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Non-Invasive Breath Analysis of Pulmonary Nodules

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Abstract

INTRODUCTION—The search for non-invasive diagnostic methods of lung cancer has led to new avenues of research, including the exploration of the exhaled breath. Previous studies have shown that lung cancer can in principle be detected through exhaled breath analysis. This study evaluated the potential of exhaled breath analysis for the distinction of benign and malignant pulmonary nodules (PNs).

METHODS—Breath samples were taken from 72 patients with PNs in a prospective trial. Profiles of volatile organic compounds (VOCs) were determined by (i) gas chromatography/mass spectrometry (GC-MS) combined with solid phase microextraction (SPME) and by (ii) a chemical nanoarray.

RESULTS—53 PNs were malignant and 19 were benign with similar smoking histories and co-morbidities. Nodule size (mean +/− SD) was 2.7±1.7 vs. 1.6±1.3 cm (p=0.004) respectively. Within the malignant group, 47 were NSCLC and 6 were SCLC. Thirty had early stage disease and 23 had advanced disease. GC-MS analysis identified a significantly higher concentration of 1-octene in the breath of lung cancer, and the nanoarray distinguished significantly between benign vs. malignant PNs (p<0.0001; accuracy 88±3%), between adeno- and squamous- cell carcinomas (p<0.0001; 88±3%) and between early stage and advanced disease (p<0.0001; 88±2%).

CONCLUSIONS—In this pilot study, breath analysis discriminated benign from malignant PNs in a high-risk cohort based on lung cancer related VOC profiles. Further, it discriminated adeno-

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Authors' contributions: All designed the research. N.P., T.C.K., J. M., J. D. M., Y.M., P.A.B.Jr., and F.R.H. collected the breath samples and investigated the effect of clinical characteristics of healthy volunteers. N.P. and M.H. performed the research; N.P., M.H. and H.H. analyzed the data; N.P. and H.H. wrote the paper; all revised the paper.

and squamous- cell carcinoma and between early vs. advanced disease. Further studies are required to validate this non-invasive approach, using a larger cohort of patients with PNs detected by CT.

Keywords

Lung cancer; pulmonary nodules; diagnosis; breath; nanoarray

INTRODUCTION

Lung cancer (LC) is the leading cause of cancer mortality, accounting for 28% of the cancer related deaths.[1, 2] The National Lung Screening Trial (NLST) demonstrated that LC screening by low dose CT (LDCT) scans reduced the lung cancer mortality rate by 20%.[3] LDCT screening programs for lung cancer are expected to be launched in many countries in the near future, so that a dramatic increase in the detection of pulmonary nodules (PNs) should be anticipated, and accordingly, a dramatic increase in invasive procedures, in the related morbidity, and the health care costs. To minimize the false positive rate in future LDCT screening programs, a complementary biomarker assay technique is needed that distinguishes benign from malignant nodules in a non-invasive and cost-effective manner.

Exhaled volatile organic compounds (VOC) are promising candidates as LC biomarkers.[4–13] These VOCs are emitted from the membrane of the cancer cells and/or from the surrounding microenvironment to the blood stream.[6, 7, 14, 15] The cancer-related changes in the blood chemistry are then reflected in measurable changes to the breath through excretion via the lungs. Previous studies with either mass spectrometry techniques[11–13, 16] or with arrays of chemical sensors[4, 5, 10, 17–20] have shown that the breath VOC profile of patients with LC differs on average from the profile of healthy subjects. However, the relationship between breath VOCs and PN features has not been studied sufficiently. Here, we perform an **explorative study** to compare benign to malignant PNs, combining complementary information from gas chromatography combined with mass spectrometry (GC-MS) and from chemical nanoarrays. GC-MS allowed the identification and quantification of a wide variety of separate breath VOCs, while the cross-reactive chemical nanoarray provided a simple-to-use and inexpensive diagnostic tool without insight into the nature and composition of the breath VOCs.

