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An electronic nose distinguishes exhaled breath of patients with Malignant Pleural Mesothelioma from controls

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ABSTRACT

Background: Malignant Pleural Mesothelioma (MPM) is a tumour of the surface cells of the pleura that is highly aggressive and mainly caused by asbestos exposure. Electronic noses capture the spectrum of exhaled volatile organic compounds (VOCs) providing a composite biomarker profile (breathprint).

Objective: We tested the hypothesis that an electronic nose can discriminate exhaled air of patients with MPM from subjects with a similar long-term professional exposure to asbestos without MPM and from healthy controls.

Methods: 13 patients with a histology confirmed diagnosis of MPM (age 60.9 ± 12.2 year), 13 subjects with certified, long-term professional asbestos exposure (age 67.2 ± 9.8), and 13 healthy subjects without asbestos exposure (age 52.2 ± 16.2) participated in a cross-sectional study. Exhaled breath was collected by a previously described method and sampled by an electronic nose (Cyranose 320). Breathprints were analyzed by canonical discriminant analysis on principal component reduction. Cross-validated accuracy (CVA) was calculated.

Results: Breathprints from patients with MPM were separated from subjects with asbestos exposure (CVA: 80.8%, sensitivity 92.3%, specificity 85.7%). MPM was also distinguished from healthy controls (CVA: 84.6%). Repeated measurements confirmed these results.

Conclusions: Molecular pattern recognition of exhaled breath can correctly distinguish patients with MPM from subjects with similar occupational asbestos exposure without MPM and from healthy controls. This suggests that breathprints obtained by electronic nose have diagnostic potential for MPM.

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1. Introduction

Malignant Pleural Mesothelioma (MPM) originates from the surface cells of the pleura and represents a highly aggressive tumour [1]. Like all mesothelioma cancers, pleural mesothelioma is mainly caused by asbestos exposure and develops when the toxic asbestos fibers are trapped in the spaces between the mesothelial cells. Although asbestos exposure can be environmental, occupational

asbestos exposure is the main factor involved in MPM pathogenesis [1]. MPM is an important public health issue with increasing incidence worldwide over the next 20 years [2]. Since 1992, high-income countries have banned asbestos usage. However, due to the long latency up to 40 years after asbestos exposure MPM will remain a serious health problem worldwide for many years to come [2].

MPM is hard to diagnose because symptoms occur considerable time after initial asbestos exposure. Moreover, symptoms of pleural mesothelioma are not typical and can be mistaken for less threatening diseases such as pneumonia [3,4]. Therefore, patients are often diagnosed not early enough for curative treatment [3,4]. The diagnosis of MPM very often requires invasive thoracoscopy, and that is why current research attempts are focusing on novel tests for the early detection of MPM [5]. Interestingly, several serum and pleural fluid markers have recently shown to be associated with the presence of MPM. In particular, osteopontin, soluble mesothelin, and

Abbreviations: MPM, Malignant Pleural Mesothelioma; CDA, canonical discriminant analysis; CVA, cross validated accuracy; FEV₁, forced expiratory volume in 1 s; GC-MS, gas chromatography–mass spectrometry; PCA, principal component analysis; ROC-curve, receiver operator curve; VOCs, volatile organic compounds.

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megakaryocyte potentiating factor (MPF) showed a link with the presence of the neoplasm in patients with MPM [1,5].

It would be attractive if MPM could be assessed by non-invasive biomarkers. Metabolomic analysis of exhaled air may be a realistic option for this. Exhaled breath contains a complex mixture of thousands of volatile organic compounds (VOCs) deriving from several metabolic pathways [6,7]. This has been established by gas chromatography and mass spectrometry (GC–MS). Indeed, when using GC–MS, we have recently shown that exhaled air from MPM patients can be discriminated from controls based on pattern recognition of VOCs [8]. Even though cyclohexane was the predominant discriminative compound in MPM, it required multiple VOCs to obtain complete separation MPM patients and controls [8]. Therefore, it appears that exhaled breath fingerprinting has potential in the differential diagnosis of MPM.

