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Integration of electronic nose technology with spirometry: validation of a new approach for exhaled breath analysis

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Abstract

New 'omics'-technologies have the potential to better define airway disease in terms of pathophysiological and clinical phenotyping. The integration of electronic nose (eNose) technology with existing diagnostic tests, such as routine spirometry, can bring this technology to 'point-of-care'.

We aimed to determine and optimize the technical performance and diagnostic accuracy of exhaled breath analysis linked to routine spirometry.

Exhaled breath was collected in triplicate in healthy subjects by an eNose (SpiroNose) based on five identical metal oxide semiconductor sensor arrays (three arrays monitoring exhaled breath and two reference arrays monitoring ambient air) at the rear end of a pneumotachograph. First, the influence of flow, volume, humidity, temperature, environment, etc, was assessed. Secondly, a two-centre case-control study was performed using diagnostic and monitoring visits in day-to-day clinical care in patients with a (differential) diagnosis of asthma, chronic obstructive pulmonary disease (COPD) or lung cancer. Breathprint analysis involved signal processing, environment correction based on alveolar gradients and statistics based on principal component (PC) analysis, followed by discriminant analysis (Matlab2014/SPSS20).

Expiratory flow showed a significant linear correlation with raw sensor deflections ($R^2 = 0.84$) in 60 healthy subjects (age 43 $\pm$ 11 years). No correlation was found between sensor readings and exhaled volume, humidity and temperature. Exhaled data after environment correction were highly reproducible for each sensor array (Cohen's Kappa 0.81–0.94).

Thirty-seven asthmatics (41 $\pm$ 14.2 years), 31 COPD patients (66 $\pm$ 8.4 years), 31 lung cancer patients (63 $\pm$ 10.8 years) and 45 healthy controls (41 $\pm$ 12.5 years) entered the cross-sectional study. SpiroNose could adequately distinguish between controls, asthma, COPD and lung cancer patients with cross-validation values ranging between 78–88%.

We have developed a standardized way to integrate eNose technology with spirometry. Signal processing techniques and environmental background correction ensured that the multiple sensor arrays within the SpiroNose provided repeatable and interchangeable results. SpiroNose discriminated controls and patients with asthma, COPD and lung cancer with promising accuracy, paving the route towards point-of-care exhaled breath diagnostics.

Introduction

Diagnostic tests are an essential part of modern medicine, including respiratory diseases. The ultimate goal of diagnosis and monitoring is to optimize the outcome or prognosis for the patient by giving the clinician directions for a clinical management strategy. Even though physiological and cell-based procedures, such as spirometry and induced sputum are often routinely available, molecular diagnostics are not widely applicable at point of care.

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Exhaled breath contains thousands of volatile organic components (VOCs) that originate from both systemic and local metabolic processes, which can be associated with normal physiology or pathophysiological inflammatory or oxidative activity [1, 2]. There are several technologies that can be applied to exhaled breath analysis, the standard being represented by identification of individual molecular compounds by analytical chemistry techniques, such as gas chromatography with mass spectrometry (GCMS) [1]. This is mandatory for pathophysiological research. Additionally, electronic noses (eNoses) are used with the objective of recognition of gas mixtures, by using a variety of cross-reactive sensors that capture the spectrum of VOCs in exhaled air without the identification of the individual components [3].

Studies conducted thus far in the research field of respiratory diseases suggest that eNose technology has potential to proceed towards a diagnostic and monitoring tool [4]. It has already been shown that by using eNoses lung cancer, inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD), and infectious diseases can be distinguished with accuracies that are approaching traditional diagnostic tests [5–13].

Recently, combined technical and medical efforts have resulted in the compilation of the 'SpiroNose' (AMC, Amsterdam; Comon-Invent BV, Delft, The Netherlands), an online and real-time, database driven eNose designed for medical purposes, which can be used in combination with routine lung function testing. Online real-time analysis offers several advantages as compared with indirect sampling, notably by retrieving immediate results, and avoiding laborious collection and storage steps. This eliminates a major source of experimental errors in exhaled breath analysis, particularly those related to the loss of compounds (e.g. compounds that change rapidly as a function of external influence or unstable compounds that decompose before being analyzed [14, 15]). To control and minimize variables known to influence exhaled breath analysis, we integrated eNose technology with spirometry. Integration of eNose with existing diagnostic tests, such as routine spirometry, brings this technology to 'point-of-care' and links it to regular clinical procedures.

We hypothesized that the SpiroNose is an accurate and valid technology to measure exhaled breath in a clinical setting. This study aimed to technically and medically validate the SpiroNose in order to proceed towards its clinical application. To that end we performed an extensive technical validation, including the development of signal-processing and data-analysis techniques and validated the diagnostic accuracy in a daily practice setting.

Methods

Subjects

Technical validation

To validate the technical performance of the SpiroNose 60 healthy adult subjects, willing to participate in this validation study, were included. The healthy controls were recruited by advertisement at the university campus of Amsterdam.

Clinical study

Adult subjects (n = 144) were included in this study. Subjects were divided into four groups according to current diagnosis: (1) patients with mild to severe asthma; (2) moderate to severe COPD; (3) lung cancer; and (4) healthy controls. Patients were recruited among those visiting the lung function departments at the Academic Medical Centre (AMC) Amsterdam and Medical Spectrum Twente (MST) Enschede, whereas controls were recruited by advertisement at the university campus of both study sites.

