

## ORIGINAL ARTICLE

## Asthma and Lower Airway Disease

# Exhaled breath condensate volatolome allows sensitive diagnosis of persistent asthma

João Cavaleiro Rufo<sup>1,2,3</sup>  | Inês Paciência<sup>1,2,3</sup>  | Francisca Castro Mendes<sup>1</sup> | Mariana Farraia<sup>1</sup> | Ana Rodolfo<sup>1,4</sup> | Diana Silva<sup>1,4</sup> | Eduardo de Oliveira Fernandes<sup>3</sup> | Luís Delgado<sup>1,4</sup> | André Moreira<sup>1,2,4</sup>

<sup>1</sup>Imunologia Básica e Clínica, Departamento de Patologia, Faculdade de Medicina, Universidade do Porto, Porto, Portugal

<sup>2</sup>EPIUnit - Instituto de Saúde Pública, Universidade do Porto, Porto, Portugal

<sup>3</sup>Grupo de Energia e Ambiente Construído, Instituto de Ciência e Inovação em Engenharia Mecânica e Industrial, Porto, Portugal

<sup>4</sup>Departamento de Imunoalergologia, Centro Hospitalar S. João EPE, Porto, Portugal

**Correspondence**

João Cavaleiro Rufo, Imunologia Básica e Clínica, Departamento de Patologia, Faculdade de Medicina, Universidade do Porto, Porto, Portugal.  
Email: jcruf@gmail.com

**Funding information**

Authors gratefully acknowledge the funding by Fundação para a Ciência e Tecnologia through the ARIA project (PTDC/DTP-SAP/1522/2012), through the scholarships SFRH/BD/108605/2015, awarded to JCR, and SFRH/BD/112269/2015, awarded to IP, and by the Project NORTE-01-0145-FEDER-000010—Health, Comfort and Energy in the Built Environment (HEBE), cofinanced by Programa Operacional Regional do Norte (NORTE2020), through Fundo Europeu de Desenvolvimento Regional (FEDER).

**Abstract**

**Background:** The diagnosis and phenotyping of paediatric asthma are particularly complex due to the lack of currently available sensitive diagnostic tools. This often results in uncertainties associated with inhaled steroid therapy prescription. Therefore, this study aimed to investigate whether volatile organic compounds measured in exhaled breath condensate can be used as biomarkers for asthma diagnosis in the paediatric population.

**Methods:** A total of 64 participants, aged 6–18 years, were recruited on a random basis during visits to an outpatient allergy clinic and to a juvenile football team training session. Lung function, airway reversibility and skin prick tests were performed. Exhaled breath condensate samples were collected, and breathprints were assessed using an electronic nose. Information on medical diagnosis of asthma, rhinitis and atopic dermatitis was retrieved for each participant. A hierarchical cluster model based on the volatolome profiles was then created.

**Results:** A two-cluster exhaled volatile organic compound-based hierarchical model was able to significantly discriminate individuals with asthma from those without the disease (AUC = 0.81 [0.69–0.93],  $P < 0.001$ ). Individuals who had persistent asthma and were prescribed corticosteroid therapy by the physician were also significantly distinguished in the model (AUC = 0.81 [0.70–0.92],  $P < 0.001$ ). Despite being less specific, the method showed higher overall accuracy, sensitivity and AUC values when compared to spirometry with bronchodilation.

**Conclusions:** Analysis of the exhaled breath condensate volatolome allowed the distinction of paediatric individuals with a medical diagnosis of asthma, identifying those in need of corticosteroid therapy.

**KEYWORDS**

asthma diagnosis, electronic nose, exhaled breath condensate, multivariate analysis, volatile organic compounds

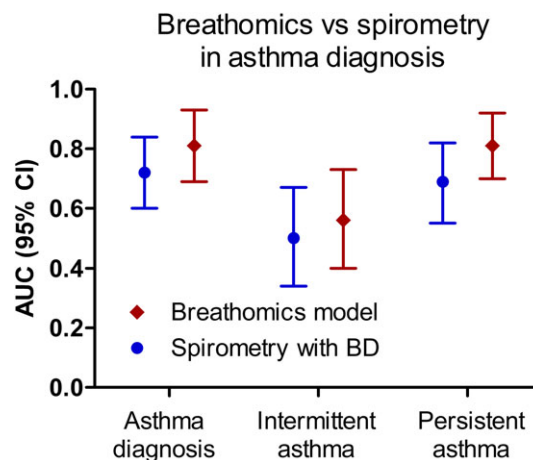
## 1 | INTRODUCTION

The diagnosis and phenotyping of asthma are particularly complex in children due to concomitant virus-induced airway symptoms, transient wheezing and the difficulty in recalling history of recurrent symptoms or exacerbations, which may lead to misdiagnosis and inappropriate treatment.<sup>1-3</sup> In the current clinical context, and according to standardized guidelines,<sup>4,5</sup> severe asthma is often diagnosed in children older than 6 years through long-term follow-up, consideration of the extensive differential diagnoses and by observing the child's response to bronchodilator through spirometry.<sup>6</sup> However, there are no specific diagnostic tools or biomarkers for detecting asthma in the paediatric population and bronchodilator response is still one of the most frequently used measurements of reversible airflow limitation.<sup>7</sup> Although highly specific, absence of significant response to a bronchodilator does not preclude asthma diagnosis.<sup>6,8-10</sup> Therefore, a more sensitive biomarker to diagnose asthma, combining versatility, noninvasiveness and accuracy is needed.

In the last decade, exhaled volatile organic compounds (VOCs) have been increasingly used as biomarkers for asthma.<sup>11-17</sup> The airway inflammation characteristically present in asthma promotes the degradation of polyunsaturated fatty acids in the lipidic structures of bronchial epithelium cells membrane, promoting the formation of volatile hydrocarbons. These VOCs enter the bloodstream and are subsequently excreted in the exhaled breath in different configurations according to their origin.<sup>18-20</sup> This type of inflammation is tendentially associated with a Th2 eosinophilic asthma and frequently responds appropriately to inhaled corticosteroid therapy.<sup>21</sup>

Several research groups were able to build VOC models or profiles (also called *breathprints*), measured through electronic nose (eNose) or gas-chromatography coupled to mass spectrometry (GC-MS), capable of accurately distinguishing individuals with asthma from healthy controls.<sup>11-14,22-26</sup> This high sensitivity method for detecting asthma inflammation might be proven useful in a real clinical context, even in childhood asthma diagnosis. Nevertheless, most of these studies were not blinded to the reference standard and missed model stability measurements, and almost all of them used exhaled breath as the VOC collection matrix. Exhaled air samples occupy large volumes, ranging from 500 mL to 10 L, and must be analysed within 6 hours of collection to avoid contamination.<sup>15,27,28</sup> In addition, sample bags are expensive, and, although reusable, they must be cleansed with multiple nitrogen flushes.<sup>29</sup> All these limitations make exhaled VOC analysis cumbersome to implement in a general clinical setting.