The use of portable electronic noses, has made the sampling of exhaled breath and the profiles of VOCs-mixtures readily available [9,10], allowing real-time analysis and discrimination of “breathprints” by composite nano-sensors arrays (“breathomics”) [10]. Such an approach is strictly based on pattern recognition without analyzing the individual molecular components [11], which is potentially suitable for diagnostic objectives [9]. Interestingly, several independent studies have recently shown that an electronic nose can distinguish the VOCs pattern in exhaled breath of lung cancer patients from subjects without it [12–16]. Based on the above we postulated that an electronic nose can discriminate exhaled breath of patients with MPM from healthy controls and from subjects without MPM but with a similar professional asbestos exposure.

Our aim was to test this hypothesis by a cross-sectional study comparing patients with an established diagnosis of MPM with healthy controls and with subjects with a certified long-term professional exposure to asbestos who did not develop the disease. As a secondary aim, we examined whether these classifications can be reproduced by repeated measurements.

2. Materials and methods

2.1. Subjects

The patients in this study have been described previously [8]. In short, 39 adult subjects were divided into 3 groups. *Group 1*: 13 patients with a histology confirmed diagnosis of MPM without current treatment by chemotherapy and/or radiotherapy. The TNM classification by the International Union Against Cancer was used to stage patients [17]. *Group 2*: 13 subjects with long-term certified professional asbestos exposure and with radiological signs of pleural plaques and/or benign asbestos pleural effusion. *Group 3*: 13 subjects each with a negative history of professional asbestos exposure, no history of smoking, and absence of any known diseases. Any subjects with cardiovascular disease, systemic or respiratory infection (<4 weeks), diabetes, any other pulmonary diseases were excluded. The study was approved by the local Ethics Committee and all the subjects gave their written informed consent.

2.2. Design

This was a cross-sectional, case-control study with two visits. Day 1 was used for checking the in- and exclusion criteria. On day 2 (within 10 days) exhaled breath was sampled. Subjects were asked to refrain from eating and drinking in the 3 h before the test. Exhaled breath was collected in duplicate within a 10 min interval and analyzed by the electronic nose.

2.3. Breath collection

Exhaled breath analysis was performed using a previously described method [18]. In short, subjects breathed tidally through a non-rebreathing valve connected to an inspiratory VOC-filter (A2, North Safety, NL) and to a silica-filled drying chamber for 5 min. Subsequently, subjects exhaled a vital capacity into a Tedlar bag, connected to the electronic nose (Smiths Detections, Pasadena, CA, USA).

2.4. Electronic nose

We used a Cyranose 320 (Smiths Detections, Pasadena, CA, USA), a handheld portable chemical vapour analyzer, containing a nanocomposite array with 32 polymer sensors. When exposed to a gas mixture the sensors swell, thereby changing the electrical resistance, resulting in a unique breathprint of differential electrical resistances [10,19].

2.5. Lung function

Spirometry (Masterlab Jaeger, Germany) was performed by a trained lung function technician according to the latest recommendations [20] and the forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) were measured for all the participants to the study.

2.6. Data analysis

Raw sensor data from the electronic nose represent a relative resistance change ($\Delta R/R$) for each of the 32 sensors (Fig. 1) [10,19]. Raw data were analyzed by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Data were reduced to a set of principal components capturing the largest amount of variance of the original 32 sensors [21]. Independent *t*-test was used to select the principal components which were discriminative between groups. Subsequently, these principal components were applied in a linear canonical discriminant analysis (CDA), to create a model that maximizes the distance between sample classes and minimizes the within-sample class distances [22]. The cross validated accuracy percentage (CVA, %) was calculated with the “leave-one-out method”. The CVA provides a percentage that reflects the amount of agreement between the clinical and model-based classification. 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 accompanying 95% confidence limits, providing the sensitivity, specificity, positive- and negative predictive values for the test. The sample size was based on our aim to limit the standard error of the estimated diagnostic measures (sensitivity, specificity) to 10% at most. Assuming 80% accuracy a sample size of 12 patients per group sufficed. For evaluating model robustness a training and test validation using 8 and 5 for bootstrapping was performed. In detail, we calculated the accuracy for the 5 cases classified by an algorithm created on the basis of PCA and CDA of the 8 other cases. 10 different permutations were applied and the average of these 10 values was used to calculate the overall accuracy. The subsequent ROC-analysis was calculated by selecting a cross validated value for each case.

