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A rapid way of breath analysis in cystic fibrosis patients

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ABSTRACT

Purpose: For easy handling and speed of lung diseases diagnostic, approaches based on volatile organic compounds (VOCs), including those emitted by pathogenic microorganisms, are considered but currently require considerable sampling efforts. We tested whether easy to handle and fast detection of lung infections is possible using Solid-Phase-Micro-Extraction (SPME) of 100 ml of exhaled breath. **Methods:** An analytical procedure for the detection of VOCs from the headspace of epithelial lung cells infected with four human pathogens was developed. The feasibility of this method was tested in a cystic fibrosis (CF) outpatient clinic *in vivo*. Exhaled breath was extracted by SPME and analyzed by gas chromatography-mass spectrometry. **Results:** The compositions of VOCs released in the infection model were characteristic for all individual pathogens tested. Exhaled breath of CF patients allowed clear distinction of CF patients and controls by their VOC compositions using multivariate analyses. Interestingly, the major specific VOCs detected in exhaled breath of infected CF patients *in vivo* differed from those monitored during bacterial *in vitro* growth. **Conclusions:** SPME extraction of VOCs from 100 ml of human breath allowed the distinction between CF patients and healthy probands. Our results highlight the importance of assessing the entire pattern of VOCs instead of selected biomarkers for diagnostic purposes, as well as the need for using clinical samples to identify reliable biomarkers. This study provides the proof-of-concept for the approach using the composition of exhaled VOCs in human breath for rapid identification of infectious agents in patients with lower respiratory tract infections.

Keywords: cystic fibrosis, exhaled breath diagnostics, volatile organic compounds, respiratory infections, solid phase micro-extraction

INTRODUCTION

Easy handling and speed are crucial for the transfer of improved infection diagnostics into the clinic [1]. For lung diseases diagnostic approaches based on volatile organic compounds (VOCs), including those emitted by pathogenic microorganisms, are considered [2]. These VOCs comprise hydrocarbons, esters, ketones, aldehydes, and alcohols [3]. VOCs were also successfully used to distinguish between asthmatic and healthy children [4]. Several studies using complex sampling procedures focussed on the characterization of compounds emitted by pathogenic microorganisms. Bacteria and fungi [5, 6] are known to produce a broad spectrum of secondary metabolites including a wealth of volatile molecules. However, most of the microbial VOCs are released in very low concentrations requiring and the exact composition of the emitted volatile compounds varies with different environmental conditions. Nevertheless, detection of microbial VOCs has great potential to be used in pathogen-

directed breath testing, as currently seen for *Mycobacterium tuberculosis* [7, 8] or the CF pathogens *Pseudomonas aeruginosa* [9] and *Aspergillus fumigatus* [10]. Exhaled VOCs analysis may be an alternative approach for the pathogen detection in cystic fibrosis (CF) healthcare, where currently cultivation of infectious agents from sputum is used. With the intention to find pathogen-specific VOCs, the analysis was concentrated to compounds with molecular weights >50 Da. The aim of this study was to develop a non-invasive, easily to handle, rapid and cost efficient method to detect lung infections of CF patients from their breath. Instead of collecting and processing large volumes of breath, only 100 ml from the deeper lung were needed, the VOCs were extracted by solid phase micro-extraction (SPME) and analysed. To prove this concept, an *in vitro* culture system was established, in which host-pathogen *in vivo* interactions were mimicked. VOCs emissions of routinely isolated microorganisms from CF sputum were assessed after their exposure to human lung epithelial cells. The feasibility of this technique was further tested in a CF outpatient clinic *in vivo*. After developing the analytical procedure for the detection of VOCs from the headspace of infected epithelial lung cells, this technique was finally tested in a CF outpatient clinic *in vivo*.

