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A breath test for malignant mesothelioma using an electronic nose

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Abstract

Rationale: Malignant mesothelioma (MM) is a rare tumour usually caused by asbestos exposure. MM is difficult to diagnose in its early stages and is invariably fatal. Earlier detection of MM could potentially improve survival. Exhaled breath sampling of volatile organic compounds (VOCs) using a carbon polymer array (CPA) electronic nose recognises specific breath profiles characteristic of different diseases. VOC breath profiling can distinguish between patients with lung cancer and controls, but there is only one prior report in MM, and the potential confounding effect of other asbestos-related diseases was not highlighted.

Hypothesis: A CPA electronic nose will distinguish patients with MM from those with benign asbestos-related diseases (ARDs) and normal subjects with high sensitivity and specificity.

Methods: Eighty patients (MM n=20, ARDs n=18, controls n=42) participated in a cross-sectional, case control study. Breath samples were analysed using the Cyranose 320, using canonical discriminant analysis and principal component reduction. Repeatability was assessed by evaluating the samples in duplicate.

Results: 20 MM, 18 ARDs and 42 control subjects could be distinguished by their breath profiles. 10 MM subjects created the training set. Smellprints from 10 new MM patients were distinguished from control subjects with an accuracy of 95%. Patients with MM, ARDs and control subjects were correctly identified in 88% of cases.

Conclusion: Exhaled breath VOC profiling can accurately distinguish between patients with MM, ARDs and healthy controls. The CPA eNOSE is a novel method for distinguishing patients with MM. This could eventually translate into a screening tool for high risk populations.

Word count: 249

Introduction:

Asbestos was extensively used worldwide over the last two centuries and exposure still continues in many countries. Asbestos exposure can lead to one of the more aggressive of human cancers, malignant mesothelioma (MM), as well as to lung cancer (1). The World Health Organisation estimates that globally 90,000 people die from asbestos related disease each year (2). Asbestos associated mortality and morbidity has been predicted to rise, peaking within the next five years. This is likely to occur even in countries where asbestos usage has been banned because of the long latency period for development of disease(3).

The pathophysiological mechanisms which result in the development of malignant disease are rapidly being elucidated(4) due to developments in understanding of basic mechanisms. Currently however there is no method for predicting which asbestos-exposed individuals will develop malignancy(5).

Worldwide, MM causes 15-20,000 deaths per year (6). The prognosis is very poor, with a median survival of only 7 months(6). Guidelines exist for screening and diagnosis of asbestos-related malignancy, yet in clinical practice diagnostic techniques remain insensitive(7-9). Conventional techniques for distinguishing between benign and malignant asbestos-related disease are inaccurate, invasive, and difficult for elderly patients(10) who frequently have a high level of intercurrent morbidity. Current treatments are largely ineffective in controlling disease. However, recently several new drugs have become available and trials are now underway to evaluate whether treatment of early disease (including combined surgery and radio/chemotherapy) will improve survival. Ideally, early detection would allow combination therapy to control or eradicate this neoplasm. A reliable, cheap and non-invasive tool for early diagnosis and/or for screening in high risk populations is urgently needed.

Because of this, there has been much recent interest in biomarkers for MM, including blood and tissue biomarkers such as soluble mesothelin-related peptide (SMRP), osteopontin and megakaryocyte potentiating factor. However, these are as yet imperfect (11-14). One novel method of biomarker analysis is through exhaled breath profiling. This has the advantage of being totally non-invasive, quick and very easy for the patient. Breath VOC profiling can distinguish lung cancer patients from healthy controls with a high degree of sensitivity and specificity (5). Over 4,000 VOCs have been found in exhaled breath, generated mainly from endogenous biochemical pathways including those of lipid peroxidation(15-21). Techniques

used for VOC analysis range from gas chromatography-mass spectrometry (GC-MS) and ion mobility spectroscopy to colorimetric and gas sensors. The CPA electronic nose relies on an array of complex nanosensors which produce a breath "smellprint," which can be distinguished from other breath patterns using principal component analysis (PCA) (15, 22).

We hypothesised that the CPA electronic nose would detect a breath profile which would accurately distinguish between patients with MM, benign asbestos-related disorders (ARDs) and healthy control subjects. If this were the case, this could represent a first step towards early detection of MM in asbestos-exposed subjects.

