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RESEARCH ARTICLE | *Translational Physiology*

Exhaled breath metabolomics reveals a pathogen-specific response in a rat pneumonia model for two human pathogenic bacteria: a proof-of-concept study

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van Oort PM, Brinkman P, Slingers G, Koppen G, Maas A, Roelofs JJ, Schnabel R, Bergmans DC, Raes M, Goodacre R, Fowler SJ, Schultz MJ, Bos LD; The BreathDx Consortium. Exhaled breath metabolomics reveals a pathogen-specific response in a rat pneumonia model for two human pathogenic bacteria: a proof-of-concept study. *Am J Physiol Lung Cell Mol Physiol* 316: L751–L756, 2019. First published February 13, 2019; doi:10.1152/ajplung.00449.2018.—Volatile organic compounds in breath can reflect host and pathogen metabolism and might be used to diagnose pneumonia. We hypothesized that rats with *Streptococcus pneumoniae* (*SP*) or *Pseudomonas aeruginosa* (*PA*) pneumonia can be discriminated from uninfected controls by thermal desorption-gas chromatography-mass-spectrometry (TD-GC-MS) and selected ion flow tube-mass spectrometry (SIFT-MS) of exhaled breath. Male adult rats (n = 50) received an intratracheal inoculation of *1*) 200 μl saline, or *2*) 1×10^7 colony-forming units of *SP* or 3) 1×10^7 CFU of *PA*. Twenty-four hours later the rats were anaesthetized, tracheotomized, and mechanically ventilated. Exhaled breath was analyzed via TD-GC-MS and SIFT-MS. Area under the receiver operating characteristic curves (AUROCCs) and correct classification rate (CCRs) were calculated after leave-one-out cross-validation of sparse partial least squares-discriminant analysis. Analysis of GC-MS data showed an AUROCC (95% confidence interval) of 0.85 (0.73–0.96) and CCR of 94.6% for infected versus noninfected animals, AUROCC of 0.98 (0.94–1) and CCR of 99.9% for *SP* versus *PA*, 0.92 (0.83–1.00), CCR of 98.1% for *SP* versus controls and 0.97 (0.92–1.00), and CCR of 99.9% for *PA* versus controls. For these comparisons the SIFT-MS data showed AUROCCs of 0.54, 0.89, 0.63, and 0.79, respectively. Exhaled breath analysis discriminated between respiratory infection and no infection but with even better accuracy between specific pathogens. Future clinical studies should not only focus on the presence of respiratory infection but also on the discrimination between specific pathogens.

biomarkers; exhaled breath analysis; infection; pneumonia

INTRODUCTION

Exhaled breath analysis of volatile organic compounds (VOCs) represents a promising new technique for diagnosing respiratory infection (12, 20, 24). Our recent review (19), however, has shown that current studies using breath analysis did not show sufficient diagnostic accuracy and lack consistency to be used for pneumonia in mechanically ventilated intensive care unit (ICU) patients.

Studies investigating individual infection related VOCs or VOC patterns in human breath encounter certain challenges, such as *1*) all possible pathogens are investigated at once; *2*) for pneumonia no gold standard is available (13); and *3*) due to coexisting factors such as comorbidities, drugs, and diet, it might be difficult to determine the biochemical origin of VOCs. The application of exhaled breath metabolomics or breathomics is rapidly expanding (6, 18). Specific VOC profiles for certain bacterial strains can be identified (5). In vitro studies using bacterial cultures (16, 17) do not take into account the host response, and bacteria appear to grow differently in culture media compared with living lung tissue (8).

To date animal studies investigating VOCs for diagnosis of pneumonia (1, 28, 29) primarily used secondary electrospray ionization-mass spectrometry (SESI-MS) as analytical platform for breath analysis, resulting in breathprint patterns associated with certain microorganisms. However, identification of specific individual VOCs is preferable, since this could guide future human studies. Capture of breath on suitable sorbent tubes followed by thermal desorption into gas chromatography-mass spectrometry (TD-GC-MS) can identify individual VOCs and is currently seen as the gold standard regarding exhaled breath analysis (8). Selected ion flow tube-mass spectrometry (SIFT-MS) offers the possibility of online breath analysis and thus might enable future application for exhaled breath monitoring at the patient's bedside.

