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# An electronic nose discriminates exhaled breath of patients with untreated pulmonary sarcoidosis from controls

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Biomarkers;
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Volatile organic compounds

**Summary**
*Background:* Sarcoidosis is a systemic granulomatous disease of unknown cause that affects the lungs in over 90% of cases. Breath analysis by electronic nose technology provides exhaled molecular profiles that have potential in the diagnosis of several respiratory diseases.
*Objectives:* We hypothesized that exhaled molecular profiling may distinguish well-characterized patients with sarcoidosis from controls. To that end we performed electronic nose measurements in untreated and treated sarcoidosis patients and in healthy controls.
*Methods:* 31 sarcoidosis patients (11 patients with untreated pulmonary sarcoidosis [age: 48.4 ± 9.0], 20 patients with treated pulmonary sarcoidosis [age: 49.7 ± 7.9]) and 25 healthy controls (age: 39.6 ± 14.1) participated in a cross-sectional study. Exhaled breath was collected twice using a Tedlar bag by a standardized method. Both bags were then sampled by an electronic nose (Cyranose C320), resulting in duplicate data. Statistical analysis on sensor responses was performed off-line by principal components (PC) analyses, discriminant analysis and ROC curves.
*Results:* Breathprints from patients with untreated pulmonary sarcoidosis were discriminated from healthy controls (CVA: 83.3%; AUC 0.825). Repeated measurements confirmed those results. Patients with untreated and treated sarcoidosis could be less well discriminated (CVA 74.2%), whereas the treated sarcoidosis group was undistinguishable from controls (CVA 66.7%)

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*Conclusion:* Untreated patients with active sarcoidosis can be discriminated from healthy controls. This suggests that exhaled breath analysis has potential for diagnosis and/or monitoring of sarcoidosis.


## Introduction

Sarcoidosis is a systemic granulomatous disease of unknown etiology, which commonly affects young and middle-aged adults throughout the world.[1] The estimated life-time risk varies between 1 and 2% in different ethnic groups.[2] Pulmonary involvement occurs in over 90% of cases.[3] Due to its wide variety in clinical presentation and subsequent disease course, the diagnosis of sarcoidosis is challenging and often requires invasive approaches such as bronchoscopy.[1] As a consequence, decisions on whether or not starting treatment often have to be taken on arbitrary grounds. Therefore, It would be desirable to have new diagnostic methods in sarcoidosis that are simple, quick, non-invasive, cost-effective and with high selectivity. In the past years, several serum markers have been associated with the presence of sarcoidosis, including angiotensin-converting enzyme, neopterin and soluble IL-2 receptor, but none of them showed an adequate sensitivity to be useful for screening for the diagnosis of sarcoidosis.[4]

During the past few years, the analysis of exhaled breath has been proposed as a novel diagnostic tool for a variety of lung diseases.[5] It is well known that exhaled breath contains thousands of volatile organic compounds (VOCs) deriving from various metabolic and inflammatory pathways in the body.[6,7] These can be measured by techniques like gas chromatography—mass spectrometry (GC—MS), Time of Flight Mass Spectrometry (TOF-MS) and Ion-Mobility spectrometry (IMS), which are the gold-standard for VOCs analysis. A feasibility study by Westhoff et al. showed characteristic peaks of volatile organic compounds in exhaled air of patients with sarcoidosis by using IMS.[8]

However, these procedures are arduous and expensive, which has limited their clinical applicability.

Electronic noses represent an innovative method of VOCs sampling, because these devices can identify a mixture of VOCs translating it into a breath profile (breathprint).[9] Differently from GC—MS, they allow a relatively inexpensive, on-site and instantaneous distinction of breathprints by pattern recognition, without identification of the individual molecular components.[10] Notably, several proof of concept studies have shown that exhaled breath molecular profiling by electronic noses could be useful in the medical diagnostics,[5] in particular in the diagnosis of several respiratory diseases such as lung cancer[11—13] asthma,[14,15] COPD[15] and pleural malignant mesothelioma.[16]

Based on the above, we hypothesized that the exhaled breath molecular profiling by an electronic nose can correctly discriminate patients with pulmonary sarcoidosis from controls with adequate repeatability.