It is possible to measure VOCs in exhaled breath condensate (EBC), a putative good matrix for transport, storage and analysis.<sup>30</sup> Yet, there are no exhaled breath condensate VOC models for asthma diagnosis or severity monitoring. Therefore, the present study aimed to investigate whether volatile organic compounds in exhaled breath condensate may be used as biomarkers for asthma diagnosis and/or monitoring in the paediatric population.



### GRAPHICAL ABSTRACT

The developed breathomics model was able to identify individuals with asthma and with persistent asthma with improved performance over spirometry with bronchodilation. The study shows that a breathomics model was able to identify individuals with a medical diagnosis of asthma with high accuracy. Individuals with asthma in need of inhaled corticosteroid therapy were significantly clustered in the model. Breathomics has the potential to assist the physician in the diagnosis of asthma and/or administration of corticosteroid therapy for paediatric patients with asthma.

## 2 | MATERIALS AND METHODS

### 2.1 | Study design and participants

The present cross-sectional study follows the updated STARD guidelines for reporting diagnostic accuracy in studies.<sup>31</sup> Sample size estimation was based on previously reported accuracy measurements obtained through meta-analysis of 6 studies focused on exhaled VOCs in asthma diagnosis,<sup>27</sup> and calculations were performed according to the classic likelihood ratio sample size estimation method for diagnostic test studies.<sup>32</sup> Therefore, estimating a sensitivity of 87% and a specificity of 86% for asthma diagnosis through exhaled VOC analysis, and considering a sample comparison ratio of 1.0 for balanced judgement (disease vs nondisease), power calculations showed that 42 individuals with asthma and 42 individuals without asthma would be needed to show that the index test at least surpasses the 2.90 positive likelihood ratio achieved by spirometry with bronchodilation in asthma diagnosis, according to published data,<sup>9</sup> with a 95% confidence interval.

Participants, aged 6-18 years, were recruited from two distinct settings: during regular appointments to a tertiary care outpatient allergy clinic (population with a high chance of having asthma) in S. João Hospital Centre, Porto, Portugal, from May to September 2016 and during a regular training session for a local juvenile football team (population with a lower chance of having asthma) in Porto, Portugal, between 5 and 13 September 2017. Eligible participants were recruited on random basis, independently of the

week day, time period of the visit and appointed physician. No information concerning the participants' medical history was obtained prior to recruitment. After obtaining the legal guardians' informed consent and participant's agreement, airway reversibility measurements and skin prick tests (SPT) were performed, followed by EBC collection. Medical diagnosis of asthma and associated severity level (based on the administered treatment), as well as medical diagnosis of allergic rhinitis and atopic dermatitis, was established by an allergy specialist, randomly selected for each participant.

A total of 57 eligible individuals were invited to participate during the outpatient clinic visits, but only 51 were included in the study since 4 refused to partake, while the remaining 2 failed to produce valid EBC samples. During the football practice recruitments, all 13 paediatric individuals that were eligible to participate accepted the invitation. Therefore, a total of 64 participants aged 6–18 years were included in the study. The recruitment flow of participants is summarized in Figure 1.

The study was approved by the Ethics Committee for Health of S. João Hospital Centre (authorized at 14 April 2016) and by the National Data Protection Agency (n° 5057/2016).

## 2.2 | Clinical assessment and reference standards

In this study, bronchodilation was performed by administering 400 µg of inhaled salbutamol, and postbronchodilator spirometry was evaluated 20 minutes afterwards. Allergic sensitization was evaluated by SPT on the participant's forearm with extracts of *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, weed pollen mix, grass pollen mix, dog dander and *Alternaria alternata*, negative control (extracts diluent) and a positive control (histamine at 10 mg/mL), all belonging to the same batches (CBF-LETI S.A., Madrid, Spain). Results were read 15 minutes afterwards. Allergic sensitization was defined by a positive SPT to at least one of the tested allergens (wheal > 3 mm) coupled to a positive histamine response (wheal > 3 mm) and no positivity in the negative control (wheal < 3 mm).<sup>4</sup>

Information on medical diagnosis of asthma, rhinitis and atopic dermatitis was retrieved for each participant. The reference standard was the medical diagnosis of asthma, based on the spirometry with bronchodilation challenge results, as well as symptoms history and physical examination, according to the Global Initiative for Asthma guidelines.<sup>4</sup> Participants diagnosed with asthma and under maintenance inhaled steroid therapy were classified as having persistent asthma, while individuals with asthma simply under reliever inhaled therapy were regarded as having intermittent asthma. Medical diagnosis of allergic rhinitis and atopic dermatitis was based on the allergic sensitization results as well as symptoms history and physical examination.

Asthma was diagnosed in 45 individuals with 29 being prescribed with inhaled steroid therapy (persistent asthma). Allergic rhinitis and atopic dermatitis were diagnosed in 49 and 6 individuals, respectively.

## 2.3 | EBC collection

The Turbo 14 DECCS condenser system (Medivac, Parma, Italy) was used to collect EBC samples from included participants.<sup>33</sup> The device was cooled to 0°C prior to collection, in accordance with manufacturer's instructions. EBC samples were obtained by at least 15 minutes of normal breathing while wearing a nose clip. Generally, 800–1500 µL of EBC was collected from each subject being a volume of at least 600 µL stipulated as the minimal requirement for a valid sample. The different sample volumes are associated with the children's tidal and minute volumes of the lungs.<sup>34</sup>

After collection, samples were transferred to capped glass tubes and stored at –80°C until analysis. This procedure was performed in controlled environment through a laminar flow cabinet to decrease possible sample contamination by environmental air.<sup>35</sup>