The asthma group consisted of 37 patients with episodic chest symptoms, smoking history $<$10 pack years, a documented reversibility in FEV1 of $\geqslant$12% predicted or 200 ml after 400 μg salbutamol or airway hyperresponsiveness (PC20 methacholine or histamine $<$8 mg ml^{-1}) [16]. The COPD group consisted of 31 patients with a smoking history $\geqslant$15 pack years, symptoms of dyspnea, chronic cough and/or sputum production, a postbronchodilator FEV1 $<$80% of predicted and FEV1/FVC ratio $<$0.70 (GOLD stage II–IV according to GOLD guidelines [17]). 31 Lung cancer patients were recruited with an established medical diagnosis of lung cancer based on current guidelines [18, 19]. The control group included 45 subjects with a negative history for chest symptoms, a postbronchodilator FEV1 $>$80% predicted and FEV1/FVC $>$0.70. Subjects were excluded in case of insulin-dependent diabetes, respiratory infection and pulmonary disease other than asthma, COPD and lung cancer.

The ethics board of AMC Amsterdam (dd. 07-05-2014) and MST Enschede (dd. 05-06-2014) concluded in writing that Dutch legislation on human participation in research was not considered to be applicable given the non-invasive nature of the study and of its integration into routine diagnostic lung function.

Design

First, the influence of integration of eNose with the pneumotachograph was assessed. Additionally, we assessed the robustness of the technique against variables potentially influencing SpiroNose measurements such as, expiratory airflow, exhaled breath volume, humidity, temperature, ambient substances and the use of a bacterial filter. Finally, signal-processing techniques and two essential eNose features, sensor stability and reproducibility, were addressed.

Second, the clinical validation had a two-centre cross-sectional case-control design merely using the diagnostic and monitoring visits of day-to-day care in clinical practice. After screening for in- and exclusion criteria, the measurements were performed as part of pulmonary function testing. Patients were not instructed to refrain from eating, drinking, smoking

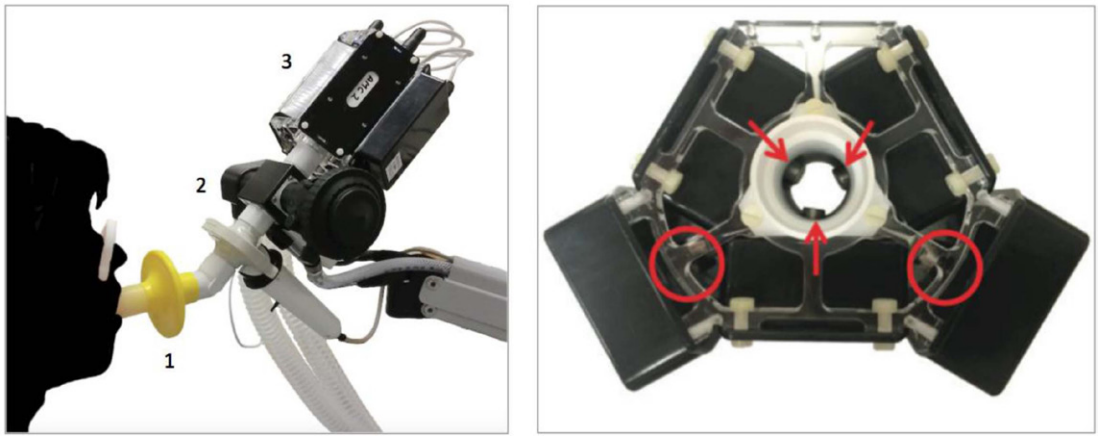

Figure 1. (Left) Patient breathing through the SpiroNose measurement setup, including: (1) a viral and bacterial filter with a soft bite mouthpiece and nose clamp (Lemon Medical GmbH); (2) the Masterscreen PFT system (Masterscreen, Jaeger, Carefusion); and (3) the SpiroNose (AMC Amsterdam, Comon Invent Delft). (Right) The SpiroNose composed of five sensor arrays, each comprising four MOS sensors. (Red arrows) three arrays of MOS sensors placed inside the tube for exhaled breath analysis. (Red circles) two arrays of MOS sensors placed at the outside of the tube, used for environment correction.

or discontinue any medication to increase applicability in clinical practice. Patients were requested to rinse their mouth thoroughly three times with water. Subsequently, exhaled breath analysis was performed in duplicate with a 2 min interval.

Measurements

Exhaled breath analysis was performed using the SpiroNose, an eNose designed for medical purposes that can be used as add-on to routine lung function testing. The SpiroNose consists of five separate sensor arrays, each comprising four metal oxide semiconductor (MOS) sensors (Figaro, Japan). The choice of Figaro-type MOS sensors was based on their good long-term performance and stability [20]. Two of the sensor arrays were used as reference arrays to detect the ambient VOCs and three sensor arrays were used to monitor the VOCs in exhaled breath (figure 1). All sensor arrays are identical and consist of four different MOS sensors with operating temperatures ranging between −40 °C and +70 °C. Using thick film techniques, the sensor material is printed on electrodes onto an alumina substrate. The four MOS sensors all used tin dioxide (SnO_2) as the main sensing material of the sensor element. The complete measurement setup used in this study consists of a soft bite mouthpiece, nose clamp and viral/bacterial filter (Lemon Medical GmbH) attached to a Masterscreen™ PFT system (Masterscreen, Jaeger, Care Fusion) and the SpiroNose (figure 1).