Methods:

Study Design

This study was approved by the Human Research Ethics Committee of St Vincent's and Prince of Wales Hospitals and subjects gave fully informed written informed consent prior to testing.

The study was of cross-sectional, case-control design in two phases: a training phase and a blinded identification phase. Subjects were assessed once with a standardised questionnaire, spirometry and exhaled breath sampling. The questionnaire included: demographics, occupational history, medical and medication history, smoking history, recent illnesses, and factors known to affect exhaled breath samples in other contexts, e.g. time since ast meal, recent alcoholic beverages, smoking and use of mouthwash. Subjects were asked to not eat or drink (excluding water) in the 90 minutes before testing. To assess reproducibility, five subjects were reassessed 3 times within 6 months.

Study Subjects

Subjects were recruited from St Vincent's and Prince of Wales Hospitals' outpatient clinics and controls from community volunteers. Patients with MM and benign ARDs were diagnosed according to current American Thoracic Society/ European Respiratory Society (ATS/ERS) recommendations (7, 23). MM patients were selected on the basis of immunohistological diagnosis (7) using tissue removed at VATS thoracoscopic biopsy. Patients were not included in the study if there was suspicious cytology and/or clinical and radiological findings without confirmatory histology. Patients with the non-malignant asbestos-related disorders were diagnosed using the following criteria (7, 23): 1. An occupational history of exposure to asbestos with an appropriate latency period for development of the relevant disease; 2. Compatible clinical findings e.g. the presence of fine end-inspiratory crackles at the lung bases in the case of asbestosis, diminished overall lung expansion in the case of DPT, lack of crackles and a normal chest examination in the case of pleural plaques; 3. The present of compatible radiological features (pleural plaques, diffuse pleural thickening, lower zone interstitial pulmonary fibrosis); and 4. Compatible features on full lung function testing including lung volumes and diffusion factor for carbon monoxide (DLCO).Controls were matched for age, gender and smoking status. Subjects were divided into four groups: 1. MM; 2. Asbestosis; 3. Pleural disease which includes both pleural

plaques (PPs) and diffuse pleural thickening (DPT);4. Control subjects. Non-smokers were defined as having never smoked cigarettes, and ex-smokers as not having smoked within the last year.

The control group consisted of subjects who reported no asbestos exposure or any lung disease, no current relevant respiratory symptoms and normal spirometry (a pre-bronchodilator forced expiratory volume in one second (FEV_1)>80% predicted and FEV_1/forced vital capacity (FVC)>70%). Subjects were selected for smoking status to match smoking history of the 2 other groups. Exclusion criteria for all groups included a history of recent respiratory tract infection, an acute exacerbation of any underlying respiratory disease in the past 4 weeks and/ or current uncontrolled other medical condition.

Lung Function

All control and ARD subjects underwent spirometry (Minato, Autospiro AS-500, Medical Science Company Ltd., Osaka, Japan) according to ATS/ERS guidelines on the same day as breath collection (24). Most MM subjects experienced significant dyspnoea, and were unable to perform repeatable spirometry. Forced vital capacity (FVC (L)), forced expiratory volume in 1 second (FEV_1 (L)) and vital capacity (VC (L)) were measured and the highest value of three manoeuvres was expressed as percentage of the predicted values (%pred), calculated using the European Coal & Steel regression equations(25).

CPA Electronic Nose (CPA eNOSE)

A handheld, portable chemical vapour analyser, the Cyranose 320 (Smiths Detection, Pasadena, CA) was used to evaluate exhaled breath samples. This consists of a composite array of 32 organic carbon polymer sensors that respond to gaseous molecules such as VOCs via a change in the electrical resistance of the sensors. The variation of change in resistance of each sensor in response to different breath samples is saved in the onboard database. These can then be compared, via pattern recognition algorithms, to distinguish different smellprint patterns.

Exhaled breath collection and sampling

This involved a training phase and a blinded identification phase conducted on different subjects. In both phases, samples were randomly introduced to the CPA electronic nose system, to prevent bias in smellprint generation. In the training phase, breath was sampled

from 10 subjects with each condition and 10 controls. In the subsequent validation phase, the exhaled breath of test subjects and controls were randomly introduced into the CPA eNOSE. After 30 seconds of sampling, the CPA eNOSE categorised them as either diseased or healthy according to the VOC smellprint. To determine baseline drift and variation over time, an assessment of reproducibility was performed on known samples to ensure the results of the training set would remain reproducible.