3. Results

The subject characteristics of the three groups are listed in Table 1. Subjects with long-term exposure to asbestos were slightly older than healthy controls ($p < 0.01$), whilst there were no significant differences in age between the three groups. FEV₁ (%pred.) was

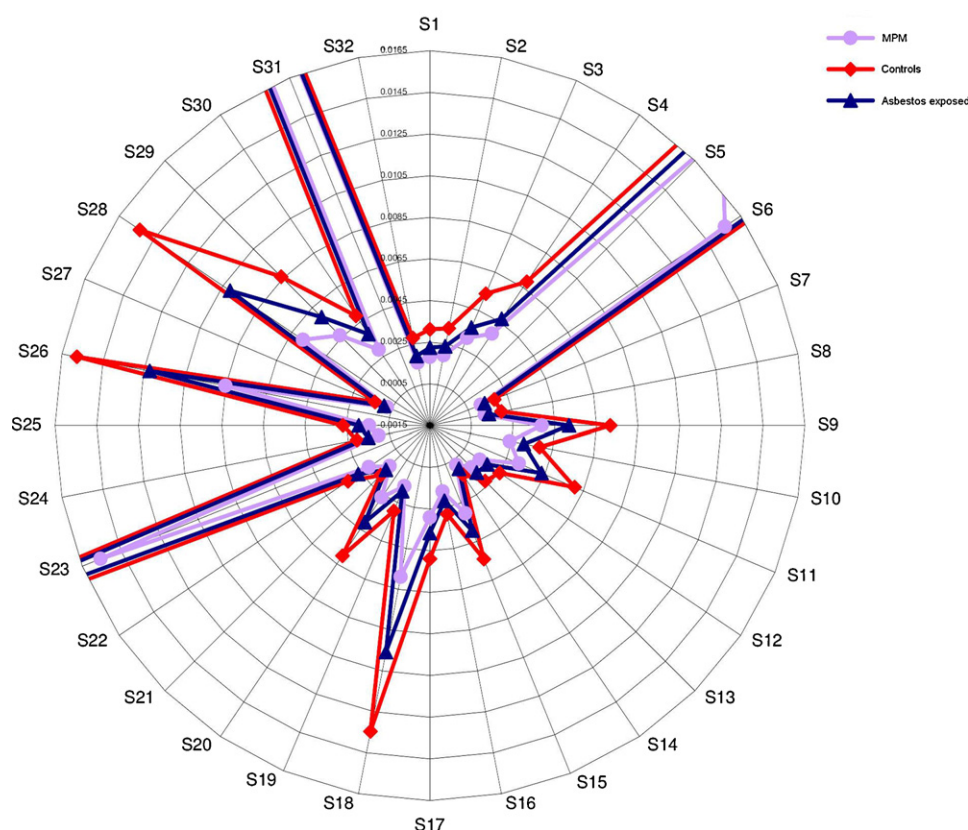


Fig. 1. Example of breathprint in the exhaled breath of a patient with MPM (purple circles), control subject (red diamonds) and asbestos exposed subject (blue triangles). S1–S32: sensor numbers. Y-axis: sensor deflection ($\Delta R/R$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

higher in healthy controls compared to patients with MPM and to those with asbestos exposure ($p < 0.05$). No significant differences in FEV_1 were observed between individuals with MPM and exposed subjects. The clinical characteristics and staging of patients with MPM are shown in Table 2. In 8 out of 13 patients the tumour was located within the pleural surface (TNM stages Ia–Ib), whereas 5 patients had locally advanced disease (stages II–III).

The two-dimensional PCA plot showed that patients with MPM could be distinguished from those subjects with professional exposure to asbestos without MPM (Fig. 2). Subsequent canonical discriminant analysis demonstrated a CVA% of 80.8 ($p < 0.001$). The area under the curve of the ROC-curve for the discrimination between MPM and subjects professionally exposed to asbestos was 0.917 (Fig. 3). Using a cut-off value for the probability of diagnosing MPM of 0.33, this model showed a sensitivity of 92.3% and a specificity of 85.7% for MPM with positive- and negative predictive values of 0.83 and 0.78, respectively (Fig. 3). Analysis of exhaled air from second bag reproduced these results (MPM vs exposed without MPM: CVA% 88.5; cut-off value 0.33; sensitivity 100%;

specificity 84.6%, positive predictive value 0.86, negative predictive value 0.92).

