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An electronic nose in the discrimination of patients with asthma and controls

Silvano Dragonieri, MD,[a,c] Robert Schot, BEng,[a] Bart J. A. Mertens, PhD,[b] Saskia Le Cessie, PhD,[b] Stefanie A. Gauw, BN,[a] Antonio Spanevello, MD,[d] Onofrio Resta, MD,[c] Nico P. Willard, PhD,[e] Teunis J. Vink, PhD,[e] Klaus F. Rabe, MD, PhD,[a] Elisabeth H. Bel, MD, PhD,[f] and Peter J. Sterk, MD, PhD[a,f] *Leiden, Eindhoven, and Amsterdam, The Netherlands, and Bari and Cassano Murge, Italy*

Background: Exhaled breath contains thousands of volatile organic compounds (VOCs) that could serve as biomarkers of lung disease. Electronic noses can distinguish VOC mixtures by pattern recognition.
Objective: We hypothesized that an electronic nose can discriminate exhaled air of patients with asthma from healthy controls, and between patients with different disease severities.
Methods: Ten young patients with mild asthma (25.1 ± 5.9 years; FEV_1, $99.9 \pm 7.7\%$ predicted), 10 young controls (26.8 ± 6.4 years; FEV_1, 101.9 ± 10.3), 10 older patients with severe asthma (49.5 ± 12.0 years; FEV_1, 62.3 ± 23.6), and 10 older controls (57.3 ± 7.1 years; FEV_1, 108.3 ± 14.7) joined a cross-sectional study with duplicate sampling of exhaled breath with an interval of 2 to 5 minutes. Subjects inspired VOC-filtered air by tidal breathing for 5 minutes, and a single expiratory vital capacity was collected into a Tedlar bag that was sampled by electronic nose (Cyranose 320) within 10 minutes. Smellprints were analyzed by linear discriminant analysis on principal component reduction. Cross-validation values (CVVs) were calculated.
Results: Smellprints of patients with mild asthma were fully separated from young controls (CVV, 100%; Mahalanobis distance [M-distance], 5.32), and patients with severe asthma could be distinguished from old controls (CVV, 90%; M-distance, 2.77). Patients with mild and severe asthma could be less well discriminated (CVV, 65%; M-distance, 1.23), whereas the 2 control groups were indistinguishable (CVV, 50%; M-distance, 1.56). The duplicate samples replicated these results.

Conclusion: An electronic nose can discriminate exhaled breath of patients with asthma from controls but is less accurate in distinguishing asthma severities.
Clinical implication: These findings warrant validation of electronic noses in diagnosing newly presented patients with asthma. (J Allergy Clin Immunol 2007;120:856-62.)

Key words: Asthma mild, asthma severe, biomarkers, diagnosis, electronic nose, exhaled breath, volatile organic compounds

In today's clinical practice, asthma is diagnosed and monitored by symptoms and physiological measurements, including lung function tests and the assessment of responses to inhaled pharmacological agents.[1] These tests have been internationally standardized and are generally considered to be reliable. However, they are rather complex, time-consuming, and not widely applicable, which has limited the implementation of the required techniques at all necessary levels of medical care.[1] Therefore, there is a need for new diagnostic methods in asthma that are simple, fast, accurate, and cost-effective.

During the past decade, cellular and molecular techniques have been added as novel options for diagnosis and monitoring in asthma. This includes eosinophil counts in induced sputum[2] and nitric oxide in exhaled air.[3] These approaches have been validated, but only the latter seems to have a realistic potential of widespread application in clinical research and practice on the basis of its simplicity.[4]

