ORIGINAL ARTICLE

Inflammatory Asthma Phenotype Discrimination Using an Electronic Nose Breath Analyzer

Plaza V[1], Crespo A[1]*, Giner J[1], Merino JL[2], Ramos-Barbón D[1], Mateus EF[1], Torrego A[1], Cosio BG[3], Agustí A[4], Sibila O[1]

[1]Department of Respiratory Medicine, Hospital de la Santa Creu i Sant Pau; Institut d'Investigació Biomédica Sant Pau (IIB Sant Pau): Universitat Autònoma de Barcelona, Department of Medicine; Barcelona Respiratory Network (BRN), Barcelona, Spain
[2]RFic group. École Polytechnique Fédérale de Lausanne, Switzerland
[3]Department of Respiratory Medicine, Hospital Son Espases, Palma de Mallorca; CIBER Enfermedades Respiratorias (CIBERES), Spain
[4]Thorax Institute, Hospital Clinic, IDIBAPS, University of Barcelona; FISIB, CIBER Enfermedades Respiratorias (CIBERES); Barcelona Respiratory Network (BRN), Barcelona, Spain
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Abstract

Background and Objective: Patients with persistent asthma have different inflammatory phenotypes. The electronic nose is a new technology capable of distinguishing volatile organic compound (VOC) breath-prints in exhaled breath. The aim of the study was to investigate the capacity of electronic nose breath-print analysis to discriminate between different inflammatory asthma phenotypes (eosinophilic, neutrophilic, paucigranulocytic) determined by induced sputum in patients with persistent asthma.
Methods: Fifty-two patients with persistent asthma were consecutively included in a cross-sectional proof-of-concept study. Inflammatory asthma phenotypes (eosinophilic, neutrophilic and paucigranulocytic) were recognized by inflammatory cell counts in induced sputum. VOC breath-prints were analyzed using the electronic nose Cyranose 320 and assessed by discriminant analysis on principal component reduction, resulting in cross-validated accuracy values. Receiver operating characteristic (ROC) curves were calculated.
Results: VOC breath-prints were different in eosinophilic asthmatics compared with both neutrophilic asthmatics (accuracy 73%; *P*=.008; area under ROC, 0.92) and paucigranulocytic asthmatics (accuracy 74%; *P*=.004; area under ROC, 0.79). Likewise, neutrophilic and paucigranulocytic breath-prints were also different (accuracy 89%; *P*=.001; area under ROC, 0.88).
Conclusion: An electronic nose can discriminate inflammatory phenotypes in patients with persistent asthma in a regular clinical setting. ClinicalTrials.gov identifier: NCT02026336.

Key words: Asthma. Electronic nose. Inflammation. Volatile organic compounds.

Resumen

Antecedentes: Pacientes con asma persistente tienen diferentes fenotipos inflamatorios bronquiales. La nariz electrónica es una nueva tecnología capaz de distinguir compuestos orgánicos volátiles (VOCs), huellas olfatorias del aire exhalado. El objetivo de este estudio fue investigar la capacidad que tiene la nariz electrónica de discriminar las huellas olfatorias en los diferentes fenotipos bronquiales de asma determinados por el esputo inducido (eosinofílicos, neutrofílicos, paucigranulocíticos) en pacientes con asma persistente.
Método: Cincuenta y dos pacientes con asma persistente fueron incluidos en un estudio transversal. Los fenotipos inflamatorios asmáticos fueron determinados a través de recuento de células inflamatorias del esputo inducido. Los VOCs fueron analizados a través de una nariz electrónica Cyranose 320™ y evaluados por un análisis de discriminación de componentes principales, resultando en valores de precisión con validación cruzada. Se calcularon las características operativas del receptor (ROC).
Resultados: Los VOCs de los asmáticos eosinofílicos fueron diferentes a los neutrofílicos (precisión 73%; p= 0.008; área bajo ROC 0.92) y de los pacientes paucigranulocíticos (precisión 74%; p= 0.004; área bajo ROC 0.79). Del mismo modo, las huellas olfatorias entre los neutrofílicos y paucigranulocíticos eran diferentes (precisión 89%; p= 0.001; área bajo ROC 0.88).

