Volatile biomarkers of pulmonary tuberculosis in the breath

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Summary Pulmonary tuberculosis may alter volatile organic compounds (VOCs) in breath because Mycobacteria and oxidative stress resulting from Mycobacterial infection both generate distinctive VOCs. The objective of this study was to determine if breath VOCs contain biomarkers of active pulmonary tuberculosis. Head space VOCs from cultured *Mycobacterium tuberculosis* were captured on sorbent traps and assayed by gas chromatography/mass spectroscopy (GC/MS). One hundred and thirty different VOCs were consistently detected. The most abundant were naphthalene, 1-methyl-, 3-heptanone, methylcyclododecane, heptane, 2,2,4,6,6-pentamethyl-, benzene, 1-methyl-4-(1-methylethyl)-, and cyclohexane, 1,4-dimethyl-.

Breath VOCs were assayed by GC/MS in 42 patients hospitalized for suspicion of pulmonary tuberculosis and in 59 healthy controls. Sputum cultures were positive for Mycobacteria in 23/42 and negative in19/42 patients. Breath markers of oxidative stress were increased in all hospitalized patients ($p < 0.04$). Pattern recognition analysis and fuzzy logic analysis of breath VOCs independently distinguished healthy controls from hospitalized patients with 100% sensitivity and 100% specificity. Fuzzy logic analysis identified patients with positive sputum cultures with 100% sensitivity and 100% specificity (95.7% sensitivity and 78.9% specificity on leave-one-out cross-validation); breath VOC markers were similar to those observed in vitro, including

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naphthalene, 1-methyl- and cyclohexane, 1,4-dimethyl-. Pattern recognition analysis identified patients with positive sputum cultures with 82.6% sensitivity (19/23) and 100% specificity (18/18), employing 12 principal components from 134 breath VOCs.

We conclude that volatile biomarkers in breath were sensitive and specific for pulmonary tuberculosis: the breath test distinguished between "sick versus well" i.e. between normal controls and patients hospitalized for suspicion of pulmonary tuberculosis, and between infected versus non-infected patients i.e. between those whose sputum cultures were positive or negative for Mycobacteria.

Introduction

The current global epidemic of pulmonary tuberculosis has highlighted the need for new screening tests that are rapid and accurate. The social burden of pulmonary tuberculosis has increased because many patients are also infected with human immunodeficiency virus (HIV), and the rates of multidrug-resistant tuberculosis are increasing.[1] However, screening technology has not changed greatly during the past several decades. Many high-burden countries depend upon sputum smears and chest radiographs, supplemented by cultures when resources permit. This approach is highly specific for active pulmonary tuberculosis, but its value in primary screening is limited by low sensitivity and high cost.

We tested the hypothesis that volatile organic compounds (VOCs) in the breath might provide new biomarkers of active pulmonary tuberculosis. The rationale of this hypothesis is based on two observations: first, Mycobacteria produce distinctive patterns of VOCs in vitro, and second, patients with active pulmonary tuberculosis suffer from increased oxidative stress which also generates distinctive patterns of VOCs. Several species of Mycobacteria produce VOC metabolites that act as chemical "fingerprints": *M. avium*, *M tuberculosis*, *M. gordonae*, *M. gastri*, *M. kansasii*, *M. szulgai*, and *M. flavescens* can be identified by their distinctive patterns of volatile metabolites, including C14–C26 fatty acids and their methylated and hydroxylated derivatives.[2–4] Also, patients with active pulmonary tuberculosis suffer from increased oxidative stress: serum markers of oxidative stress, including lipid peroxidation products, conjugated dienes, malondialdehyde, and allantoin are generally increased in patients with active pulmonary tuberculosis, and decrease following a course of antituberculous therapy.[5–7] Oxidative stress also liberates distinctive VOCs into the breath, particularly C4–C20 alkanes and methylated alkanes comprising the breath methylated alkane contour (BMAC).[8] We have previously reported altered patterns of breath markers of oxidative stress in different diseases, including heart transplant rejection,[9,10] lung cancer,[11,12] breast cancer,[13] ischemic heart disease,[14] preeclampsia of pregnancy,[15] and diabetes mellitus.[16]

We analyzed VOCs derived from Mycobacteria cultures, as well as VOC markers of oxidative stress in the breath of patients undergoing evaluation for Mycobacterial infection. Two different mathematical techniques, pattern recognition analysis and fuzzy logic, were employed to address two questions: First, could breath VOCs distinguish between healthy controls and all hospitalized patients undergoing evaluation for Mycobacterial infection (culture positive as well as culture negative)? Second, could breath VOCs distinguish between hospitalized patients whose sputum cultures for Mycobacteria were positive or negative?