MATERIAL AND METHODS

Cell culture and microbial strain preparation

Microorganisms were grown overnight in Luria Broth (LB) liquid medium and diluted with fresh LB medium. *Staphylococcus aureus* DSM 20231, *P. aeruginosa* PaO1, *Achromobacter xylosoxidans* DSM 2402, and *Candida albicans* SC 5314 were grown to mid-log phase. Cultures were incubated at 37°C on a shaking platform at 120 rpm. Microbial suspensions were used to determine colony forming units (CFU) and *in vitro* VOCs extractions. For determination of CFUs, suspensions were diluted; triplicates (200 µl) were plated on LB agar plates and incubated at 37°C overnight or 3 days (*A. xylosoxidans*) before colony counting. The A549 human epithelial cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; PAA, Pasching, Austria), with 10% foetal bovine serum (FCS) and 2 mM L-glutamine without antibiotics. Cells were incubated in culture flasks (37°C, growth area of 25 cm², humidified atmosphere with 5% CO₂). Cells were trypsinized and sub-cultured before reaching confluence.

Microbial *in vitro* models for measurements of VOC emissions

Host-pathogen interaction cultures were defined as A549 cell cultures inoculated with microbial pathogens. Bacterial suspensions (1 ml) and fungal suspensions (2 ml) in the mid-log phase were taken and centrifuged at

10,000 rpm for 8 min. Pellets were resuspended in 1 ml Dulbecco's modified Eagle's medium. Bacterial (200 µl) and fungal (2 ml) suspensions, respectively, were added to a confluent monolayer and a solid phase micro-extraction (SPME) fibre was exposed to the headspace at different time points (Table S1). Pathogen inocula: the CFUs added to A549 cells were 7.6×10^8 (7.6×10^7) for *A. xylosoxidans*, 4.8×10^8 (4.8×10^6) for *S. aureus*, 2.7×10^7 (2.7×10^6) for *C. albicans*, and 9.5×10^8 (9.5×10^6) for *P. aeruginosa* (in brackets CFU for overnight incubations). All cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Solid phase micro-extraction

VOCs were extracted using SPME [11]. For optimal VOCs extractions different fibre coatings were tested. Major criteria for the optimal fibre were: (i) molecular weight and size of the analytes, (ii) polarity, (iii) concentration level and (iv) complexity of sample [12], but also storage and transport between the clinic (Hannover Medical School; MHH) and the institute (Helmholtz Centre for Infection Research). A 75 µm Carboxen/PDMS fibre (Portable Field Sampler) from Supelco (Bellefonte, PA, USA) was finally selected. Here, the fibre coated with the polymers carboxen and polydimethylsiloxan (PDMS) is secured with a protective needle for transport (Portable Field Sampler, Supelco Bellefonte, PA, USA). All fibres were preconditioned for 20 min at 250°C. The sorbent coated fibre was exposed for 10 min to the sample and, subsequently, VOCs were released by thermal desorption in the GC at 200°C. Each SPME fibre showed an individual peak pattern even after preconditioning and, to minimize bias in the data analysis, all fibres used for *in vivo* applications were numbered and labelled. Where possible for *in vitro* experiments, the same fibre was taken for a series of associated VOCs extraction.

Gas chromatography/mass spectroscopy (GC/MS) parameters

For analysis Varian 450-GC gas chromatograph coupled with ion-trap mass spectrometer Varian 240-MS (Varian, Palo Alto, USA) and a forte GC Capillary Column 50 m x 0.32 mm x 1 µm (SGE Analytical Science, Ringwood, Australia) was used; manual injection of SPME fibre at 200°C and with splitless mode for 2 min, followed by a split ratio of 100 for another 2 min and a final split ratio of 20. Temperatures of ion trap, manifold and transfer line were 150°C, 50°C and 180°C, respectively. The GC oven temperature: 35°C (2 min), then heating to 75°C at 3°C min⁻¹, 15°C min⁻¹ to 250°C (hold for 5 min), helium flow (1 ml min⁻¹), EI full scan mode with a mass range *m/z* 40-205 was used.

CF cohort

Nine CF patients (30 - 48 years old, 6 male, 3 female) were recruited in the CF outpatient clinic of the MHH (Hannover, Germany), after ethical approval by the local health authority ethics committee. The study was approved by the local ethic committee (No: 1002-2011) and written informed consent was obtained from all study participants. Breath samples were taken during their routine examination in the ambulance. Anonymized clinical data of patients was collected, including culture-based microbiological diagnostics by routine cultivation methods which were applied according to the German Quality Standards in Clinical Microbiology and Infectious Diseases [13]. Additionally, breath samples of two healthy volunteers (30 years old; male and female) were taken in the Helmholtz Centre for Infection Research.