METHODS

Test Population, Study Design and Collection of Breath Samples

The study was a prospective trial including 74 patients attending subspecialty referral clinics at the University of Colorado Cancer Center (Aurora, CO, USA) and Denver Veterans Affairs Medical Center (Denver, CO, USA) from March 2009 to May 2010. The Colorado IRB granted approval for the study, and the clinical trial was registered at Clinicaltrials.gov (registration no.: NCT01386203). All patients were under investigation for PNs and were recruited after written informed consent. The clinical details of the study population are given in Table 1.

Alveolar exhaled breath was collected in chemically inert Mylar bags (Eco Medics) in a controlled way, following a 3 minute procedure of lung washout as described elsewhere[9, 10, 17] and in the Online Data Supplement, Section 1.1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A320). The procedure was designed to avoid ambient contaminants and nasal entrainment of gas from entering the sampling bags. Each subject provided at least one Mylar bag of 750 ml. The content of each bag was transferred immediately through an off-line procedure to a tenax sorbent tube (SKC Inc; USA). All

breath samples were tagged with a barcode at the collection site. The tubes were stored at 4°C in a clean environment. The sorbent tubes were then shipped to the labs in the Technion-IIT (Haifa, Israel) for analysis by both GC-MS and chemical nanoarray (see below).

Unrelated to the breath sampling, all patients underwent clinical investigation including bronchoscopy, wedge resection and/or lobectomy as was required for final diagnosis (*see* Table 1). Patients without a definitive histologic diagnosis were followed by serial CT imaging, and final diagnosis was reconfirmed in August 2011. Nodules which either regressed or remained stable over a 24 month period were considered benign.

Breath Samples Analysis and Statistical Treatment

In the first phase of this study, the constituent VOCs in the breath samples were analyzed using GC-MS as described in the Online Data Supplement, Section 1.2 (Supplemental Digital Content 1, http://links.lww.com/JTO/A320). GC-MS chromatograms were analyzed using GC-MS Postrun Analysis (Shimadzu Corporations) version 2.53. The data was processed using the open source XCMS (version 1.22.1) package (http://metlin.scripps.edu/download/) which provides mass/charge (m/z) and retention time. Statistical tests were performed by SAS JMP, Version 8.0 (SAS Institute Inc., Cary, NC, USA, 1989–2005) for Wilcoxon/Kruskal-Wallis tests.

In the second phase of this study, the breath samples underwent a blind test-run with a chemical nanoarray of eighteen cross-reactive sensors, as described in the Online Data Supplement, Section 1.3 (Supplemental Digital Content 1, http://links.lww.com/JTO/A320). Two of the sensors were chemiresistors based on random networks of single-wall carbon nanotubes with polycyclic aromatic hydrocarbons (PAH/SWCNT).[21–24] The PAH molecules contained a hydrophobic mesogen and were terminated with (i) an ether group and with (ii) an 2-ethyl-hexane hydrophobic group.[21–24] Sixteen sensors were chemiresistors based on spherical gold nanoparticles (GNPs; 3–4 nm core diameter) coated with hexanethiol, 2-ethylhexanethiol, 3-methyl-1-butanethiol, octadecylamine, decanethiol, dodecanethiol, 2-mercaptobenzoazole, 4-methoxy-toluenethiol, *tert*-dodecanethiol, 2-amino-4-chlorobenzenethiol, 2-mercaptobenzimidazole, benzylmercaptan, 2-nitro-4-trifluoro-methylbenzenethiol, 2-naphthalenethiol, 2-nitro-4-trifluoro-methylbenzenethiol and 2-mercaptobenzoazole. The wide variety of these cross-reactive organic ligands assured responses from a wide spectrum of VOCs.[4, 5, 8–10, 25] The GNPs were synthesized as described in ref. [26].

Wilcoxon test was applied to identify the features that show statistically significant differences of the average sensing signals for the different study groups. Data classification was attempted by employing Discriminant Factor Analysis (DFA).[27] The accuracy of DFA was further confirmed by employing leave-one-out cross-validation. Statistically significant differences between the first-discriminant scores were studied using the Wilcoxon test. Sensitivity, specificity and accuracy were calculated through leave-one-out cross validation. Statistical tests were performed by SAS JMP, Version 8.0.