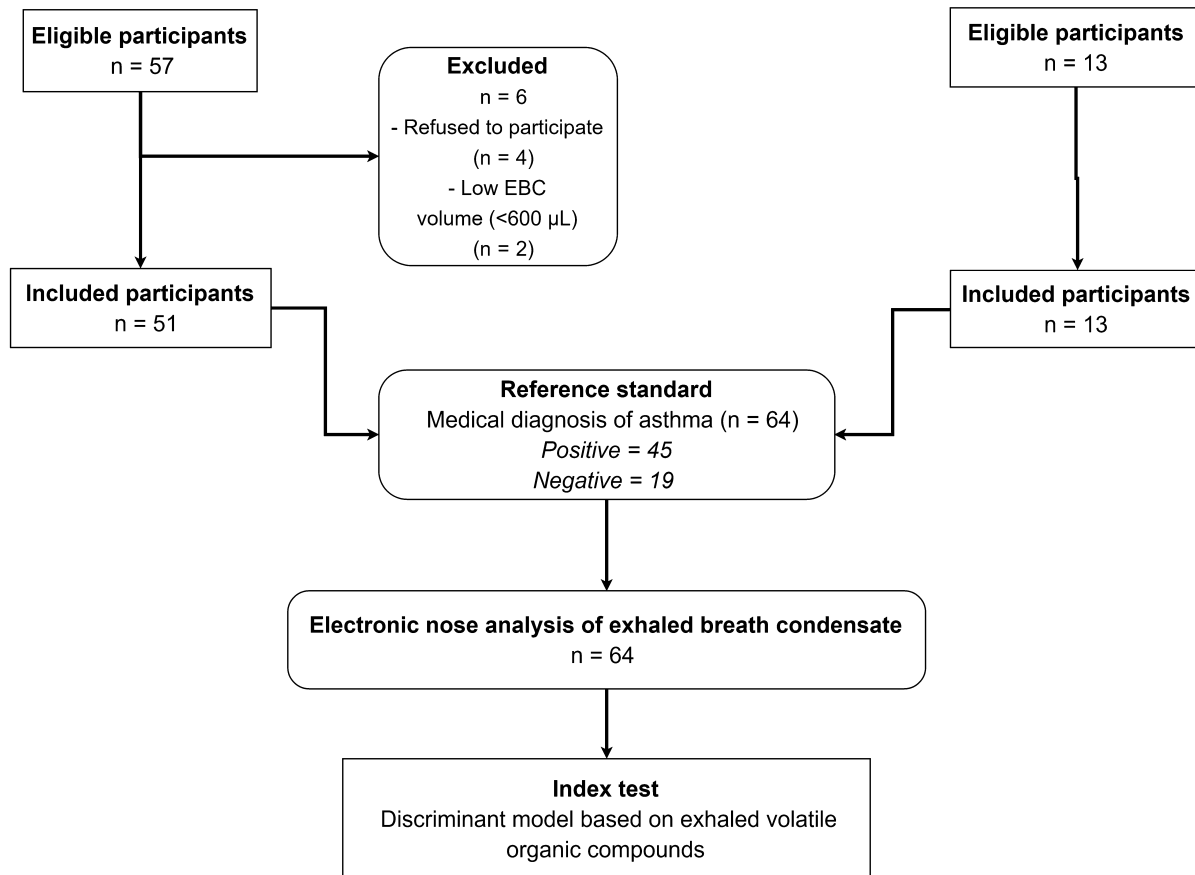
## 2.4 | Electronic nose measurements

To measure breathprints in EBC samples, the Cyranose 320 (Sensigent, Baldwin Park, CA, USA) electronic nose (eNose) was used. The device uses the NoseChip® nanocomposite array of 32 conducting polymer sensors. Sensors are composed by films of carbon black dispersed into a polymer matrix, deposited across two metal electrodes which form electrical connections capable of measuring relative resistance changes when exposed to VOC. Conductivity is given by the carbon black films, while selectivity to analytes arises from the matrix polymer. The signal is obtained by conducting air through the sensor array exposing the analyte to the sensors. Attachment of molecules is measured as a bulk relative resistance change which is unique for each sensor. Consequently, patterns of VOC can be obtained from the vapour of interest. The settings used for the current study are presented in Table S1.

Samples were defrosted at ambient temperature prior to processing. The whole measurement procedure was conducted in a laminar flow cabinet to prevent ambient air contamination, and the cabinet's temperature and relative humidity were continually measured. For breathprint analysis, 600 µL of EBC was transferred to 12 × 75 mm glass assay tubes which were then covered with laboratory film. The tubes were subsequently heated in dry bath at 37°C for 2 minutes to increase the gas phase, and an exhaust was created in the laboratory film, as previously described.<sup>30</sup> Afterwards, a 20-g needle was attached to the Cyranose 320 snout and used to pierce the laboratory film while holding the device above the surface. The laminar flow cabinet's air was used as reference air while baselining for 10 seconds, and then, a sample was drawn for another 10 seconds. This procedure was repeated for all samples.

## 2.5 | Model construction

The 32 sensor resistance values measured by eNose were retrieved and imported as a dataframe object to the R software v3.4.2 (R Foundation, Vienna, Austria). In a first step, principal component analysis

**Flow of participants through outpatient clinic visits****Flow of participants through soccer team****FIGURE 1** Flow of participants through the study according to STARD<sup>31</sup>

(PCA) was applied for exploratory observation of the data dimension and distribution (Figure S1). To assess clustering tendency, the Hopkin's index was calculated and a dissimilarity matrix was drawn (Figure S2). Since the Hopkin's statistic retrieved a value of 0.89, data were considered as highly clusterable.<sup>36</sup> Internal validation and cluster stability methods were performed and optimal scores were achieved in both cases through hierarchical clustering with  $k = 2$ . To further confirm the optimal number of clusters, the total within sum of squares and the average silhouette width methods were applied. Both methods confirmed the optimal cluster score of  $k = 2$  (Figures S3, S4). Therefore, a hierarchical model with two clusters following an agglomerative approach was created using the 32 sensor resistance values, blinded to the reference standard results. In this type of clustering algorithm, each observation starts in its own cluster, and pairs of clusters are merged as one moves up the hierarchy.<sup>37</sup> In this specific situation, Euclidean distances were used for sample dimensioning. Results are graphically expressed in Figure 2.

## 2.6 | Statistical analysis

The SPSS<sup>®</sup> statistical package software v20.0 (IBM, New York, NY, USA) was used for statistical analysis. The  $k = 2$  hierarchical model

constructed with the 32 eNose resistance sensors was compared to the diagnostic standards. There were no missing values for any of the sensors composing the hierarchical model or individuals missing the reference standard results, in accordance with the STARD guidelines.<sup>31</sup>

Student's  $t$  test was used to find whether there were differences in participants' characteristics between groups, for continuous variables. Chi-square tests were used for inferential statistics between two categorical variables and risk estimation was performed to assess the tendency of asthma severity according to each cluster of the hierarchical model. Discriminant analysis was performed to identify the most relevant variables in the model. Receiver operating characteristic (ROC) curves were built, and accuracy, sensitivity, specificity and areas under the ROC (AUC) corresponding to the performance of EBC breathprints in identifying asthma diagnosed by a physician were calculated according to standardized methods.<sup>38,39</sup>

## 3 | RESULTS

The exhaled breath condensate VOC-based hierarchical model was able to discriminate individuals with asthma ( $P < 0.001$ ) and those

**FIGURE 2** Dendrogram of the hierarchical model. All presented conditions have been independently diagnosed by a physician according to standardized guidelines. It is possible to observe a tendency for more asthma cases in cluster B (left branch). On the other hand, individuals without a medical diagnosis of asthma were tendentially agglomerated in cluster A (right branch)