***In vivo* breath sampling**

A modified BIO-VOC[®] sampler (Markes International Ltd, Rhonda Cynon Taff, UK) [14] was used for breath sampling with optimized autoclavable mouthpieces and adapter (polyvinyl chloride), ensuring a sterile procedure and easy handling. Patients and healthy volunteers were asked to rest and, when they feel relaxed, to exhale completely a single slow breath into the sampler. The last portion of the exhaled air, originating from the alveolar space, was trapped (approx. 100 ml) and immediately exposed to the SPME fibre. No cross-contamination of exhaled VOCs was observed when breath sampler was kept uncapped for two hours to release the remaining analytes. In addition, ambient air was sampled for VOCs.

Data processing and statistical analyses

GC-MS data was analyzed using MS Data Review (version 6.9.3, Varian). All peaks of a chromatogram were aligned by their retention times and mass spectra. Parameters for peak integration of host-pathogen interaction samples (in brackets: *in vivo*) were: Peak width 4.0 sec, slope sensitivity 20 (2) SN, tangent 30% (10%). Minimum peak size was 10,000 units for most microorganisms and 5,000 units for *P. aeruginosa* respectively. Peaks were rejected if they: i) appeared in several controls and only with smaller peak size in breath samples, ii) were detected in controls and only from healthy individuals, iii) were present in all samples without significant changes. All *in vivo* data were divided by minimum peak size and normalized. Heatmaps were calculated using the software Package R [15] from the colour palettes, the topo.colors Package was chosen without scaling. Further statistical analyses were carried out using PRIMER (Version.6.1.6, PRIMER-E, Plymouth Marine Laboratory, UK). Resemblance matrices were calculated using Euclidean distance algorithm, including all variables. Samples were ordinated in non-metric multidimensional scaling (MDS) plots (50 random restarts) reflecting differences in their VOC compositions. Trajectories were used to connect associated data points in

chronological order. 2D stress values indicate the differences between ideal distances in higher dimensional space and the actual distances in two dimensional space.

RESULTS

***In vitro* discrimination of CF pathogens by VOC emission**

To establish the analytical protocol, human epithelial cells were infected with four different pathogens (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Achromobacter xylosoxidans*, *Candida albicans*). In these host-pathogen interaction cultures the VOCs were changing characteristically over time. Retention times and mass spectra differentiated the signals sufficiently, rendering further identification for diagnostic purposes unnecessary. Only steadily increasing signals were considered as culture-specific VOCs. Signals P13, P14, P15 were only lacking in *A. xylosoxidans* and P7 in *P. aeruginosa*. Characteristic VOCs were P16 and P23 for *A. xylosoxidans*, P11 and P33 for *C. albicans* and P32 for *P. aeruginosa*, however, *S. aureus* had no unique signal. Similarity values between the VOCs were calculated and visualized in a MDS plot after logarithmic transformation. The starting points (T0) of all four pathogens ordinated close together but the distance increased during incubation, indicating a continuous differentiation of VOCs in each interaction culture (Figures S1 and 1). Therefore, not just presence and absence of released VOCs, but also the relative intensities were characteristic for each host-pathogen interaction culture. This allows discrimination between the different pathogens *in vitro* and even between different time points during incubation of the interaction cultures.

***In vivo* analyses of VOC emissions in exhaled breath of CF patients**

With this optimized protocol, breath of nine CF patients (30 - 48 years old, 6 male, 3 female) from the CF outpatient clinic of the Medical School Hannover (MHH), were sampled. Anonymized clinical data of patients were collected, including cultured pathogens. Additionally, breath samples of two healthy volunteers were taken. Proband exhaled completely a single breath into the sampler where the last portion, originating from the alveolar space, was trapped and immediately extracted by SPME. Ambient air was also sampled. For all samples a rather complex signal composition was observed, characterized by the presence of 33 different signals (hP1 - hP33, Figure 2). Signals hP3 and hP5 were present in high intensities in all breath extractions, whereas controls showed no such signals or only in low intensities. Signals hP4 and hP6 were detected in all samples extracted at the clinic, but never in those made at the institute. Signal hP10 was ubiquitously present in all chromatograms and intensities were higher in all samples stored or transported for more than 1 hour after exposition.