Methods

Breath collection and assay

The method has been described.[17] Subjects breathed in and out through the disposable mouthpiece of a portable breath collection apparatus for 2.0 min, and the VOCs in 1.0 l alveolar breath and 1.0 l room air were captured onto separate sorbent traps. VOCs captured on the sorbent traps were analyzed in the laboratory by automated thermal desorption, gas chromatography and mass spectroscopy (ATD/GC/MS).

Identification of VOCs produced by *M. tuberculosis* in vitro

Reference samples of *M. tuberculosis* were cultured in vitro (by VLB) utilizing VersaTREK Myco bottles (Trek Diagnostic Systems, Cleveland, OH) at Saint Vincent's Medical Center, New York, NY. The

Myco bottles containing 1.0 ml of Growth Supplement were inoculated with 0.5 ml of a 1.0 McFarland suspension in sterile saline prepared from isolates grown on Lowenstein Jensen medium. Myco bottles containing growth supplement and inoculated with 0.5 ml of sterile saline served as the control. VOCs in 1.0 ml aspirated head space were captured by injection onto a sorbent trap similar to those employed for breath collections. Samples incubated an additional 2 days after the Myco bottle yielded a positive signal, were found to yield optimal results. Head space samples were collected from different isolates: Fresh clinical isolates of *M. tuberculosis* ($n = 12$) and *M. tuberculosis* H37RV, the pan-sensitive control strain of used for susceptibility tests ($n = 8$) were assayed. Matching control samples were drawn from uninoculated Myco bottles incubated under the same conditions as the test bottles. VOCs in the sorbent traps were analyzed by ATD/GC/MS employing the same method described for analysis of breath samples.[18] The abundance of a VOC was determined as abundance in the test sample minus abundance in the uninoculated sterile control. VOCs were ranked by multiple *t*-tests comparing mean abundance in all samples to sterile incubation containers.

Human subjects: pulmonary tuberculosis

Technically usable breath VOC samples were obtained from 42 patients admitted to an isolation ward on the in-patient Chest Service of Bellevue Hospital to rule out suspected pulmonary tuberculosis. Criteria for admission were chronic constitutional symptoms (cough, night sweats, fever, and weight loss for more than 1 week) and/or an abnormal chest X-ray (infiltrates, nodules, cavities, or pleural effusions). A PPD test was performed in eligible patients on admission and read at 48 h. Sputum was induced daily for 3 days and sent for staining for acid fast bacilli and culture for Mycobacteria.

Human subjects: healthy controls

Breath samples were obtained in a similar fashion from members of the general population in Staten Island, NY with no history of tuberculosis or other chronic disease.[8] An age-matched subgroup ($n = 59$) was selected to serve as a control group for the patients admitted for screening for pulmonary tuberculosis. The institutional review boards of all participating institutions approved the research.

Masking procedures

Clinicians and pathologists at Bellevue Hospital collected and cultured sputum samples with no knowledge of the breath test results. Breath samples were collected (by MIM) and analyzed in the laboratory (by RNC and JG) without knowledge of the sputum smears or culture results.

Identification of breath VOC markers of oxidative stress

The BMAC was constructed for each subject using alveolar gradients of C4-C20 n-alkanes and mono-methylated alkanes.[8] The oxidative age, an age-corrected value for the abundance of these VOCs[16] was compared in hospitalized patients and age-matched healthy controls with a *t*-test.

Analysis of data

Two forms of multivariate analysis—fuzzy logic and pattern recognition analysis—were employed in order to correlate the patients' breath VOCs with their clinical status.