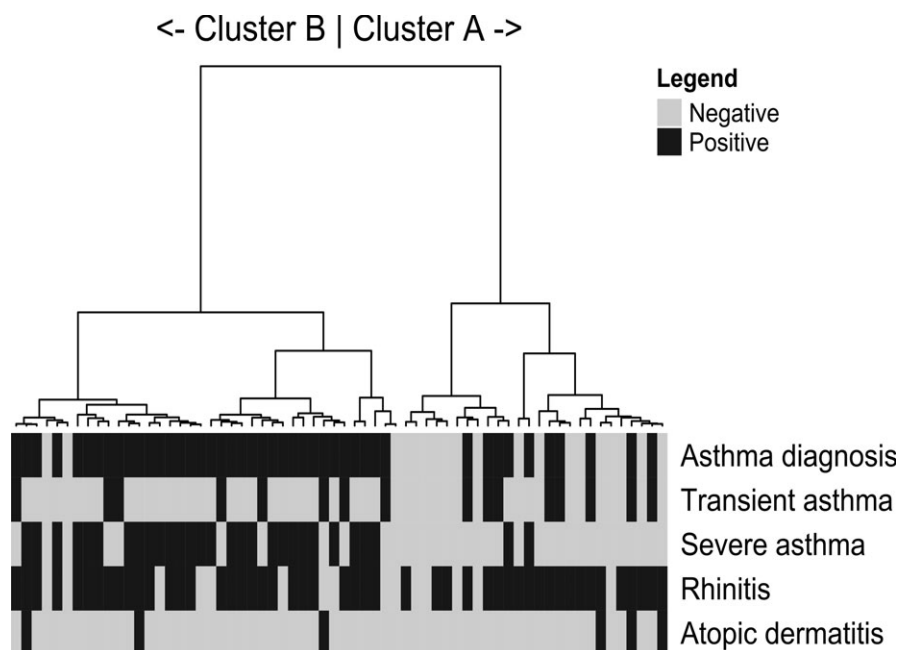


**TABLE 1** Differences between the hierarchical model clusters, created using the 32 sensor resistance values measured by the eNose, according to participants' characteristics

| Characteristics                 | Cluster A           | Cluster B           | P      |
|---------------------------------|---------------------|---------------------|--------|
| No. (males)                     | 26 (17)             | 38 (24)             | 0.855  |
| Age (years, mean $\pm$ SD)      | 10.7 ( $\pm$ 3.5)   | 12.1 ( $\pm$ 3.1)   | 0.223* |
| Height (cm, mean $\pm$ SD)      | 148.2 ( $\pm$ 19.3) | 155.2 ( $\pm$ 15.2) | 0.050* |
| Weight (kg, mean $\pm$ SD)      | 43.3 ( $\pm$ 15.4)  | 51.4 ( $\pm$ 14.9)  | 0.920* |
| Positive BD (n)                 | 0                   | 20                  | <0.001 |
| Positive SPT (n)                | 22                  | 29                  | 0.418  |
| Medical diagnosis of asthma (n) | 10                  | 35                  | <0.001 |
| Intermittent asthma (n)         | 8                   | 8                   | 0.378  |
| Persistent asthma (n)           | 2                   | 27                  | <0.001 |
| Allergic rhinitis (n)           | 21                  | 28                  | 0.511  |
| Atopic dermatitis (n)           | 3                   | 3                   | 0.623  |

P values in bold correspond to significant differences between clusters. Differences calculated through chi-square tests, except for continuous variables (marked with \*), which were calculated through Student's t tests. BD, spirometry with bronchodilation; SPT, skin prick tests.

with persistent disease ( $P < 0.001$ ), but not those with allergic rhinitis or atopic dermatitis (Table 1, Figure 2). Classification estimates showed a tendency to include individuals with asthma (OR = 18.68; 95% CI: 4.50-77.24) and with a persistent disease (OR = 29.50; 95% CI: 5.92-146.50) in cluster B (Figure 3).

Discriminant analysis showed that the highest correlation between the hierarchical model and standardized canonical discriminant functions was achieved by the persistent asthma variable ( $\rho = 0.602$ ), followed by bronchodilation challenge positivity ( $\rho = 0.544$ ). These results show that the analysed exhaled VOC

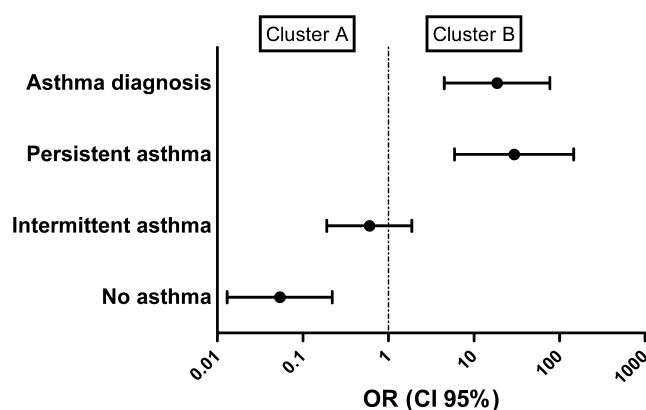


**FIGURE 3** Classification rates estimates (OR with 95% confidence interval) between the two clusters of the hierarchical model. Results represent the chance of participants with specific characteristics (represented in the y-axis) being aggregated in a cluster of the developed hierarchical model

profiles were hierarchized mainly according to the asthma status of the patients, with those prescribed with inhaled corticosteroids (persistent asthma) presenting significantly different VOC profiles from those with intermittent asthma or without asthma at all (Table 2).

Diagnostic accuracy showed AUC values of 0.81 for both asthma identification and for persistent asthma diagnosis ( $P < 0.001$ ) and achieved accuracy, sensitivity and specificity values ranging from 68.6% to 93.1%. Although specificity values were generally lower, accuracy, sensitivity and AUC parameter values obtained from exhaled breath condensate VOC analysis surpassed those from spirometry with bronchodilation in all cases (Table 3). Cluster distributions were not influenced by the participants' baseline lung function as there were no significant associations between clusters for

**TABLE 2** Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables are ordered by absolute size of correlation within function

| Discriminating variables | $\rho^a$ |
|--------------------------|----------|
| Persistent asthma        | 0.602    |
| Positive bronchodilation | 0.544    |
| Weight                   | 0.156    |
| Age                      | 0.146    |
| Height                   | 0.134    |
| Intermittent asthma      | -0.077   |
| Skin prick tests         | -0.052   |
| Allergic rhinitis        | -0.042   |
| Sex                      | 0.035    |
| Atopic dermatitis        | -0.028   |
| Recruitment setting      | -0.009   |

<sup>a</sup>Correlation coefficient for standardized canonical discriminant functions.

pre- and postbronchodilation FEV<sub>1</sub> and FEF<sub>25-75</sub> spirometry variables (Table S2).