Technical performance

To verify that the addition of the SpiroNose to the pneumotachograph did not influence spirometry results healthy controls performed three reproducible FVC maneuvers for three different device setups; (1) regular pneumotach flow and volume calibration by a 3 L calibration syringe (2) regular calibration before the addition of the SpiroNose and (3) calibration after adding the SpiroNose to the setup. The three device setups were used in random order to exclude the influence of fatigue and habituation to the exercise.

By using available and specialized software (J-scope, Jaeger) included on the PFT system, flow, volume, time and moment of in/expirations during measurements were determined. The humidity and temperature were registered before every measurement by the Masterscreen™ PFT system with the additional SentrySuite software.

To gain insight in the ambient gases/VOCs that are present during exhaled breath measurements, the ambient VOCs were detected by the reference sensor arrays of the SpiroNose.

Furthermore, the influence of a bacterial filter was addressed by comparing SpiroNose sensor deflections with and without a bacterial filter in both a patient and control group (10 asthma patients (age 30 ± 6.9 years, FEV1 93 ± 11.2% predicted) versus 10 healthy controls (age 28 ± 7.4, FEV1 105 ± 7.2)). Bacterial filters placed after the mouthpiece represent a routine procedure for hygiene purposes. The SpiroNose measurements were performed in duplicate with a 2 min time interval. After two weeks, the healthy controls and asthma patients performed the same measurements to determine both within-day and between-day repeatability.

SpiroNose sensor stability was verified using a test gas (Lindegas, for the measurement of pulmonary diffusion capacity) as quality control (QC) gas before every session. This QC gas is a standard mixture of acetylene (0.300 ± 0.015%), carbon monoxide (0.300 ± 0.015%), methane (0.300 ± 0.015%), oxygen (20.7 ± 1.0%) and nitrogen used as a balance gas. To verify sensor stability over the course of this study, each morning before exhaled breath measurements the demand valve was placed at the rear end of the SpiroNose. Subsequently, the gas mixture was flushed for 5 s with a mean flow of 3 L s^{-1} through the SpiroNose, which was measured with the pneumotachograph. In addition, we assessed the sensor variability relative to

the effect size observed when discriminating subject groups to obtain the signal-to-noise ratio.

Finally, the exhaled breath results of the 60 healthy controls, obtained in triplicate by the three identical sensor arrays that monitor VOCs in exhaled breath, were used to determine reproducibility of the four MOS sensors. We compared the unprocessed (figure 3(A)) and processed (figure 3(B)) sensor deflections of the three identical sensor arrays for sensor-to-sensor reproducibility.

Clinical validation

Exhaled breath measurements were performed right before regular spirometry. All patients were instructed to start the measurement by performing 10 tidal breaths. After a single deep inspiratory capacity maneuver and a 5 s breath hold the patient exhaled, with an expiratory flow <0.4 L s^{-1}, a vital capacity volume into the measurement setup. Exhaled breath was directly sampled in triplicate by the SpiroNose, which is connected to a GSM-module that ensures that the obtained data (changes in electrical voltage) are real-time transmitted and stored at the online server of Comon Invent BV, Delft, without the addition of patient-related data. Here, data could be downloaded, whereupon it was further processed and analyzed using offline pattern-recognition software.

Data-analysis

Clinical validation

The primary analysis of the clinical study was done by comparing the exhaled breathprints between patients with asthma, COPD, lung cancer and healthy controls. Secondarily, the exhaled breathprints between asthma patients measured at different study sites (AMC versus MST) were compared to investigate whether the used data sampling and analysis techniques are appropriate for multi-center purposes. Offline analysis of raw sensor data was performed using Matlab (MathWorks, 2014) and SPSS (version 20). First, data were pre-processed and corrected for ambient VOCs. Subsequently, the data was normalized and reduced by principal component analysis (PCA) after which cases were classified into a categorical division.

Environment correction

The environment correction was based on the calculation of alveolar gradients [21–23]. Alveolar gradients were obtained by subtracting inspiratory (reference signal) from expiratory VOC concentrations (patient signal) [22, 23]. Reference sensor signals were filtered using a low-pass Butterworth filter to extract the overall shape of the signal, followed by linear trend removal based on a least squares fit. Subsequently, both reference signals were averaged. Cross-correlation was used to obtain the highest correlation between reference and patient signal, as both showed a similar but delayed pattern. Finally, the patient sensor signals were filtered using a low-pass Butterworth filter before subtracting the processed and averaged reference signal. Following this environment correction, linear trends and offsets were removed from the patient signals. The three processed and corrected patient signals were not averaged.

Table 1. Subject characteristics and pulmonary function of healthy controls included for the technical validation of the SpiroNose.

	Controls ($n = 60$)
Age, years	42.7 (11.1)
Gender (M/F)	23/37
BMI	24.0 (4.3)
FEV1 postbronchodilator (%pred)	101.9(7.5)
FEV1/FVC	0.8(0.07)
Current / ex- / never-smoker	4/29/27
Pack years	5.5 (9.4)
ICS-use (n)	0

Note: values are expressed as mean (SD). BMI—body mass index; FEV1—forced expired volume in 1 s; FVC—forced vital capacity; ICS—inhaled corticosteroids.