RESULTS

Seventy-two patients with pulmonary nodules were examined by GC/MS and/or the chemical nanoarray. Baseline epidemiology, smoking history and co-morbidities were similar in both groups. The two sub-populations were relatively well-matched with respect to their ages: 64.9±7.2 years for the malignant nodules vs. 60.8±6.8 years for the benign nodules ($p = 0.038$). Thirty-six patients underwent bronchoscopy and 36 underwent a surgical procedure. Nineteen had benign findings and 53 had cancer. Forty-seven patients

had non-small cell lung cancer (NSCLC) (30 adenocarcinomas, 13 squamous cell carcinoma, 2 large cell carcinoma and 2 poorly differentiated carcinoma) and 6 patients had small cell lung cancer (SCLC). Thirty patients had early stage disease (Stage I–II or limited SCLC) and 23 had advanced disease (Stage III/IV or extensive SCLC; *see* Table 1). Among the benign group (N=19), histological examination identified 13 patients with granulomas or fibrotic tissue, one with alveolar hyperplasia, and 5 with infectious/inflammatory histologies. Nodule size was significantly higher in the malignant group (mean diameter+/−SD = 2.7±1.7 for LC vs. 1.56±1.3 cm for benign, p = 0.004). Due to the relatively small number of samples, the results of this study could be validated only using leave-one-out cross validation. Blinded external validation in an independent validation set is the next step to study this approach in the future.

Chemical Analysis of Breath Samples

In the first phase of this study, we identified the VOCs that could serve as biomarkers for PNs and determined their relative compositions, using GC-MS in conjugation with solid phase microextraction (SPME). Thirty-nine samples were analyzed, out of which 28 were malignant (25 NSCLC and 3 SCLC) and 10 were benign. Twenty three were advanced disease and 30 were early. Tumor size was 2.7±1.7cm for the malignant vs. 1.56±1.3 for the benign (p=0.004). GC-MS analysis identified approximately 200 VOCs with main masses in the range of 33 to 282 m/z and retention times between 1.8 and 42.8 minutes. Non-parametric Wilcoxon/Kruskal-Wallis tests identified one VOC that appeared in higher concentrations in LC patients than in patients with benign nodules (*see* Figure 1 and Table 2). This VOC was 1-octene (CAS number: 111-66-0; p = 0.049) – see footnote [28] and refs. [11, 12, 29]. Comparative GC-MS analysis failed to show any discrimination between early stage and advanced disease of the NSCLC patients and also for sub-histologies of NSCLC. Comparative GC-MS analysis was not performed for the NSCLC and SCLC groups due to the insufficient number of the GC-MS samples of the SCLC group.

Nanoarray Analysis of Breath Samples

In the second phase of the study, 69 breath samples were exposed to the chemical nanoarray. Of the 69 samples, 50 were malignant (45 NSCLC and 5 SCLC) and 19 were benign. One of the five SCLC samples was excluded from the analysis with the chemical nanoarray, due to technical failures of the electrical readout of the sensing signals during the analysis. All sensors responded rapidly and reversibly and provided a consistent output specific to a given breath exposure.

The feasibility of the tailor-made nanoarrays to distinguish between the collective VOC patterns of malignant and benign lung nodules was demonstrated by building a Discriminant Factor Analysis (DFA) model[27] based on the 68 breath samples collected for the chemical nanoarray. The chemical nanoarray analysis discriminated well between the patients with malignant nodules and the patients with benign nodules (*see* Figure 2A). Full separation was achieved along the first canonical score ($p < 0.0001$). Leave-one-out cross-validation was performed in order to check the accuracy of the DFA model. The first DFA model had a classification success of 86±4% for sensitivity, 96±4% for specificity, and 88±3% for accuracy (*see* Table 3). ROC analysis has yielded AUC of 0.986 (*see* Figure 2B; Table 3) for the discrimination between benign and malignant conditions. The identification of the collective patterns was supported by the observation of statistically significant differences between the sub-populations in the average composition of the exhaled breath.