## 4 | DISCUSSION

The developed hierarchical model based on exhaled breath condensate VOC analysis by eNose was able to distinguish individuals with a medical diagnosis of paediatric asthma. In addition, persistent asthma patients that were under the need of inhaled corticosteroid therapy were significantly discernible with high accuracy, sensitivity and AUC values. These results show that volatilome analysis is significantly more sensitive than spirometry with bronchodilation challenge for asthma diagnosis. Moreover, although in need of future external validation, evidence suggests a possible application of the studied methodology as a complementary diagnostic methodology to assist the physician's decision in administering corticosteroid therapy for paediatric patients with asthma. These results suggest that breathomics may be a future solution to help deliver precision therapy, answering recently proposed recommendations from an expert commission to "evolve from the use of umbrella terms to disease labels that allow for treatment guidelines to be more precise".<sup>40</sup>

To our knowledge, this is the first study showing a methodology capable of distinguishing individuals with asthma through eNose exhaled breath condensate analysis. Overall, it is shown that breathprints in exhaled breath condensate provide similar AUC values to those shown in other studies using gas-phased exhaled breath and/or gas-chromatography methodologies for asthma diagnosis.<sup>11-14,22,23</sup> In fact, the measured VOC profiles allowed the inclusion of 93.1% individuals with persistent asthma and all of those with positive bronchodilation in the same cluster of the developed model. Still, considering the high specificity of spirometry, both techniques could be used in combination to achieve a significantly reliable asthma diagnosis.

As expected, the accuracy measurements obtained by eNose VOC analysis in EBC were different from those obtained by GC-MS analysis of gaseous samples. For instance, Dallinga and coworkers (2010) were able to discriminate individuals with asthma using 8 distinct components, with 89% sensitivity and 95% specificity.<sup>12</sup> Although GC-MS provides more specific results, it is more difficult to implement as a point of care technology and the lack of specificity of the eNose approach is compensated by a higher sensitivity, which may allow the detection of false negative results provided by spirometry.

The population size in the present study may be seen as a limitation since the total required population of 84 participants to surpass the 2.90 positive likelihood ratio reported for spirometry with bronchodilation was not achieved within its 95% confidence interval. Nevertheless, despite falling slightly short on individuals without asthma, the study managed to surpass the 42 mark for minimum individuals with asthma to be included, still reaching high sensitivity values, though not allowing an inhaled steroid daily dosage stratified analysis. Secondly, although participants with and without inhaled steroid treatment have been included, the developed breathomics clusters were constructed through an unsupervised machine-learning algorithm completely blinded to whether the participant was under inhaled steroids treatment. Finally, our findings were limited by the cross-sectional nature of the study. It is possible that individuals with intermittent asthma who were clustered among individuals with persistent asthma may indeed be diagnosed with persistent asthma in future appointments.

Despite the aforementioned limitations, the present study has several important traits. Although several studies focused on asthma diagnosis through eNose technologies have already been

|                     | Method | Accuracy (%) | Sensitivity (%) | Specificity (%) | AUC (CI 95%)     | P                |
|---------------------|--------|--------------|-----------------|-----------------|------------------|------------------|
| Asthma diagnosis    | BD     | 61.3         | 44.4            | 100             | 0.72 (0.60-0.84) | <b>0.005</b>     |
|                     | eNose  | 79.7         | 77.8            | 84.2            | 0.81 (0.69-0.93) | <b>&lt;0.001</b> |
| Intermittent asthma | BD     | 59.4         | 31.3            | 68.8            | 0.50 (0.34-0.67) | 1.000            |
|                     | eNose  | 40.6         | 50              | 37.5            | 0.56 (0.40-0.73) | 0.457            |
| Persistent asthma   | BD     | 70.3         | 51.7            | 85.7            | 0.69 (0.55-0.82) | <b>0.010</b>     |
|                     | eNose  | 79.7         | 93.1            | 68.6            | 0.81 (0.70-0.92) | <b>&lt;0.001</b> |

BD, spirometry with bronchodilation; eNose, exhaled VOC model measured by electronic nose. Values in bold represent statistical significant differences.

**TABLE 3** Diagnostic accuracy parameters of the developed hierarchical model based on EBC VOC analysis through eNose in comparison with spirometry with bronchodilation

published,<sup>27</sup> most of these studies had either small sample sizes, biased analysis associated with the study design or statistical methods, leading to methodologies inappropriate for real clinical application. In the present survey, recruitment was performed in two different settings, an outpatient allergy clinic and a football team training session, which hamper bias associated with the exclusive inclusion of individuals with allergic or respiratory conditions. Also, despite the fact that only football players were recruited during the training sessions, it was the variable that least influenced the model, showing that neither the recruitment setting nor the participants' exercise activities have interfered with asthma diagnosis results. Stability and internal validation methods were adopted prior to model construction to assess clustering tendency. Moreover, although an exploratory analysis was performed, through PCA, the model itself was constructed blindly to the diagnostic standards (the 32 eNose sensor resistance values were the only variables used to create the model), thus reducing possible bias associated with prelabelled group comparisons. The STARD guidelines for reporting diagnostic accuracy were followed throughout the study to avoid eventual reporting bias.<sup>31</sup> The reference standard results for asthma diagnosis were provided by physician's evaluation, according to standardized guidelines.<sup>4</sup> Finally, breathomics were performed by eNose on EBC samples, which allowed the storage and freezing of samples, a considerably inexpensive 10 second per sample analysis, and the possibility of rapidly discriminating individuals with asthma, even in a hard-to-diagnose paediatric population.

The proof of principle for loss of asthma control monitoring by eNose technology has already been showed by Brinkman and coworkers (2017).<sup>17</sup> The present study's results further support this evidence, as individuals with persistent asthma were significantly distinguishable from those with intermittent asthma, which may be associated with the different degree of airway inflammation at sample collection. The odds ratio results also showed a significant tendency for medical asthma diagnosis and persistent asthma to be included in the same cluster. As expected, individuals without asthma were significantly associated with the opposite cluster of the model, thus providing a clear opposing tendency for asthma diagnosis between clusters. These results were not influenced by the participants' baseline lung capacity.

Although the developed hierarchical model was already blindly built to the diagnostic standard results, an external validation of the model, tested on a new sample of individuals, should be performed to complete the STARD recommendations. Nevertheless, evidence suggests a promising future for breathomics towards a rapid and complementary asthma diagnosis and therapy administration assistance in clinical settings.