Peak detection

The exhaled breath analysis was performed in duplicate. Peak values of the sensor signals were detected using an automatic peak detection algorithm implemented in Matlab for both measurements. This algorithm automatically finds the local maxima in a vector (patient signal). In order to create exhaled breathprints, the highest sensor peak of both measurements for each sensor signal was selected and normalized with respect to the most sensitive sensor (range: 1–30 ppm) with the highest corrected sensor values, sensor 4. The study focused on sensor-to-sensor ratios rather than absolute sensor values, to minimize inter-array differences.

Statistical analysis

Pre-processed exhaled breath data were restructured by PCA from the original 12 sensors monitoring exhaled breath (three sensor arrays of four MOS sensors) to four principal components according the Kaiser criterion [24]. The obtained PCA factors were used to perform a univariate ANOVA analysis to select the principal components that were discriminative between groups. Breathprints were internally validated by multiple iterations of bootstrap. Furthermore, PCA was used as an exploratory analysis plotted in 3D graphs to visualize between-group separations. Subsequently, linear canonical discriminant analysis was performed to plot cases on a linear classifier. Based on the differentiating PCA factors, a discriminant function was calculated that best distinguished between categories. The accuracy of this model was defined as the percentage of correctly classified patients, cases and controls combined. Cross-validation using the leave-one-out method was used to calculate the cross-validated accuracy value (CVV, %). The discriminant

Table 2. Subject characteristics and pulmonary function in patients with asthma, COPD, lung cancer and healthy controls.

	Controls	Asthma	COPD	Lung cancer
No.	45	37	31	31
Age, years	41.5(12.5)	41.4(14.2)	66.6(8.4)	62.5(10.8)
Gender (M/F)	19/26	15/22	15/16	18/13
BMI	24.7(4.2)	26.1(5.4)	27.9(5.5)	27.3(4.8)
FEV1 postbronchodilator (%pred)	103.9(8.5)	85.3(18.9)	49.9(20.3)	71.4(19.1)
FEV1/FVC	0.79 (0.05)	0.67(0.08)	0.41(0.11)	0.69(0.15)
GOLD-stage (II/III/IV)	NA	NA	9/15/7	2/6/1
GINA-classification				
Mild / moderate / severe	NA	10/18/9	NA	NA
Lung cancer histologic subtype				
SCLC /NSCLC	NA	NA	NA	12/19
Lung cancer stage II/III/IV	NA	NA	NA	2/8/21
Current / ex- / never-smoker	1/21/23	0/8/29	8/23/0	10/18/3
Pack years	5.5 (9.4)	1.9(6.4)	36.1(12.7)	31.9(16.6)
ICS-use (*n*)	0	35	26	6

Note: values are expressed as mean (SD). NA—not applicable; BMI—body mass index; FEV1—forced expired volume in 1 s; FVC—forced vital capacity; SCLC—small cell lung cancer; NSCLC—non-small cell lung cancer; ICS—inhaled corticosteroids.

functions were used to construct receiver operator characteristic (ROC) curve. The area under the curve (AUC) and single spot test sensitivity and specificity were determined. Finally, reproducibility was assessed by comparison of the three identical sensor arrays monitoring patient's exhaled breath using Cohen's kappa analysis.

Results

Subject characteristics of healthy controls included for technical validation of the SpiroNose are presented in table 1. The subject characteristics and pulmonary function of patient groups included for clinical validation of the SpiroNose are described in table 2. Healthy controls and patients with asthma were significantly younger than patients with COPD and lung cancer ($p < 0.01$). There were no significant differences in age between healthy controls and asthma patients and between patients with COPD and lung cancer. The postbronchodilator FEV1 (% predicted) in asthma patients was significantly lower than in healthy controls ($p < 0.01$), but significantly higher than in COPD ($p < 0.01$) and lung cancer patients ($p < 0.01$). Furthermore, nine lung cancer patients were also diagnosed with COPD.

Technical performance

A strong linear correlation ($R^2 = 0.84$) was found for sensor 1 between expiratory flow and absolute sensor deflections (figure 2). Between 0.19 and 0.38 L s^{-1} absolute sensor values were not significantly influenced by the expiratory flow rate ($p > 0.5$). Similar results were found for sensor 2–4. In addition, a significant linear correlation ($R^2 = 0.75$) was found between sensor-to-sensor ratio (S1/S4) and the expiratory flow. The sensor ratios S2/S4 and S3/S4 again showed comparable results.

No correlation was observed between the sensor readings and the exhaled volume for sensor 1 (figure 2). Comparable results were found for sensors 2–4.

There were no significant differences in spirometry results between the three device setups related to adding the SpiroNose to the pneumotachograph ($p > 0.5$). Furthermore, no significant correlation was found between sensor-to-sensor ratios and temperature or humidity ($p > 0.5$) (figure S1 of the supplementary data).

Distinct breathprints were found for a subgroup of healthy controls and patients with asthma between using ($p < 0.01$, CVV 83%) and not using ($p < 0.01$, CVV 78%) a bacterial filter. In addition, the SpiroNose established intraclass correlation coefficient (ICC) values ranging between 0.81–0.86 for within-day repeatability and 0.69–0.80 for between-day repeatability (supplementary data; 1.3 and 1.4).