Furthermore, SPME fibres showed individual signal patterns. No VOCs from the CF patients matched with the characteristic VOCs of the host-pathogen interaction cultures. For a detailed analysis, the samples were ordinated in a MDS plot (Figure 3). The MDS plot revealed clustering, clearly separating healthy individuals from CF patients and from control samples. The VOCs datasets were further compared with individual clinical data of CF patients as well as with individual results from microbiological diagnostics (Table S2), however, no further correlations were observed.

DISCUSSION

The diagnostic potential of biomarkers in exhaled breath is attracting growing attention and different approaches were tested in recent years. Several thousand different VOCs from diverse origins and biochemical pathways have been detected in levels of parts-per-billion (ppb) with various methods in human exhaled air [16]. Studies in the past concentrated on the search for single biomarker molecules for a certain respiratory pathogen, which leads to several difficulties further discussed below. Several studies investigated VOCs released by *P. aeruginosa* [17, 18] and *S. aureus* [19] *in vitro* revealing numerous compounds in the headspace of these microorganisms. Recently, 2-aminoacetophenone was getting into the focus as biomarker molecule for *P. aeruginosa* and was used in a first breath analysis study to identify *P. aeruginosa*-colonized CF patients [20, 21]. However, other studies detected this compound also in the headspace of different respiratory pathogens [22] and in food [23]. Furthermore, microbial VOCs production and intensity are strongly influenced by growth media and conditions [24].

Interestingly, none of the VOCs from *in vitro* cultures were detected in exhaled breath of CF patients, although colonization of patients with pathogens was confirmed by cultivation. Potential explanations for this discrepancy may be the emission of different microbial VOCs due to limitation of the *in vitro* cultures due to considerable different conditions in the human lung. Further reasons may be VOCs present at very low concentrations and the relatively healthy status of the studied patients. This highlights the importance of carrying out studies in humans to identify VOCs which are reliable biomarkers for the clinical management of infected patients. In our study evidence was provided that no single molecule but rather the global VOCs pattern is an appropriate target in the search for volatile biomarkers. The MDS plots used here have the advantage of featuring multivariate similarity and simultaneously providing the underlying variables responsible for certain groupings. A clear influence by the ambient air was observed demanding *a priori* calibration. In a comparative study comprising 12 VOCs in

exhaled breath from CF patients and healthy individuals, slightly altered compositions were observed between both groups identifying no distinct biomarkers [25]. Nevertheless, in our study differences in exhaled air profiles were observed and resulted in a grouping of VOCs profiles separating CF patients from healthy volunteers.

By extracting large volumes 1099 VOCs in the breath of CF patients were analysed and 100% identification of CF patients and healthy individuals was shown [26]. Even further discrimination of *P. aeruginosa* colonized patients was achieved. These results strongly suggest that the composition of exhaled compounds is superior to single biomarkers for the differentiation between respiratory infections. Our study demonstrated that large breath volumes combined with time-consuming extraction procedures are not required for breath analyses. The SPME approach applied here has the advantage of easy handling as it has been demonstrated by its successful application in the outpatient clinic. Furthermore, it demonstrated the potential to deliver results faster with reduced effort and costs in comparison to sputum based diagnostics (Table 1).

CONCLUSIONS

This study showed that the simplified extraction procedure for VOCs allowed differentiation of microbial species and underlines that human breath delivers information on the health status of the lung. The profile of exhaled VOCs in combination with multivariate analyses rather than single molecules [27] was found to be more informative for clinical diagnostics [28]. Despite the great potential of such a non-invasive diagnostic method, the technical issues are demanding [29] and breath sampling and VOCs extraction have to be further optimized. Overall, exhaled VOCs analysis for the detection of pulmonary infectious diseases has the potential to become a rapid and efficient diagnostic tool in the future.