Similarly, the chemical nanoarray analysis discriminated well between groups of LC patients with different sub-histology. As seen in Figure 3, excellent distinction could be achieved between adeno- and squamous- cell carcinomas within the test group with

malignant nodules. The two sub-populations were completely separated along the first canonical score, with no overlap between the clusters ($p < 0.0001$). Cross-validation of the analysis yielded an accuracy of 88±3% (*see* Table 3).

Within the NSCLC group, excellent distinction could be achieved between early and advanced stages of NSCLC ($p<0.0001$) – *see* Figure 4. Cross-validation of the analysis yielded 86±3% sensitivity, 88±6% specificity and 88±2% accuracy (*see* Table 3).

DISCUSSION

Previous studies with exhaled breath have compared lung cancer to COPD patients or to healthy subjects, with either GC-MS[9, 10, 12, 13] **or** chemical sensors[4, 5, 9, 10, 18, 20]. In contrast, this study used both techniques and compared exhaled breath of patients with either malignant or benign (>10 mm) PNs that were validated either through histological examination or through follow-up serial imaging. The results of this study showed that exhaled breath testing might have future potential for the management of patients with pulmonary nodules. In particular, the results indicate the breath testing could be used to discriminate between benign and malignant PNs detected by CT, to reduce the false positive rate and minimize the risk of morbidity related to invasive diagnostic procedures and to reduce the costs of lung cancer screening.

Generally speaking, GC-MS yields a wealth of information for basic research.[9, 10, 12, 13, 18] However, this method has several prominent disadvantages for use as a clinical point-of-care application. First and foremost, it requires sophisticated, expensive, and bulky equipment that would only be available in large, well-equipped laboratories. Second, the interpretation of the GC-MS results is challenging and requires much expertise. Third, the analysis of breath VOCs at ppbv/pptv concentrations requires pre-concentration prior to GC-MS – for example, onto solid-phase microextraction fibers or other suitable absorption media.[30] The pre-concentration methods selectively enhance the signals of certain VOCs, while potentially missing others. These disadvantages were expressed, amongst the rest, in the failure of the GC-MS analysis to detect altered VOC production between early stage and advanced NSCLC cases and between the NSCLC sub-histologies through exhaled breath samples (Table 3), in contrast to our recent *in-vitro* observations of similar (sub-)histologies.[14] Recently, we have collected and analyzed headspace samples from of cell lines with different (sub-)histology characteristics.[14] Statistical analysis has identified three substances (decanal, acetophenone and 1,3-bis (1,1-dimethylethyl)-benzene) as main contributors to the separation between *in vitro* NSCLC from SCLC with 100% sensitivity and 75% specificity.[14] Nine VOCs (two aldehydes, one alkane, two ketones, one alcohol and three benzene derivatives) showed differences between the sub-types of NSCLC, *viz.* between adenocarcinoma and squamous cell carcinoma. Among these VOCs, we identified 2-ethyl-1-hexanol, 1,3-dimethyl-benzene and 1,3-bis (1,1-dimethylethyl)-benzene as key distinguishing VOCs, which are all found at higher concentration in the headspace of adenocarcinoma than in the headspace of squamous cell carcinoma.[14] The difference between the in vivo and *in vitro* observations could be explained, therefore, by one or by a combination of the following possible reasons: **(i)** the VOCs emitted from the LC cells could be diluted on their way to the exhaled breath so that their final concentration in the breath is lower than the GC-MS/SPME limit of detection; **(ii)** the emitted VOCs expressed a combination with the environmental (host) response, rather than the pure cells metabolism (e.g., inflammation); and/or **(iii)** the analysis of the breath samples by GC-MS/SPME was affected by confounding factors (variations in lifestyle, culture, geographic, etc.), whereas in vitro findings did not take into account these effects.

