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DIAGNOSTICS

Point-of-care breath test for biomarkers of active pulmonary tuberculosis

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SUMMARY

Rationale: Volatile organic compounds (VOCs) in breath provide biomarkers of tuberculosis (TB) because *Mycobacterium tuberculosis* manufactures VOC metabolites that are detectable in the breath of infected patients.
Objectives: We evaluated breath VOC biomarkers in subjects with active pulmonary TB, using an internet-linked rapid point-of-care breath test.
Methods: 279 subjects were studied at four centers in three countries, Philippines, UK, and India, and data was analyzed from 251 (130 active pulmonary TB, 121 controls). A point-of-care system collected and concentrated breath and air VOCs, and analyzed them with automated thermal desorption, gas chromatography, and surface acoustic wave detection. A breath test was completed in 6 min. Chromatograms were converted to a series of Kovats Index (KI) windows, and biomarkers of active pulmonary TB were identified by Monte Carlo analysis of KI window alveolar gradients (abundance in breath minus abundance in room air).
Measurements and main results: Multiple Monte Carlo simulations identified eight KI windows as biomarkers with better than random performance. Four KI windows corresponded with KI values of VOCs previously identified as biomarkers of pulmonary TB and metabolic products of *M. tuberculosis*, principally derivatives of naphthalene, benzene and alkanes. A multivariate predictive algorithm identified active pulmonary TB with 80% accuracy (area under curve of receiver operating characteristic curve), sensitivity = 71.2%, and specificity = 72%. Accuracy increased to 84% in age-matched subgroups. In a population with 5% prevalence, the breath test would identify active pulmonary TB with 98% negative predictive value and 13% positive predictive value.
Conclusions: A six-minute point-of-care breath test for volatile biomarkers accurately identified subjects with active pulmonary TB.

An estimated 2 billion people, one third of the world's population, are infected with *Mycobacterium tuberculosis*.[1] Tuberculosis (TB) remains a leading cause of death from infectious disease, with an estimated 9.4 million new cases throughout the world every year.[2] Sputum smear microscopy remains the mainstay of diagnosis in resource-poor countries with a high TB burden, but the low sensitivity of this test results in patients with smear-negative but culture-positive pulmonary TB passing undetected through the health care system.[3] The high incidence of smear-negative TB in patients infected with HIV has further highlighted the clinical need

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for tests for TB that are not only sensitive and specific, but also rapid, non-invasive, and cost-effective.[4]

A breath test for volatile organic compounds (VOCs) could provide a rational test for active pulmonary TB because the causative organism, *M. tuberculosis,* manufactures VOC metabolites *in vitro*, and a number of these VOCs have been detected in the breath as apparent biomarkers of infection.[5,6] Breath biomarkers identified active pulmonary tuberculosis (TB) with 85% accuracy in a multicenter international study employing a breath collection apparatus (BCA) and VOC analysis with automated thermal desorption, gas chromatography and mass spectrometry (ATD-GC-MS).[7] Breath testing for active pulmonary TB appears rational and feasible, but clinical application has been limited by the cost of ATD-GC-MS and the requirement for highly trained technical staff.

However, recent advances in sensitive and cost-effective analytical instruments have enabled breath VOC microanalysis at a clinical point-of-care without the requirement for specialized laboratory resources. We report here an analytical system that was developed for rapid point-of-care collection and analysis of breath VOCs, and the evaluation of this system in a multicenter international study of breath VOC biomarkers in patients with active pulmonary TB.

1. Materials and methods

1.1. Clinical sites

Four tuberculosis treatment centers participated in the study, in the Philippines (University of Santo-Tomas, Manila, and De La Salle Health Sciences Institute, Cavite), UK (Homerton University Hospital, London) and India (Hinduja Hospital, Mumbai & Sir JJ Group of Hospitals, Mumbai).

1.2. IRB approval and informed consent

An Institutional Review Board (IRB) at each collaborating site approved the research. All subjects gave their signed informed consent to participate. Assent from adolescent subjects and consent from a parent or legal guardian was obtained for subjects 13–16 yr in England or younger than 18 yr at sites in other countries.