Breathprints of patients with MPM also differed from those of healthy subjects (Fig. 4), with cross-validated accuracy of 84.6% ($p < 0.001$). The area under the curve of the ROC-curve for the discrimination between MPM and healthy controls was 0.893. When using a cut-off value for the probability of diagnosing MPM of 0.31 the electronic nose had 92.3% sensitivity and 69.2% specificity for MPM in this model with positive- and negative predictive values of 0.91 and 0.80, respectively (Fig. 5). Analysis of the second collected bag replicated these results (MPM vs healthy controls: CVA% 88.5; cut-off value 0.31; sensitivity 92.3%; specificity 92.3%, positive predictive value 0.92, negative predictive value 0.86).

When performing a three-way classification of MPM patients, asbestos exposed and healthy controls we obtained a CVA% of 79.5%

Table 1
Clinical characteristics of the study population.

	MPM	Exposed to asbestos	Healthy controls
Subjects (n)	13	13	13
Age (years) [*]	61 ± 12	67 ± 10	52 ± 16
Male (n/total)	11/13	9/13	5/13
FEV_1 (%pred) [§]	81.5 ± 12.1	77.4 ± 8.9	97.2 ± 10.7
Smoking history (n/total)	5/13	4/13	0/13

Patient characteristics [8]. Values are expressed as mean ± SD.

^{*} $p < 0.01$ by analysis of variance.

[§] $p < 0.05$ by analysis of variance.

Table 2
Histology and stage for the group of patients with MPM.

Patient.number	Histology	Stage
1	Epithelial	Ib
2	Epithelial	Ib
3	Epithelial	Ia
4	Epithelial	II
5	Epithelial	III
6	Biphasic	II
7	Epithelial	Ib
8	Epithelial	II
9	Desmoplastic	Ib
10	Desmoplastic	Ib
11	Epithelial	Ib
12	Biphasic	Ib
13	Epithelial	III

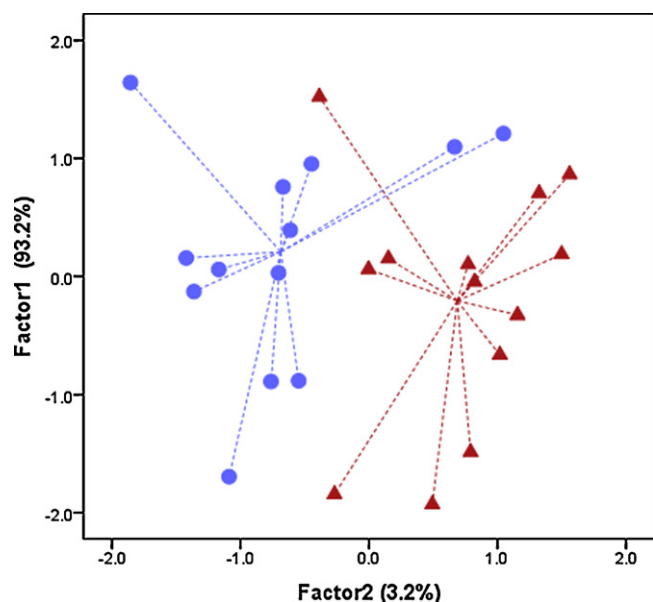


Fig. 2. Two-dimensional principal component analysis with 2 composite factors showing the discrimination of breathprints between patients with MPM (red triangles) and subjects professionally exposed to asbestos (blue circles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

($p = 0.001$) (Fig. 6). The area under the curve of the ROC-curve was 0.885.

Internal validation by bootstrapping using 8 and 5 patients as training and test sets, respectively, reproduced the results above. In particular, the comparison between MPM and asbestos exposed led to a CVA% of 82.9 ($p < 0.05$). The accompanying area under the curve of the ROC-curve was 0.88. The analysis between MPM and healthy controls resulted in a CVA% of 85.0 ($p < 0.05$), the area under the curve of the ROC-curve being 0.83.