Within the scope of this study, exhaled breath in a rat pneumonia model was investigated for two common causative pathogens of pneumonia: *Streptococcus pneumoniae* (*SP*) and *Pseudomonas aeruginosa* (*PA*). It was hypothesized that *1*) rats with *SP* or *PA* pneumonia can be discriminated from unin-

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fected controls, and *2*) the different pathogens can be distinguished using exhaled breath analysis.

METHODS

The study was approved by the Animal Welfare Body at the AMC Amsterdam, the Netherlands (project number LEICA125AD-1).

Experimental groups. Male adult specific pathogen-free Sprague-Dawley rats ($n = 50$) weighing ~350 g (Envigo, Netherlands) received an intratracheal inoculation of either: *1*) a total of ~1×10^7 colony-forming units (CFU) of *SP* (6303; ATCC, Rockville, MD) ($n = 18$); or *2*) a total of ~1×10^7CFU of *PA* (PA103; Iglewski Laboratory) ($n = 16$), under light anesthesia using isoflurane 3%; or *3*) 200 μl saline ($n = 16$) for the control group.

Anesthesia and mechanical ventilation. Twenty-four hours after inoculation, an anesthetic mixture (0.15 ml/100 g body wt) of 1.8 ml ketamine (100 mg/ml; Eurovet Animal Health), 0.5 ml dexmedetomidine (0.5 mg/ml; Vetoquinol), 0.2 ml atropine (0.5 mg/ml; Eurovet Animal Health), and 0.5 ml NaCl 0.9% was injected. The rats were weighed, tracheotomized, and connected to a mechanical ventilator (Dräger). The rats were pressure controlled ventilated with 16 cmH_2O over 2 cmH_2O positive end-expiratory pressure, using a fraction of inspired oxygen of 32%.

Exhaled breath collection. For breath sampling, a stainless steel tube filled with sorbent material (for GC-MS: Tenax GR 60/80; Interscience; and for SIFT-MS Carbograph 1TD/Carbopack X; Markes International) was inserted between the expiratory ventilator tubing and a pump (Markes). For 10 min, VOCs were absorbed onto the steel sorbent tube with a flow of 100 ml/min. The sorbent tubes were stored at 4°C for a maximum of 14 days until analysis.

Other samples. Directly after collection of the exhaled breath samples, the rats were euthanized. For the bronchoalveolar lavage (BAL) sample, three 2-ml aliquots of saline were instilled and directly withdrawn from the right lung. The upper lobe of the left lung was fixed in 4% buffered formaldehyde for later paraffin embedding, sectioning, and staining at the pathology department. The middle and lower lobes of the left lung were homogenized.

Thermal desorption gas chromatography–mass spectrometry. Sorbent tubes were placed within a TD unit (TD100; Markes) and heated (250°C for 15 min, flow 30 ml/min). The VOCs were captured on a cold trap (5°C), which was rapidly heated to 300°C for 1 min, after which the molecules were splitless injected through a transfer line at 120°C onto an Inertcap 5MS/Sil GC column (30 m, 0.25-mm diameter, 1-μm film thickness, and 1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane; Restek) at 1.2 ml/min. The oven temperature was isothermal at 40°C for 5 min and then increased to 270° at 10°C/min and kept isothermal at 270°C for 5 min.

Molecules were ionized using electron ionization (70 eV), and the fragment ions were detected using a quadrupole mass spectrometer (GCMS-GP2010) with a scan range of 37–300 Da. Ion fragment peaks were used for statistical analysis. The predictive fragment ions were manually checked in the raw chromatograms, and corresponding metabolites were tentatively identified using National Institute of Standards and Technology library (NIST, Gaithersburg, MD); we followed the Metabolomics Standards Initiative for metabolite identification (26).