Competing interests

RK, ASH, CAG, MGH and WRA declare that they have no competing interests. TW is on the advisory board of Bayer, NOVARTIS, Basilea and lecturing for Bayer, NOVARTIS.

Authors' contribution

RK established the analytical methods, analyzed the samples, carried out the interpretation of data, and contributed to the writing of the article. ASH selected the patients, sampled their breath and contributed medical data of the patients. WRA oversaw all aspects, contributed to the conception and design of the article, conceptualized and oversaw analyses, contributed to the interpretation of data, and wrote the article. CAG, MGH, TW oversaw all aspects and reviewed the article for important intellectual content. All the co-authors critically revised the manuscript and approved the final draft before submission.

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Table legend

Table 1: Schematic comparison between sputum and exhaled breath based diagnostics. In respect to the broad range of phenotypic and molecular techniques in diagnostics, the individual specifications are approximate estimates.

Respiratory specimen	Methods	Additional expertise needed	Rapidness of results	Overall costs
Exhaled breath	VOCs extraction	low to moderate	within 2 hours	low
Sputum	Culture based	moderate to high	over night	low to moderate
	Culture independent	highly trained professionals only	within 1 or 2 days at best	high

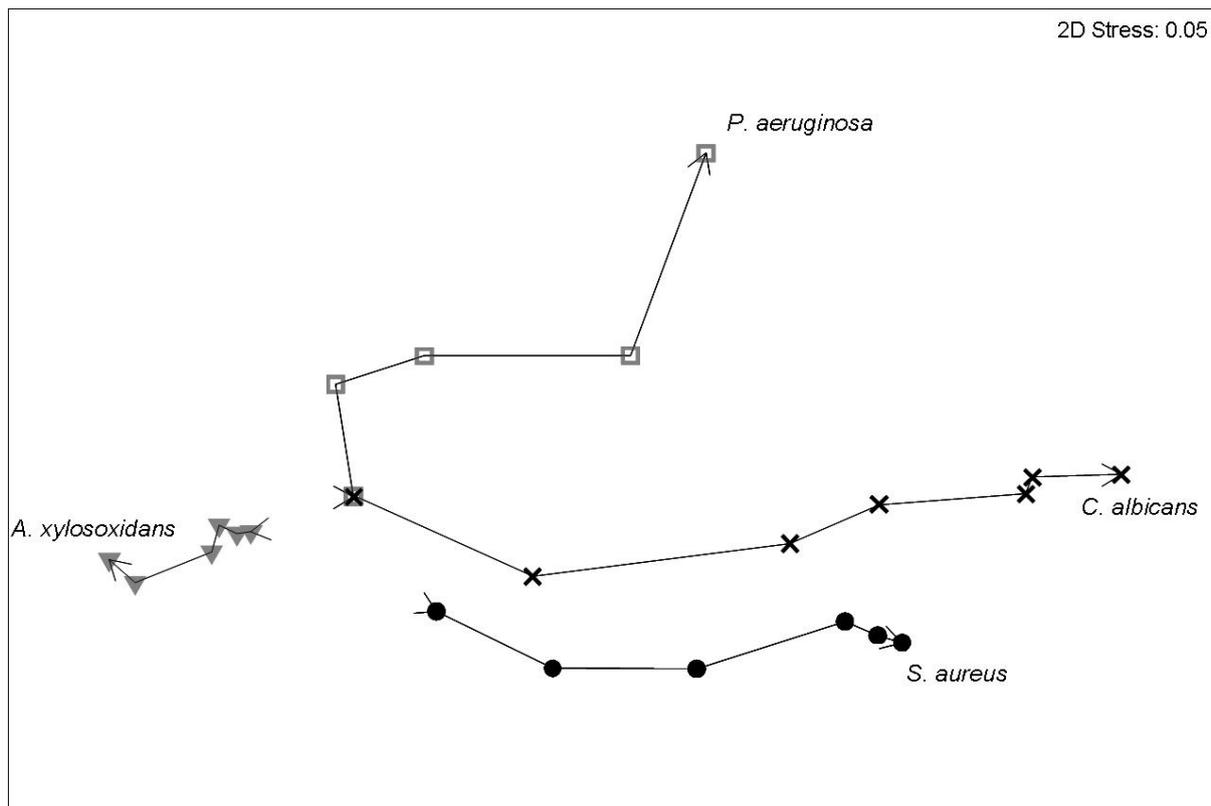


Figure 1: MDS plot with dimensionless axes of the characteristic VOCs from host-pathogen interaction cultures during different incubation time points. Trajectories connect the series of VOCs from each host-pathogen interaction culture.