Breath collection was based on previously validated methods(15). All subjects sat at rest for 20 minutes before sample collection. Subjects first rinsed their mouth with distilled water before breathing tidally through a mouthpiece connected to a 1-way non-rebreathing valve (Vitalograph, Buckingham, England). Neither a nose clip nor VOC filter were used. After 5 minutes of tidal breathing the expiratory port was connected to a 2 litre gas impermeable bag (Rapak, Mulgrave, Victoria, Australia). The subject then inhaled to inspiratory capacity and immediately exhaled from full vital capacity into the bag.This was connected to the CPA electronic nose within 5 minutes and the sample was drawn across the sensors at a flow rate of 120mL/min, for 30 seconds.

Data Analysis

Smellprints were analysed using the Cyranose 320 on-board learning software. Savitzky–Golay filtering (performs a local polynomial regression (of degree k) on a series of values) was utilised to process the sensor response data and baseline corrections were applied to improve signal-to-noise ratio(26). To reduce the data from 32 individual sensors to a set of principal components, principal component analysis (PCA) was used(27). This determined factors that captured the largest variance in the data. PCA factors were then used to perform a linear canonical discrimination analysis for the construction of a pattern recognition algorithm. This was achieved by enhancing the ratio of pooled within-class scatter to between-group distance. A cross validation value (CVV%) was calculated, which gave an estimate of error, or in other words, the accuracy in distinction between the smellprints from different subject groups. The Mahalanobis distance (M-distance) between group means, in units of standard deviation, was then calculated (27). This was used to quantify the discrimination between sample groups, providing a measure of dissimilarity between two samples. Thus, M-distance and the ability to discriminate are directly related, so that values >3 are indicative of a high probability of discrimination ($p<0.01$).

Results

Subject characteristics and lung function

Eighty subjects were studied. Demographic data are shown in Table 1. All healthy controls had normal spirometry. FEV_1, FVC and FEV_1/FVC ratio were significantly reduced in subjects with benign ARD compared with healthy control subjects ($p<0.05$). The most significant difference was FEV_1 and FVC between controls and patients with asbestosis (100.1%±11.1 vs 72.2± 9.4 and 94.4%±9.4 vs78.9± 10.4 respectively, $p<0.001$). Subjects with MM were unwell and unable to perform spirometry.

The MM group consisted of 20 subjects with histologically diagnosed MM. Nineteen subjects were IMIG stage II, in which the mesothelioma has spread to both layers of the pleura on one side of the body, and has enlarged to form a tumour mass on the pleural tissue around the lungs, or has started to spread into the diaphragm muscle or the lung tissue. One patient was stage 1b. All were non-smokers (n=8) or ex-smokers (n=12) with previous asbestos exposure. The benign ARD group (pleural disease and asbestosis) comprised 18 subjects, all of whom were non-smokers (n=6) or ex-smokers (n=12). The healthy control group comprised 42 subjects, who were non-smokers (n=30) or ex-smokers (n=12).

CPA Electronic Nose

Each of the 32 carbon polymer sensors in the CPA electronic nose were responsive to exhaled breath, with a recordable change in sensor resistance elicited by VOC mixtures in breath. The exhaled breath sample of each individual resulted in a unique pattern of sensor responses, which characterised their ‘smellprint’ (Fig. 1).

Mesothelioma and healthy controls

In the training phase, 10 subjects with MM and 10 controls were introduced to the CPA eNOSE to create two breath recognition classes. Control subjects were recruited at the same time. Subjects were seen in random sequence and timeframe, depending upon their availability to come to the hospital for testing. Smellprints from MM patients clustered distinctly from control subjects when analysed by PCA (Fig. 2). Subsequent canonical discriminant analysis showed a cross validated accuracy value of 95% (Fig 3). Canonical

discriminant analysis on the data set showed an M-distance of 4.59 between the two groups, suggestive of a high probability of discrimination ($p<0.01$).