Thermal desorption selected ion flow tube–mass spectrometry. The discriminatory power of the GC-MS and SIFT-MS full-scan VOC patterns was compared. SIFT-MS (Voice200; Syft Technologies) was used as an offline instrument in combination with a TD unit (UNITY; Markes). A full scan was performed in the mass-to-charge (m/z) ratio of 15+ to 200+, without the limitation of changing VOC levels throughout breathing maneuvers, as would be the case when analyzing online. Sorbent tubes were placed in an autosampler (ULTRA; Markes) connected to the TD unit. TD was performed in tube conditioning mode and the tubes were heated to 270°C (flow 30 ml/min) for 10 min. The VOCs were recollected in a 1-liter Tedlar gas sampling bag (Sigma-Aldrich) at the split outlet. The Tedlar bag was placed at the sample inlet of the SIFT-MS (Voice200; Syft Technologies) and full scan was initiated with a scan range from m/z 15+ to 200+ for 3 precursor ions (H_3O+, NO+, and O_2+), a dwell time of 100 ms, a count limit of 10,000, and 8 repeats. Raw data in counts/second of all scanned ions were corrected for the instrument calibration function (ICF) of the measurement day. The ion counts were multiplied by the ion-specific instrument calibration function. The ICF-corrected data were then used for statistical analysis.

Infection assessment. Serial 10-fold dilutions of the homogenized lung and the BAL fluid were plated on blood agar plates and incubated overnight at 37°C. The number of CFUs was counted the next morning. Cell counts in the BAL fluid were measured (Z2 Coulter Particle Counter; Beckman Coulter), and neutrophils were counted (Cytospin 4 Cytocentrifuge; Thermo Scientific).

Histologic examination of the 4-μm hematoxylin and eosin-stained lung sections was performed by a pathologist blind to group identity. Lung inflammation and damage were determined using a lung infection scoring system as described previously (4).

Data analysis. All statistical analyses were performed in R statistics through the R-studio interface (22). A $P \leq 0.05$ was considered statistically significant for single comparisons. P values were corrected for multiple testing by Benjamini-Hochberg correction (2). Diagnostic accuracy was measured by the area under the receiving operating characteristics curve (AUROCC).

The allocation of an animal to pneumonia or control group was the primary dependent variable. All analyses were repeated for *SP* versus control, *PA* versus control and *SP* versus *PA*, to study the interpathogen variance. The VOCs measured by TD-GC-MS and SIFT-MS were used as two separate predictor matrices for pneumonia status.

First, high-dimensional data sets with VOCs were reduced by principal component (PC) analysis. The first six PCs were retained, capturing 57% of variance. A conservative number of PCs was used because of the relatively low number of animals. Mann-Whitney U-test was used to test differences in PCs between groups. PCs with a $P \leq 0.1$ were used for logistic regression (LR) analysis (14). Second, individual VOCs were compared using the "limma" package, and P values and fold changes are reported and shown in a volcano plot. VOCs with an adjusted $P \leq 0.05$ were identified. Third, sparse partial least square discriminant analysis (sPLS-DA; MixOmics package) with leave-one-out cross validation was used to identify the most discriminatory VOCs and estimate the accuracy of such a selected data set. We could not use bootstrap analyses due to low sample number so we employed leave-one-out where data from an individual animal was left out of the modeling. The correct classification rate (CCR) was calculated by comparing the AUROCC of the leave-one-out cross-validated model to a similarly constructed model for 1,000 randomly permutated labels, as is recommended (27).