The effect size obtained when discriminating the various subject groups was 5.2 till 7.4-fold higher than the normal sensor variability, indicating a good signal-to-noise ratio. More detailed information about these results can be found in the supplementary information.

The results obtained by the specified data analysis techniques used to create breathprints were compared to the unprocessed sensor data analysis for 60 healthy controls (figure 3). Data of four MOS sensors are presented for sensor array 1, 2 and 3. Analysing the unprocessed data showed significant differences in breathprints between the three identical sensor arrays for sensor 1 ($p < 0.001$) and sensor 3 ($p < 0.05$), while each sensor array monitored the same exhaled breath (figure 3(A)). When data were processed and corrected for ambient VOCs, breathprints did not show significant differences for sensor 1–4 (figure 3(B)) and a

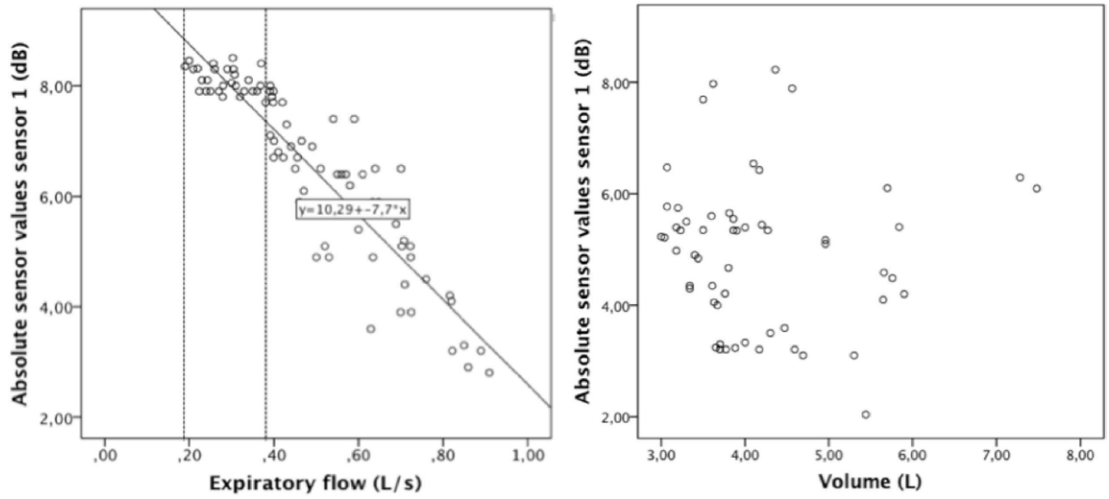

Figure 2. (Left) Linear relation between expiratory flow and absolute sensor values for sensor 1. (Right) The relation between expiratory volume and absolute sensor values for sensor 1.

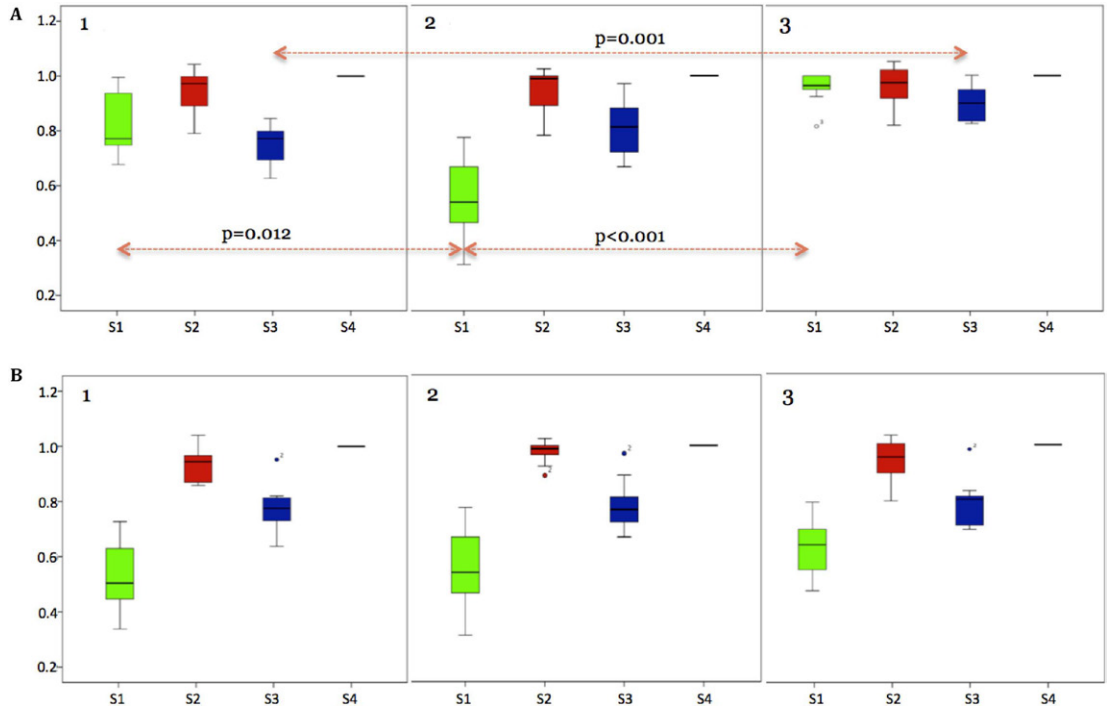

Figure 3. (A) Normalized but *unprocessed* sensor values (sensor 1–4) given for sensor array 1, 2 and 3 (monitoring the exhaled VOCs). (B) Normalized sensor values (sensor 1–4) *after signal pre-processing and correction for ambient VOCs*, for sensor array 1, 2 and 3. Sensor values are normalized with respect to sensor 4.