## Methods

### Patients

A total number of 56 patients volunteered to participate to this study. All individuals were never-smoking adults (18—75 years). The study population included 3 groups of patients: patients with untreated pulmonary sarcoidosis, patients with treated pulmonary sarcoidosis and a healthy control group. Measurements were performed in May 2011. During January—March 2011 otherwise unselected patients, however fulfilling the inclusion criteria, were approached during regular visits to the outpatient clinic of the Amsterdam University Medical Center. Those who volunteered participated in the study, whilst controls were recruited amongst personal contacts.

The untreated pulmonary sarcoidosis group was composed of 11 patients with a more recently established diagnosis or longer standing stable disease without previous or current medical treatment. The treated pulmonary sarcoidosis group consisted of 20 patients with stage 0—IV pulmonary sarcoidosis currently under inhaled and/or systemic therapy (corticosteroids alone, corticosteroids combined with azathioprine, methotrexate or, hydroxychloroquine). Sarcoidosis was defined as presence of histological evidence of non-caseating granulomas in patients with bilateral hilar adenopathy on the chest roentgenogram, except for those with particular conditions where a diagnosis was based on clinical-radiographic findings alone, such as Löfgren syndrome, Heerfordt syndrome, bilateral hilar gallium-67 uptake and positive PET scan.[1] Patients were radiologically staged using currently accepted consensus criteria.[1] Patients with clinically established conditions affecting the exhaled VOCs spectrum were not eligible for participation, in particular Diabetes Mellitus, respiratory disease other than sarcoidosis, autoimmune disease, renal dysfunction, cardiac failure, prior or current malignancies and respiratory tract infections requiring antibiotics and/or oral steroids in the 4 weeks preceding the study.

The control group was composed by 25 subjects with a negative history of chest symptoms and without of any known disease.

The study was approved by the Amsterdam University Medical Centre Ethics Committee and all patients gave their written informed consent.

### Study design

The study had a cross-sectional case-control design. The measurements were performed at one visit. Patients were asked to refrain from eating and drinking at least for 3 h before the study. Exhaled breath was collected in duplicate and sampled by the electronic nose.

## Exhaled breath collection

Exhaled breath analysis was performed as previously described.[14] In short, patients breathed tidally for 5 min through a 3-way non-rebreathing valve connected to an inspiratory VOC-filter (A2, North Safety, NL). Afterward, patients exhaled a single vital capacity volume into a Tedlar bag, connected to the electronic nose. The entire maneuver was repeated after 5 min resting, which provided duplicate samples.

## Electronic nose

We used a commercially available handheld electronic nose, (Cyranose 320, Smith Detections, Pasadena, CA, USA) with a nano-composite array of 32 organic polymer sensors. When the sensors are exposed to a mixture of VOCs the polymers swell, inducing a change in their electrical resistance.[10] The raw data are captured as the changes in resistance of each of the 32 sensors in an onboard database, thereby producing a distribution (breathprint) that describes the VOC mixture and that can be used for pattern-recognition algorithms.[9]

## Data analysis

Raw data were analyzed by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA), using analysis strategies that purposely limit false-discoveries.[17] Data were reduced to a set of principal components capturing the largest amount of variance of the original 32 sensors. Univariate ANOVA analysis was used to select the principal components that best discriminated among groups. Afterward, these principal components were used to perform a linear Canonical Discriminant Analysis (CDA), to classify cases into a categorical partition. We used the "leave-one-out method" to calculate the Cross Validated Accuracy percentage (CVA, %). The CVA provides a percentage that estimates how accurately a predictive model will perform in practice. For each case the probability of a positive diagnosis was calculated on basis of the canonical discriminant function. These probabilities were subsequently used to create a receiver operator curve (ROC-curve) with 95% confidence limits. The sample size calculation was based on estimating the standard error (SE) of the percentage correctly classified patients. The reliability of the percentage correct classification $C$ is dependent on SE, whilst SE itself is a function of $p$: $SE = \sqrt{[C(100 - C)/n]}$. If C is between 85% and 100% the current sample sizes per subgroup (11, 20 and 25 patients, respectively) provide SE values between 7 and 9%. A $p$-value of $<0.05$ was considered significant.