RESULTS

All animals survived the 24 h postinoculation and 1-h period of mechanical ventilation. Median BALF white cell count was (in cells/ml) 13.8×10^5 [interquartile ratio (IQR): 8.7×10^5–16.7×10^5) for the *SP* rats, 5.9×10^5 (IQR: 4.0×10^5–11.2×10^5) for the *PA* rats, and 1.3×10^5 (IQR: 1.2×10^5–1.5×10^5) for the control rats ($P < 0.001$). The CFU counts of the BALF samples differed significantly between the groups: no CFUs were seen on the agar plates for BALF of the *PA* and control rats, compared with a median of 4.8×10^6 (IQR: 1.2–8.8×10^6) CFU/ml for the *SP* animals ($P < 0.001$). Only the homogenate of the *SP* group showed significant growth ($P < 0.001$; 1.0×10^9 (IQR: 7.4–1.0×10^9) CFU/ml), compared with 650 (IQR: 0–4.4×10^3) CFU/ml for the *PA* rats and 0 (IQR: 0–1.4×10^3) CFU/ml for the controls. Microscopic counts of the percentages of neutrophils present

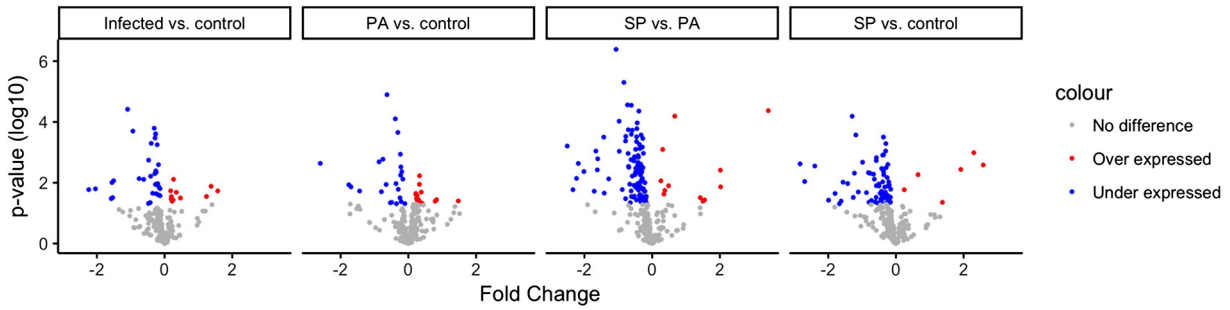

Fig. 1. Volcano plots for the group comparisons.

on the stained cytospin preparations differed between groups ($P < 0.001$), with a median of 88.5 (IQR: 72.5–95.3) for the *SP* animals, 81 (IQR: 68.5–89) for the *PA* group, and 2.5 (IQR: 0–5) for the controls.

The percentage of pneumonia on histopathological investigation was significantly higher in the *SP* rats ($P < 0.001$). Pneumonia scores were significantly higher in the infected versus the noninfected animals: median pneumonia score was 8 (IQR: 6–10.5) for the *SP* rats and 5.5 (IQR: 3–6.5) for the *PA* rats, compared with 3 (IQR: 2–4) for the controls ($P < 0.001$).

Thermal desorption gas chromatography–mass spectrometry The analysis of significant PCs (using PCs 1, 4, and 5) and subsequent LR model for infected versus noninfected animals showed an AUROCC of 0.93 [95% confidence interval (CI): 0.85–1]. The AUROCC (using PC 1, 4, and 5) was 0.93 (95% CI: 0.84–1) for *SP* versus controls, 0.98 (95% CI: 0.94–1) for *PA* versus controls using PC 4 and 5, and 0.99 (95% CI: 0.97–1) for *SP* versus *PA* using PC 1, 3, and 5.