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Conclusión: La nariz electrónica puede discriminar los fenotipos inflamatorios bronquiales en los pacientes con asma persistente en un entorno clínico regular. ClinicalTrials.gov: NCT02026336.

Palabras clave: Asma. Nariz electrónica. Inflamación. Compuestos orgánicos volátiles.

Introduction

Chronic airway inflammation is a key pathogenic mechanism in asthma. Yet, there is significant variability in the inflammatory phenotype across individuals. In fact, several guidelines already recommend tailored management approaches for patients with different inflammatory phenotypes, especially those with severe, refractory asthma [1,2]. This is particularly relevant with the emergence of a variety of biological anti-inflammatory therapies targeting both eosinophilic [3,4] and noneosinophilic asthma phenotypes.

Two noninvasive methods are commonly used in clinical practice to assess airway inflammation in asthma: quantification of exhaled nitric oxide (FeNO) and analysis of induced sputum. Measuring FeNO is an inexpensive, simple technique that provides readily available readouts, but it is not informative of the specific inflammatory profile present. Induced sputum analysis can certainly assess the specific airway inflammatory phenotype present [5,6], but it is time-consuming, costly, and requires technical expertise. Furthermore, 10% of patients fail to provide adequate samples. Therefore, there is a lack of methods able to classify asthmatic individuals according to their airway inflammatory profile in an easy, cost-effective fashion suitable for generalized use in regular clinical practice.

The electronic nose (e-nose) is an emerging technology that detects volatile organic compounds (VOCs) in exhaled gas. It uses an array of sensors that react with different VOCs and generate a specific "breath-print" for each individual [7]. The exhaled gas contains a complex mix of VOCs derived from various metabolic and inflammatory pathways in the lung. Previous studies have shown that some respiratory diseases, including lung cancer, malignant mesothelioma, pulmonary arterial hypertension, chronic obstructive pulmonary disease (COPD), and asthma, are associated with specific breath-prints that can be detected by an e-nose [8-12]. Here we hypothesized that the breath-print of asthmatic individuals may reflect their airway inflammatory profile, and that the e-nose may therefore qualify as a novel tool of potential clinical value for the inflammatory phenotyping of asthma. Accordingly, this proof-of-concept study sought to use an e-nose to compare VOC profiles generated from different asthma inflammatory phenotypes.

Methods

Study Design and Participants

This was a cross-sectional study designed to analyze the concordance of sputum inflammatory phenotypes with e-nose VOC profiles. Fifty-two adults with persistent asthma, as per the Global INitiative for Asthma (GINA) criteria [13], were consecutively enrolled from the outpatient clinic at the asthma unit of our institution, a tertiary referral university hospital, between January and December 2013. All the patients had a positive bronchodilator test or a daily peak expiratory flow variability greater than 20%, or a positive methacholine challenge test documented in their case history. Patients were excluded if they had a respiratory tract infection or asthma exacerbation within 30 days prior to inclusion or if they were receiving oral corticosteroids or immunosuppressive treatments. Smoker and ex-smokers were included. Patients who had stopped smoking more than 12 months earlier were considered ex-smokers. Only patients with good-quality sputum were selected.