Cohen's kappa ranging from 0.81 to 0.94 was achieved, thereby showing high between-sensor reproducibility.

Clinical validation

Principal component analysis with ANOVA showed that exhaled breath data from patients with asthma and COPD could be well distinguished ($p = 0.001$). Subsequent discriminant analysis showed a cross-validated accuracy value of 81% (figure 4). The ROC-AUC ±95% confidence interval after internal cross-validation reached 0.81 ± 0.09. Breathprints of COPD patients could also be discriminated from those of lung cancer patients ($p = 0.002$; CVV: 80%), with an ROC-AUC of 0.88 ± 0.11 (figures 4 and 5). When excluding those lung cancer patients with co-morbid COPD from the latter analysis, the COPD and lung cancer patients could still be distinguished ($p < 0.001$) with a cross-validated accuracy of 87% and a ROC-AUC of 0.91 ± 0.12. For completeness, exhaled breath data from lung cancer and asthma patients were discriminated ($p = 0.045$) with a cross validated accuracy of 68%. The ROC-AUC reached 0.71 ± 0.09.

Breathprints of asthma patients were distinguished from those of healthy controls ($p < 0.001$) with a cross-validated accuracy value of 87% and an ROC-AUC of 0.94 ± 0.15. Exhaled breath results of COPD

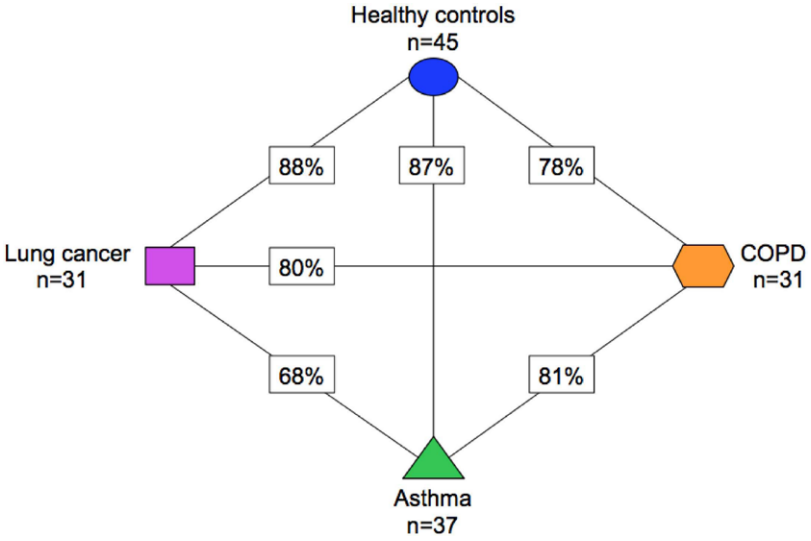

Figure 4. Cross-validation values (%) for the discrimination between healthy controls and patients with asthma, COPD and lung cancer.

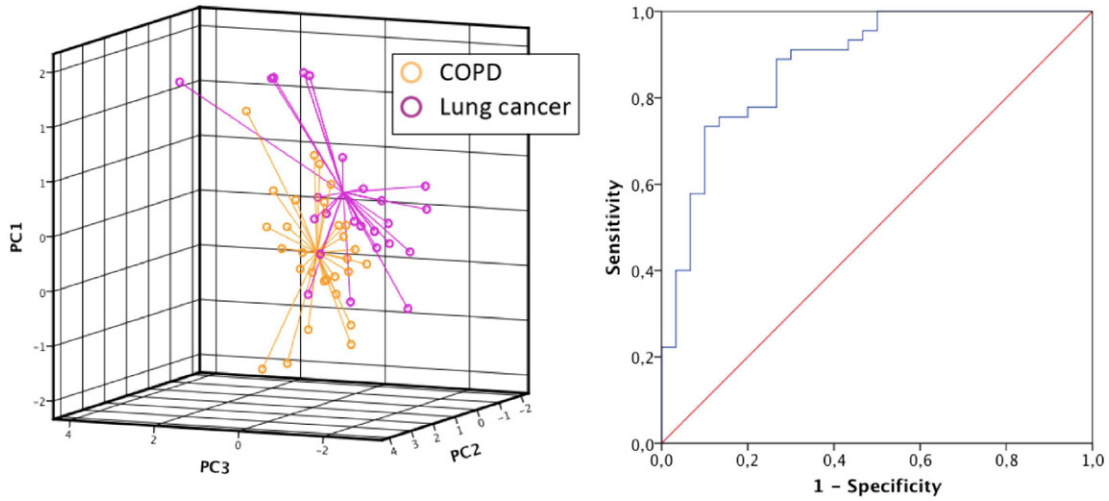

Figure 5. (Left) 3D PCA plot showing the discrimination of breathprints between patients with COPD and lung cancer along discriminative principal components (CVV:80%, $p = 0.002$). (Right) ROC-curve with line of identity of the breathprint discriminant function (representing PC 1, 2 and 3), predictive for the differential diagnosis of asthma and COPD (AUC 0.88).

patients and controls were discriminated ($p = 0.001$) with a cross validated accuracy of 78%. The ROC-AUC reached 0.80 ± 0.10. Furthermore, breathprints from patients with lung cancer and healthy controls were very well distinguished ($p < 0.001$) and a cross-validated accuracy value of 88% was achieved with an ROC-AUC of 0.95 ± 0.11 (figure 4).