Figure 1 shows the group comparisons. Comparing infected versus noninfected animals, 16% of VOCs were significantly different between groups, resulting in a false discovery rate (FDR) of 31.3%. For *SP* versus controls, the significant rate was 30% (FDR 16%), for *PA* versus controls 15% (FDR 33%), and for *SP* versus *PA* 42% (FDR 12%). Table 1 shows identified VOCs, with an adjusted $P < 0.05$ to limit chances of false discovery.

sPLS-DA with leave-one-out cross-validation at the animal level followed by LR showed an AUROCC of 0.85 (95% CI: 0.73–0.96) for infected versus noninfected animals, with a CCR of 94.6% (Fig. 2*A*). *SP* versus controls had an AUROCC of 0.92 (95% CI: 0.83–1) (CCR 98.1%), *PA* versus controls an AUROCC of 0.97 (95% CI: 0.92–1) (CCR 99.9%), and *SP* versus *PA* an AUROCC of 0.98 (95% CI: 0.94–1) (CCR 99.9%) (Fig. 3*A*).

Thermal desorption selected ion flow tube–mass spectrometry The analyses were repeated for the SIFT-MS data. For infected versus noninfected animals, the significant PCs (PC 1 and 4) had an AUROCC of 0.78 (95% CI: 0.62–0.94). For *SP* versus controls the AUROCC (using PC 1, 2, and 4) was 0.82 (95% CI: 0.67–0.96), for *PA* versus controls the AUROCC was 0.85 (95% CI: 0.69–1) using PC 4, and for the *SP* versus *PA* animals the AUROCC was 1.0 (95% CI: 1–1) using PC 1 and 2.

The aforementioned method for sPLS-DA analysis resulted in an AUROCC of 0.54 (95% CI: 0.38–0.71) for infected versus noninfected animals (Fig. 2*B*) (CCR 1.6%), an AUROCC of 0.63 (95% CI: 0.43–0.83) (CCR 26.9%) for *SP* versus controls, an AUROCC of 0.79 (95% CI: 0.62–0.96) (CCR 77.6%) for *PA* versus controls, and an AUROCC of 0.89 (95% CI: 0.77–1) (CCR 19.6%) for *SP* versus *PA* (Fig. 3*B*).

DISCUSSION

The exhaled breath of rats with *SP* or *PA* pneumonia can be discriminated from uninfected controls with good accuracy using GC-MS. The discriminative accuracy was even higher for the discrimination between the two specific pathogens. Overall, GC-MS results provided better results than SIFT-MS as analytical platform for this purpose.

This is the first study that demonstrates an evidently better discriminative performance of breath analysis when used for discrimination between pathogens instead of distinguishing healthy from diseased. So far, clinical studies have been aiming to show a potential for breath analysis to diagnose a variety of lung diseases, e.g., acute respiratory distress syndrome (ARDS; 10) and chronic obstructive pulmonary disease (COPD; Ref. 26a).

Table 1. *Identified VOCs with an adjusted P value of <0.05, per comparison*

Group Comparison	VOC
Infected vs. noninfected	**Octane, 4-methyl-**
	Octane, 2-5-dimethyl
	Unidentified naphthalene compound
	Unidentified cyclic compound
	Unidentified
	Unidentified branched aldehyde
	Tetra chloroethylene
	Unidentified cyclic compound
SP vs. control	**Octane, 4-methyl-**
	Octane, 2-5-dimethyl
	Unidentified naphthalene compound
	Unidentified
	Unidentified cyclic compound
	Hexadecane
	Unidentified
	Unidentified
	Hexane, 2-,4-dimethyl-
	2-Propanol, 1-methyloxy-
	Nonane, 2-methyl-
	Heptane, 2-,4-dimethyl
	Unidentified cyclic compound
	Unidentified
PA vs. control	**Unidentified branched aldehyde**
	2-Propenoic acid, 2-ethylhexyl ester
	Unidentified cyclic compound

In bold: identical volatile organic compounds (VOCs) showing overlap between the group comparisons. *SP*, *Streptococcus pneumoniae*; *PA*, *Pseudomonas aeruginosa*.