Measurements

The tests were all performed during a single visit in the morning. Patients stopped their medications 10 hours before the visit. In the 3 hours before the visit, they rested, and did not smoke, eat, or drink. The following tests were performed in the order shown: 1) the asthma control test using the validated Spanish version [14], 2) allergy skin prick tests to usual local allergens [15], 3) FeNO testing, 4) e-nose VOC profiles, 5) sputum induction and processing, 6) forced spirometry, and 7) venipuncture for total serum IgE. FeNO levels were measured using a chemiluminescence analyzer (NO Vario Analyser, FILT GMBH) at a flow rate of 50 mL/s, and, following the recommendations of the American Thoracic Society and the European Respiratory Society [16], we used the mean of 3 measurements. For the e-nose VOC profiles, exhaled gas was collected as described before [8,10,17-18]. Briefly, patients breathed through a mouthpiece into a 2-way nonrebreathing valve (Hans Rudolph 2700, Hans Rudolph) with an inspiratory VOC filter (Compact Air Plus, North) and an expiratory silica reservoir (replaced after each patient) to dry the expired air. Expiratory air was collected in a 10-L Tedlar bag that was flushed with ambient air after each use by filling and emptying the bag twice. After 10 minutes, which is the time needed to verify that all the sensors were within the reference range specified by the manufacturer, the bag was connected to the e-nose device (Cyranose 320, Smith Detections) and fitted with a 32-organic polymeric nano-composite sensor array for 5 minutes. Changes in the nano-sensor electrical resistance generated a breath-print VOC profile. The measurement is based on a resistance variation in each sensor when exposed to a VOC mixture. The differential responses across the array (resistance shifts) are presented as patterns and analyzed by pattern recognition algorithms [18]. All breath samples were collected in the same room. Sputum induction and processing

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was performed as previously described [19,20]. Briefly, mucus plugs were manually selected and weighed, incubated (for 15 minutes at room temperature) in 4 times the weight (in mL) of the selected plug (in mg) in 0.1% dithiothreitol (DTT) (Calbiochem), washed with 4 times the plug weight (in mL) in Dulbecco's PBS, and gravity-filtered through a 41 µm–pore nylon net filter (Millipore). After DTT homogenization, each specimen was aliquoted into 2 parts of equal volume. Total cell counts were performed using a Neubauer hemocytometer. Visually identifiable squamous epithelial cells were not counted or included in the total cell count. Samples that did not produce adequate sputum cell numbers ($<1000 \times 10^6$ cell/g) were excluded. Cell viability was determined by light microscopic assessment using trypan blue exclusion staining. After centrifuging the cell preparation, we obtained cell pellet and supernatant. The cell pellet was used for the differential cell count (macrophages, eosinophils, neutrophils, lymphocytes, and bronchial epithelial cells) with Wright-Giemsa staining. Differential leukocyte analysis of nonsquamous cells (Diff-Quik stained) was performed on a minimum of 400 cells. Differential cell counts were expressed as a percentage of total nucleated nonsquamous cells [19].The inflammatory asthma phenotypes were classified as neutrophilic (>61% neutrophils), eosinophilic (>3% eosinophils), or paucigranulocytic (<61% neutrophils and <3% eosinophils) according to previous studies [21]. Forced spirometry (Datospir-500, Sibelmed SA) was performed according to the guidelines of the Spanish Respiratory Society (SEPAR) [22], using reference values from a Mediterranean population [23].

Data Analysis

Categorical variables are expressed as absolute and relative frequencies and quantitative variables as mean and SD. Groups were compared using analysis of variance and the χ^2 or Fisher exact test as appropriate. A *P* value of less than .05 was considered statistically significant. Breath-print data were analyzed using a pattern-recognition application of the MATLAB software (v.R2012a), and were represented by logarithmic regression as single- or 2-dimensional graphs following previously published algorithms [10,17]. Raw data were first reduced by principal component analysis (PCA) to 3 principal factors. These PCA factors entered a univariate ANOVA followed by a post-hoc least significant difference test. Linear canonical discriminant analysis, using the PCA factors, was then performed to classify patients into categories. Based on the differentiating PCA factors, a discriminating function was calculated that best distinguished the different inflammatory phenotypes. The accuracy of the model is defined as the percentage of correctly classified patients using induced sputum results as the reference. It was obtained using the leave-one-out method [17]. In this method, the discriminant function is trained using all subjects minus one, and then the function is tested with the 4 samples (the PCA factors) from the "left-out" individual. If 3 of the samples are correctly allocated, the discriminant function is considered to be valid for this individual. The process is repeated for all individuals and results are used to calculate the cross-validated accuracy percentage value. Sensitivity, specificity, and positive predictive and negative predictive values were calculated for the 3 inflammatory phenotypes. A receiver operating characteristics (ROC) curve was generated using the results of the discriminant function and combining all the samples of 1 individual. If one tested individual (true 'class 1') has 3 samples allocated to 'class 1' by the identifier and 1 sample allocated to 'class 0', the true positive rate is considered to be 0.75. The area under the ROC curve (AUC) was calculated