Fuzzy logic (Interrelation Miner, SystAim, Zürich, Switzerland) creates a membership score T_{pos} for membership in the group with *disease present* and a second score T_{neg} for membership in the group with *disease not present*. Fuzzy logic was employed to address two questions: (1) Can breath VOCs distinguish between patients with a high suspicion of pulmonary tuberculosis (and hence hospitalized) from healthy controls? (2) Can breath VOCs distinguish between hospitalized patients with a positive sputum culture for Mycobacteria from hospitalized patients with a negative sputum culture? Values for T_{neg} and for T_{pos} were obtained in two sets of data:

	T_{neg}	T_{pos}
Can breath VOCs be used to distinguish hospitalized patients from healthy controls?	Typicality of a healthy control	Typicality of a patient with high suspicion of pulmonary TB (and hence hospitalized)
Can breath VOCs be used to distinguish hospitalized sputum culture positive patients from hospitalized sputum culture negative patients?	Typicality of a hospitalized patient who is sputum culture negative	Typicality of a hospitalized patient who is sputum culture positive

In both cases, a similar analysis procedure was applied: In the training set, fuzzy functions were constructed for the candidate breath VOCs in order to create a typicality matrix for the two groups being compared in the table. In the prediction set, these typicality matrices were employed to predict the outcome by generating two numerical values from the breath VOCs: T_{neg}, the typicality for *disease not present* and T_{pos}, the typicality for *disease present*. Employing a leave-one-out method, this procedure was iterated n times, employing $n-1$ subjects in the training set and one subject in the prediction set. The resulting values of $T_{pos}-T_{neg}$ were employed as predictors of the diagnosis *disease present* or *disease not present*, and the accuracy of prediction was displayed in a receiver operating characteristic (ROC) curve.

Pattern recognition analysis of breath VOCs (Pirouette, Version 3.11, Infometrix, Inc. Bothell, WA 98011) was employed for multivariate exploratory data analysis, category classification, and continuous dependent variable modeling. Exploratory data analysis methods include hierarchical cluster analysis (HCA) and principal component (PC) analysis, category classification methods include *K*-nearest neighbor (KNN) and soft independent modeling of class analogy (SIMCA), and continuous dependent variable methods include PC regression and partial least squares path modeling. Subjects were assigned to three diagnostic groups: class 1 (age-matched healthy controls), class 2 (sputum culture negative for *M. tuberculosis*), and class 3 (sputum culture positive for *M. tuberculosis*). Exploratory evaluations using PC factor analysis (PCA) and HCA were performed to investigate data structure and similarities between subjects and between variables. Data were autoscaled for all procedures in order to put each variable on the common footing of zero mean and unit variance over the sample set. Following exploratory analysis and creation of subsets excluding potential outliers, classification modeling procedures KNN and PC proximity modeling (SIMCA) were tested to assess classification accuracy into diagnostic models.

The goals of exploratory analysis include assessing the relationships amongst the variables and the dimensionality of the problem. Exploratory analysis also helps assess the relationships amongst the test subjects to see if mathematically derived clusters reflect the diagnostic class assignments. Exploratory data analysis can reveal potential outlier subjects so that they may be reviewed to determine which measured values are unusual. Outliers may also be excluded from derivation of models to predict class assignments. Exploratory PC analysis and factor analysis methods help assess the underlying dimensionality of the alveolar gradient data using orthogonal (mathematically independent) components that contain significant portions of the data variance. The calculated PCs are ordered by largest to least variance, allowing retention of a few PCs containing the majority of data variance and discarding the bulk of remaining components that contain small amounts of variance that may be measurement noise. Dimensionality assessment is also a guide for sufficiency of sample size for each category of subjects. Since breath alveolar gradients for over 130 VOCs were measured for each test subject, the number of variables far exceeded the number of cases in each diagnostic category. A general rule is to require at least three times as many class members as variables or dimensions. The number of subjects in the diagnostic groups suggested limiting models to fewer than 12 PCs.

Results

In vitro studies

One hundred and thirty different VOCs were consistently detected in *M. tuberculosis* cultures in vitro, predominantly derivatives of benzene, naphthalene, and alkanes. The 10 most abundant VOCs are shown in Table 1.

Human studies

Patient characteristics are shown in Table 2.

Clinical course of hospitalized patients

All patients had three induced sputum for AFB. A total of 23/42 patients had sputum that was culture positive for *M. tuberculosis*. These patients were referred to the Bellevue Chest clinic/DOT clinic and followed. Of these patients, 16 had sputum that was smear positive with appropriate clinical setting. Four patients had bronchoscopy to confirm the diagnosis. All four had post-bronchoscopy sputum smears that were positive. Three patients had empiric therapy initiated and later confirmed by positive culture.