## 5 | CONCLUSION

Our findings suggest that the analysis of exhaled breath condensate by an eNose system coupled to a postsampling discriminant analysis may be able to distinguish paediatric individuals with asthma and to

identify those in the need of inhaled maintenance steroid therapy. Moreover, the method showed higher overall accuracy, sensitivity and AUC values when compared to spirometry with bronchodilation challenge.

## ACKNOWLEDGMENTS

Authors gratefully acknowledge the participants and their families, as well as the clinical staff at the Immunoallergy Department, in particular Alice Coimbra, Mário Miranda, José Luís Plácido, Leonor Leão and Fabícia Carolino, for their contribution on recruitment and clinical evaluation of the participants.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

JCR and AM were involved in all tasks associated with the study. IP participated in the recruitment of participants and data processing. FCM, MF, AR and DS were involved during fieldwork and data collection phases. Finally, EOF and LD provided their expert revision of the manuscript.

## ORCID

João Cavaleiro Rufo  <http://orcid.org/0000-0003-1175-242X>

Inês Paciência  <http://orcid.org/0000-0001-5762-7814>

## REFERENCES

1. Baena-Cagnani CE, Badellino HA. Diagnosis of allergy and asthma in childhood. *Curr Allergy Asthma Rep*. 2011;11:71-77.
2. Contoli M, Papi A. When asthma diagnosis becomes a challenge. *Eur Respir J*. 2010;36:231-233.
3. Konradsen JR, Caffrey Osvald E, Hedlin G. Update on the current methods for the diagnosis and treatment of severe childhood asthma. *Expert Rev Respir Med*. 2015;9:769-777.
4. Global Initiative for Asthma. Global strategy for asthma management and prevention. 2017.
5. Chung KF, Wenzel SE, Brozek JL, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J*. 2014;43:343-373.
6. Agache I, Ciobanu C. Predictive value of lung function trend and FeNO for difficult asthma in children. *J Investig Allergol Clin Immunol*. 2012;22:419-426.
7. Miller MR, Crapo R, Hankinson J, et al. General considerations for lung function testing. *Eur Respir J*. 2005;26:153-161.
8. Tantisira KG, Fuhlbrigge AL, Tonascia J, et al. Bronchodilation and bronchoconstriction: predictors of future lung function in childhood asthma. *J Allergy Clin Immunol*. 2006;117:1264-1271.
9. Schneider A, Gindner L, Tilemann L, et al. Diagnostic accuracy of spirometry in primary care. *BMC Pulm Med*. 2009;9:31.
10. Tse SM, Gold DR, Sordillo JE, et al. Diagnostic accuracy of the bronchodilator response in children. *J Allergy Clin Immunol*. 2013;132:554-559.

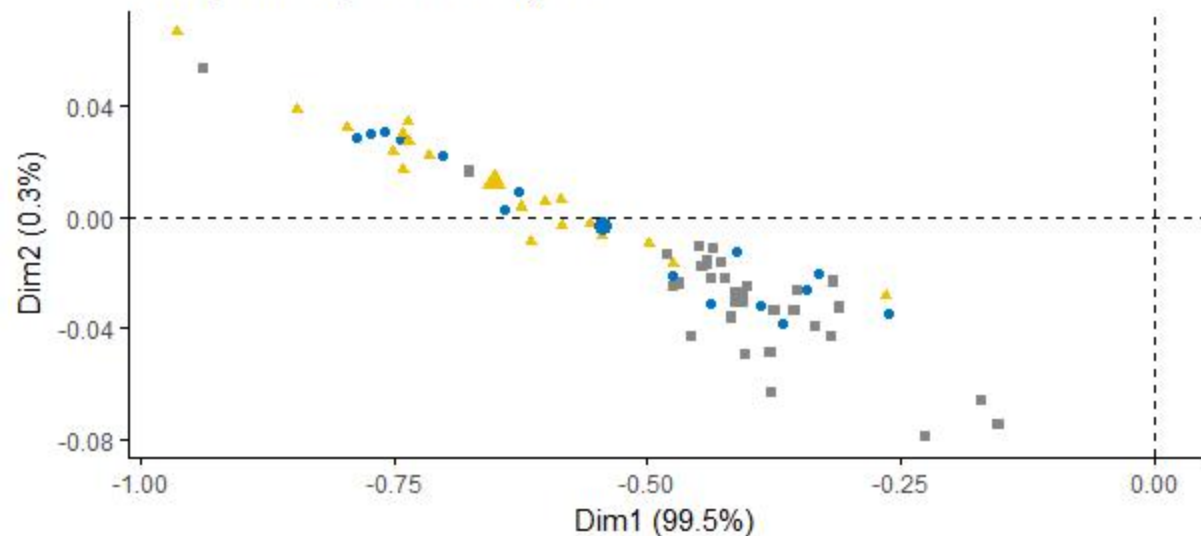
11. Caldeira M, Perestrelo R, Barros AS, et al. Allergic asthma exhaled breath metabolome: a challenge for comprehensive two-dimensional gas chromatography. *J Chromatogr A*. 2012;1254:87-97.
12. Dallinga JW, Robroeks CM, van Berkel JJ, et al. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. *Clin Exp Allergy*. 2010;40:68-76.
13. Ibrahim B, Basanta M, Cadden P, et al. Non-invasive phenotyping using exhaled volatile organic compounds in asthma. *Thorax*. 2011;66:804-809.
14. Smolinska A, Klaassen EM, Dallinga JW, et al. Profiling of volatile organic compounds in exhaled breath as a strategy to find early predictive signatures of asthma in children. *PLoS One*. 2014;9:e95668.
15. Couto M, Barbosa C, Silva D, et al. Oxidative stress in asthmatic and non-asthmatic adolescent swimmers-A breathomics approach. *Pediatr Allergy Immunol*. 2017;28:452-457.
16. Fitzpatrick AM, Park Y, Brown LA, Jones DP. Children with severe asthma have unique oxidative stress-associated metabolomic profiles. *J Allergy Clin Immunol*. 2014;133:258-261.
17. Brinkman P, van de Pol MA, Gerritsen MG, et al. Exhaled breath profiles in the monitoring of loss of control and clinical recovery in asthma. *Clin Exp Allergy*. 2017;47:1159-1169.
18. Buszewski B, Keszy M, Ligor T, Amann A. Human exhaled air analytics: biomarkers of diseases. *Biomed Chromatogr*. 2007;21:553-566.
19. van de Kant KD, van der Sande LJ, Jobsis Q, van Schayck OC, Dompeling E. Clinical use of exhaled volatile organic compounds in pulmonary diseases: a systematic review. *Respir Res*. 2012;13:117.
20. Miekisch W, Schubert JK, Noeldge-Schomburg GF. Diagnostic potential of breath analysis—focus on volatile organic compounds. *Clin Chim Acta*. 2004;347(1-2):25-39.
21. Wagener AH, Zwinderman AH, Lutter R, et al. Exhaled air volatile organic compounds and eosinophilic airway inflammation in asthma. *Eur Respir J*. 2012;40(suppl 56):4695.
22. Montuschi P, Santonico M, Mondino C, et al. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma. *Chest*. 2010;137:790-796.
23. van der Schee MP, Palmay R, Cowan JO, Taylor DR. Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. *Clin Exp Allergy*. 2013;43:1217-1225.
24. Dragonieri S, Schot R, Mertens BJ, et al. An electronic nose in the discrimination of patients with asthma and controls. *J Allergy Clin Immunol*. 2007;120:856-862.
25. Fens N, Roldaan AC, van der Schee MP, et al. External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease. *Clin Exp Allergy*. 2011;41:1371-1378.
26. Schivo M, Seichter F, Aksenov AA, et al. A mobile instrumentation platform to distinguish airway disorders. *J Breath Res*. 2013;7:1752-1755.
27. Rufo JC, Madureira J, Fernandes EO, Moreira A. Volatile organic compounds in asthma diagnosis: a systematic review and meta-analysis. *Allergy*. 2016;71:175-188.
28. Mochalski P, Wzorek B, Sliwka I, Amann A. Suitability of different polymer bags for storage of volatile sulphur compounds relevant to breath analysis. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877:189-196.
29. Caldeira M, Barros AS, Bilelo MJ, Parada A, Camara JS, Rocha SM. Profiling allergic asthma volatile metabolic patterns using a head-space-solid phase microextraction/gas chromatography based methodology. *J Chromatogr A*. 2011;1218:3771-3780.
30. Hüttmann E-M, Greulich T, Hattesoil A, et al. Comparison of two devices and two breathing patterns for exhaled breath condensate sampling. *PLoS One*. 2011;6:e27467.
31. Bossuyt PM, Reitsma JB, Bruns DE, et al. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *Radiology*. 2015;277:826-832.
32. Simel DL, Samsa GP, Matchar DB. Likelihood ratios with confidence: sample size estimation for diagnostic test studies. *J Clin Epidemiol*. 1991;44:763-770.
33. Montuschi P. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. *Ther Adv Respir Dis*. 2007;1:5-23.
34. Liu J, Thomas PS. Relationship between exhaled breath condensate volume and measurements of lung volumes. *Respiration*. 2007;74:142-145.
35. Yang X, Chen Q, Zeng J, Zhang JS, Shaw CY. A mass transfer model for simulating volatile organic compound emissions from 'wet' coating materials applied to absorptive substrates. *Int J Heat Mass Transf*. 2001;44:1803-1815.
36. Banerjee A, Dave RN. Validating clusters using the Hopkins statistic. Paper presented at: 2004 IEEE International Conference on Fuzzy Systems (IEEE Cat. No. 04CH37542); 25-29 July 2004, 2004.
37. Rokach L, Maimon O. Clustering methods. In: Maimon O, Rokach L, eds. *Data Mining and Knowledge Discovery Handbook*. Boston, MA: Springer US; 2005: 321-352.
38. Baratloo A, Hosseini M, Negida A, El Ashal G. Part 1: simple definition and calculation of accuracy, sensitivity and specificity. *Emerg (Tehran)*. 2015;3:48-49.
39. Carter JV, Pan J, Rai SN, Galandiuk S. ROC-ing along: evaluation and interpretation of receiver operating characteristic curves. *Surgery*. 2016;159:1638-1645.
40. Pavord ID, Beasley R, Agusti A, et al. After asthma: redefining airways diseases. *Lancet*. 2018;391(10118):350-400.