Validation on the training set was performed. The capacity of the CPAe NOSE to correctly distinguish subjects' smellprints was performed on 10 patients with MM and 32 control subjects. Exhaled breath samples were plotted against MM and control breath recognition classes; an example plotted 'X' in Figure 4.

Identification results were correct in 38 of 42 subjects, with 9 of 10 MM and 29 of 32 controls being correctly identified (sensitivity 90%, specificity 91%). The incorrect samples were measured again within 2 hrs and were correctly identified on the second analysis.

Mesothelioma and other asbestos-related disorders

Subjects with benign ARD (pleural disease and asbestosis; n=18) were used in a second validation phase of the MM versus healthy set. Exhaled breath samples were correctly identified as healthy in 15 of 18 subjects (specificity 83.3%). Of patients with pleural disease alone and no asbestosis, 12 of 13 patients were correctly identified as healthy (92% specificity). Three of five patients with asbestosis smellprints were correctly identified (60% specificity).

For smellprint identification between MM, benign ARD and control subjects the CPA eNOSE had a sensitivity of 90% and specificity of 88%. The positive predictive value and negative predictive values were 60%and 97.8% respectively when compared with the gold standard of histologically proven MM.

Reproducibility and intra-subject variability

To assess reproducibility of results, 5 subjects' breaths were measured on three occasions. Two MM patients and 3 control subjects had their exhaled breath profile validated against the MM training set on 3 occasions over a period of 6 weeks. Breath sample validation was performed on healthy subjects at a 2, 4 and 6 week time points and the MM subjects at a 1, 4 and 6 week intervals from the initial breath sample collection date. These time frames were

due to the availability of the subjects to come in for testing. This supported the hypothesis that results of the model were reproducible, with subjects being assigned to the correct class in 13 of 15 trials (86%). CPA eNOSE breathprints have been previously shown to be repeatable in other studies with a kappa ranging between 0.75 and 0.91 (28, 32).

Discussion

Our study has demonstrated that CPA electronic nose breath profiling allows accurate discrimination between patients with MM, healthy control subjects and subjects with benign asbestos-related diseases. This is the second published report of use of electronic nose breath profiling in MM and substantiates the promise of this technique as a simple, easy way for detecting malignancy which has previously been reported for lung cancer(15, 17-19). A study by Dragonieri et al, published very recently and while our paper was under review, also reports high levels of discrimination between patients with MM and those with long-time asbestos exposure using an electronic nose system (28), with results almost identical to those of our study. This work was performed contemporaneously with ours, substantiating the validity of both groups' results. In our study, we carefully took into account the type of asbestos-related disease and a wider range of asbestos disorders, because this is known to affect exhaled breath biomarkers (4,5), and this is an important potential confounding factor for MM detection. This work exemplifies the novel use of this technology for differentiating individuals with asbestos exposure and benign conditions from those with malignant disease.

Asbestos inhalation is an important cause of disease, yet is associated with the development of malignancy in only a small percentage of occupationally exposed workers(9). However, these patients subsequently suffer very high morbidity and mortality. Subjects with asbestos-related lung cancer are potentially curable, with reported 5 year survival rates for Stage 1A lung cancer now achieving 80%, compared with < 15% for Stages II-IV(29, 30). The same is not true however for MM, where survival rates have not changed significantly over the last 20 years(1, 3). However, new drugs and combined multimodality treatment offer hope for cure in selected cases. MM is usually diagnosed at a late stage and it is theoretically possible that earlier disease might be more responsive to treatment.

Our study is an early pilot approach, but has demonstrated that MM can be distinguished from normal controls and subjects with ARDs with promising accuracy. Asbestos-exposed subjects have a high rate of benign pleural disorders, especially pleural plaques. Also, they may have interstitial pulmonary fibrosis (asbestosis), although this is becoming increasingly rare. Thus, it is important that these co-existing conditions are taken into account in order to ensure that they are not a confounder for the diagnosis of malignancy. In our study, the CPA eNOSE distinguished accurately between benign and malignant asbestos-related disease, implying that the breath profile of VOCs in MM is very different. Although it might have

been expected that MM, a pleural disease rather than an endobronchial disease, would not produce such a change in breath profile, our results are similar to one previous unpublished abstract report (31) and also to the reports of Phillips et al, whose work on lung cancer suggests that the change in VOC profile is related to a systemic alteration in metabolism which occurs with malignancy(20, 32-34) rather than due to a localised event.