4. Discussion

The current study shows that an electronic nose can discriminate the molecular profile in exhaled breath of patients with

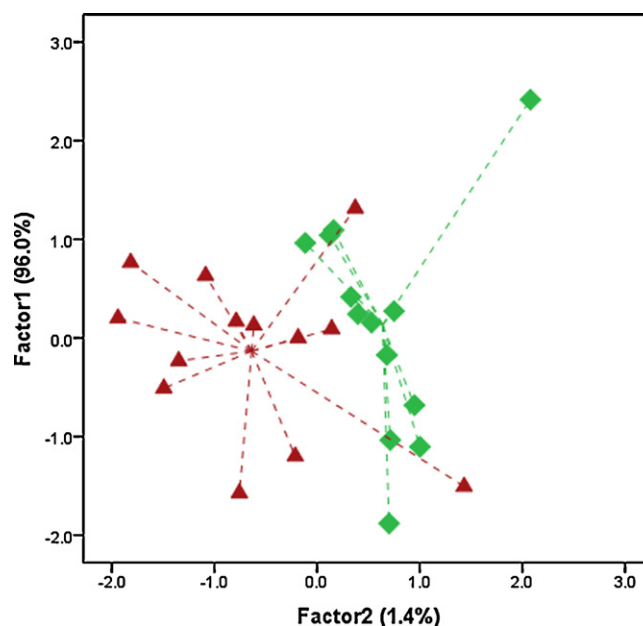


Fig. 4. Two-dimensional principal component analysis with 2 composite factors showing the discrimination of breathprints between patients with MPM (red triangles) and healthy controls (green diamonds). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

MPM from subjects with similar professional asbestos exposure but without MPM. MPM could also be distinguished from healthy controls. These distinctions were confirmed when analyzing exhaled breath from repeated samples. This indicates that exhaled breath of patients with MPM has distinct molecular characteristics from that of subjects without it, and that such molecular patterns can be captured by an electronic nose. These findings are therefore providing the first step towards diagnostic validation of electronic nose in MPM.

To the best of our knowledge this is the first study applying exhaled breath molecular pattern recognition by electronic nose in patients with MPM. Notably, we observed a nearly complete separation of breathprints between the MPM group and the asbestos

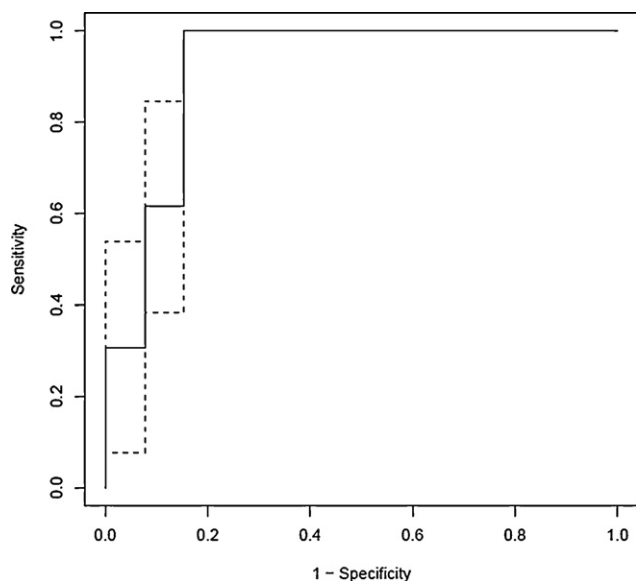


Fig. 3. ROC-curve with 95% confidence interval for diagnosis of MPM compared to subjects professionally exposed to asbestos. AUC was 0.917.

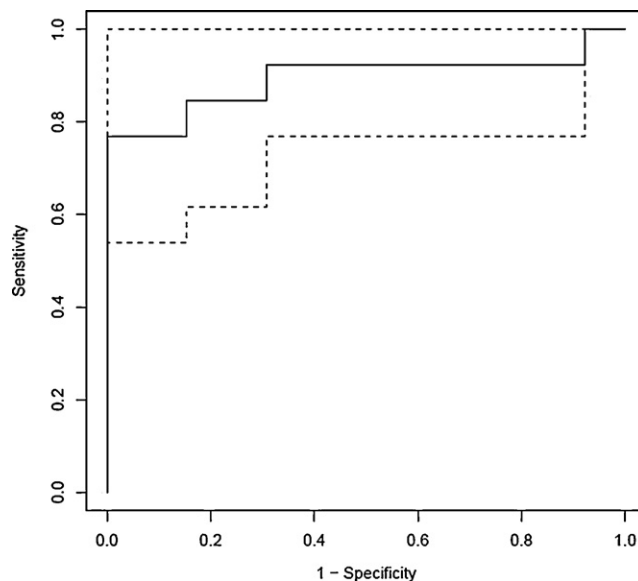


Fig. 5. ROC-curve with 95% confidence interval for diagnosis of MPM compared to healthy controls. AUC was 0.893.