1.3. Human subjects

279 subjects were recruited according to the following criteria:

1.3.1. Control group – inclusion criteria

1. Subject is older than 13 years of age
2. Subject is undergoing screening for pulmonary TB without clinical evidence of active TB

1.3.2. Exclusion criteria

1. Clinical suspicion of pulmonary TB based on: symptoms and signs e.g. cough, sputum production, night sweats, weight loss or hemoptysis
   OR: history of known recent exposure to infection
   OR: chest X-ray abnormalities consistent with active pulmonary TB
2. Positive sputum smear test or positive sputum culture.

1.3.3. Disease group – inclusion criteria

1. Subject is older than 13 years of age
2. Clinical suspicion of pulmonary TB based on: symptoms and signs e.g. cough, sputum production, night sweats, weight loss or hemoptysis
   OR: history of known recent exposure to infection
   OR: chest X-ray abnormalities
   OR: positive sputum smear consistent with active pulmonary TB
   OR: sputum culture results positive or pending

1.3.4. Exclusion criteria

1. Subject is currently taking anti-TB therapy or has received more than 7 days of anti-TB therapy in the past six months

1.4. Point-of-care breath test

The BreathLink system developed for this study comprised three main components:

1. *Breath VOC sample collector and concentrator (BCA)*: The front end of the system, the BCA method for collection and concentration of alveolar breath VOC samples has been described.[7] In summary, a subject wore a nose-clip and respired normally for 2.0 min, inspiring room air from a valved mouthpiece, and expiring into a breath reservoir through with a bacterial filter. The valved mouthpiece and the bacterial filter were disposed after use. A one-way outlet valve in the mouthpiece prevented backflow of breath into the mouth, and the 6-micron bacterial filter blocked transmission of *Mycobacteria* or other microorganisms. The mouthpiece and filter presented low resistance to expiration, so that subjects could donate breath samples without effort or discomfort. Alveolar breath VOCs were pumped from the breath reservoir through a sorbent trap where they were captured and concentrated. VOCs in a similar volume of room air were separately collected and concentrated in the same fashion.
2. *Breath VOC analyzer:* An analyzer was developed for the BreathLink system, employing a portable gas chromatograph coupled to a surface acoustic wave (SAW) detector. The VOC sample was thermally desorbed from the sorbent trap in a stream of helium carrier gas and separated on a GC column with thermal ramping. VOCs were detected with a single non-functionalized SAW solid-state mass-sensitive detector with picomolar sensitivity and universal selectivity; the principles have been reported.[8] The analyzer was calibrated daily with an external standard, a mixture of C6 to C22 n-alkanes (Restek Corporation, Bellefonte, PA 16823, USA). Each breath test comprising collection and analysis of separate samples of breath and room air was completed within 6 min.
3. *Control software:* Custom software was developed for the BreathLink system and installed on a secure computer at the point-of-care where it performed the following functions:
   a *Control of instrument functions*. The software automatically controlled breath and air sample collections with the BCA, and analysis with the breath VOC analyzer.
   b *Electronic Case Report Form (eCRF):* Collaborators at clinical sites entered subject data into an electronic case report form (eCRF) with a unique identification number. Subject names were not recorded, except in a separate confidential log maintained at the clinical site. A menu-driven program prevented collection of a breath sample unless all inclusion and exclusion criteria were fulfilled. The eCRF comprised demographic data and clinical information including chest X-ray reports and results of sputum smear microscopy and culture.
   c *File storage, encryption, and transmission:* Files containing de-identified chromatographic raw data and eCRFs were stored locally on the computer, then encrypted and transmitted via the internet to a server at the Menssana Research

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Breath Research laboratory in Newark, NJ, USA, where they were decrypted and stored for analysis. An industry standard hypertext transfer protocol secure (https) connection ensured data security.

Instrument detection limit is defined as the analyte concentration that is required to produce a signal greater than three times the standard deviation of the noise level[9] and was determined for tridecane (Sigma Aldrich, St. Louis, MO 63103) serially diluted in methanol.