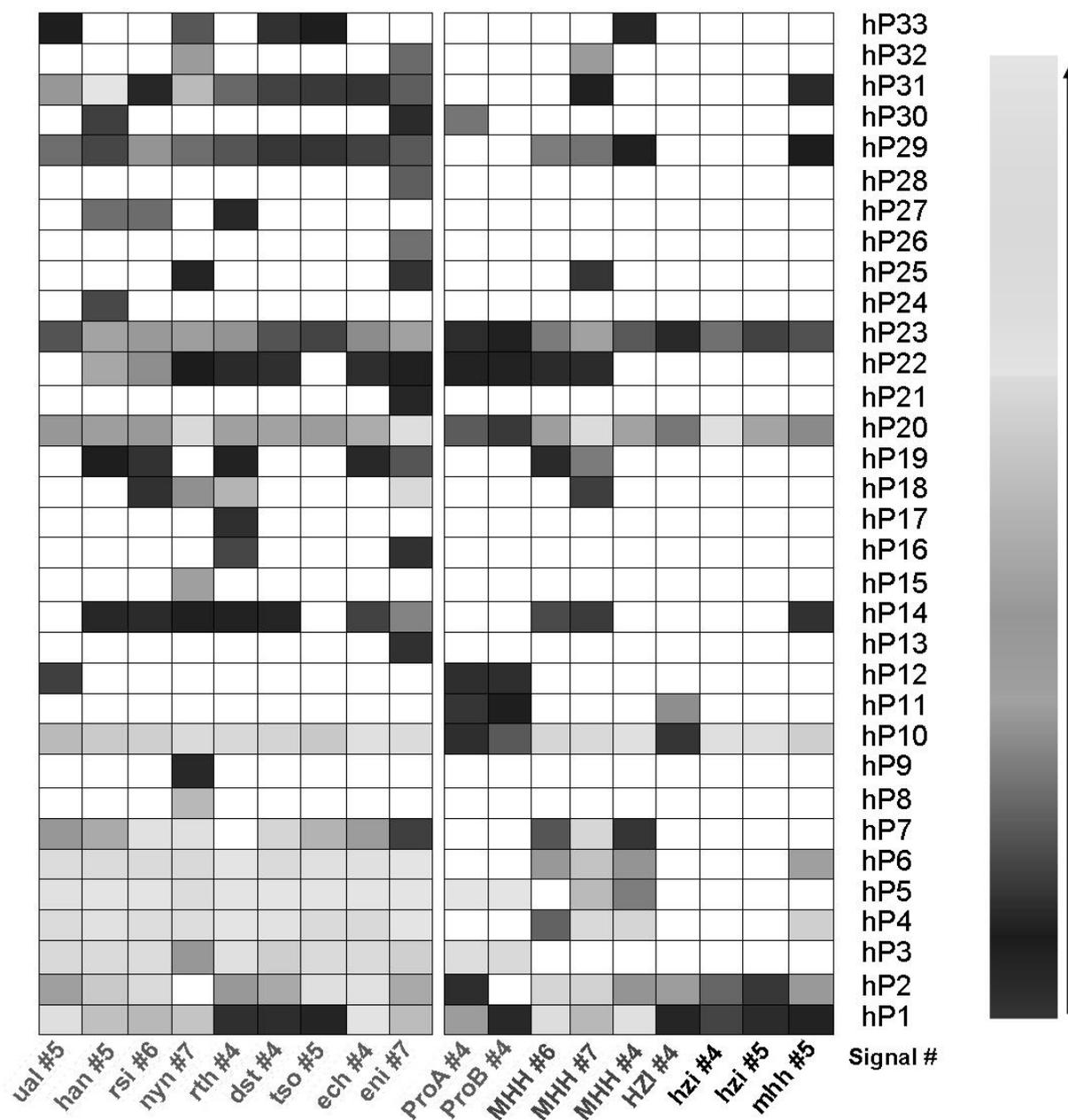


Figure 2: Heatmap of VOC compositions from breath extractions. VOCs extractions from exhaled breath of CF patients were taken at MHH, whereupon ProA and ProB were extractions from healthy non-CF individuals, performed at Helmholtz Centre for Infection Research (HZI). Room samples are indicated as MHH and HZI samples and SPME fibres without exposition are marked mhh or hzi, respectively. Signals are numbered hP1 - hP33. Relative intensities of signals are indicated by a grey gradient in which the intensity increases from dark to bright grey whereas blank boxes indicate that no signal was detected. Locations of sampling were given