Table 1. Demographic Characteristics of Patients Who Completed 3 Years of Follow-up

	Eosinophilic Phenotype (n=24)	Neutrophilic Phenotype (n=10)	Paucigranulocytic Phenotype (n=18)	*P* Value
Age, mean (SD), y	47.8 (16.4)	54.8 (18.0)	42.9 (10.6)	NS
Sex, % female	67	40	61	NS
BMI, mean (SD), kg/m^2	26.1 (4.3)	25.7 (4.0)	27.2 (6.8)	NS
Active smokers, %	12	10	5	NS
Ex-smokers, %	30	30	23	NS
IgE, mean (IQR), IU/mL	148 (404.6)	45.25 (100.7)	238.5 (565.58)	NS
Patients with positive skin prick test, %	71	60	83	NS
Patients with ACT ≥20, %	58	60	78	NS
ACT score, mean (SD)	19.8 (4.4)	20.9 (5.9)	20.9 (4.5)	NS
FEV_1, mean (SD), % of reference values	86.9 (19.2)	78.8 (17.9)	89.9 (14.9)	NS
FEV_1/FVC, mean (SD)	69.4 (15.2)	65.5 (13.1)	71.5 (10.6)	NS
FeNO, median (IQR), ppb	32 (38.75)	25 (26)	21.5 (37.25)	NS
Patients with beclomethasone (or equivalent ICS) ≥800 µg/d, %	29	30	33	NS

Abbreviations: ACT, asthma control test; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; ICS, inhaled corticosteroid; IQR, interquartile range; NS, nonsignificant.

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using the pairs of X, Y values and a trapezoidal approximation for the space between points.

Ethics Statement

The study was conducted in accordance with the principles of the Declaration of Helsinki (18th World Medical Assembly, 1964) and was approved by the clinical research ethics committee at our institution (approval number, IIBSP/10/122/1161). The participants signed an informed consent form to participate in this study and all personal identification data were anonymized. ClinicalTrials.gov identifier: NCT02026336.

Results

Characterization of Participants

Table 1 presents the main demographic, clinical, and functional characteristics of the participants. According to the induced sputum results (Table 2), 24 patients (46%) had an eosinophilic phenotype, 10 (19%) a neutrophilic phenotype, and 18 (35%) a paucigranulocytic phenotype. Age, sex, body mass index (BMI), smoking status, serum IgE concentration, proportion of individuals positive to the standard skin prick test series, response to the asthma control test questionnaire, spirometry values, and FeNO or inhaled corticosteroid usage was similar in the 3 groups (Table 1).

Breath-Print Analysis

PCA of the e-nose data showed that the breath-prints of the 3 inflammatory asthma phenotypes were different. As shown in Figure 1, plotted breath-prints from neutrophilic versus paucigranulocytic phenotypes were clearly distinct on visual assessment (panel A) and canonical discriminant analysis showed a cross-validated accuracy of 89% (P=.001). Likewise, the breath-prints of eosinophilic versus neutrophilic phenotypes (panel B), and eosinophilic versus paucigranulocytic (panel C), were also fairly distinguishable, with accuracy values of 73% (P=.008) and 74% (P=.004), respectively. Finally, a comparison of eosinophilic versus noneosinophilic asthmatics (ie, neutrophilic plus paucigranulocytic phenotypes) showed a reduced but still significant cross-validated accuracy (61%, P=.008).

Table 3 presents the results of the ROC analysis, as well as the sensitivity, specificity, and positive and negative predictive values, and the AUC for the 3 breath-print comparisons of the 3 inflammatory asthma phenotypes studied.

Discussion

The results of the proof-of-concept study presented here show that different inflammatory asthma phenotypes based on induced sputum analysis can be readily recognized by their breath-prints using an e-nose device.