Our subjects were matched for smoking status, and were never or ex-smokers, so the potentially confounding effect of smoking was not formally assessed. However, MM is not related to previous smoking habit, therefore smoking as a confounder is not as relevant as it would be for lung cancer diagnosis. The CPA eNOSE is capable of picking up differences due to smoking status (22), but because training is specific for the VOC pattern profile relevant for a particular disease, irrelevant compounds are ignored. This implies that the effects of smoking are unlikely to have a significant confounding effect, as has been found in other studies in lung cancer and also in obstructive lung disease(15, 35, 36).

CPA electronic noses do not quantify which specific VOCs are responsible for any observed difference in exhaled breath patterns. Each of the 32 polymer sensors responds to a different fraction of the VOC mixture, based on features such as molecular mass, dipole moment and hydrogen binding capacity (36). Significant differences in resistance are then selected by PCA. Hence, smellprint pattern recognition by CPA electronic noses is purely based on a statistical approach, providing empiric evidence (37). However, studies are underway to identify individual breath components and it is likely that these will be characterised in the near future. For example, cyclopentane and cyclohexane are likely to prove important molecules which distinguish between MM patients and subjects with asbestos exposure (38).

CPA electronic noses are a novel technology in medical diagnostics, and technical developments in this area are likely to increase understanding and improve methodology in the future. Because the Cyranose 320 was not developed for medical diagnostics, it has inherent limitations. It was originally designed for industrial use, assessing the identity of gaseous exposure in chemical spills, and only allows 10 samples (10 subjects) in the training set for each class. The number of MM cases we studied was small, but we were limited by the fact that the Cyranose 320 limits test validation to 10 subjects at a time. Ideally, larger numbers would be included in both training and validation sets to avoid potential type 1 error.

VOCs are produced both from endogenous and exogenous processes, so there is also a possibility of background environmental contamination. We did not use a VOC filter, but this

is likely to improve accuracy (15, 37, 39). However, our patients were all studied in an identical air conditioned environment typical of usual clinical practice, and there are few published data regarding optimal filter type or methods in this area. Our diagnostic accuracy was still high in these circumstances, while background contamination should in theory have resulted in dilution of the signal.

In our study, all subjects with MM had advanced disease, diagnosed on surgical biopsy. Thus, our study results might not be so accurate with early stages of MM. However, currently few cases of MM are detected at an early stage and histological confirmation was important as a gold standard for our study. Ideally, a prospective study design would be employed in screening an asbestos-exposed cohort, similar to work which has been performed with other biomarkers(40). When initial case-controlled studies are translated into prospective cohort screening studies, diagnostic accuracy usually falls. However, technological developments in this area seem likely to improve both accuracy and ease of testing in this area of research. Although our data are early and require further evaluation in larger studies, our work demonstrates the potential for a convenient, hand-held non-invasive device such as a CPA electronic nose for early diagnosis of MM.

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Table 1. Subject demographic and lung function data

	Controls	Mesothelioma	Asbestosis	Pleural disease
Subjects (n)	42	20	5	13
Age (mean & SD, years)	66.5 ± 14	69 ± 10	70 ± 10.5	70.9 ± 8.2
Sex (male/ female)	34 men 8 women	18 men 2 women	5 men	13 men
Smokers (non, ex, smoker)	30 non 12 ex	8 non 12 ex	1 non 4 ex	5 non 8 ex
FEV_1 (%predicted)	100.1 ± 11.1	ND	72.2± 9.4 ***	90.2± 17.5 *
FVC (%predicted)	94.4 ± 9.4	ND	78.9± 10.4 ***	82.7± 18.6 *
FEV_1/FVC (%predicted)	93.4 ± 14.3	ND	76.2± 7.8 *	80. 1± 12.7 *
IMIG Stage	NA	19subjects stage 2; 1 stage 1b	NA	NA

SD; standard deviation. ND; not determined. NA; not applicable. n; number of subjects. Data presented as means ± standard deviations. Significant differences between subjects with asbestosis orpleural plaques compared with normal controls (***p<0.001, *p<0.05). Smoking status defined in manuscript. FEV_1; forced expiratory volume in 1 second, FVC; full vital capacity. IMIG; International Mesothelioma Interest Group Mesothelioma Staging System; NA = not applicable.