For breath analysis to become a clinical reality, the tailor-made nanoarray reported in this study would be more appropriate. First, the chemical nanoarray is significantly smaller, easy-to-use, inexpensive, and can detect VOCs in the presence of water vapor, without requiring pre-concentration and/or dehumidification techniques.[4, 5, 8–10, 14, 26] Indeed, the nanoarray could differentiate between benign and malignant tumors with higher accuracy than the GC-MS. Moreover, the nanoarray could differentiate between the sub-histologies of LC as well as between the early stages and advanced stages of NSCLC, whereas GC-MS could not. The higher detection capabilities of the nanoarray, compared to the GC-MS, could be attributed to the fundamentally different sensing mode of the two methods. The chemical nanoarray is broadly cross-reactive and responsive to all (or part of) the LC specific VOCs of interest. The responses of the chemical nanoarray to a specific VOC at a certain concentration differ individually between the constituent (nanoarray) sensors due to the chemical diversity of the organic ligands and/or nanomaterials used (*cf.* Online Data Supplement, Section 1.3; Supplemental Digital Content 1, http://links.lww.com/JTO/A320.). On the other hand, the signals of the same chemical nanoarray to the mixed VOCs that are present in the breath sample are additive, so that the overall signal of one sensor stems from a total ~ppm amount of breath VOCs of interest. Hence, the sensors' responses are less affected by noise than the detected (sub) ppb concentrations of the separate VOCs in the GC-MS/SPME analysis.[4, 5]

Discrimination between sub-populations of NSCLC, *i.e.* adeno- vs. squamous- cell carcinoma via the nanoarray is not surprising, as our previous studies showed that this discrimination is robust also in the in-vitro setting.[14] In this study, the nanoarray showed a clear discrimination between the adenocarcinomas and the squamous cell carcinomas (Figure 3). This finding might have a future potential clinical implication, mainly related to the treatment algorithm in advanced LC. In patients with adenocarcinomas of the lung invasive diagnostic procedures are often performed, as adenocarcinomas have a high rate of gene alterations with related therapies. In addition, our future effort is directed also to discriminate between NSCLC and SCLC. It would be reasonable to expect a higher quantity and variety of metabolites released by the SCLC, because SCLC cells are rapidly dividing cells that require more adenosine triphosphate (ATP), nucleotides, fatty acids, membrane lipids and proteins.[31] This assumption is based on our previous report of pronounced differences in the headspace atmosphere of SCLC and NSCLC cell lines.[14] Neither the discrimination between the NSCLC and SCLC nor the distinction of the NSCLC sub-types via breath samples would replace tissue histology. However, in many cases, tissue is unreachable and a non-invasive tool might help to manage such LC patients.

In summary, this study offers an inexpensive and portable tool to further improve the non-invasive biomarker-based investigation of patients who are not candidates for invasive procedures or in cases where the tissue is hard to sample. More specifically, breath analysis with nanoarray could serve as a primary and/or a secondary screener for PN-positive patients after LDCT, and might avoid delay in performing an invasive investigation when cancer is suspected rather than proceeding with follow-up imaging. Hence, the recommendation for an invasive procedure can be made immediately with a relatively high level of confidence. The reported breath test in this study could have significant impact on reducing unnecessary investigation and reducing the risk of procedure-related morbidity and costs. Additionally, it could facilitate faster therapeutic intervention, replacing time-consuming clinical follow-up that would eventually lead to the same intervention. Further studies using a larger cohort of patients are underway and will be published elsewhere.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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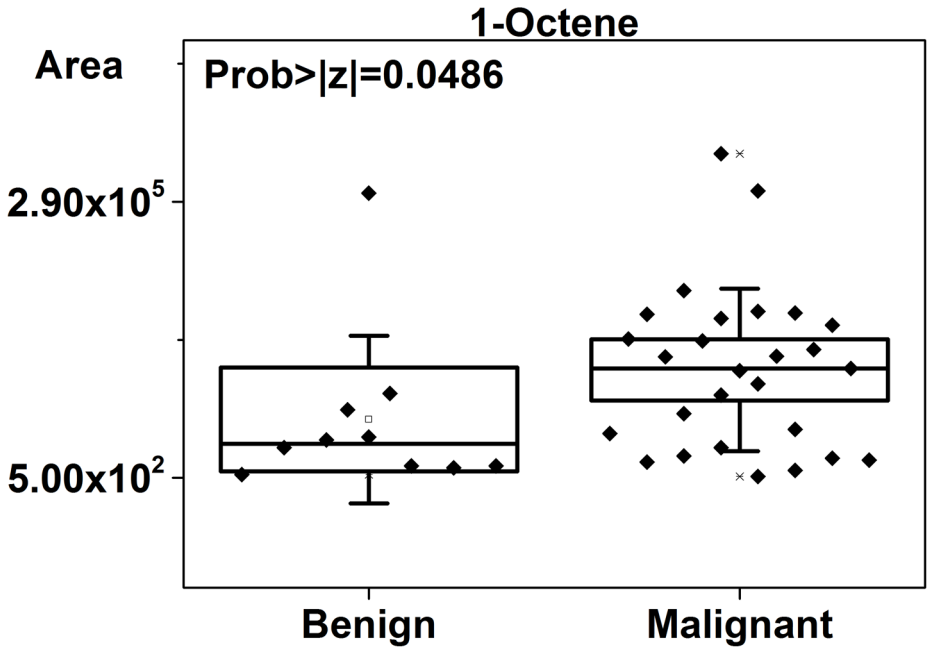