Table 2. Inflammatory Cell Counts Observed in the 3 Inflammatory Groups

	Eosinophilic Phenotype (n=24)	Neutrophilic Phenotype (n=10)	Paucigranulocytic Phenotype (n=18)	*P* Value
Lymphocytes, mean (SD), %	0.8 (0.6)	0.9 (0.5)	1.0 (0.6)	.516
Macrophages, mean (SD), %	56.4 (17.8)	19.2 (9.3)	61.1 (20.0)	<.001
Eosinophils, mean (SD), %	13.2 (14.0)	1.3 (0.9)	0.9 (0.6)	<.001
Neutrophils, mean (SD), %	29.4 (16.4)	77.3 (8.1)	36.5 (20.4)	<.001
Squamous cell, median (IQR), %	7.21 (5.87)	3.17 (2.38)	4.53 (3.78)	.064
Cell concentration, mean (SD), x10^6 cell/g	3.26 (2.16)	3.96 (1.72)	3.5 (1.74)	.682

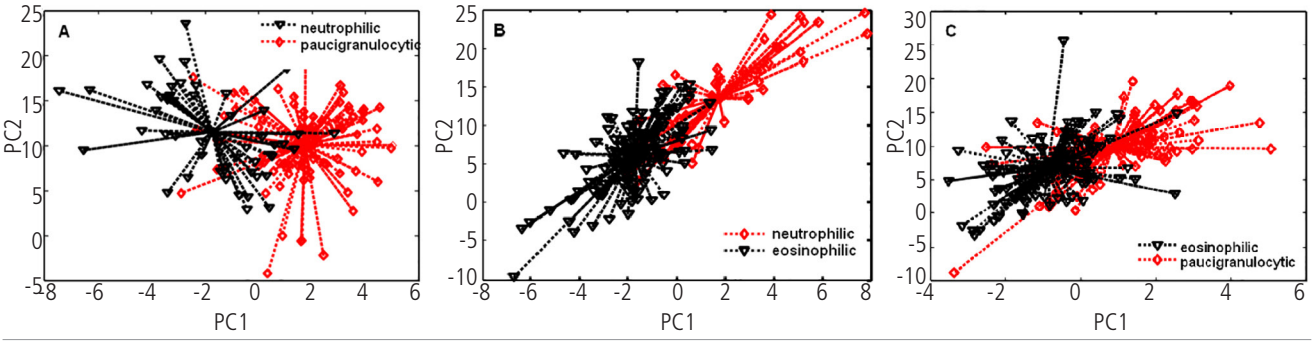

Figure. Comparison of e-nose breath-prints between asthma inflammatory phenotypes defined by differential leukocyte counts in induced sputum. The graphs depict 2-dimensional principal component analysis plots showing breath-print discrimination between patients with A) neutrophilic vs paucigranulocytic phenotypes (accuracy 89%; P=.001; AUROC, 0.88); B) neutrophilic vs eosinophilic phenotypes (accuracy, 73%; P=.008; AUROC, 0.92), and C) eosinophilic vs paucigranulocytic phenotypes (accuracy 74%; P=.004; AUROC, 0.79). Axes represent discriminative composite principal factors. Symbol legends indicate phenotype representating in each plot. There are 4 samples represented for each patient. AUROC indicates area under the receiver operating characteristics curve.

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Table 3. Receiver Operating Characteristics Analyses for the Comparisons Between Breath-Prints of the 3 Asthma Inflammatory Phenotypes Studied

	Neutrophilic vs Paucigranulocytic Phenotype	Neutrophilic vs Eosinophilic Phenotype	Eosinophilic vs Paucigranulocytic Phenotype
Cross-validation accuracy, %	89 (*P*=.001)	73 (*P*=.008)	74 (*P*=.004)
Sensitivity	0.94	0.6	0.55
Specificity	0.80	0.79	0.87
Positive predictive value	0.89	0.54	0.61
Negative predictive value	0.72	0.83	0.68
Area under the receiver operating characteristic curve	0.88	0.92	079

Prior studies have provided evidence supporting the use of the e-nose to aid in the diagnosis of asthma by discriminating asthmatics from healthy controls (100% accuracy) [17], asthma from COPD (96% accuracy) [10], and asthma with fixed airflow obstruction from COPD (88% accuracy) [24]. It has also been seen to be more accurate than FeNO measurements in this diagnostic setting, with an accuracy of 95.8% [25]. Overall, these previous reports suggest that e-nose VOC analysis holds promise as an accurate, noninvasive procedure for the diagnosis of different respiratory diseases, and may potentially serve to discriminate between different airway inflammatory phenotypes [26].