Nitric oxide may not be the only diagnostically relevant molecule in exhaled breath. It is well known that exhaled human breath contains thousands of volatile organic compounds (VOCs) in gas phase, which can individually be detected by gas chromatography and mass spectrometry (GC-MS).[5,6] These include mixtures of, for example, hydrocarbons, such as formaldehyde, methanol, ethanol, hydrogen sulfide, benzene, acetaldehyde, propanal, acetone, dimethyl sulfide, isoprene, toluene, phenol, xylene, and many others.[6] Such VOCs can potentially be used as noninvasive biomarkers of various biochemical pathways that are operative in health and disease. Interestingly, it has been demonstrated that there is a link between exhaled VOCs and human lung disease, in particular regarding lung cancer.[7,8] However, the requirement of

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Abbreviations used
       CVV:  Cross-validation value
       FVC:  Forced vital capacity
     GC-MS:  Gas chromatography and mass spectrometry
M-distance:  Mahalanobis distance
       PCA:  Principal component analysis
       SPT:  Skin prick test
       VOC:  Volatile organic compound
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GC-MS has thus far limited the medical applications of this approach.

Electronic noses represent an innovative method of VOCs sampling because these devices allow online recognition of complex VOCs mixtures by composite nanosensor arrays in combination with learning algorithms.[9] Each sensor represents different fractions of the VOC mixture, and the arrays exhibit good discrimination performance along with high sensitivity, short response time, and reversible behavior.[9] Hence, electronic noses use an essentially different concept from GC-MS. They principally follow an empirical approach, allowing the distinction of ''smell-prints'' obtained from various gaseous sources by pattern recognition, providing discrimination of gas mixtures irrespective of the individual molecular components.[10] The first proof of concept studies of electronic noses in respiratory medicine have provided high accuracy in the *ex vivo* classification of bacterial infection[11-13] and promising discrimination of exhaled breath obtained from patients with lung cancer and controls.[14]

In the current study, we postulated that an electronic nose can discriminate exhaled breath of patients with asthma from healthy controls. Our aim was to test this hypothesis by a cross-sectional study comparing patients with an established diagnosis of asthma with healthy controls. As a secondary aim, we examined whether an electronic nose can distinguish different degrees of asthma severity, and whether these classifications are reproducible when performing repeated measurements.

METHODS
Subjects

A total number of 40 subjects volunteered to participate to this study. All the subjects were nonsmoking adults (18-75 years) without any other acute or chronic disease than asthma. The study population included 4 groups of subjects based on current standard diagnostic procedures[1]: patients with intermittent-mild asthma and patients with moderate-severe persistent asthma, each with their own healthy control group. Patients were recruited among those visiting the outpatient clinic of the Leiden University Medical Center, whereas controls were recruited by advertisements in the hospital, the university, and public newspapers. Patients on medications other than short-acting or long-acting β_2-agonists and/or inhaled steroids or those who had a history of upper or lower respiratory tract infection during the 4 weeks before to the measurements were excluded from the study.

The mild asthma group was composed of 10 patients with episodic chest symptoms, prebronchodilator FEV_1 >80% predicted, documented reversibility in FEV_1 by 400 μg inhaled salbutamol >12% predicted or airway hyperresponsiveness (PC_{20} methacholine < 8 mg/mL),[15] atopy by positive skin prick tests (SPTs) in response to common airborne allergen or by RAST, treatment by as necessary use of inhaled short-acting β_2-agonists only, no use of inhaled corticosteroids for 3 months before the study, and no exacerbations (requiring oral steroid therapy) during the past 12 months.

The severe asthma group consisted of 10 patients who had episodic or chronic chest symptoms, documented reversibility for FEV_1 >12% predicted or PC_{20} methacholine (<8 mg/mL),[15] positive SPTs or positive RAST test, need of high doses of inhaled corticosteroids (≥ 1000 μg/d beclomethasone or equivalent) and long-acting β_2-agonists for more than 12 months, and at least 1 asthma exacerbation requiring oral steroid therapy during the past 12 months.