## Results

The patient characteristics of the three groups are described in Table 1. Patients with untreated and treated sarcoidosis were slightly older than controls ($p < 0.05$ and $p < 0.01$, respectively). Serum ACE was higher in untreated patients with sarcoidosis compared to those treated ($p < 0.01$).

**Table 1** Clinical characteristics of the study population.

| | Untreated sarcoidosis | Treated sarcoidosis | Healthy controls |
|---|---|---|---|
| Patients ($n$) | 11 | 20 | 25 |
| Age (yrs, mean ± SD)* | 48.4 ± 9.0 | 49.7 ± 7.9 | 39.6 ± 14.1 |
| Males | 4 | 13 | 11 |
| Females | 7 | 7 | 14 |
| FVC %pred | 87.8 ± 11.2 | 88.5 ± 16.3 | n.d. |
| DLCO %pred | 68.2 ± 12.4 | 62.9 ± 13.3 | n.d. |
| Serum ACE¥ (u/mg) | 111.6 ± 58.5 | 72.6 ± 37.4 | n.d. |
| Radiostage 0/1/2/3/4 | 1/2/5/2/1 | 2/2/2/1/13 | n.d. |
| Time since diagnosis (yrs, mean ± SD) | 6.8 ± 5.5 | 11.1 ± 6.5 | |
| Treatment**: C/C + A/M/H | n.d. | 12/4/1/3 | |

* = $p < 0.05$ untreated p.s. vs. controls; $p < 0.01$ treated vs. controls.
¥ = $p < 0.05$ by independent samples $t$-test.
**C: corticosteroids; C + A: corticosteroids and azathioprine; M: methotrexate; H: hydroxychloroquine.

The radiological stages of patients with sarcoidosis are also shown in Table 1. Eight out of eleven untreated patients had hilar and mediastinal lymphadenopathy and/or pulmonary infiltrates without signs of lung fibrosis (stage 1−3), whereas fourteen out of twenty treated patients had advanced disease with reticular opacities and evidence of pulmonary fibrosis (stage 4).

The two-dimensional PCA plot showed that patients with untreated sarcoidosis could be distinguished from healthy controls (Fig. 1). Canonical discriminant analysis was then performed on the data and showed a CVA% of 83.3 ($p < 0.001$). The area under the curve of the ROC-curve for the discrimination between untreated sarcoidosis and healthy controls was 0.825 (Fig. 2). Analysis of exhaled air from the second bag reproduced these results (untreated sarcoidosis vs. controls: CVA% 86.1, $p < 0.001$, AUC 0.853).

However, breathprints of untreated sarcoidosis patients were barely separated from those of the treated sarcoidosis group, with cross-validated accuracy of 74.2%. Analysis of the second collected bag confirmed these findings (untreated sarcoidosis vs. treated sarcoidosis: CVA% 71.1).

Finally, the comparison between breathprints of treated patients with healthy controls did not reach statistical significance for both duplicate measurements (CVA% 66.7 and 64.4, respectively).

## Discussion

Our study shows that an electronic nose can discriminate the exhaled breath of patients with untreated sarcoidosis from healthy controls. The electronic nose could less adequately distinguish patients with untreated sarcoidosis from those with treated sarcoidosis. These distinctions were confirmed when analyzing exhaled air from repeated samples. However, the electronic nose could not discriminate treated sarcoidosis from healthy controls. These