In the culture negative group, the most common diagnosis was bronchiectasis, followed by bronchitis. Three patients had sputum cultures positive for *M avium* intracellulare and were co-infected with HIV. Two patients were begun on TB treatment while awaiting culture results. In both cases, TB therapy was discontinued when cultures were negative at 2 months and an alternative diagnosis of bronchiectasis was assigned to these patients. Of the culture negative groups, 10 patients have had

Table 1 VOC markers of *M. tuberculosis* observed in culture and in breath.

Culture (in vitro)	Breath (fuzzy logic)	Breath (pattern recognition)
Naphthalene, 1-methyl-	Cyclohexane, 1,3-dimethyl-, trans-	Factor 1
3-Heptanone	Benzene, 1,4-dichloro-	Benzene, ethyl-
Methylcyclododecane	Cyclohexane, 1,4-dimethyl-	Benzene, methyl-
Heptane, 2,2,4,6,6-pentamethyl-	1-Octanol, 2-butyl-	Benzene, propyl-
Benzene, 1-methyl-4-(1-methylethyl)-	2-Butanone	Heptane, 3-methyl-
Cyclohexane, 1,4-dimethyl-	Naphthalene, 1-methyl-	Propane, 2-methoxy-2-me
3,5-dimethylamphetamine	Camphene	Factor 2
Butanal, 3-methyl-	Decane, 4-methyl-	1-Octene
2-Hexene	Heptane, 3-ethyl-2-methyl-	Cyclohexane
Trans-anti-1-methyl-decahydronaphthalene	Octane, 2,6-dimethyl-	Heptanal
	Benzene, 1,2,3,4-tetramethyl-	Heptane, 2,4-dimethyl-
	Bicyclo_3_1_1_hept-2-ene, 3,6,6-trimethyl-	Heptane, 4-methyl-
	Cyclohexane, 1-ethyl-4-methyl-, trans-	Nonanal
	l-_beta_-Pinene	Pentane, 2-methyl-
		Styrene
		Tridecane

The ''culture'' column lists the 10 most abundant VOCs observed in cultures of Mycobacteria in vitro, ranked by their increased abundance compared to sterile control vials. The ''breath'' columns lists the VOCs in breath identified by fuzzy logic analysis and by pattern recognition analysis as the best discriminators between patients whose sputum cultures were positive or negative for Mycobacteria. Breath VOCs identified by fuzzy logic are ranked by lambda value, and comprise the VOCs employed as markers of Mycobacterial infection in Fig. 4. Breath VOCs identified by pattern recognition analysis are listed as components of Factor 1 or Factor 2, and comprise the VOCs employed as markers of Mycobacterial infection in Fig. 3. Naphthalene, 1-methyl- and cyclohexane, 1,4-dimethyl- were observed both in Mycobacterial culture and in the fuzzy logic breath discriminators of infection. There were structural similarities among VOCs in all three groups, particularly derivatives of heptane and benzene.

Table 2 Patient characteristics.

	No. in group	PPD status			Mean age
		Pos	Neg	ND	Year (SD)
Hospitalized patients					
Sputum culture					
Positive for Mycobacteria	23	10	2	11	39.5 (14.2)
Negative for Mycobacteria	19	8	4	7	47.2 (8.9)
Healthy controls	59	59			41.1 (16.1)

PPD status is indicated as positive (pos), negative (neg) or not done (ND). There were no significant differences between ages of subjects in the three groups (one-way ANOVA, NS).

long-term follow-up through the Bellevue Health Care system without evidence of tuberculosis, and nine were lost to follow up.

Breath VOC markers of oxidative stress

Mean BMACs in normal controls, sputum culture negative patients and sputum culture positive patients are shown in Fig. 1. The intensity of oxidative stress as shown by oxidative age was significantly increased in all hospitalized patients

undergoing evaluation for Mycobacterial infection compared to normal controls (Fig. 2), but there was no significant difference between patients whose sputum was culture positive or culture negative for *M. tuberculosis*.