The 2 control groups also had 10 subjects each with a negative history of chest symptoms, prebronchodilator FEV_1 >80% predicted, and FEV_1/forced vital capacity (FVC) >70%, absence of atopy by negative SPTs or RASTs, and absence of airway hyperresponsiveness (PC_{20} methacholine > 8 mg/mL). The 2 control groups differed with respect to age (18-45 years and 46-70 years, respectively) to be compatible with the different age ranges that were observed in the 2 asthma groups of the study.

The study was approved by the Leiden University Medical Center Ethics Committee, and all the subjects gave their written informed consent.

Study design

The study had a cross-sectional case-control design with 2 visits within a 10-day period. The first day was a screening day to check all the inclusion and exclusion criteria. On the second day, exhaled breath was collected in duplicate and sampled by the electronic nose.

Lung function

Spirometry (Masterlab Jaeger, Hoechberg, Germany) was performed by a trained lung function technician according to the latest recommendations,[16] and the FEV_1 and FVC were measured before and 20 minutes after 400 μg inhaled salbutamol per metered dose inhaler with a spacer (Volumatic, GSK, Brentford, United Kingdom [UK]). Airway hyperresponsiveness was assessed by methacholine challenge tests performed by the tidal breathing method, using doubling inhaled doses (0.6-80 μmol/mL) at 5-minute intervals, until the PC_{20} was reached.[15] Patients withheld short-acting β_2 agonists for >8 hours and long-acting β_2 agonists for >12 hours before all lung function measurements.

SPT

Skin prick tests were performed by using a standardized set of 12 common airborne allergen extracts (ALK-Abelló, Hørsholm, Denmark). Atopy was indicated by positivity (>3 mm wheal) to 1 or more allergens or by positive RAST.

Exhaled breath collection

The patients breathed through a mouthpiece with the nose clipped into a 2-way nonrebreathing valve (Hans Rudolph 2700, Hans Rudolph, Kansas City, Mo) with an inspiratory VOC filter (A2, North Safety, Middelburg, NL) and an expiratory silica reservoir to dry the expired air. The breathing maneuvers were based on validation experiments in our laboratory. After 5 minutes of equilibration by tidal breathing with VOC-filtered air, the expiratory port was connected to a 10-L Tedlar bag. The subject then performed an inspiratory capacity maneuver and exhaled the full expiratory vital capacity into the bag with an expiratory resistance of 20 cmH_2O to close the soft palatum and to obtain an expiratory flow of 0.1 to 0.2 L/s. Within 10 minutes, the bag was connected to the electronic
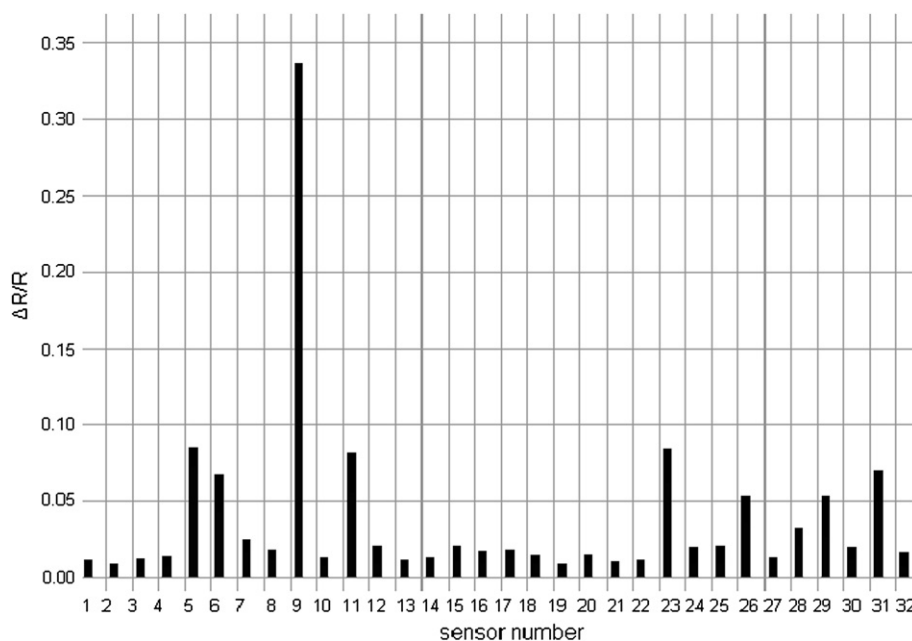
FIG 1. Example of pattern of relative differential electrical resistance (ΔR/R) of an array of 32 polymer sensors of the electronic nose, which represents the smellprint of a VOC mixture in exhaled breath of a single volunteer with asthma.