Finally, no significant difference was found between exhaled breathprints obtained from asthma patients measured at different study sites ($p = 0.892$) (figure 6).

Discussion

The present study shows that integration of an eNose with spirometry is feasible without influencing lung function results ($p > 0.5$). Exhaled breath analysis with this technique, by SpiroNose, provided adequate discrimination between healthy controls, patients with asthma, COPD and lung cancer. Repeated measurements showed adequate reproducibility and confirmed this distinction. This indicates that the SpiroNose and the associated data analysis techniques are suited for exhaled breath profiling in a point-of-care setting. Our findings warrant further validation of the SpiroNose regarding its ability to correctly identify patients with various (respiratory) diseases in independent, external populations.

To our knowledge this is the first study that aimed to integrate eNose technology with spirometry to conduct exhaled breath measurements in daily clinical practice, without additional restrictions in eating, drinking, smoking and medication. We were not able to integrate the two completely, since the required flow for exhaled breath analysis is not the same as obtained during spirometry. Our findings are showing the need for a desired flow range for SpiroNose assessment that

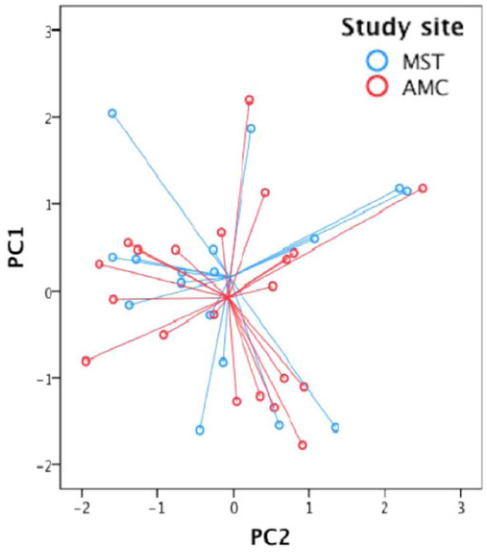

Figure 6. 2D PCA plot showing no significant discrimination of breathprints from asthma patients measured at the Academic Medical Center (AMC), Amsterdam and Medical Spectrum Twente (MST), Enschede.

can easily be monitored by spirometry, thereby ensuring quality control of exhaled breath analysis. In doing so, patients can conveniently perform exhaled breath measurements before spirometry within less than 2 min, making this technology suitable for point of care.

Our work extends the results of many research groups who performed exhaled breath analyses in patients with asthma [8, 10, 12, 25], COPD [7, 9, 13, 25] and lung cancer [5–7, 11] and obtained similar cross-validated accuracies. Nevertheless, complete separation of these disease could not be accomplished. First, COPD is also a frequent comorbidity in lung cancer. Therefore, it may not be surprising that exhaled breath profiles by electronic noses have shown definite overlap between lung cancer and COPD [7]. When we excluded lung cancer patients with co-morbid COPD from the clinical analysis between COPD and lung cancer patients the discrimination became even better. This may suggest that the patients with a double diagnosis were correctly allocated in the overlap area. Second, the obtained CVV for the distinction between asthma and lung cancer patients was surprisingly low. This outcome has no clinical implication as asthma versus lung cancer is not a differential diagnosis, but the result requires further studies using GC-MS on the proliferative, oxidative and inflammatory mechanisms involved in both diseases.

In this study, we performed both a clinical and technical validation of the newly developed SpiroNose, which exhibited strengths and limitations. For the clinical validation we included three relevant groups of patients as commonly seen in daily practice and one asymptomatic control group. International guidelines for evaluating diagnostic accuracy of a novel test, recommend evaluation of the discriminative ability between predefined, gold standard diseased and non-diseased subjects as the initial step [26]. Although our subjects were well characterized, there were some inevitable differences between the groups. First, healthy controls and patients with asthma were significantly younger than COPD and lung cancer patients. These differences potentially introduced an age-bias in the between-group separations, although Dragonieri *et al* showed that VOC profiles in exhaled breath from young (26 ± 6 years) and older (57 ± 7 years) healthy controls could not be separated [8]. Second, the difference in medication use (ICS) between COPD and lung cancer patients could have influenced the exhaled VOC-profiles. We have previously shown that the difference in VOC-profiles between asthma and COPD patients (CVV: 97%, $p < 0.0001$) remained significant after removal of the difference in ICS-use (CVV: 95%, $p < 0.0001$), by restricting both groups to those patients using ICS [25]. Although these results were obtained with a different eNose, we believe that the differences in exhaled breathprints between patients with asthma, COPD and lung cancer, found with the SpiroNose, are disease related.