1.5. Analysis of data

In summary, the retention time of each chromatographic peak was normalized according to its Kovats Index (KI) (retention time relative to known n-alkane standards)[10,11] and the chromatogram was converted into a series of data points by segmenting it into a series of 100 KI windows. The alveolar gradient of each KI window (i.e. abundance in alveolar breath minus abundance in ambient room air) was determined as: alveolar gradient $= V_b/I_b - V_a/I_a$ where V_b = integrated abundance of VOCs in breath observed with SAW detector, and I_b = area under the curve (AUC) of the chromatographic peak associated with the external control standard. V_a and I_a were corresponding values observed in the associated sample of ambient room air. The alveolar gradient varies with rate of synthesis of a VOC minus its rate of clearance, so that a positive value indicates that a VOC was synthesized at a greater rate than it was cleared from the body, and vice versa for a negative value.[12,13]

1.6. Identification of biomarkers and construction of predictive algorithm

Multiple Monte Carlo simulations were employed to identify the KI windows that identified disease with greater than random accuracy. The method has been described.[7] In summary, the alveolar gradients of all KI windows were compared in the disease and control groups and ranked as candidate biomarkers according to their C-statistic values i.e. the AUC of the receiver operating characteristic (ROC) curve.[14] The average random behavior of each chromatographic KI window was determined with multiple Monte Carlo simulations by randomly assigning subjects to the disease or control group, and performing 40 estimates of the C-statistic value. Differences between the C-statistic values obtained with correct diagnosis and random diagnosis identified the KI windows that were true biomarkers, because they identified the disease group with better than random accuracy.[15,16] The KI windows identified as biomarkers of disease were employed to construct a multivariate predictive algorithm with weighted digital analysis (WDA).[17]

1.7. Comparison of KI windows to previously reported biomarkers

KI window values were compared to KI values of previously reported VOC biomarkers of active pulmonary TB[5,7] employing a database maintained by the National Institute of Standards and Technology (NIST Standard Reference Database Number 69).[18] Also, pure samples of benzene, 1,3,5-trimethyl- and toluene were analyzed with the BreathScanner GC-SAW.

2. Results

2.1. Human subjects

279 subjects fulfilled recruitment criteria and 251 were entered into data analysis. Characteristics and exclusions are shown in Table 1. No adverse effects of the breath test were reported.

Instrument detection limit requirements were fulfilled by 0.1 μL of 0.1 ppt tridecane solution, which was equivalent to less than 10^{-12} mol tridecane in a breath sample.

Table 1
Characteristics of human subjects.

Control group		Technical quality of breath test	
Site	No. recruited	Unsatisfactory	Satisfactory
Cavite	47	5	42
London	17	4	13
Manila	52	6	46
Mumbai	22	2	20
Total	138	17	121
Male			53
Female			68
Total			121

Disease group	Technical quality of breath test			Smear positive	Culture-positive	Chest X-ray positive	Total positive**
Site	No. recruited	Unsatisfactory	Satisfactory				
Cavite	30	0	30	30	30	30	30
London	3	0	3	1	3	3	3
Manila	60	4	56	56	55	56	56
Mumbai	48	7	41	38	7	41	41
Total	141	11	130	126	96	130	130
Male			86				
Female			44				
Total			130				

Age (yr)	Controls	Disease group
Mean	33.31	40.86
SD	13.46	16.57

$p < 0.0001$.
2-tailed *t*-test.
All subjects with technically unsatisfactory breath test samples were excluded from analysis. Subjects who were initially recruited to the control group but were found to have a positive sputum culture or a positive sputum smear, or a chest X-ray consistent with active pulmonary TB were transferred to the disease group. Inclusions in the disease group. **Subjects were included as positive for active pulmonary TB and entered into the analysis of data if they had a positive sputum culture and/or positive sputum smear microscopy and/or chest X-ray consistent with active pulmonary TB.

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2.2. Identification of KI window biomarkers

Multiple Monte Carlo simulations identified eight chromatographic KI windows as biomarkers with better than random performance (Figure 1a). Figure 2 displays KI values of these biomarkers in controls and in subjects with pulmonary TB in a heatmap of chromatographic alveolar gradients. Four KI window biomarkers corresponded with KI values of VOCs previously reported as biomarkers of pulmonary TB and in vitro metabolites of *M. tuberculosis*[5–7] (Table 2).

2.2.1. Predictive algorithm and ROC curve

The WDA multivariate predictive algorithm identified active pulmonary TB with 80% accuracy. Cumulative accuracy of the algorithm and ROC curve are shown in Figure 1b and c.