nose, followed by 1 minute of bag sampling in parallel with a second Tedlar bag containing background VOC-filtered room air. All subjects performed these maneuvers in duplicate by repeating the same procedure after 2 to 5 minutes of resting.

Electronic nose

Exhaled breath samples were analyzed by a commercially available handheld electronic nose (Cyranose 320; Smith Detections, Pasadena, Calif) with a nanocomposite array of 32 organic polymer sensors.[9] When the sensors are exposed to a mixture of VOCs the polymers are swelling, which induces a change in their electrical resistance. The raw data are captured as the changes in resistance of each of the 32 sensors in an onboard database, producing a distribution (smellprint; Fig 1) that describes the VOC mixture and that can be used for pattern-recognition algorithms.

Exploration of specific VOCs in exhaled breath was performed by sampling 1 L gas from the Tedlar bag through an adsorption tube filled with Tenax GR (Scientific Instruments Services, Ringoes, NJ). After sampling, the analytes were collected in a cryo-trap for refocusing and subsequently injected into a gas chromatography column (HP 5890 series II, Hewlett Packard, Palo Alto, Calif) and identified by mass spectrometry (HP 5972 MSD, Hewlett Packard), using a calibration mixture to check average sensitivity.

Breathing maneuvers

Exhaled breath was collected by an expiratory vital capacity maneuver after 5 minutes of tidal breathing with VOC-filtered room air. We validated the breathing maneuvers by 3 preliminary experiments to examine the effects of (1) inspiring VOC-filtered air, (2) expiratory lung volume, and (3) expiratory flow. First, we examined whether previous steady-state washin of VOC-filtered air is required, or whether a single inspiration with VOC-filtered air suffices preceding the collection of exhaled breath. Ten nonsmoking healthy subjects (mean ± SD age, 35.2 ± 10.1 years) performed 2 maneuvers in random order with a 30-minute interval: equilibration with

inspiratory VOC-filtered air by 5 minutes of tidal breathing or by 1 single inspiratory VC. The results were analyzed by principal component analysis (PCA) and canonical discriminant analysis. The smellprints obtained by the single inspiratory VC clustered distinctly from those after 5 minutes of washin (cross-validation value, 95%; Mahalanobis distance [M-distance], 4.24), indicating that a single inspiration of VOC-free air is not sufficient to equilibrate the exhaled breath VOCs. This led us to choosing the 5-minute tidal washin method. Second, we investigated whether collecting an expiratory VC and expiratory tidal breaths provided the same smellprints. This was also addressed in 10 nonsmoking healthy subjects (age, 39.6 ± 11.7 years) for whom a single expiratory VC and 1-minute tidal breaths were collected in random order with a 30-minute interval. The PCA data showed that tidal breath samples could be discriminated from expiratory VC samples (cross-validation, 85%; M-distance, 2.51). We chose to use the single expiratory VC sampling on the basis of the relatively smaller contribution of the anatomical dead space. Third, we examined the possible influence of expiratory flow during expiratory VC sampling in 10 nonsmoking healthy subjects (age, 29.4 ± 7.5 years) by using 0.1 to 0.2 L/s and 0.3 to 0.5 L/s in a random order with a 30-minute interval. The smellprints could not be discriminated well (cross-validation value, 65%; M-distance, 1.17), suggesting a limited influence of expiratory flow within this flow range. For our studies, we chose to standardize flow at 0.1 to 0.2 L/s.