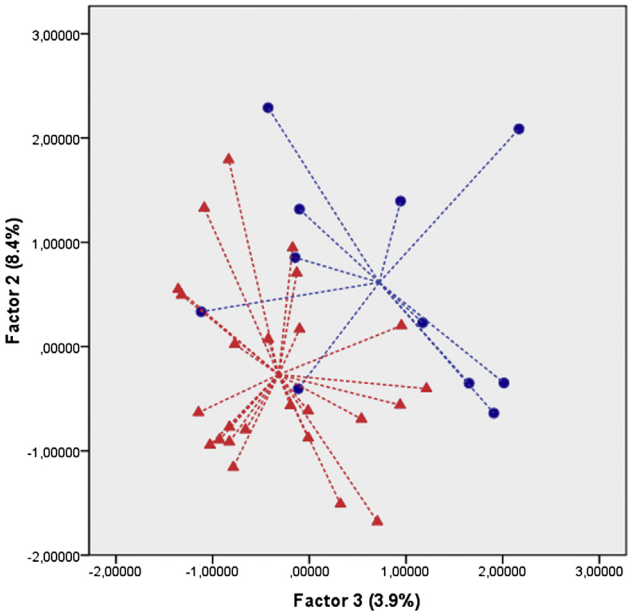


**Figure 1** Two-dimensional principal component analysis with 2 composite factors showing the discrimination of breathprints between patients with untreated sarcoidosis (blue circles) and controls (red triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

findings indicate that the VOC-profile in human exhaled breath differs between untreated pulmonary sarcoidosis as compared and controls, warranting further diagnostic validation of electronic noses in sarcoidosis.

To the best of our knowledge this is the first study using exhaled breath molecular pattern recognition by electronic noses in the field of sarcoidosis. Notably, we observed an adequate separation between the untreated sarcoidosis

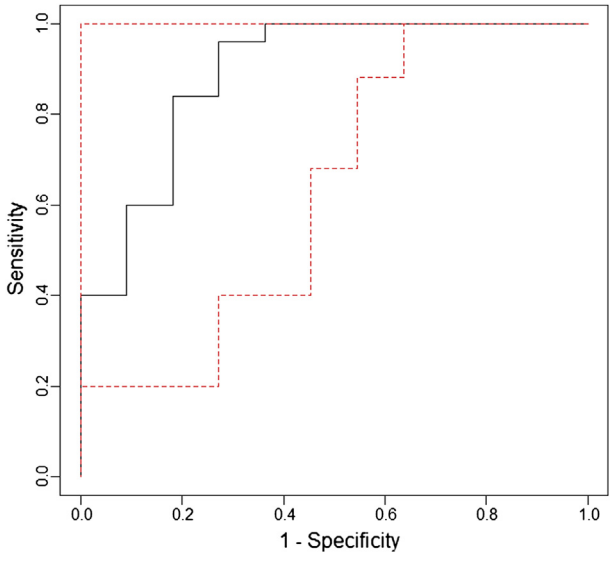


**Figure 2** ROC-curve with 95% confidence interval for diagnosis of untreated sarcoidosis compared to controls. AUC was 0.825.

group and healthy controls. This was confirmed when using duplicate measurements. To date, in clinical research the potential of electronic nose technology has already been tested for detecting a variety of other diseases, such as bacterial vaginitis, venous leg ulcer infections, sinusitis, cerebrospinal fluid leak, urinary tract infections, diabetes mellitus and renal dysfunction.[5,9] The application of electronic noses in respiratory medicine showed its potential in diagnosing, phenotyping and monitoring obstructive diseases like bronchial asthma and chronic obstructive pulmonary disease (COPD). Several studies demonstrated that patients with asthma could be discriminated from healthy controls.[14,15,18,19] Interestingly, patients with COPD were also discriminated from asthmatics.[15,18] Moreover, a number of studies with different electronic nose technologies suggest that exhaled breath profiling may be applicable in the diagnosis of lung cancer,[11–13,20,21] as well as in pleural malignant mesothelioma.[16,22] Finally, several groups have undertaken efforts to detect *Mycobacterium tuberculosis*[8] and ventilator-associated pneumonia.[23,24] The present findings suggest that the detection of pulmonary sarcoidosis by electronic nose may also be accomplished.

We paid particular attention in considering methodological issues such as the selection of groups. All the patients were carefully selected by generally accepted consensus criteria.[1] This discrimination of *a priori* diagnosed gold-standard groups is essential as first step in the validation of novel tests according to current guidelines.[25–27] In addition, we used previously validated sampling techniques and breathing maneuvers[14] to minimize any influence on the exhaled VOC-profile by environmental VOCs, humidity, expiratory flow-rate or contaminated material. We excluded smokers and ex-smokers because tobacco smoking is known to change the level of several VOCs in exhaled breath.[28] Moreover, we ruled out patients with any comorbidities, such as diabetes mellitus, renal failure and heart disease which have been shown to interfere with the VOCs spectrum.[9,29]