Pattern recognition analysis of breath VOCs

Figure 3 displays a scatter plot of test subjects showing their PC scores for factor 1 versus factor 2 in the 134 VOC measurement space. Exploratory PC
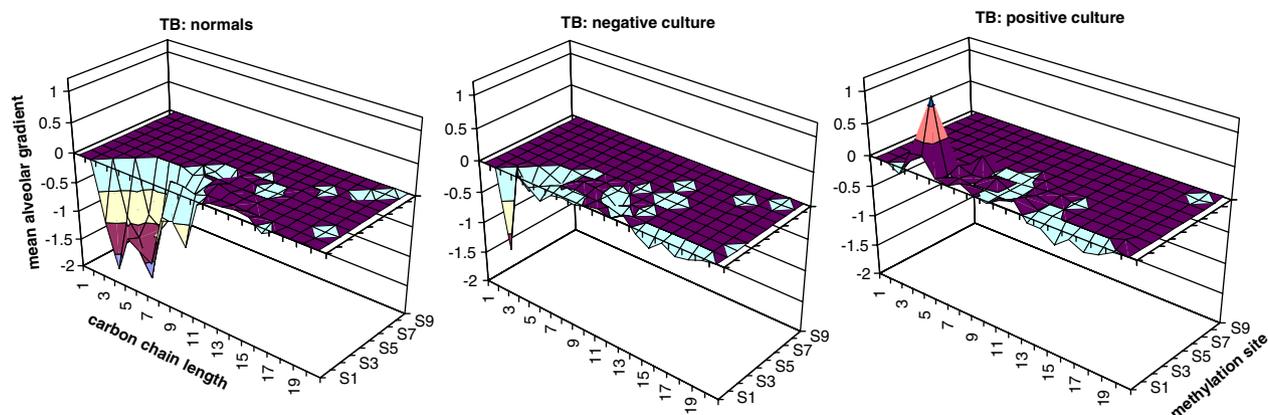
Figure 1 Effect of Mycobacterial infection on breath markers of oxidative stress: the breath methylated alkane contour (BMAC) is a display of oxidative stress markers in breath comprising C4–C20 alkanes and their monomethylated derivatives. Mean BMACs (from left to right) are shown in normal controls, sputum culture negative patients and sputum culture positive patients. The mean alveolar gradient (concentration in breath minus concentration in room air) is shown on the vertical axis. The horizontal axes identify the specific VOC (e.g. the combination of carbon chain length = 7 and methylation site = 3 corresponds to 3-methylheptane). The peaks are predominantly negative in the normal controls, and predominantly positive in both culture positive patients and culture negative patients.

plots and cluster dendograms indicated two cases (one normal, one culture-positive) that were potential outliers. Evaluation of the alveolar gradient values for these cases confirmed highly unusual numbers and they were excluded from model development. The PC plot of Fig. 3 is for the reduced subset which has the two outliers excluded. In the full data set, these two outliers are main determinants of the first two PCs due to the large variance in their alveolar gradients.

Several KNN and SIMCA models exhibited either high specificity or high sensitivity. The best single model used SIMCA classification based on 10–12 PCs for each class model. Correct classification of class 3 culture positive subjects was[19] out of 23, sensitivity = 82.6%. No class 1 controls or class 2 negative culture subjects were incorrectly classified as class 3 positive for specificity = 100% (Table 3).

Fuzzy logic analysis of breath VOCs

The major breath VOCs that were used to distinguish hospitalized patients from healthy controls are shown in Table 4. The major breath VOCs that identified hospitalized patients with positive sputum cultures are listed in Table 1. ROC curves with sensitivity and specificity values are shown in Fig. 4. When applied to distinguish hospitalized patients from healthy controls (left panel) the cross-validation with the leave-one-out procedure
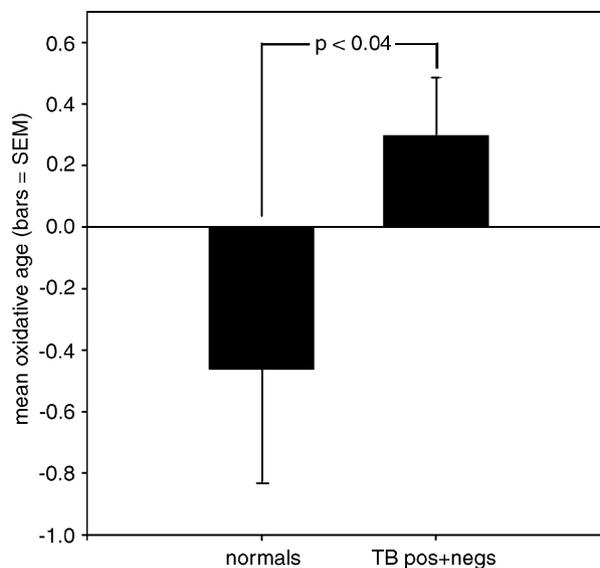