Data analysis

First, the smellprints were analyzed online by the on-board learning software of the Cyranose 320. Subsequently, an offline confirmatory analysis was performed by double cross-validatory implementation of linear discriminant analysis on principal component reduction using Matlab software (version 7; MathWorks Benelux, Gouda, NL) as previously described.[17] The data were processed through Savitzky-Golay filtering and baseline correction.[18] Then they were analyzed by PCA to reduce the data from 32 individual

TABLE I. Clinical characteristics of the study population*

	Mild asthma	Severe asthma	Younger controls	Older controls
No. of patients	10	10	10	10
Age (y)	25.1 ± 5.9	49.5 ± 12.0	26.8 ± 6.4	57.3 ± 7.1
Sex, male/female	1/9	8/2	2/8	4/6
FEV_1 prebronchodilator, % predicted	99.9 ± 7.7	62.3 ± 23.6	101.9 ± 10.3	108.3 ± 14.7
FEV_1 postbronchodilator, % predicted	111.9 ± 9.2	74.3 ± 21.8	ND	ND
FVC, % predicted	109 ± 8.1	80.9 ± 17.3	101.9 ± 7.2	102.6 ± 17.4

ND, Not determined.
*Values are expressed as means $\pm$ SDs.

sensors to a set of principal components aimed to finding the factors capturing the largest variance in the dataset.[19] PCA was used as an exploratory analysis and was plotted in 2-dimensional or pseudo–3-dimensional graphs to visualize between-group separations. Once the PCA factors had been calculated, these factors were used to perform a linear canonical discriminant analysis for the construction of a pattern recognition algorithm by maximizing the ratio of pooled within-class scatter to between-group distance. The online software calculated a cross-validation estimate of error: the cross-validation value (CVV).[20] M-distance was used to quantify the discrimination between 2 sample groups.[19] The M-distance provides the nonsimilarity of a set of values derived from 2 samples. It takes the sample variability into account and reflects the distance between group means in units of standard deviations. The M-distance value and the ability to discriminate are directly related, so that M-distance values >3 are indicative of high probability of discrimination ($P < .01$).

RESULTS

The subject characteristics of the 4 groups are described in Table I. Patients with severe asthma were older than patients with mild asthma ($P < .01$), which was the reason for using 2 control groups with ages below and above 45 years, respectively. Two samples of exhaled air could be obtained in all subjects.

First, we examined whether exhaled breath from patients with asthma could be discriminated from controls. The plots of the online PCA of the smellprints obtained from the first bags in the patients and controls are shown in Fig 2. Smellprints of patients with mild asthma were separated from those of the young controls (Fig 2, *upper left*). Subsequent canonical discriminant analysis demonstrated a cross-validation of 100% correct with a M-distance of 5.32. Similarly, smellprints from severe asthmatics could be distinguished from those of old controls (Fig 2, *upper right*). The cross-validation value was 90% correct with a M-distance of 2.77.

However, discriminant analysis could discriminate less well smellprints from patients with mild and severe asthma (Fig 2, *lower left*). This led to a cross-validation value of 65% correct and a M-distance of 1.23. There was no difference in smellprints between the 2 control groups (Fig 2, *lower right*), with a cross-validation value of 50% and a M-distance of 1.56.

Analysis of exhaled air from the second bags replicated these results (patients with mild asthma vs young controls:

CVV, 100%; M-distance, 4.86; patients with severe asthma vs old controls: CVV, 95%; M-distance, 5.72; patients with mild asthma vs patients with severe asthma: CVV, 60%; M-distance, 1.87; old controls vs young controls: CVV, 60%; M-distance, 1.20).

The offline discriminant analysis on PCA reduction using double cross-validation confirmed the onboard discrimination between the groups (data not shown).