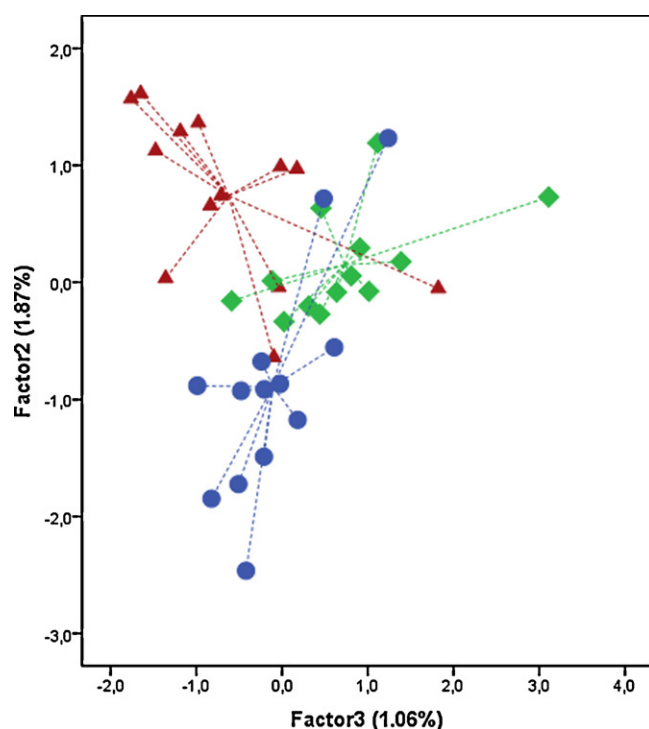


Fig. 6. Two-dimensional principal component analysis with 2 composite factors showing the discrimination of breathprints in the three-way analysis among MPM (red triangles), subjects professionally exposed to asbestos (blue circles) and controls (green diamonds). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

exposed group. Notably, this concerns two groups with similar long-term professional exposure to asbestos. All subjects of the exposed group did have pleural plaques, which is a marker of asbestos exposure [1]. Even though these plaques do not undergo malignant degeneration [1], there is evidence suggesting that individuals exhibiting pleural plaques do have an increased risk for developing MPM [23]. This underlines the need of accurate diagnosis in MPM exposed subjects. Our data suggest that exhaled molecular profiling should be considered as a non-invasive method for this.

To date the application of electronic nose in the respiratory field showed promising results in the detection of lung cancer [12–16,24], asthma [18,25,26], COPD [25], in the *in vitro* diagnosis of mycobacterium tuberculosis infections [27] and in the assessment of ventilator associated pneumonia [28,29]. With regard to lung cancer, we previously showed with the same methodology that an electronic nose can distinguish the VOCs pattern in exhaled breath of well-characterized Non-Small Cell Lung Cancer patients from that of subjects with established COPD as well as healthy controls [15]. This suggests that the use of an electronic nose in the analysis of exhaled breath may qualify as a diagnostic tool for lung cancer in the future [12–16,24]. Our present data are extending these into the difficult assessment of MPM.

We carefully considered methodological aspects such as the selection of groups. All the participants were well-characterized by worldwide accepted guidelines [17] and recruited by the same operator and from the same out-patient clinic. Moreover, the subjects were workers of asbestos-cement factories and asbestos mines located in a narrow geographical area. MPM and exposed group included a number of ex-smokers. Current smokers were excluded from the study because tobacco smoking is known to alter VOCs profile in breath [30]. Nevertheless, despite the fact that our patients were carefully checked to exclude smoking-related diseases, such as COPD, we cannot exclude that ex-smoking may

have influenced our results. Finally, we used a previously validated breathing pattern, inspiratory VOC-filtering, drying of the air and sampling exhaled breath [18]. This is a fundamental aspect and standardization of air collection and sampling should always be attempted.