with MHH/mhh for clinical samples and HZI/hzi for the institute. Numbers in the sample labels indicate the individual SPME fibre used for the experiments.

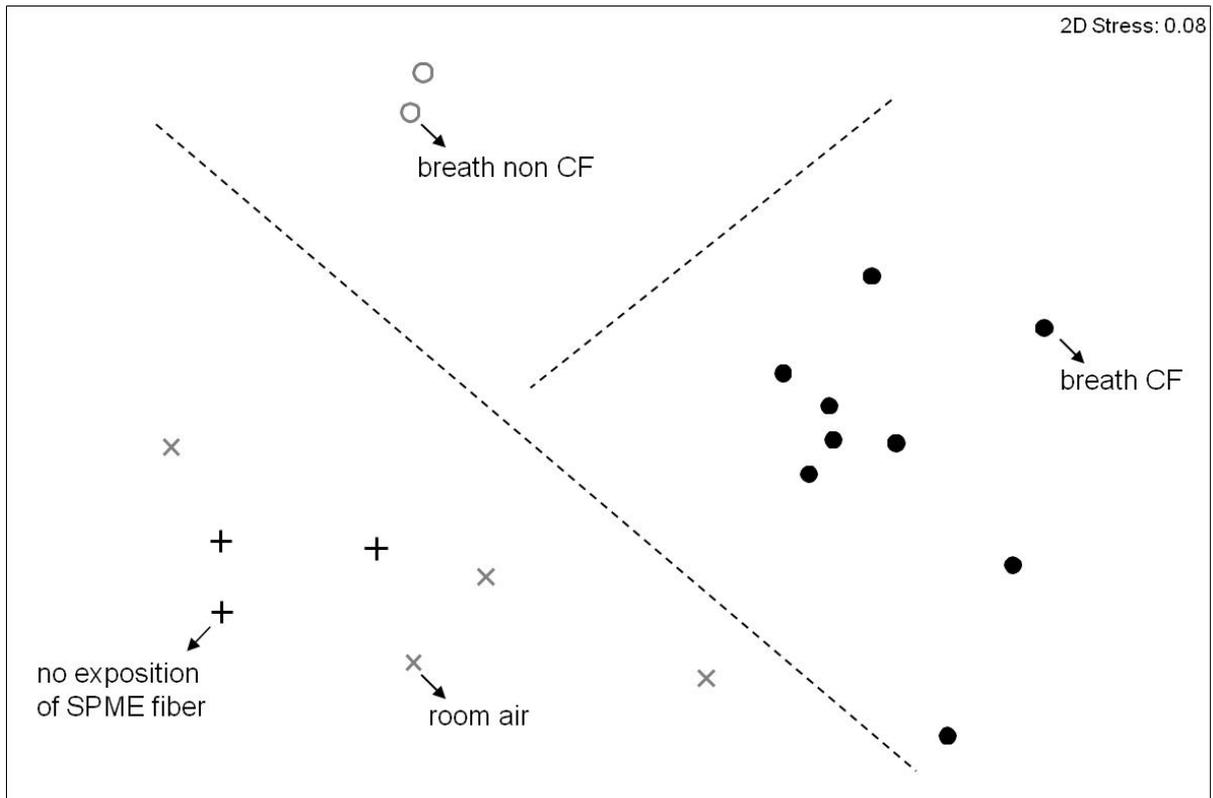


Figure 3: MDS plot of VOCs extraction from breath analyses. VOC compositions of different samples were compared and samples ordinated according similarity values. Symbols are affiliated to samples and indentified in the figure. Dashed lines indicate the three different clusters of samples within the plot. Stress value indicates the differences between ideal statistical distances of samples in higher dimensional space and the actual distances in this two dimensional space.

