PAPER

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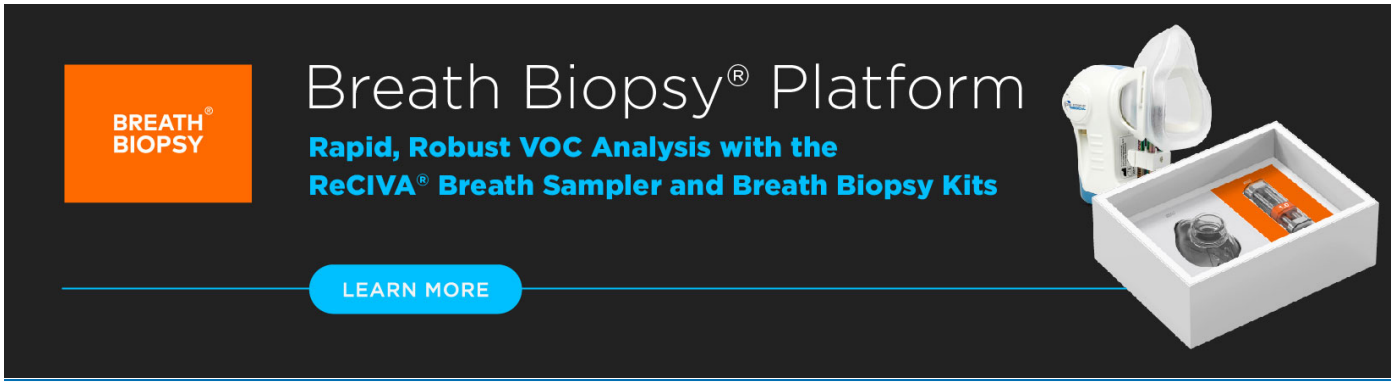

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Supplementary material for this article is available online

Abstract

We aimed at defining profiles of volatile organic compounds in exhaled breath from patients with cystic fibrosis (CF) using a novel real-time mass spectrometry technique. In this prospective matched case-control study, 30 patients with CF, and 30 healthy control subjects were matched one-to-one according to age, gender, and smoking state. We performed exhaled breath analysis by untargeted secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS). Patients with CF (mean age 26.0 ± 13.0 years) and controls (mean age 27.9 ± 14.0 years) were analyzed using SESI-HRMS. 49 exhaled breath features were found to be altered (p-value < 0.05/q-value < 0.1) in CF patients, in comparison to healthy controls. The two most discriminating features showed a prediction AUROC of 77.1% (95% CI 62.2%–87.8%) with a specificity of 80.0% and a sensitivity of 63.3%. Levels of oxidative stress metabolites such as fatty acids were found to differ significantly between patients with CF and healthy controls. Furthermore, in patients with CF, 11 features correlated with the mucus concentration of *Stenotrophomonas maltophilia* bacteria. Exhaled breath analysis with SESI-HRMS allows the identification of CF specific compounds in real-time and may trace bacterial strains in affected patients with CF.

Introduction

Respiratory disease is the major cause of morbidity and mortality in cystic fibrosis (CF). Nowadays, neonates are routinely screened for CF in many countries, and in older patients, sweat chloride and genetic testing is conducted if suspicious symptoms are present [1, 2]. With the recent development of disease-modifying treatments for CF [3], there is a growing demand for minimally-invasive and radiation-free techniques to monitor disease development and progression [4]. The proposed breath-analysis specifically aims to explore aspects of respiratory disease, such as lung infection, in patients with CF.

A simple, non-invasive analysis of breath may not just offer a cost-effective alternative for diagnosis/monitoring, but also uncover further information about pathophysiology and disease phenotypes, which is in line with current efforts towards individualized medicine [5]. Analysis of so-called breathprints (i.e. the exhalome) via mass spectrometry is a highly promising, but little explored area of translational research. Recent advances in mass spectrometry have enabled real-time assessment of a large number of volatile and semi-volatile compounds in the exhalome. Rather than comparing only small numbers of pre-selected compounds (e.g. fractional exhaled nitric oxide [FeNO]), breathprints represent relative intensity