Figure 2 Oxidative age in healthy controls and hospitalized patients: oxidative age is the intensity of oxidative stress expressed in standard deviations from the mean observed in normal humans of the same age. The value of oxidative age was determined in all subjects as $(O–E)/S$ where: VUC = volume under curve of BMAC, O = observed VUC of BMAC in the study subject, E = expected BMAC of VUC in a normal subject of the same age,[13] and S = standard deviation of $O–E$ in all normal subjects. Oxidative age was significantly increased in all hospitalized patients (TB pos+negs) regardless of whether their sputum was culture positive or culture negative for *M. tuberculosis*. However, oxidative age was not significantly different in culture positive and culture negative patients.

was 100% sensitive (42/42), and 100% specific (52/52). When applied to distinguish hospitalized patients with positive and negative sputum cultures the cross-validation with the leave-one-out procedure was 95.7% sensitive (22/23), and 78.9% specific (15/19).
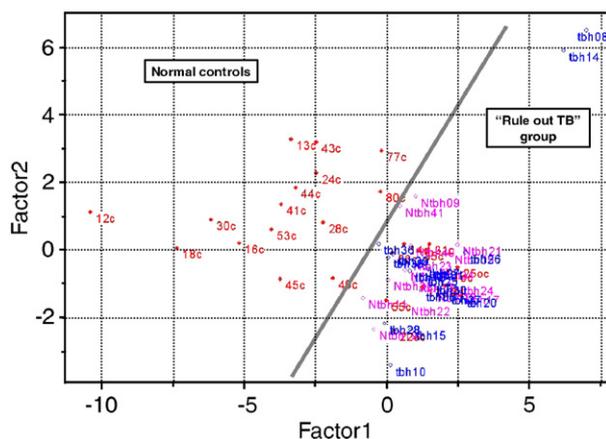

Figure 3 Pattern recognition analysis of breath VOCs: scatter plot of test subjects per their principal component scores for factor 1 versus factor 2, containing 20.94% of the original variance in the 134 VOC measurement space. Principal components (factors) are orthogonal (independent) dimensions calculated from the measured variables, ranked by decreasing amounts of variance contained in each factor. Factor 1 contains 14.48% and factor 2 contains 6.46% of the variance, respectively. The scatter plot shows separation between the majority of normal (control) test subjects (diagnostic class 1) and those with symptoms (diagnostic classes 2 and 3). SIMCA classification models for each class used from six to twelve principal components to describe the location and dispersion of subjects for the three diagnostic groups.

Discussion

There were two main conclusions from this study: First, a set of breath VOCs accurately distinguished between normal controls and hospitalized patients undergoing screening for Mycobacterial infection. Second, another set of breath VOCs distinguished between hospitalized patients whose sputum cultures were positive or negative for Mycobacterial infection. Two different mathematical techniques—fuzzy logic analysis and pattern recognition analysis—independently generated similar conclusions.

These findings appear to have resulted from two different pathophysiologic processes. Breath markers of oxidative stress distinguished between normal controls and hospitalized patients, but not between hospitalized patients whose sputum cultures were positive or negative for Mycobacterial infection (Figs. 1 and 2). Oxidative stress markers apparently distinguished the "sick" from the "well", because all of the hospitalized patients had an abnormal chest X-ray and complained of cough, night sweats, fever, and weight loss. However, the best discriminators between hospitalized patients with positive or negative sputum cultures comprised a group of breath VOCs that were structurally similar to the most abundant VOCs observed in cultures of Mycobacteria (Table 1, Figs. 3 and 4).

Fuzzy logic and pattern recognition analysis are powerful problem-solving methodologies that are employed widely in industry and increasingly in clinical medicine. Fuzzy logic has been employed to identify tuberculous pleural effusions based upon the immunoreactive concentrations of interleukins in blood,[20] and also for the detection of lung cancer by combining the contributions of multiple

Table 3 Classification with pattern recognition analysis.