Figure 1. One potential biomarker for differentiating malignant lung nodules from benign ones found by means of GC-MS

1-octene shows a statistically significant difference in the peak area the benign and malignant samples ($p = 0.0486$). The positions of the mean values for benign and malignant states are marked with □, the boxes correspond to their 95% confidence limits, and the error bars corresponds to the standard deviation

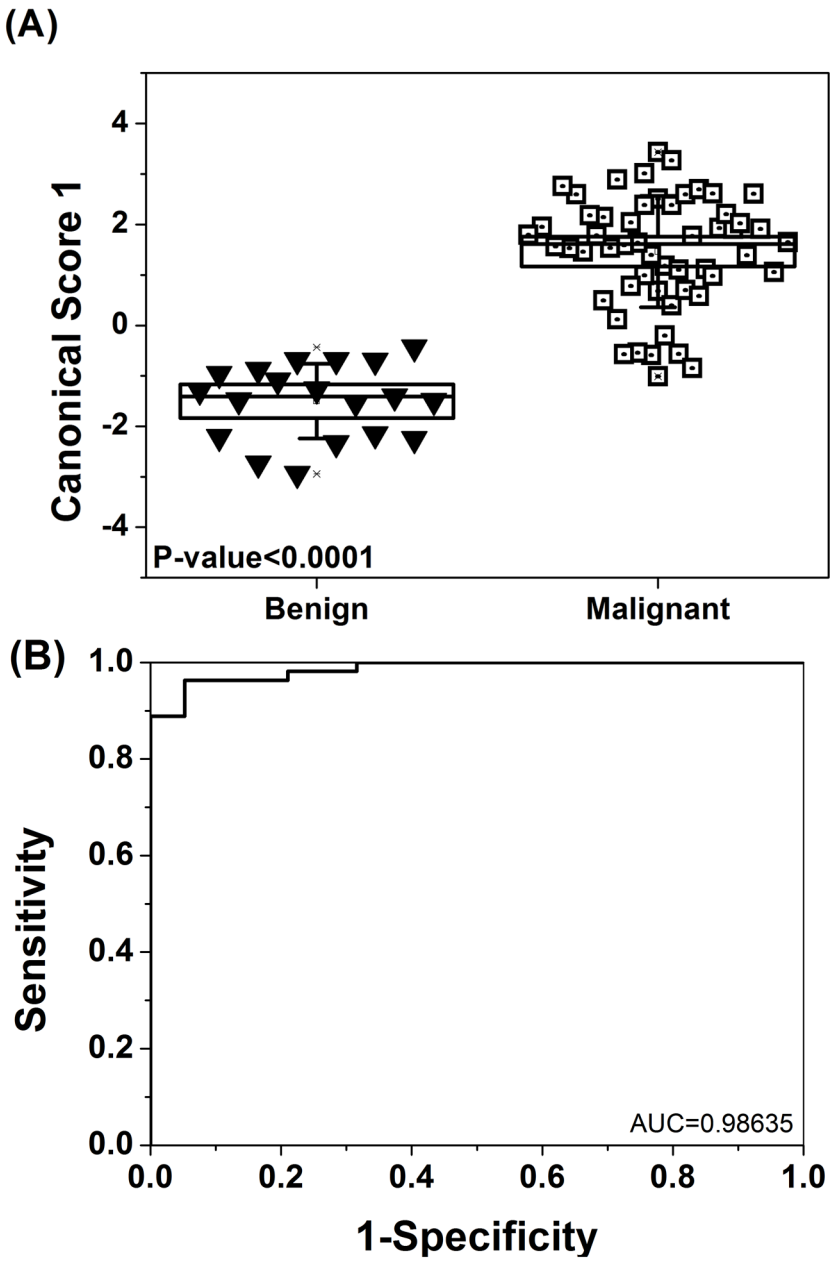

Figure 2. Graphical representation of the first canonical score values for patients with benign nodules and malignant nodules that were obtained from a chemical nanoarray containing 5-nm gold nanoparticle sensors and an organically-functionalized carbon nanotube sensor