Explorative GC-MS analysis showed that the predominant VOCs (>15 ng/L) in asthma were 4-methyloctane, 2,4-dimethylheptane, isopropanol, toluene, isoprene, alkane, acetic acid, acetone, 2,6,11-trimethyl dodecane, 3,7-dimethyl undecane, and 2,3-dimethyl heptane.

DISCUSSION

Our study shows that an electronic nose can discriminate exhaled breath of patients with asthma from healthy controls. This distinction was replicated when analyzing exhaled air from repeated samples. However, the electronic nose could not adequately discriminate mild from severe asthma. These findings indicate that the mixture of exhaled volatile organic compounds is different in asthma compared with controls. Our findings warrant further validation of electronic noses regarding their ability to correctly identify newly presented patients with asthma.

To our knowledge, this is the first study using pattern analysis of exhaled VOC mixtures by an electronic nose in the field of asthma. Interestingly, we observed a complete separation of smellprints between patients with mild asthma and healthy controls as well as between patients with severe asthma and controls. This was confirmed when using duplicate measurements. To date, electronic noses have been used in a variety of medical fields, including the detection of sinusitis, cerebrospinal fluid leak, urinary tract infections, bacterial vaginosis, and diabetes mellitus.[10] The application of electronic noses in respiratory medicine showed moderate sensitivity but high specificity in the detection of lung cancer from exhaled breath[14] and high accuracy in the *in vitro* diagnosis of *Mycobacterium tuberculosis* infections.[13] Recent studies further suggest that an electronic nose may be applicable in the diagnosis of ventilator-associated pneumonia.[21,22] Therefore, the VOCs present in the exhaled breath may become a powerful source of biomarkers for the diagnosis of respiratory diseases, including asthma.
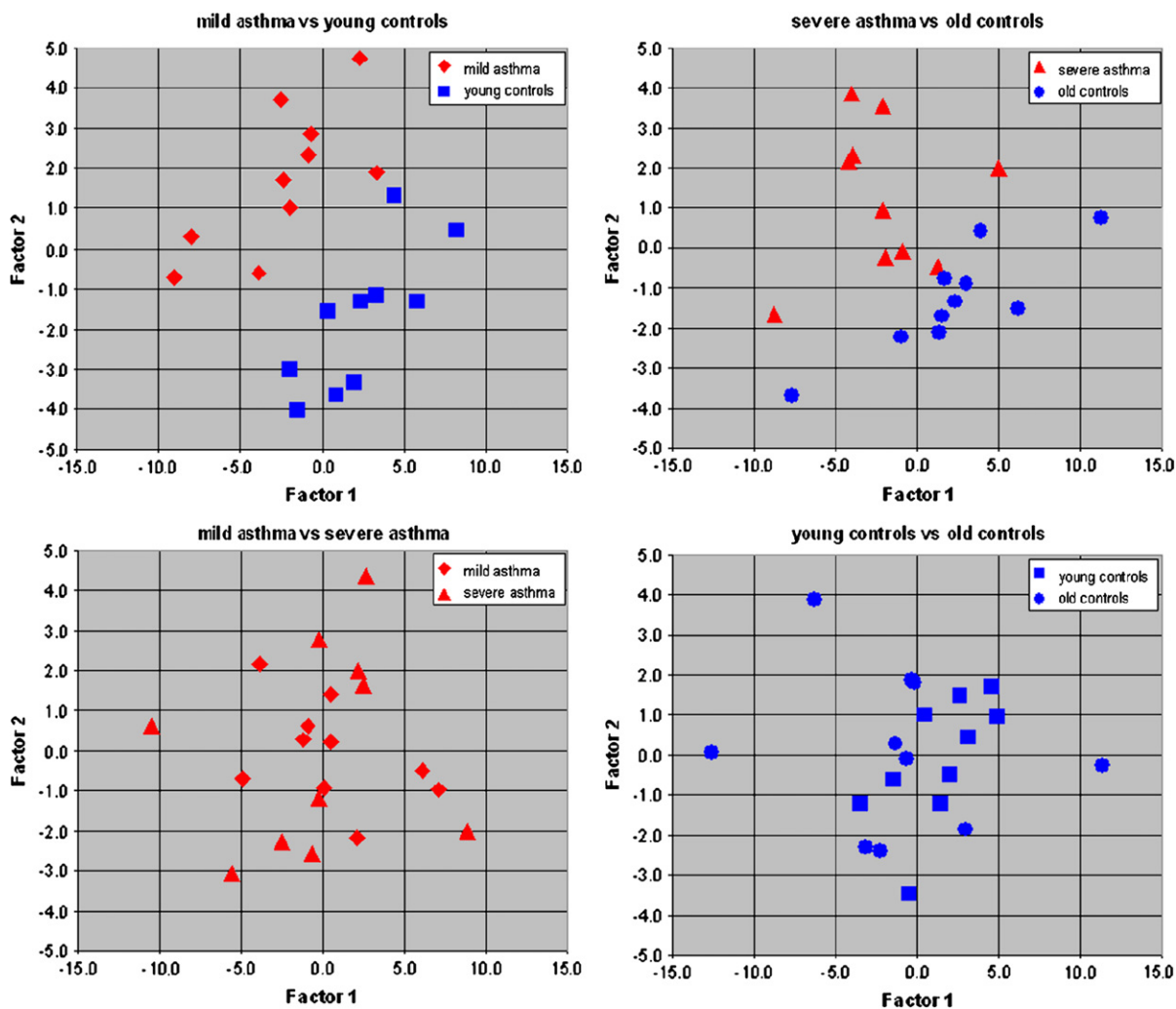
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FIG 2. Two-dimensional PCA plots with 2 composite factors (factors 1 and 2) maximizing the discrimination of smellprints between patients with mild asthma *(diamonds)* from their controls *(squares, upper left)*, patients with severe asthma *(triangles)* from their controls *(bullets, upper right)*, patients with mild asthma *(diamonds)* from patients with severe asthma *(triangles, lower left)*, and young controls *(squares)* from older controls *(bullets, lower right)*.