Figure 1.Pattern of relative differential electrical resistance (ΔR/R) of the 32 polymer sensors of the CPA electronic nose.

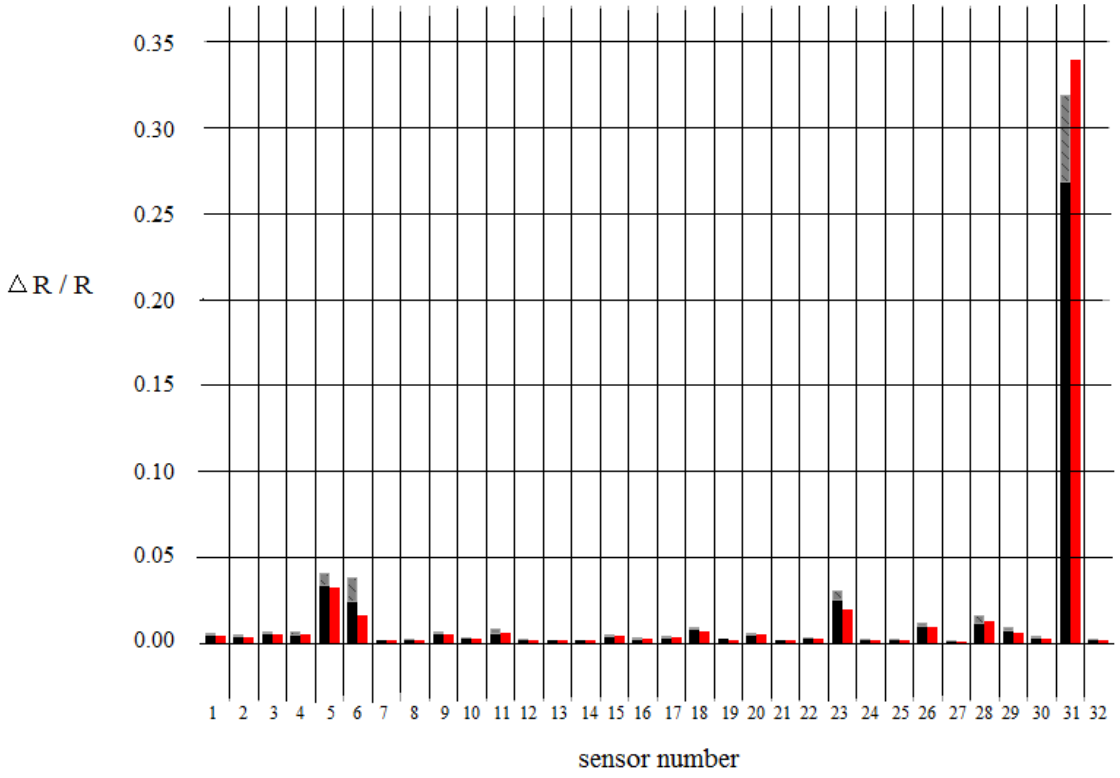

Figure 2.Three-dimensional PCA plot classifying subjects with malignant mesothelioma and healthy controls.