The sample size of our study was relatively limited. This is essentially due to the fact that MPM is a rare tumour. Nevertheless, we had access to patients in a geographical focus area of MPM, due to long-term professional exposure encountered in a previous big factory. The sample size of 13 subjects per group appeared to be sufficient for obtaining a clear separation among breathprints of MPM, asbestos exposed and healthy controls. Our results were confirmed by duplicate measurements. In view of these positive results, the (adequate) statistical power of our study is not of primary relevance. In contrast, the 95% confidence limits of our findings (Figs. 2 and 4) are of major importance in order to demonstrate the reliability of our findings and to exclude false-positive results [31]. In addition, training and test validation by bootstrapping established the robustness of our results. Regarding the percentage of correct classification for MPM vs healthy controls of 84.6% and for MPM vs exposed without MPM of 80.8% we calculated an accompanying standard error of 10.0% and 10.9%, respectively. Nevertheless, our sample size was not sufficient for discrimination of different stages of MPM (5 out of 13 of patients with MPM had a locally advanced tumour: stages II–III). This is particularly relevant, since diagnosis by eNose may be most applicable in patients with less advanced disease with resectable tumours [32]. This indicates that the next step is to obtain training sets for electronic noses in subgroups of patients with different stages of MPM.

How can we interpret our findings? Approximately 3000 different VOCs have been detected in human breath, and most breath samples contain over 200 VOCs [6,7]. Although the source and the physiological function of most of VOCs are still unknown, these compounds are likely to represent metabolites from systemic as well as local origin [7]. The most abundant compounds in human breath include acetone, methanol, ethanol, propanol and isoprene [30]. Interestingly, several authors have previously shown by GC–MS analysis that levels of pentane, isoprene, acetone and benzene were altered in the exhaled breath of patients with lung cancer as compared to controls [16,24,33–35].

Recently, we showed by explorative GC–MS analysis that levels of cyclohexane, toluene, xylene, benzaldehyde, trimethylbenzene, limonene, 2-ethyl-1-hexanol, and acetophenone, were altered in the exhaled breath of subjects with long-term exposure to asbestos, with and without MPM [8]. Furthermore, cyclohexane, cyclopentane, dodecanoic, xylene, toluene, decane, methylcyclohexane, dimethyl-nonanoic, benzylaldehyde, limonene and b-pinene were most discriminative between MPM and exposed or non-exposed controls [8]. Hence, part of the compounds might be related to mechanical and/or fibrogenic injury to the pleural surface caused by the long-term inhalation of asbestos fibers, leading to chronic inflammation and generic oxidative stress thereby altering the VOCs profile in exhaled breath [36,37]. However, other components may be reflective of the malignancy in MPM patients. In particular, oxydrilated compounds are fitting in with the hypothesis of the presence of a cytochrome p450 polymorphism as proposed by Phillips et al. [34], whereas methylated compounds are generally related to methylation reactions that seem to be involved in the development of neoplasms [38].

Although the goal of using electronic noses in medicine is to obtain empiric diagnostic accuracy by VOCs pattern recognition rather than identifying the individual breath constituents, VOCs identification will be essential for examining specific pathophysiological pathways involved. In addition, GC–MS analysis can also be used to develop future tailor-made electronic noses with more

specific sensors for discriminating VOCs in a given disease [10,16,24]. Hence, the current electronic nose data and those by GC–MS [8] will be complementary in the medical and pathophysiological research of MPM.

What are the clinical implications of our findings? Our data indicate that an electronic nose can discriminate exhaled breath from patients with and without MPM, despite similar long-term occupational exposure to asbestos. This provides a so-called training-set and represents the very first step by showing internal validation. Our results warrant the next step towards external validation of an electronic nose in diagnostic assessment of MPM by firmly following the current guidelines to assess the accuracy of a new diagnostic test [39–41]. Therefore, future studies should include newly recruited and not ‘a priori’ diagnosed patients. If validated using this way, electronic nose can have the potential for a quick and non-invasive diagnostic tool for targeted populations. The electronic nose could either become a diagnostic tool for excluding MPM in individuals professionally exposed to asbestos or a diagnostic tool for selecting patients for additional, more invasive diagnostic procedures.

Conflict of interest statement

None of the authors have any actual or potential conflict of interest.

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