Actual	Predicted					
	Normal controls	Sputum culture negative	Sputum culture positive	No match	Sensitivity	Specificity
Normal controls	58	0	0	0		58/58 = 100%
Sputum culture negative	4	14	0	0		18/18 = 100%
Sputum culture positive	4	0	19	0	19/23 = 82.6%	

The following table shows classification results for SIMCA employing 130 of 134 VOC variables, 2 outliers not included. The rationale for exclusion of outliers is described in the Results section. Outlier cases were excluded in model development and classification analysis, based on PCA and HCA results. There were 12 principal components in the model for control subjects, ten principal components in the model for negative culture subjects and 12 principal components in the model for positive culture subjects.

Table 4 Major breath VOC markers in hospitalized patients versus healthy controls.

VOCs	Goodman/Kruskal λ
1,3-Isobenzofurandione	0.905
Pentane, 2,3-dimethyl-	0.881
Acetaldehyde	0.857
Benzenemethanol, _alpha_,_alpha_-dimethyl-	0.81
Cyclohexane	0.643
1,1′-Biphenyl, 2,2′-diethyl-	0.595
1H-Indene, 2,3-dihydro-1,1,3-trimethyl-3-phenyl-	0.571

Fuzzy logic identified these VOCs that distinguished ''sick'' from ''well'' subjects.
VOCs are shown ranked by Goodman/Kruskal λ because higher values indicate an increased likelihood that the VOC was distinctive in a hospitalized patient.
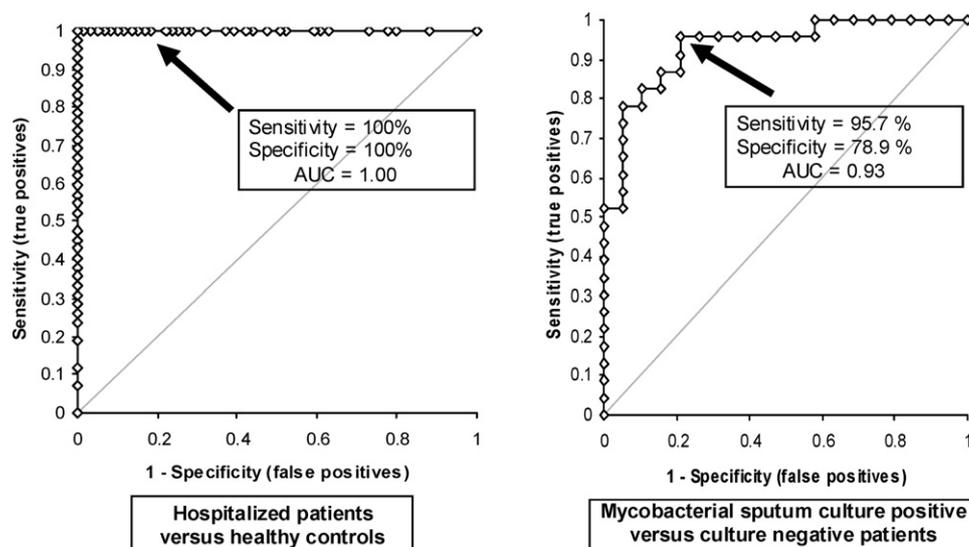

Figure 4 Fuzzy logic analysis of breath VOCs: these ROC curves display the sensitivity and specificity of the breath test in two groups. Left panel: hospitalized patients undergoing evaluation for Mycobacterial infection (42) versus age-matched healthy controls (59). Right panel: hospitalized patients with a positive sputum culture for Mycobacteria (23) versus hospitalized patients with a negative sputum culture (19). In both cases (left panel and right panel), a leave-one-out procedure was employed, where n–1 subjects were employed in the training set to construct the typicality matrices, and the outcome was predicted in one subject. The process was iterated n times, in order to predict the outcome in every subject. AUC = area under curve.

different tumor markers in serum.[21] Pattern recognition software has been employed for the rapid identification of Mycobacteria based upon their mycolic acid patterns detected by high-performance liquid chromatography.[22]

It is not yet known if the results of the breath test are affected by concomitant infection with HIV. This pilot study was insufficiently powered to resolve that concern, and larger future studies will be required to provide a definitive answer.

We conclude that breath testing, combined with multivariate analysis of data employing fuzzy logic or pattern recognition analysis, could potentially provide a new method for rapid, accurate, and non-invasive identification of patients at high risk of active pulmonary tuberculosis, and to distinguish between those with positive or negative sputum cultures. However, since these findings were derived from a comparatively small pilot study, confirmation will require additional studies in larger numbers of patients.

Acknowledgements

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