2.2.2. Positive and negative predictive values (PPV and NPV)

Figure 1d displays the expected variation of PPV with NPV of the test in a high-burden setting with 5% prevalence of active pulmonary TB, and Figure 3 displays the expected outcome when a combination of sensitivity and specificity values was selected to result in NPV = 98%.

2.3. Effect of age on accuracy of predictive algorithm

Patient subsets were selected in the age range 30–65 yr (controls $n = 45$, mean = 43.89 yr, SD = 10.9, disease group $n = 72$, mean = 46.1 yr, SD = 10.14, 2-tailed t-test $p = 0.26$, NS). When the same predictive algorithm was applied to these subsets, AUC of ROC curve = 0.84.

3. Discussion

The main finding of this study was that a model based on a point-of-care breath test for volatile biomarkers identified active pulmonary TB with 80% accuracy overall, increasing to 84% accuracy in age-matched subsets. This was consistent with our previous

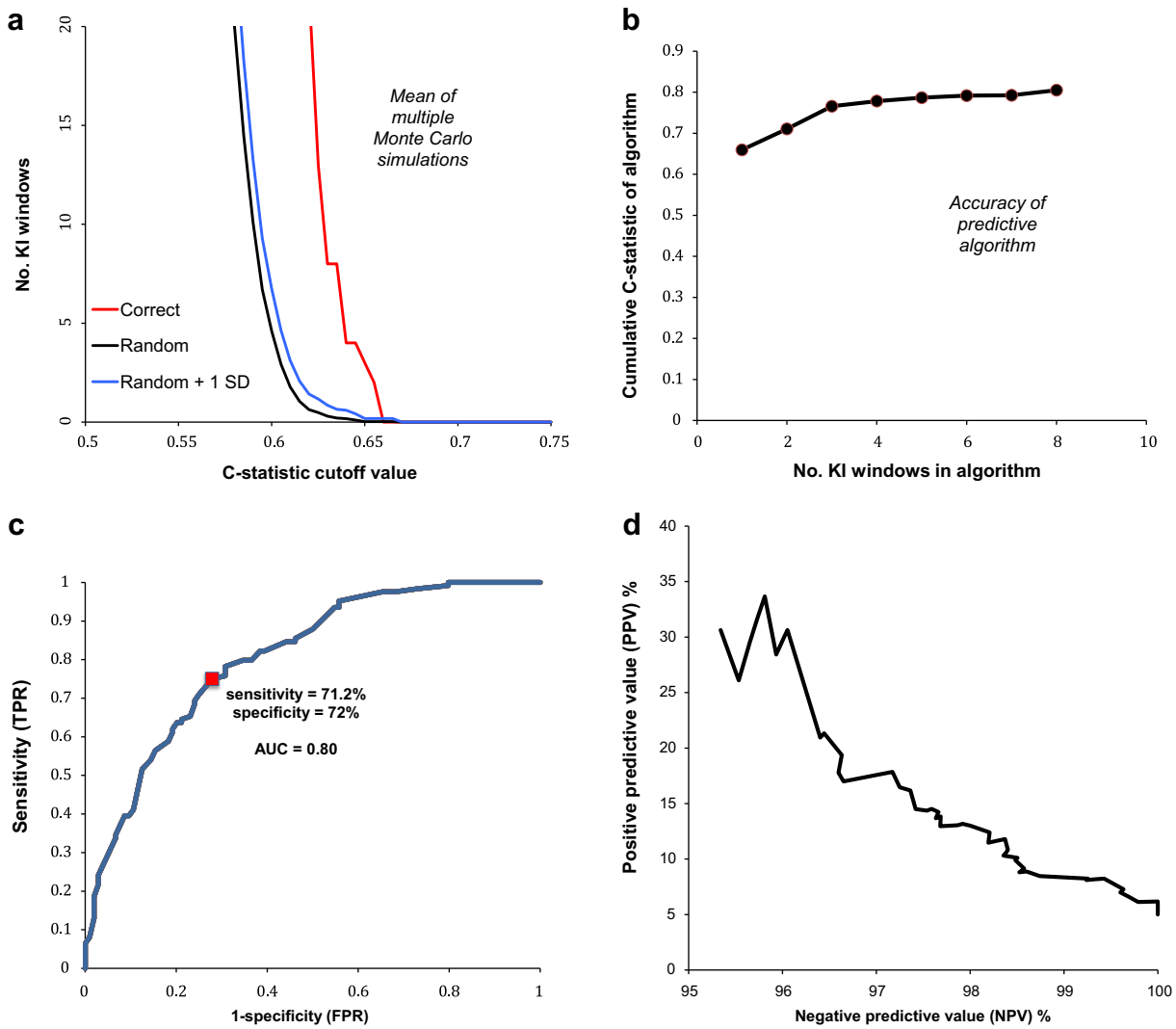

Figure 1. Analysis of breath VOC data. Top left panel 1a: Monte Carlo identification of significant biomarkers. The "correct" curve shows the number of KI windows with the highest C-statistic values that correctly identified pulmonary TB. The C-statistic is the AUC of the ROC curve of a KI window when it was employed alone as a biomarker of disease. The "random" curve shows the number of KI windows with