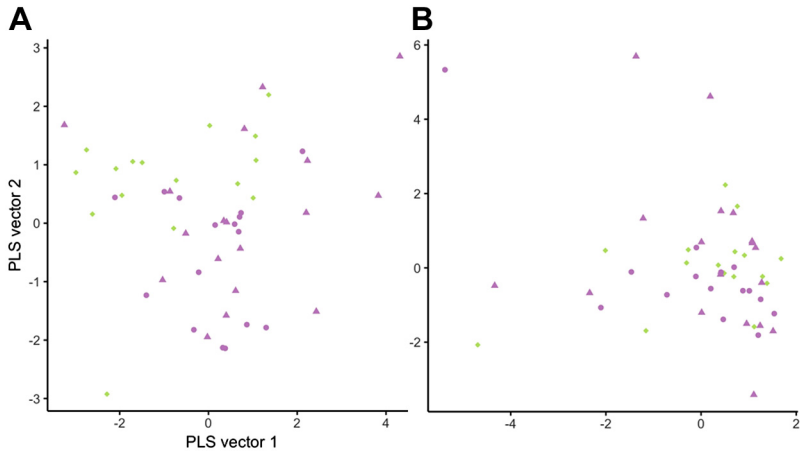

Fig. 2. Sparse partial least squares discriminant analysis (sPLS-DA) with leave-one-out cross-validation: infected [purple triangles: *Streptococcus pneumoniae* (*SP*); purple dots: *Pseudomonas aeruginosa* (*PA*)] vs. noninfected (green rhombus) animals: *A*: GC-MS results. *B*: selected ion flow tube-mass spectrometry results.

Clinical studies investigating breathomics for the diagnosis of respiratory infection showed a general focus on the identification of distinctive individual VOCs or breathprints to be served as biomarkers for pneumonia (12, 20, 24) and not specifically for the causative pathogens. In contrast, our results demonstrate that breath analysis can differentiate bacteria with a higher diagnostic accuracy. In retrospect, this finding seems to be more in line with the available in vitro data. A meta-analysis of all available studies linked more VOCs to one or a few pathogens and rarely found VOCs in the headspace of all studies (8).

Among the identified VOCs were several alkane hydrocarbons (Table 1). Alkanes are associated with oxidative stress (16) yet have been linked to pneumonia as well (19). The abundance of octane may be secondary to peroxidation of oleic acid (16). The other identified hydrocarbons, hexadecane (previously linked to lung infection; Ref. 28), 2-,4-dimethylhexane, 2-methylnonane, and 2-,4-dimethylheptane (previously associated with *Staphylococcus aureus* and *Escherichia coli* infection; Ref. 11), were mainly produced by *SP*. 2-Propanol is, as endogenous compound, suggested to be a product of an enzyme-mediated reduction of acetone (25) and, like octane, might serve as a possible biomarker (7). Tetrachloroethylene is used primarily in the dry cleaning industry and likely to be a contaminant. 2-Propenoic acid is known to derive from ventilator and tubing (7). Table 1 shows that presently many of our discovered VOCs could not be named and remained unidentified, which does not limit them to be of value, for their specific combination of retention time and mass spectrum enables future recognition of these markers in clinical studies and therefore they might still serve as markers for the presence of a specific bacterium.

Animal models provide a controlled environment free of genetic or behavioral influences, allowing selected pathogens to be studied without coexisting microorganisms or diseases contaminating the breath signal. Several studies in murine models focused on the differentiation between individual pathogens by detecting selective VOC patterns (29, 30). Since the present study used GC-MS, individual VOCs could be identified as opposed to the recognition of patterns. These VOCs could serve as specific markers for particular pathogens and could thus be applied for future human exhaled breath studies (21). However, the diagnostic accuracy of single markers provides less accuracy than composite signals. Pathogen identification by VOC analysis in exhaled breath may be most feasible by breathprint analysis and not solely by the analysis of one or several specific VOCs.