The SpiroNose consists of five identical sensor arrays (sensor array 1–3 monitoring exhaled breath and sensor arrays 4–5 are reference arrays) containing four different MOS sensors. Romain *et al* tested a large number of sensors with different sensor technologies and showed that metal oxide-based gas sensors (Figaro, JP) exhibited the best long-term stability [20]. When developing the SpiroNose we reasoned that correction for ambient VOCs based on two reference sensor arrays would be required for optimal performance. Because of the large similarity of responses between sensor arrays in the present study, in retrospect this strategy might have been overly cautious.

Reproducibility data for sensor arrays 1, 2 and 3 showed significant improvement after signal processing and correction for ambient VOCs, with Cohen's kappa results ranging from 0.81 to 0.94. This suggests that the location of the sensors in the tubing does not affect exhaled breath results. Furthermore, this signal processing demonstrates that the data analysis techniques used are appropriate for exhaled breath sampling by SpiroNose. Interestingly, with this small number of MOS sensors comparable accuracy levels were achieved as previously described in literature with other eNoses, including arrays using far more sensors [4]. Apparently, increasing the sensor array number does not necessarily improve eNose classification performance as that could amplify the noise and/or generate redundancy of information [3, 27].

Regarding the technical validation we found a linear correlation between exhaled breath sensor deflections and the expiratory flow. Dragonieri *et al* [8] studied the possible effects of expiratory flow during vital capacity in healthy non-smokers by using 100–200 ml s^{-1} and 300–500 ml s^{-1} in a random order with a 30 min interval. Within the 100–200 ml s^{-1} flow range; expiratory flow had a limited effect on eNose measurements. In addition, Pedroletti *et al* [28] studied the NO levels

at six different exhalation flow rates in 15 asthmatic schoolchildren with elevated NO levels and 15 age-matched controls. They found that a targeted flow rate of approximately 50 mL s^{-1} seems to be most appropriate for single-breath measurements when considering discriminatory power, reproducibility and patient comfort. Taken together, our data suggest that expiratory flow rates between 0.19 and 0.38 L s^{-1} are suitable for SpiroNose measurements. Nevertheless, further studies are required to address the effect of expiratory flow on eNose measurements, particularly after a period of normal breathing (as opposed to single-breath sampling), and in patients with various respiratory diseases [29]. When using the SpiroNose setup connected to spirometry, it is not possible to regulate expiratory flow by means of a resistor. The expiratory flow can only be influenced by instructing the patient, which if needed can be achieved by e.g. visual feedback from pneumotachograph measurements. We asked patients to exhale the expiratory vital capacity very slowly, preferably with a flow between 0.19 and 0.38 L s^{-1}, at which the MOS sensors likely become saturated thereby limiting the effect of expiratory flow at this flow range during SpiroNose measurements in practice (figure 2).

Due to technical feasibilities sensor stability was validated by flushing pulmonary diffusion test gas with 3 L s^{-1} through the SpiroNose. For this we conveniently used the demand valve, which is part of the Masterscreen PFT system, but the latter did not allow us to manually flush the test gas at a lower flow rate. Even though it would have been most optimal to determine sensor stability with a flow <0.4 L s^{-1}, we believe sensor stability results regarding the absence of linear trends and assessment of the sensor variability relative to the effect size are representative for these MOS sensors. For the exhaled breath measurements we asked patients to exhale vital capacity very slowly, preferably with a flow between 0.19 and 0.38 L s^{-1}. Here, sensor readings suggested that the MOS sensors became saturated. Therefore, we expect sensor stability to increase when measured at a flow rate <0.4 L s^{-1} instead of 3 L s^{-1} since the influence of flow will be minimal.

In the present study we decided to sample an integrative exhaled air sample, as it is yet unknown whether the discriminative VOC signals arise from the airways and/or the alveoli [12]. In addition, even alveolar samples are trafficking through the conducting airways and oropharynx, which may complicate interpretations of separated airways and alveolar samples. The SpiroNose uses real-time analysis in order to measure the integrative exhaled air sample, implicating that dead space air samples can be separated in time compared to the alveolar air samples.

The applied real-time approach of breath sampling has the advantage of increasing breath VOC concentrations and stability of the total exhaled breath sample [29]. Direct breath sampling is a very appealing option, since it makes the widely used collecting bags unnecessary. Tedlar bags, which are recommended by the US Environmental Protection Agency [30] for ambient gas sampling, are widely used for breath sampling in patients with respiratory disease. However, if bags are exposed to direct sunlight and over time Tedlar bags can release hydrocarbons N,N-dimethylacetamide and phenol [31]. Furthermore, there may be considerable carryover effects from previous measurement. Therefore, SpiroNose real-time sampling and analysis may not only be practical for clinical usage, but may also merit unaffected VOC assessment.

In conclusion, we have shown that eNose analysis of exhaled biomarkers can be integrated with spirometry. This provides a simple approach to exhaled breath analysis we have shown to be able to discriminate between patients with asthma, COPD and lung cancer. Furthermore, we have shown adequate reproducibility and transferability of algorithms between devices. The latter will allow exchange of results between different labs and locations, which is a prerequisite for clinical implementation [32]. Therefore, the SpiroNose may strongly contribute to bringing the promise of exhaled breath diagnostics to clinical practice, particularly when linked to an online database (www.breathcloud.org).

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