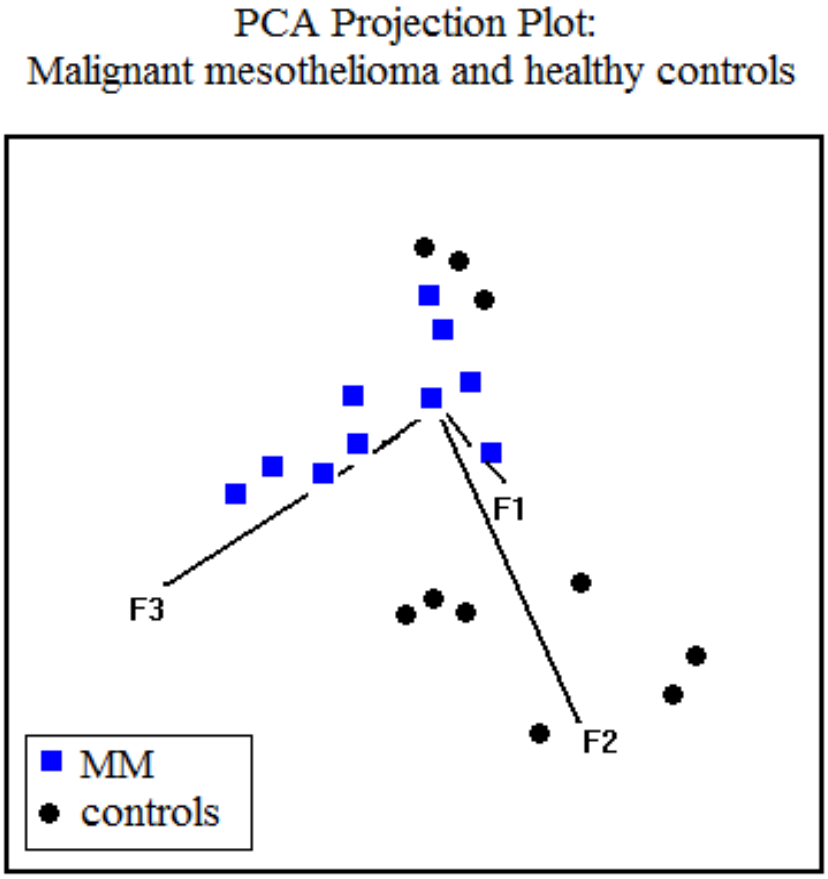

Figure 3. One-dimensional canonical discriminant analyses plot graphing the smellprint of subjects with mesothelioma and healthy controls.

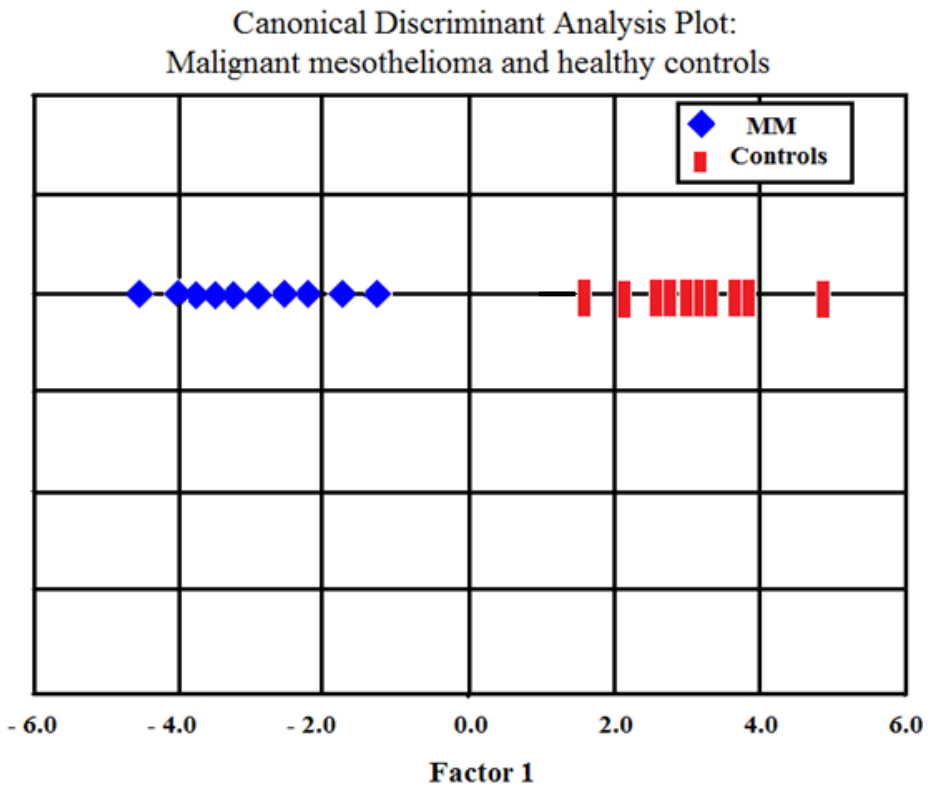

Figure 4. One-dimensional canonical discriminant analysis plot showing the correct identification of a randomly introduced malignant mesothelioma subject.