Our results indicate that e-nose VOC analysis can reliably discriminate airway inflammatory phenotypes in asthma patients with similar clinical manifestations. This observation is in line with some previous reports. For instance, Ibrahim et al [27] used gas chromatography-mass spectrometry in a smaller number of patients (n=18) and reported an accuracy of 83% for distinguishing eosinophilic from noneosinophilic asthma phenotypes and of 72% for separating neutrophilic from non-neutrophilic phenotypes; it should be noted, however, that paucigranulocytic patients were not included. More recently, Wagener et al [28] used the e-nose to discriminate breath-prints from 27 patients with eosinophilic versus noneosinophilic asthma with an accuracy of 85% and an AUC of 99% (95% CI, 0.9752-1). Finally, van der Schee et al [29] found that e-nose patterns predicted response to corticosteroids in 25 asthma patients with greater accuracy (mean [SD] AUC, 0.883 [0.16]; *P*=.008) than sputum eosinophil counts (0.610 [0.29]; *P*=.441) or FeNO measurements (0.545 [0.28]; *P*=.751). All in all, these and our results indicate that the e-nose is a simple, easy-to-use technology that can identify inflammatory asthma phenotypes in the clinical setting, as has already been suggested for COPD [30-31].

The identification of specific immunological pathways [1], now termed endotypes [32], is particularly relevant in the management of patients with difficult-to-treat asthma [6] because it can guide personalized treatment [33]. For instance, several recent studies [7] and systematic reviews [5] have shown that therapeutic strategies based on eosinophilic counts in induced sputum were highly effective in preventing exacerbations in difficult-to-treat asthma. However, the analysis of induced sputum is not currently recommended in clinical practice guidelines [13] because it is a complex, time-consuming technique and requires trained personnel. Additionally, assessable samples are not always obtained, and special care must be taken in patients with uncontrolled or severe asthma. Our data support that the theory that the e-nose may be a simple alternative to identify airway inflammatory phenotypes in clinical practice.

The inclusion of phenotypically well-characterized asthma patients (eosinophilic, neutrophilic, and paucigranulocytic) and the use of an e-nose in a regular clinical practice setting are clear strengths of our study. It has, however, some limitations. First, because it was a proof-of-concept study, we investigated a relatively small number of patients in a single center. Further investigations are needed in larger, multicenter asthma cohorts. Second, we did not investigate the reproducibility of our results. Nevertheless, previous studies using the same methodology have reported good reproducibility with the same e-nose device [10]. Third, smoking and inhaled corticosteroid treatment may theoretically alter VOC patterns. However, in our study there were no differences between groups regarding the proportion of smokers or patients receiving high doses of inhaled corticosteroids. In addition, the e-nose was able to differentiate VOC patterns despite these potential confounding factors. And fourth, unlike a recent study [27], we did not investigate which VOC species formed the e-nose pattern, and our data analysis was limited to a discriminant analysis approach. Future studies may use more advanced data analysis techniques suitable for the e-nose data complexity, such as Support Vector Machines [34] or Back Propagation Neural Networks [35] to immediately differentiate between different asthma phenotypes in a clinical setting.

The use of an e-nose in a regular clinical setting can reliably discriminate different inflammatory asthma phenotypes in patients with persistent asthma.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Vicente Plaza

Servei de Pneumologia
Hospital de la Santa Creu i Sant Pau
C/ Sant Antoni Mª. Claret, 167
08025 Barcelona, Spain
E-mail: vplaza@santpau.cat

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