In our study, particular attention was paid to methodologic aspects such as the selection of patients and controls. All the patients were well characterized by using subjective and objective criteria of the presence and severity of atopic asthma.[1] This included the presence of symptoms, reversible airways obstruction or airways hyperresponsiveness to methacholine, and positive SPTs. The controls of the 2 asthma groups were carefully screened to exclude all these features. The downside of this strict selection is that we cannot deduce the separate contributions of atopy, lung function, and hyperresponsiveness to the current discrimination. This requires studies focused on subphenotyping. Smokers and exsmokers were excluded because tobacco smoking may well change the exhaled VOCs profile. However, we had an imbalance in sex between the asthma groups and cannot exclude that this affected the exhaled breath

molecular profiles. Furthermore, patients with mild and severe asthma were selected on the basis of their medication usage. The mild asthma group was free of steroids, whereas the severe asthma group was taking inhaled corticosteroids and long-acting bronchodilators. We cannot exclude that this (inevitable) difference in drug regimen affected the VOCs profiles. However, the electronic nose was not able to make a clear distinction between mild and severe asthma. This may suggest that drug usage is not a major determinant of exhaled breath smellprints.

By using sample sizes of 10 subjects per group, the electronic nose was able to make a full separation between patients with asthma and controls (Fig 2). However, our results suggest that higher sample sizes may be required to obtain optimal training sets for electronic noses in patients with various severities of asthma. Finally, it seems

unlikely that the current findings can be explained by accident or by error, because the duplicate samples led to the closely similar results. Moreover, offline statistics[17] confirmed the onboard analysis of the smellprints.