## SUPPORTING INFORMATION

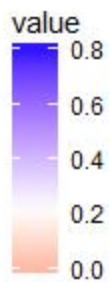
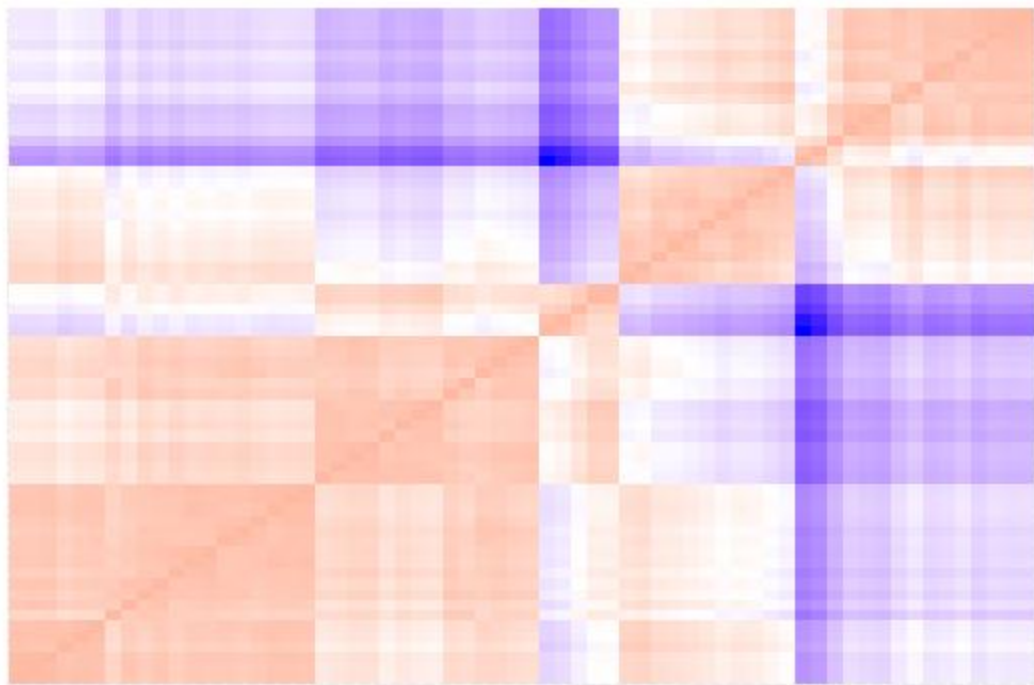
Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Cavaleiro Rufo J, Paciência I, Mendes FC, et al. Exhaled breath condensate volatolome allows sensitive diagnosis of persistent asthma. *Allergy*. 2019;74:527-534. <https://doi.org/10.1111/all.13596>