(A) Each point represents one patient. The positions of the mean values are marked with □, the boxes correspond to the 95% confidence limits, and the error bars correspond to the standard deviations. The confidence intervals are significantly separated (p-values<0.0001). (B) ROC curve for the discrimination between malignant and benign SPN, AUC 0.986

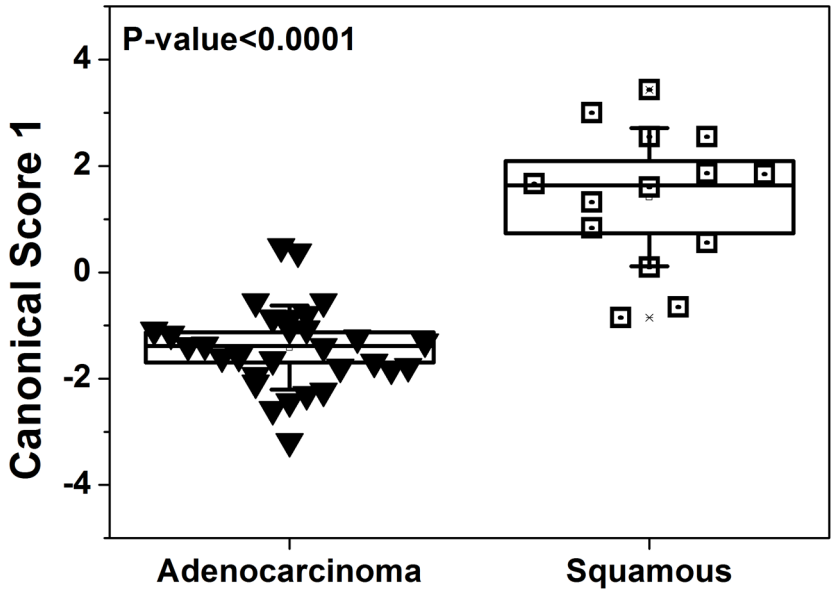

Figure 3. Graphical representation of the first canonical score values for patients with malignant nodules that differentiate between adenocarcinoma and squamous cell lung cancer that were obtained from a chemical nanoarray containing gold nanoparticle sensors

Each point represents one patient. The positions of the mean values are marked with □, the boxes correspond to their 95% confidence limits, and the error bars corresponds to the standard deviations. The confidence intervals are significantly separated ($p < 0.0001$).