the highest C-statistic values that identified pulmonary TB by chance alone. The random C-statistic values of all KI windows were determined in 40 Monte Carlo simulations in which subjects were randomly assigned to the disease group or the control group. Where C-statistic = 0.64, the "random" curve fell to <1, but eight KI windows in the "correct" curve identified the disease group with better than random accuracy. SD = standard deviation. Top right panel 1b: Accuracy of predictive algorithm. The significant biomarker KI windows identified with Monte Carlo simulations were entered into a multivariate predictive algorithm. The algorithm achieved 80% accuracy with eight biomarkers, and achieved near-maximal accuracy with only three biomarkers. Bottom left panel 1c: Receiver operating characteristic (ROC) curve of predictive algorithm. The figure displays true-positive rate (TPR) (sensitivity) versus false-positive rate (FPR) (1-specificity), and the point on the curve where the sum of sensitivity plus specificity was maximal. Bottom right panel 1d: Variation in PPV with NPV. This figure displays the expected outcome PPV and NPV of the breath test in a high-burden setting with 5% prevalence of active pulmonary TB, based on the sensitivity and specificity values shown in the ROC curve.

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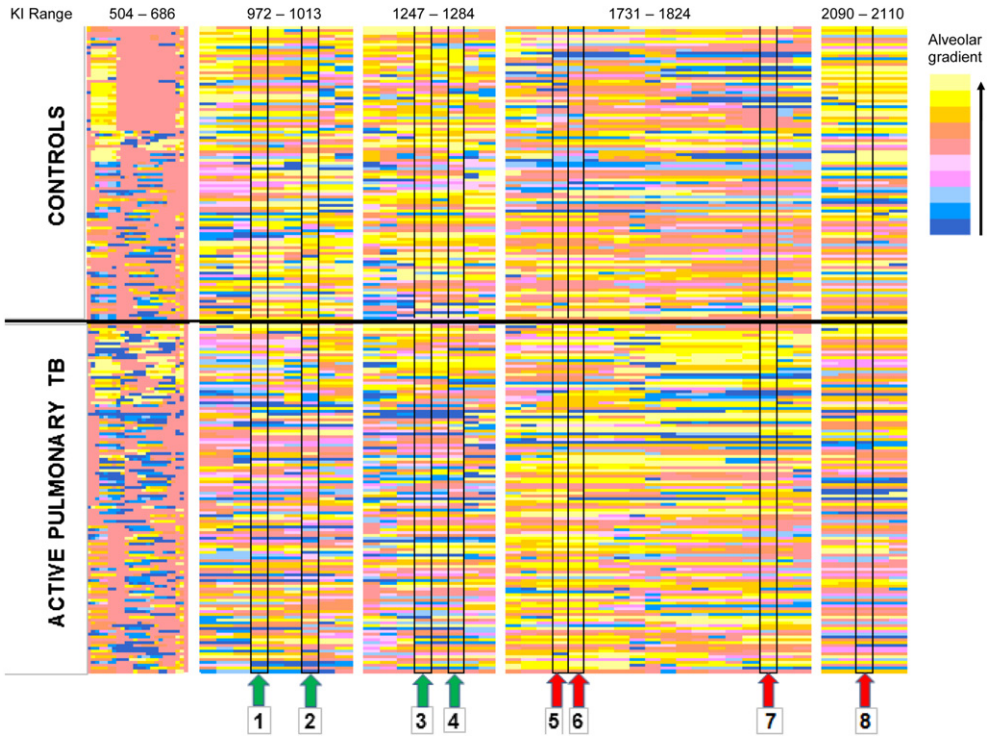