The sampling technique and breathing maneuvers were optimized by pilot experiments. The measurements were made in the same room with fixed temperature and humidity. We dried the exhaled breath through a silica filter to limit the influence of variable humidity on the sensor signals. In addition, we made an attempt to avoid any acute effects by food, coffee, or other drinks by not allowing those during the 2 hours before the test. Finally, our pilot experiments showed that the conditioning of inspiratory air and the expiratory breathing maneuver both influence the VOC pattern. The 5-minute steady-state washin with VOC-free air was based on recommendations for helium washin during lung volume measurements. It cannot be excluded that this needs to be prolonged in patients with severe airways obstruction. Our experiments indicate that careful methodological standardization is required for exhaled breath analysis by electronic nose.

How can the present findings be interpreted? GC-MS analysis has shown that human exhaled breath contains more than 3000 different VOCs.[5,6] When using an inspiratory VOC filter, the detected VOCs are most likely derived from physiologic and pathophysiologic metabolic pathways.[5,6] These can arise from the airways and the lungs or can represent systemic metabolites from elsewhere in the body. Interestingly, before the application of electronic noses, GC-MS analysis had already demonstrated changes in specific VOCs in exhaled breath from patients with lung cancer that are compatible with increased production of reactive oxygen species and enhanced alkane metabolism by cytochrome P450.[23] Chronic airways inflammation in asthma may change several metabolic pathways that affect molecular markers in exhaled breath.[24] For instance, asthma is associated with elevated levels of pentane[25] in exhaled air, and with increased concentrations of markers of oxidative stress[26] and eicosanoids in exhaled breath condensate.[27] It remains to be established whether such markers are associated with airways inflammation and airway remodeling in asthma,[28,29] which indeed has been reported recently.[27]

It needs to be emphasized that our study does not reveal which specific VOCs are responsible for the discrimination of exhaled breath between patients with asthma and controls. Our explorative GC-MS analysis showed the presence of similar VOCs in asthma compared with normal subjects[6] or patients with lung cancer.[14] However, the determination of the specific, discriminative VOC concentrations between these diseases will require subsequent studies. The electronic nose that we used detects VOC mixtures with a polymer nanocomposite sensor array.[9] Each sensor signal represents partly different fractions of the complete VOC mixture on the basis of, for example, molecular mass, shape, dipole moment, and hydrogen binding capacity.[14] Therefore, unlike GC-MS, an electronic nose principally does not chemically identify and separate VOCs.[9,30] Hence, the high relatively differential

resistance in sensor 9 (Fig 1) represents a mixture of VOCs. Whether it contributes to the discrimination between patient groups depends on any differences in this signal between the groups and its subsequent selection by the PCA. Therefore, pattern recognition of smellprints by electronic noses is purely based on a statistical approach, providing empirical evidence. Such procedure is hypothesis-free and potentially powerful. It resembles other high-throughput methods that are based on the analysis of molecular profiles of complex biological samples by a single measurement (omics techniques).[31] Interestingly, the principle of electronic noses exactly mirrors biological olfaction in mammals, in which multisensitive olfactory receptor cells appear to be coupled to pattern recognition systems in the brain, leading to unique odors.[32] Hence, our current observations by using an electronic nose system can be considered complementary to GC-MS analysis and warrant further studies by GC-MS to identify the critical VOCs.

What are the clinical implications of our findings? The electronic nose appears to be able to discriminate exhaled breath from well characterized subjects with and without asthma. Such discrimination of established cases and controls represents the first step in the cross-sectional validation of diagnostic tests.[33] Our data warrant an external validation of the electronic nose by testing its diagnostic accuracy in a sample of newly presented patients with various severities and subcategories of asthma. Such study should be performed according to international recommendations.[34] If successful, electronic noses have the potential to become convenient devices for physicians and nurses for handheld, noninvasive, and rapid diagnosis of asthma. In addition, validation of electronic noses in other respiratory diseases, such as chronic obstructive pulmonary disease or lung cancer, seems to be mandatory.[14,35]

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