could distinguish uncontrolled patients from both the healthy controls and asthmatics with good control of the disease (area under the curve, AUC 0.814).[6] The criteria used to define uncontrolled asthma focused on the significant changes in the PEF, increased use of reliever medicines exceeding the average daily use and awakenings due to asthma more than two times a week. Recently, the exhaled breath of patients with asthma was evaluated in a longitudinal study using two different approaches (GC-MS and eNose technology).[37] Both techniques distinguished the breath-prints collected at different times (baseline, loss of control and recovery). The eNose achieved a higher accuracy than the GC-MS (86%-95% and 68%-77%, respectively). The participants exhibiting a poor control over the asthma-like symptoms exhibited a specific smell-print, possibly due to inflammation and oxidative stress, leading to lipid peroxidation and excretion of the VOCs in the exhaled breath.[39] These studies corroborate our results, as the main discriminative VOCs were alkanes, methyl alkanes and alcohols. Additionally, more than 80% of the participants presented a diagnosis of allergic rhinitis, which might have contributed to the presence of

VOCs derived from oxidative stress and, consequently, lipid peroxidation.[40] To our knowledge, there have been no studies that have reported the type of VOCs present in the exhaled breath of patients with rhinitis; however, breath-prints analysed using an eNose could differentiate the patients from the controls.[41] Our study stands out from the previous ones because the eNose was tested in a clinical setting, in the presence of confounders and comorbidities potentially responsible for the asthma-like symptoms.

Our study is the first to test an eNose exclusively in a real clinical setting and included participants with asthma and those suspicious of asthma. The eNose could differentiate the uncontrolled asthma-like symptoms in a clinical setting, indicating that the exhaled breath exhibits differences due to inflammation of the airways, despite the presence of confounders and comorbidities. Other studies have suggested that this technology can differentiate inflammatory markers (eosinophilia and neutrophilia). This study extends the results of the other research groups who observed differences in the exhaled breath of asthmatics using GC-MS and eNose technology, particularly in those with uncontrolled symptoms. In practice, these results are important because this fast and noninvasive approach can screen individuals with uncontrolled asthma symptoms, thus leading to faster monitoring, and enhanced management and treatment. It would be interesting to follow-up with patients in a longitudinal study and evaluate the effect of therapy on the breath-prints and symptoms. The GC-MS studies should be continuously performed to provide information on the altered pathways. In conclusion, our study encourages the design of further surveys for testing the eNose in clinical settings.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

MF, JCR, IP, FCM and AM were involved in investigation. MF, JCR, IP, FCM, AR, TR, SMR, LD, PB and AM gave constructive criticism of the study manuscript. MF, JCR, IP, FCM and AM were involved on study conceptualisation and interpretation. MF, TR and AR were involved in data collection. MF and JCR were involved in the development of breath pattern algorithm that was revised by PB. MF, JCR, IP, PB and AM contributed to the statistical analysis. MF and AM wrote the manuscript with input from all authors. AM obtained funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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