Figure 2. Heatmap of chromatographic KI windows. The heatmap displays KI windows in controls (upper panel) and in subjects with active pulmonary TB (lower panel). The horizontal axis indicates the value of each KI window. The color of each KI window varied on a scale from one to ten (scale shown at right). The scale was calculated for each KI window by dividing the range between the highest and lowest observed value of the alveolar gradient into ten equal parts. Vertical black lines with adjacent numbered arrows indicate eight KI windows that were identified as biomarkers of active pulmonary TB because they were predominantly darker in the controls than in active pulmonary TB, or vice versa, and multiple Monte Carlo simulations identified these differences in alveolar gradient as greater than predicted by chance alone. The KI windows indicated with green arrows corresponded with the KI values of VOCs previously reported as biomarkers of active pulmonary TB and metabolic products of *Mycobacterium tuberculosis* (see Table 2).

report of a laboratory-based assay for breath biomarkers that identified active pulmonary TB with 85% accuracy.[7] Four KI windows corresponded with KI values of VOCs previously identified as breath biomarkers of pulmonary TB and metabolic products of *M. tuberculosis*, principally derivatives of naphthalene, benzene and alkanes.[5–7] This correspondence provides presumptive evidence from two independent clinical studies that these breath VOCs are biomarkers of active pulmonary TB.

This study also provided proof of concept that breath biomarkers identified in the laboratory with advanced analytical instruments may be successfully identified with a point-of-care system employing a faster and less expensive instrument platform. In practice, the rapid point-of-care breath test combined with internet transmission of clinical and chromatographic data was convenient and effective. Clinical sites in countries widely separated by geography were able to complete a subject's breath test in a standardized fashion in 6 min, and pool their data promptly.

The biological origins of breath VOC biomarkers of active pulmonary TB were consistent with metabolic products derived from the infective organism, the host, or both. *M. tuberculosis* manufactures a spectrum of VOCs *in vitro*, including methylated derivatives of n-alkanes, naphthalene and benzene.[5,6] The metabolic source of these products and their biological significance are unknown.

Infection of the host with TB causes increased oxidative stress, which can result in increased excretion of n-alkanes and their derivatives in the breath.[19–21] The heatmap (Figure 2) demonstrates that different VOCs in the biomarker KI windows were either increased or decreased in abundance in active pulmonary TB.

Table 2
Correspondence between biomarkers of active pulmonary TB.

Biomarker number	KI window	Breath biomarker VOCs	*Mycobacterium tuberculosis in vitro* VOCs
1 & 2	965–1030	camphene; l-beta-pinene; benzene, 1,3,5-trimethyl-	heptane,2,2,4,6,6-pentamethyl-
3 & 4	1243–1313	naphthalene,1-methyl-; tridecane; 1-octanol, 2-butyl; dodecane, 4-methyl	naphthalene, 1-methyl-

The point-of-care breath test identified a group of KI windows as biomarkers of active pulmonary TB (Figure 2). Four of these KI windows corresponded with the KI values of VOCs previously identified as breath biomarkers of active pulmonary TB, or VOC products of *M. tuberculosis in vitro*.[5–7] Biomarkers 1, 2, 3 and 4 in this table correspond with the KI windows indicated by green arrows 1, 2, 3 and 4 in the heatmap (Figure 2). Based on their chromatographic retention times, this correspondence provides presumptive evidence, though not definitive proof, that the listed VOCs are similar to those identified in the heatmap. KI values were obtained from the NIST Standard Reference Database Number 69.[18] GC-SAW analysis of pure samples of benzene, 1,3,5-trimethyl- and toluene yielded similar KI values. The presence of more than one candidate VOC in a single KI window may have arisen from their coelution in a single peak in the gas chromatogram. The VOC biomarkers listed above are similar, but not identical to those identified in previous reports.[5–7] These differences may have arisen in part from differences in experimental design, diagnostic criteria and assay methodology. In particular, the assay parameters and instrumentation employed with the rapid point-of-care ATD-GC-SAW using a short 1 M column would be expected to yield different selectivity and sensitivity values for breath VOCs compared to the much slower assays with laboratory-based ATD-GC-MS using a 30 M column.