Canonical Discriminant Analysis Plot:
Identification of a mesothelioma subject

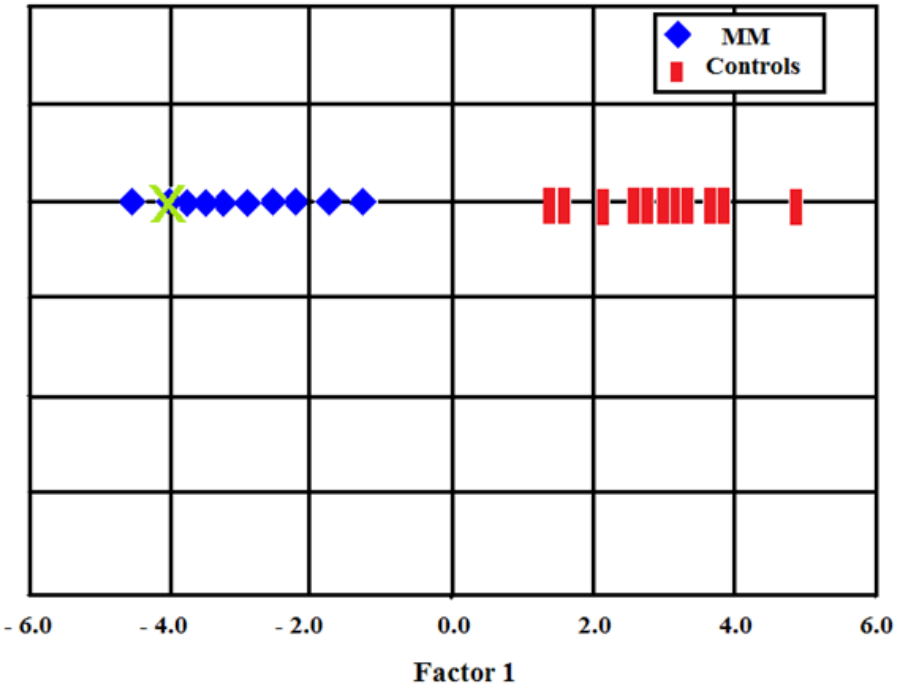

Figure legends

Figure 1. Pattern of relative differential electrical resistance (ΔR/R) of the 32 polymer sensors of the CPA electronic nose.This illustrates a smellprint created by the volatile organic compound mixture in exhaled breath of a single volunteer with mesothelioma (MM). ΔR/R represents the change in resistance of an individual sensor. The vertical axis represents sensor response or ΔR/R, where ΔR is the difference between sensor resistance recorded during 'Sample Draw' and 'Baseline Purge' stages of the sampling cycle and R is the resistance recorded at the end of the 'Baseline Purge' stage (see Fig. 2). Sensor numbers are plotted along the horizontal axis.. The view allows comparison of a new smellprint (red) with a smellprints previously stored during training (black and grey). The grey area is maximum exposure, and black area is minimum exposure of an individual sensor in a training set.

Figure 2. Three-dimensional PCA plot classifying subjects with malignant mesothelioma and healthy controls. Three principal component composite factors (Factors F1, F2 and F3) were discerned from the reduced data of the 32 sensors, maximising the discrimination of smellprints between patients with MM (n=10; blue squares) and control subjects (n=10; black circles). The reduction to 3 principal components shows marked clustering of MM smellprints.

Figure 3. One-dimensional canonical discriminant analyses plot graphing the smellprint of subjects with mesothelioma and healthy controls. This illustrates discrimination of subjects with MM (n=10; blue, diamonds) from healthy control subjects (n=10; red, oblongs) along an arbitrary composite factor (factor 1). Factor 1 represents a single principal component, formed from the reduced data of the 32 sensors, on which canonical discriminant analysis was carried out.

Figure 4. One-dimensional canonical discriminant analysis plot showing the correct identification of a randomly introduced malignant mesothelioma subject. This illustrates discrimination of subjects with MM (n=10; blue diamonds) from healthy control subjects (n=10; red oblongs) along an arbitrary composite factor (Factor 1). The smellprint of a single MM subject, marked as an X (green), validates the capacity of anCPA electronic nose to classify newly introduced subjects into the correct subject group, bases on breath analysis alone. Factor 1 represents a single principal component on which canonical discriminant analysis was carried out.

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