SUPPLEMENTARY MATERIAL

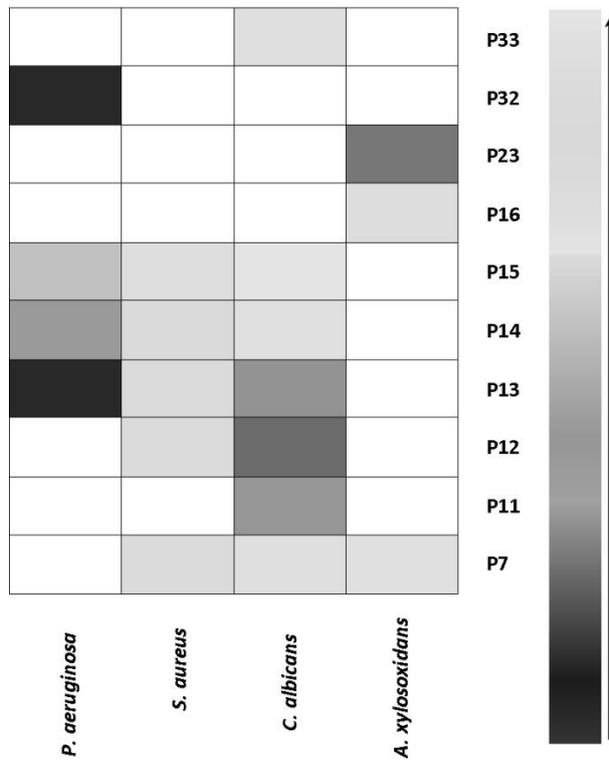
Table S1: Time points of SPME fibre exposition in host-pathogen interaction cell cultures. A series of VOCs extractions (T0 to Tmax) was performed at different time points post injection (min = minutes; h = hours) for each culture. The pathogen of each interaction culture is given in italics above the corresponding rows.

<i>C. albicans</i>						
T0	T1	T2	T3	T4	T5	Tmax
0 min	95 min	195 min	275 min	390 min	470 min	20.5 h
<i>P. aeruginosa</i>						
T0	T1	T2	T3	T4	T5	Tmax
0 min	115 min	215 min	295 min	410 min	520 min	22.5 h
<i>A. xylosoxidans</i>						
T0	T1	T2	T3	T4		Tmax
0 min	120 min	210 min	300 min	390 min	no extraction	21 h
<i>S. aureus</i>						
T0	T1	T2	T3	T4		Tmax
0 min	120 min	210 min	300 min	390 min	no extraction	21 h

Table S2: Individual data of CF patients. For microbiological diagnostics, sputum samples were immediately processed in-house at the microbiological laboratory of the MHH and the diagnostic report was copied to this table.

patient	gender	age (in years)	isolated species
tso	female	36	<i>P. aeruginosa, Candida albicans</i>
dst	male	35	<i>P. aeruginosa, Candida albicans</i>
han	male	31	<i>P. aeruginosa, S. aureus, Penicillium sp.</i>
rth	male	40	<i>P. aeruginosa, S. aureus, Aspergillus fumigatus</i>
rsi	female	42	<i>P. aeruginosa, Yeast species</i>
ech	male	48	<i>P. aeruginosa, Pseudoallescheria boydii</i>
eni	female	41	<i>P. aeruginosa, Streptococci</i>
nyu	male	30	<i>P. aeruginosa, Candida albicans, S. aureus</i>
ual	male	33	<i>P. aeruginosa, Candida albicans, S. aureus, Enterococcus sp.</i>

Figure S1: Heatmap of VOCs compositions from different host-pathogen interaction cultures. Only the intensities of the characteristic VOCs after 24 hours of incubation are shown. Relative intensities of signals are indicated by a grey gradient in which the intensity increases from dark to bright grey whereas blank boxes indicate that no signal was detected.



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