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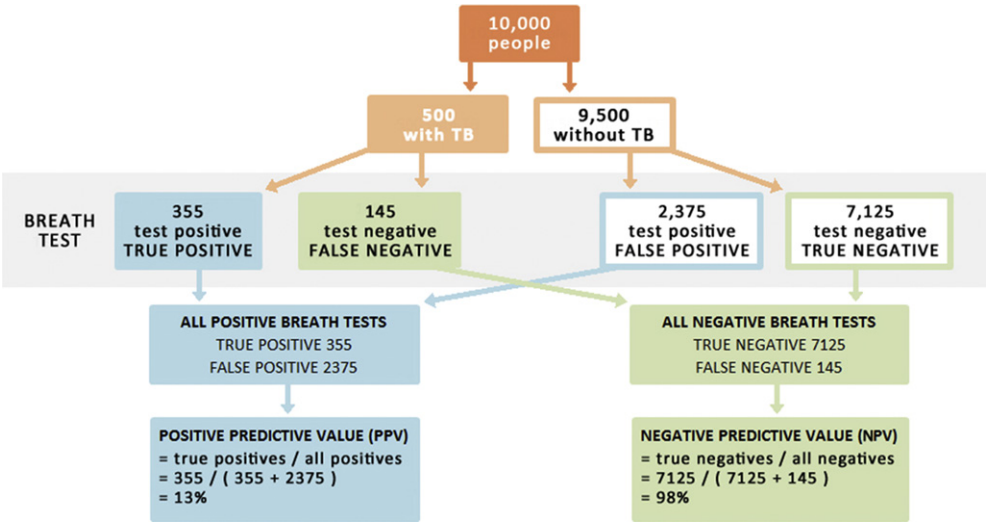

Figure 3. Predicted outcome of breath test for active pulmonary TB: A specified target value of NPV or PPV may be achieved by selecting a cutoff point on the ROC curve (Figure 1) with an appropriate combination of sensitivity and specificity. In this example, breath testing could achieve a desired target value of NPV = 98% in a population of 10,000 people with a high (5%) prevalence of active pulmonary TB by selecting a cutoff point on the ROC curve where sensitivity = 71% and specificity = 75%.

Decreased abundance of breath VOCs may have resulted from *M. tuberculosis* catabolizing human metabolic products as nutrients. This hypothesis is consistent with mycobacterial catabolism of other host-derived nutrients including carbohydrates, amino acids, phosphate, and cholesterol,[22,23] as well as with reports that alkanes, alkane derivatives or benzene derivatives can all sustain mycobacterial growth as nutritional substrates.[24–26]The potential clinical utility of the point-of-care breath test for active pulmonary TB may be estimated from its positive and negative predictive values, which will vary with the prevalence of disease in the study population. Figure 1 displays the expected values of PPV and NPV in a population with a high prevalence of active pulmonary TB. In the example shown in Figure 3, an NPV of 98% was associated with a PPV of 13%. When the NPV was increased to 99%, the PPV declined to 10.1%. These findings illustrate a conundrum that public health physicians frequently encounter when planning to screen a large population for a disease: PPV increases as NPV decreases, and vice versa. The choice of a test cutoff point affects costs as well as patient benefits. Ideally the NPV should be set as high as possible in order to minimize the number of infected persons who test as false-negatives and whose disease goes undetected. However, the PPV should also be set as high as possible in order to minimize the much larger number of non-infected persons who test as false-positives, and who might potentially overwhelm the health care system. In practice, breath testing might be employed in resource-poor environments as a cost-effective first step in a two-stage evaluation process: Subjects with a negative test result could be reassured that they not have active pulmonary TB with 98% certainty, so that no further testing is necessary, while those with a positive test result could be referred for more costly evaluation with sputum culture and nucleic acid amplification testing (NAAT). Although costs have not yet been determined, a rapid point-of-care breath test would probably cost significantly less than sputum culture or NAAT because neither sputum collection nor expensive reagents are required.