A strength of this study is the controlled environment of the established animal model, using a breath sampling technique that had been proven successful in rat experiments (9). Genetically identical rats were used, and a precisely regulated amount of bacteria was inoculated. Another strength is the use of two independent analytical platforms that showed similar trends in results. Limitations of the study were the small panel of

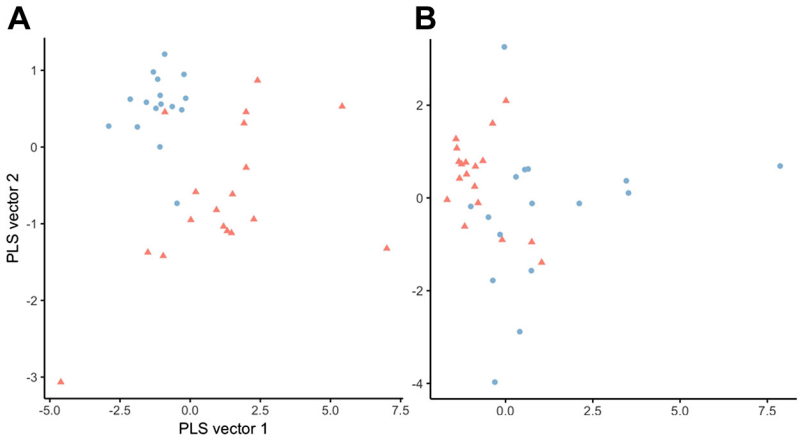

Fig. 3. Sparse partial least squares discriminant analysis (sPLS-DA) with leave-one-out cross-validation: *Streptococcus pneumoniae* (*SP*; red triangles) vs. *Pseudomonas aeruginosa* (*PA*; blue dots) animals: *A*: GC-MS results. *B*: selected ion flow tube-mass spectrometry results.

pathogenic bacteria that was studied and the relatively limited amount of animals that was used. Due to the small number of animals used in these experiments, cross validation had to be performed at the leave-one-animal-out level. Another limitation is the number of VOCs of interest that remained unidentified.

To date, GC-MS is seen as the gold standard for exhaled breath analysis (8). SIFT-MS has the advantage of being quick (few minutes), without requiring calibration standards for the measured VOCs. Furthermore, it can be used as an online instrument enabling real-time measurements, without the need of sample preconcentration. However, an offline approach was used in the current study, involving a rather novel variation of coupling a TD unit upfront the instrument, as earlier described for detection of selected compounds in ambient air (23). In this offline confirmation, full scan mode is more feasible: a chosen range of ions with defined *m*/*z* ratio can be scanned for a chosen time, without the limitation of online sampling, including changing VOC levels throughout breathing maneuvers. An additional advantage of using SIFT-MS offline in combination with the TD unit is the possibility to preconcentrate and potentially measure trace elements in exhaled breath which would fall below the detection limit without preconcentration.

In the present study, both the GC-MS and the SIFT-MS technique delivered adequate accuracies regarding the ability of VOCs to differentiate between causative pathogens, but only GC-MS could discriminate between infected and noninfected rats. GC-MS data for infected versus noninfected animals could have been overfit, as indicated by the high FDRs in the univariate analysis. Nevertheless, the GC-MS results were proven to be superior to SIFT-MS results before in gaseous samples containing large numbers of VOCs at high concentrations (15).

In conclusion, the current focus of exhaled breath metabolomics might have to be reconsidered: in addition to the aim to detect the general presence of respiratory infection, clinical studies should concentrate more on the discrimination between pathogens.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.M.v.O. and L.D.B. conceived and designed research; P.P.v.O. and A.M. performed experiments; P.M.v.O., P.B., G.S., G.K., and L.D.B. analyzed data; P.M.v.O., J.J.R., and L.D.B. interpreted results of experiments; P.M.P.v.O. and L.D.B. prepared figures; P.M.v.O. drafted manuscript; P.M.v.O., R.S., D.C.B., M.R., R.G., S.J.F., M.J.S., and L.D.B. edited and revised manuscript; P.M.v.O., P.B., G.S., G.K., A.M., J.J.R., R.S., D.C.B., M.R., R.G., S.J.F., M.J.S., L.D.B., and B.C. approved final version of manuscript.

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