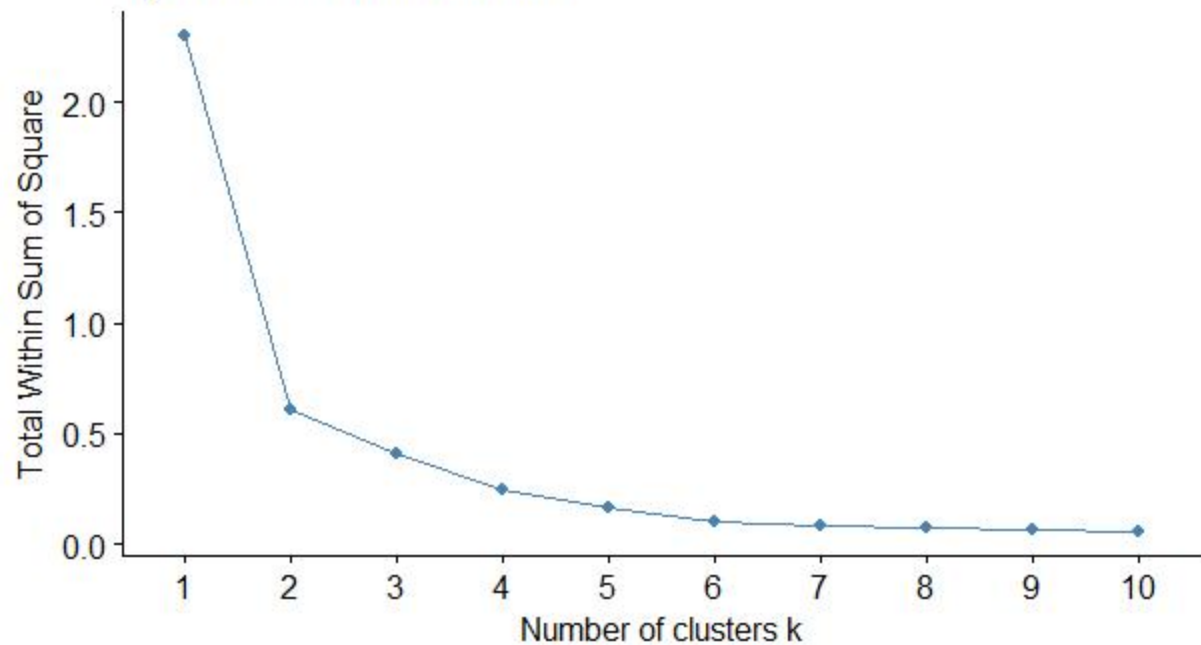
# Principal component analysis



Groups ● Intermittent asthma ▲ No asthma ■ Persistent asthma



Optimal number of clusters



Optimal number of clusters

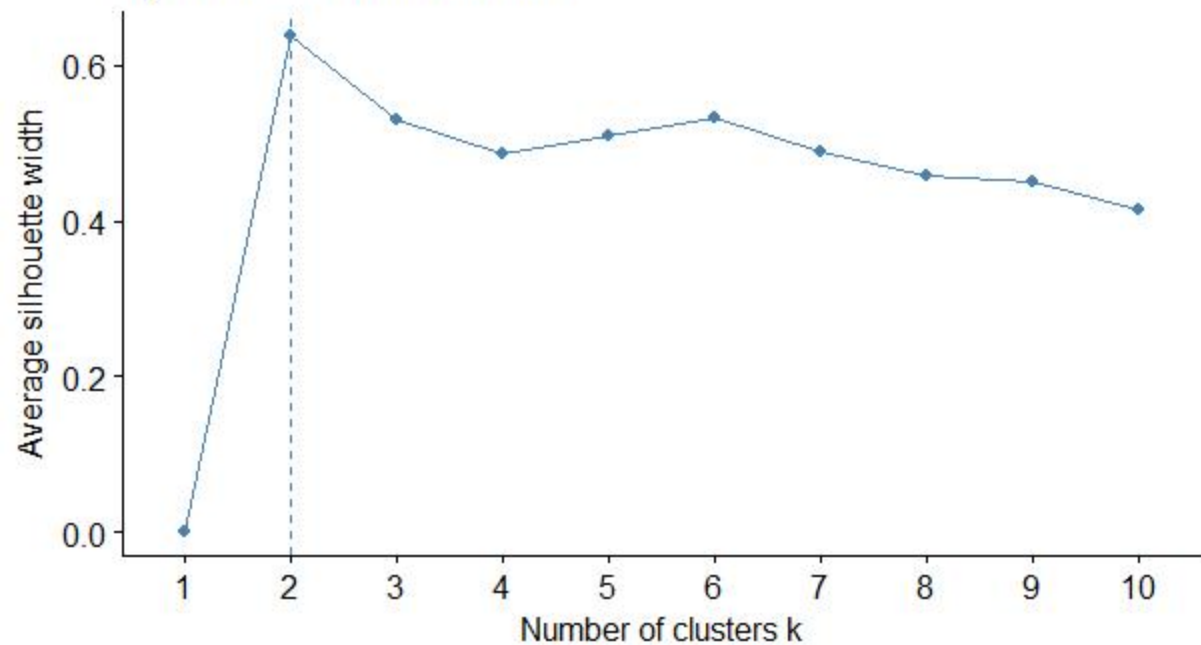


**Table S1** – Cyranose 320 eNose settings for EBC analysis.

| Setting              | Time | Speed       |
|----------------------|------|-------------|
| Baseline purge       | 10s  | Medium      |
| Sample draw 1        | 10s  | Medium      |
| Sample draw 2        | 0s   | <i>n.a.</i> |
| Snout removal        | 10s  | <i>n.a.</i> |
| 1st sample gas purge | 20s  | High        |
| 1st air intake purge | 30s  | High        |
| 2nd sample gas purge | 20s  | High        |
| 2nd air intake purge | 0s   | <i>n.a.</i> |

*n.a.*: not applicable

**Table S2** – FEV<sub>1</sub> and FEF<sub>25-75</sub> spirometry parameter distribution between clusters.

| <b>Spirometry parameters</b>      | <b>Cluster A</b>  | <b>Cluster B</b>  | <b><i>p</i></b> |
|-----------------------------------|-------------------|-------------------|-----------------|
| FEV <sub>1</sub> pre-BD (L)       | 2.5 (1.8 : 3.0)   | 2.4 (1.7 : 3.1)   | 0.987           |
| FEV <sub>1</sub> pos-BD (L)       | 2.5 (1.9 : 3.1)   | 2.7 (2.1 : 3.6)   | 0.286           |
| FEV <sub>1</sub> Change (%)       | 3.5 (0.7 : 6.2)   | 13.7 (4.5 : 22.4) | <0.001          |
| FEF <sub>25-75</sub> pre-BD (L/s) | 2.4 (2 : 3.3)     | 2.1 (1.7 : 3.4)   | 0.333           |
| FEF <sub>25-75</sub> pos-BD (L/s) | 2.8 (2.3 : 3.5)   | 2.8 (2.3 : 4.0)   | 0.735           |
| FEF <sub>25-75</sub> Change (%)   | 16.3 (8.1 : 24.3) | 32.4 (8.8 : 57.5) | 0.037           |

Results presented as median (27 : 75 percentile).