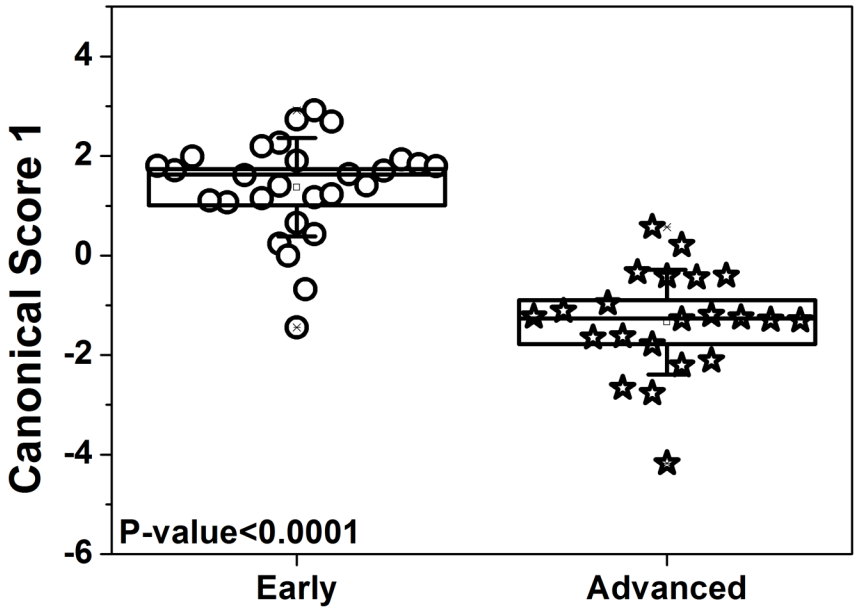

Figure 4. Graphical representation of the first canonical score values for patients with early stage or advanced NSCLC nodules that were obtained from a chemical nanoarray containing seven gold nanoparticle sensors and an organically-functionalized carbon nanotube sensor

Each point represents one patient. The positions of the mean values are marked with □, the boxes correspond to their 95% confidence limits, and the error bars corresponds to the standard deviations. The confidence intervals are significantly separated ($p < 0.0001$).

Table 1

Clinical characteristics of the tested patients

	Benign Nodules (N=19)		Malignant Nodules (N=53)		p-value (<0.05)
Age (years)	60.8 ± 6.8		64.9 ± 7.2		0.038
BMI (kg/m^2)*(1)*	26.2 ± 5.1		27.4 ± 7.2		NS*(5)*
Males	15 (79%)		31 (58%)		NS*(5)*
Active Smokers	6 (32%)		19 (36%)		NS*(5)*
Former Smokers	11 (58%)		26 (49%)		NS*(5)*
Never Smokers	2 (11%)		8 (15%)		NS*(5)*
PY (Total) *(2)*	40.6 ± 34.4		40.5 ± 27.0		NS*(5)*
COPD*(3)*	10 (52%)		26 (49%)		NS*(5)*
IHD*(4)*	7 (37%)		22 (42%)		NS*(5)*
Nodule Size (cm)	1.56±1.3		2.7±1.7		0.004
Diagnostic Procedure					
Bronchoscopy	16		20		
Wedge/Lobectomy	3		33		
Etiology	Non-Cancerous	13	**NSCLC**	47	
	Atypical Alveolar		Adenocarcinoma	30	
	Hyperplasia	1	Squamous	13	
	Infectious/Inflammation	5	Large Cell	2	
			Poorly Diff.	2	
			SCLC	6	
Stage			**NSCLC**		
			Stage I	23	

	Benign Nodules (N=19)	Malignant Nodules (N=53)		p-value (<0.05)
		Stage II	4	
		Stage III	10	
		Stage IV	10	
		SCLC		
		Limited	3	
		Extensive	3	

(1) BMI: Body Mass Index

(2) PY: Pack Years

(3) COPD: Chronic Obstructive Pulmonary Disease

(4) IHD: Ischemic Heart Disease

(5) NS: No significant changes between the groups were identified

Table 2

Breath VOC that showed a significant statistical difference in benign and malignant nodules (N=38; Malignant = 28; Benign N=10)

Retention time (min)	m/z	Tentative name	Trend in Cancer	p-values[(1)]
11.18	83	1-Octene	Up	0.049

[(1)] Wilcoxon non-parametric method

Table 3

Sensitivity, Specificity and Accuracy of the chemical nanoarray (from leave-one-out cross-validation) and the area under curve of the ROC curve analysis

	Sensitivity	Specificity	Accuracy	ROC AUC
Malignant/Benign	86±4%	96±4%	88±3%	0.986
Adeno/Squamous	92±8%	78±7%	88±3%	0.974
Early/Advanced NSCLC	86±3%	88±6%	88±2%	0.961