Historically, most advances in breath testing resulted from innovation in analytical technology. The invention of chemical colorimetry in the 19th century enabled Anstie to develop the first breath test for alcohol in 1874.[27] Nearly 100 years later, advances in gas chromatography enabled Linus Pauling to discover large numbers of low-molecular weight VOCs in concentrated human breath[28] Subsequent research has identified more than 3000 different VOCs in human breath,[29] and demonstrated breath VOC biomarkers in diseases including lung cancer[17] and breast cancer.[30] Tests employing array technologies have also been proposed to identify lung cancer and other diseases based on their response to a unique pattern of breath VOCs[31,32]; their advantage is simplicity because they do not employ GC separation of VOCs, however their disadvantage is that they do not identify or quantify specific VOCs in a breath sample. Recent advances in ATD-GC-SAW now enable breath testing at the clinical point-of-care with sensitivity comparable to laboratory-based ATD-GC-MS, but with instruments that are smaller, faster, less expensive, and simpler to operate. A laboratory-based ATD-GC-MS fully equipped for breath VOC analysis currently costs nearly $200,000, while a point-of-care ATD-GC-SAW costs only one-tenth as much. Analysis of a breath VOC sample with laboratory-based ATD-GC-MS employing a 30 M column may require 60 min of instrument time, while the point-of-care ATD-GC-SAW employed in this study analyzed breath VOC samples in less than one-tenth the time. Despite the disadvantages of lower selectivity and the lack of MS identification of breath VOCs, the point-of-care ATD-GC-SAW instrument delivered diagnostic sensitivity and specificity for active pulmonary TB that was comparable to laboratory-based ATD-GC-MS. This finding illustrates that laboratory-based and point-of-care analytical technologies may be complementary rather than competitive.

Although sputum culture is commonly employed as the gold standard of diagnosis of active pulmonary TB, the test is frequently inaccurate. Published reports of its accuracy vary widely, with sensitivity ranging between 40 and 81.5% and specificity between 85 and 98.4%.[33–35] In resource-limited settings, TB is often diagnosed with high accuracy by non-physicians based on acid-fast bacilli smear, chest X-ray, and clinical history. TB frequently presents a complex diagnostic problem in which different tests contribute important information to clinical risk assessment.[36,37] For this reason, we ranked subjects as positive for active pulmonary TB if they had positive sputum culture and/or positive sputum smear microscopy and/or chest X-ray consistent with active pulmonary TB. The diagnostic criteria employed in this study may have introduced a potential source of error, because the observed accuracy of a biomarker cannot exceed the accuracy of the diagnostic gold standard to which it is compared. This may be

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illustrated by considering a hypothetical Biomarker X that is 100% accurate. In a clinical study, all subjects with disease will test positive for Biomarker X. However, an imperfect gold standard will generate some false-negatives, so the positive test results from Biomarker X in these subjects will be scored as false-positives, even though they were correct in reality. Similarly, false-positive results with the imperfect gold standard will be scored as false-negatives for Biomarker X. Consequently, the observed total number of false-positives and false-negatives will be the same for both the imperfect gold standard and for Biomarker X, so that the observed accuracy of Biomarker X will underestimate its true value. Consequently, the 80% accuracy of the breath test for active pulmonary TB observed in this study may have been an underestimate of its true value.

We conclude that this is the first report of a rapid point-of-care breath test for VOCs exhaled in picomolar concentrations, and that this test detected volatile biomarkers of active pulmonary TB consistent with biomarkers previously reported using laboratory-based instrumentation. The point-of-care breath test was rapid, accurate, and cost-effective, and could potentially provide a clinically valuable new tool for detection of active pulmonary TB.

Author's contributions

Michael Phillips and Jaime Blais designed and supervised the study and drafted the manuscript. Anirudh Chaturvedi and Urvish Patel constructed and maintained the BreathLink systems. Anirudh Chaturvedi, Urvish Patel & Mauli Pandya performed quality control of the chromatograms and clinical data. Peter Schmitt and Anirudh Chaturvedi developed the software for the BreathLink system. Peter Schmitt analyzed the data statistically. Victoria Basa-Dalay, Graham Bothamley, Kinjal D. Modi, Maria Piedad R, Natividad Nagsen N Ramraje and Zarir F Udwadia collected data for the study. All authors reviewed the manuscript and participated in its revisions.

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Competing interests: None declared.

Ethical approval: Ethical approval was obtained from IRBs at all sites.

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