Nevertheless, several limitations of our study need to be acknowledged. First, patients with sarcoidosis were older than controls, thus potentially introducing an age-bias in the discrimination of groups. We previously showed that exhaled breath from young and older patients was not distinguishable by an electronic nose.[14] However, we cannot exclude that age-related factors have affected our results. Second, we divided patients with sarcoidosis into two groups according to their medication usage. Exhaled breath of the treated sarcoidosis group was not clearly discriminated from that of untreated patients and controls. The treatment of our patients with sarcoidosis included inhaled and/or systemic corticosteroids and other immunosuppressants such as azathioprine and methotrexate. These medications may affect the VOC-pattern by locally and systemically modifying inflammatory and metabolic pathways, potentially leading to the poor discrimination between treated sarcoidosis patients and controls. Nevertheless, we did not observe a sharp discrimination between treated and untreated sarcoidosis, suggesting that medication usage may not be a key element in the lack of differentiation between these groups. Not unexpectedly, there were many more patients with advanced disease (stage 4) in treated group. Therefore, our data raise the

hypothesis that eNose assessment may only discriminate between early stage granulomatous inflammation and control conditions, whereas it cannot distinguish late stage fibrotic inflammation or (partly) medically suppressed inflammation from control.

Third, even though we had 56 patients in the study, the current sample size was relatively limited. Nevertheless, it appeared to be sufficient for obtaining a well-defined separation between breathprints of untreated sarcoidosis and controls. This is unlikely to be explained by accident or error, since repeated sampling confirmed our findings. The 95% confidence limits of the ROC-curve are in line with this. Undoubtedly, further investigations with a larger and independent population are required for testing exhaled breath profiling in various stages of sarcoidosis.

Fourth, we did not include a control group of patients with chest diseases having a similar clinico-radiological presentation, such as tuberculosis, hypersensitivity pneumonitis, fibrotic lung diseases or even Beryllium disease, which is notably difficult to distinguish from sarcoidosis. This is mandatory for the next step in the validation of electronic noses as diagnostic tools for sarcoidosis.

How can we interpret our results? It appears that the exhaled breath of patients with untreated pulmonary sarcoidosis is different from that of healthy controls. Sarcoidosis is characterized by a non-caseating granulomatous inflammation, caused by an unknown "sarcoid" antigen which leads to an amplified Th-1 like lymphocytic response and to the release of a variety of cytokines and chemokines, including pro-fibrotic factors, pro-inflammatory agents and macrophage-derived substances.[30] It is likely that these inflammatory processes may change several metabolic pathways, thereby providing different VOCs in the exhaled air. There are only few data about exhaled breath analysis in sarcoidosis. Interestingly, elevated levels of $H_2O_2$ and 8-isoprostane (markers of oxidative stress) and ethane (marker of lipid oxygenation) were detected in the exhaled breath condensate of patients with sarcoidosis.[30] It is essential to emphasize that electronic noses do not identify which specific VOCs are responsible for the distinctive patterns between sarcoidosis and controls. This requires GC—MS analysis, which needs to be applied in follow-up studies, when aiming to identify the molecular pathways that are driving the currently observed discrimination. Such studies can also be helpful in the development of specific sensors tailored for a given disease, as has already been done for lung cancer.[12]

What are the clinical implications of our findings? Using an electronic nose, it appears to be feasible to distinguish exhaled breath from patients with untreated pulmonary sarcoidosis from healthy subjects. Diagnostic potential based on probabilistic assessment is highly valuable in medicine, provided that the sensitivity and specificity of the eNose meets the demands for the confirmation and/or the exclusion of the disease.[25] Our data warrant external validation studies in larger cohorts which will better enable the comparison of breathprints across radiographic stages. This needs to be compared with other granulomatous diseases, as previously mentioned. If successful, electronic noses may have the potential to become a relatively inexpensive, easy to use and non-invasive diagnostic tool for sarcoidosis. They may either qualify as screening devices (with maximal sensitivity) aimed to exclude sarcoidosis amongst patients with suspected sarcoidosis, or as diagnostic instrument for selecting patients for further, more specific diagnostic procedures.

## Conflict of interest statement

All authors have not conflict of interest to declare.

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