patterns for a multitude of compounds reflecting robust subject-specific metabolic signatures. Secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) has been developed for that purpose and allows high-resolution breathprint profiling (>3000 compounds) in real-time without the need for storage and thus avoiding contamination of the breath sample and loss of information [6]. Its ability to detect differences between individual breathprints that are reasonably stable over time [7], follow predictable diurnal patterns [8], and detect levels of volatile organic compounds (VOCs) down to the parts-per-trillion level [9] make it a highly attractive tool for everyday clinical use. As opposed to similar techniques such as proton transfer reaction and selected ion flow tube mass spectrometry, SESI can be adapted to any atmospheric pressure ionization mass spectrometer, depending on the user needs. Thus, one can benefit of ultra-high resolution, mass accuracy and fragmentation capabilities of state-of-the-art commercial mass spectrometers [10, 11]. However, selected-ion flow-tube mass spectrometry and proton-transfer-reaction mass spectrometry are capable of providing absolute quantification of the detected volatiles, whereas SESI-HRMS requires post-calibration to translate signal intensities into gas-phase concentrations [9]. Recent efforts in phenotyping other lung diseases with SESI-HRMS have shown promising results using an untargeted approach and the technology is capable of detecting physiological compounds which diffuse from the blood into the alveoli such as a large number of amino acids [12–14]. When it comes to CF, there is ample evidence that breath composition reflects aspects of CF pathology or airway colonization [15–22].

The main objective of this study was to explore CF specific breathprints and their association with clinical findings. We further aimed to investigate whether bacterial infections in CF are detectable and how substances in the exhalome correspond to other markers such as bacterial metabolites.

Methods

Study design and participants

For this prospective case-control study, 30 CF-patients (children, adolescents and young adults) in a stable state from two university-affiliated CF adult and pediatric centers were recruited according to the established guidelines for diagnosis of CF [2]. Diagnosis was confirmed by genetic testing in each individual. 30 healthy participants were then matched one-to-one for sex (exact), smoking status (no-smoker versus smoker versus ex-smoker; exact), pack years of smoking (caliper of 5 pack years maximum), and age (normally distributed; caliper of 10 years maximum). Exclusion criteria for both groups were: (1) previous lung transplantation; (2) pulmonary exacerbation within the preceding six weeks (defined as inpatient treatment for respiratory complication or intravenous antibiotic use [23]); (3) moribund or severe disease prohibiting protocol adherence; (4) physical or intellectual impairment precluding informed consent or protocol adherence; or (5) pregnancy. Clinical data including height, weight, smoking status, bacterial colonization (not older than 3 weeks), and lung function were obtained by a structured interview or on-site testing. Spirometry results are expressed in percentage of predicted values according to the European reference equations [24]. The study protocol was reviewed and approved by the cantonal ethics committee of Zurich (KEK-ZH-Nr. 2014-0076). The study was conducted according to the Declaration of Helsinki and registered at ClinicalTrials.gov (NCT02209571). Written informed consent was obtained from each participant (parental authority/legal guardian if applicable) before participation in the study.

Sample size

Traditional methods for estimating sample sizes needed to detect a minimum meaningful difference cannot be applied to an exploratory study. Tentative estimations can be made based on sample size requirements for factor analysis [25]: assuming a variable-to-factor ratio of at least seven, the minimum necessary sample size for good (>90%) model agreement is 55–58 subjects. Based on our prior experience of exploratory breath analysis [6, 8, 13, 26], we aimed to recruit 60 subjects. Furthermore, we applied a one-to-one matching design (dependent/matched groups) to increase statistical power and decrease type-I-error rate.

Breath analysis

The methods used for sampling, processing, and analyzing the data in this study have been published in detail elsewhere [13]. In short, participants were examined in the fasting state in the same room with ambient air and were asked to abstain from smoking, chewing gum, alcohol, or caffeine 1 h before the measurements, according to the recommendations [13, 27]. For SESI-HRMS a standardized protocol [13] was applied to all participants in order to exclude influence of breathing manoeuvres on exhaled compounds and to keep artefacts to a minimum level [13]. Breath exhalations at a pressure level of 10 mbar via a mouthpiece with a saliva trap were repeated six times and directly analyzed with SESI-HRMS in real-time (figures 1, 2). SESI-HRMS spectra were acquired in positive and negative ion mode. Lung mucus was sampled simultaneously and bacterial infection markers were analyzed by the accredited university hospital laboratory. Exhaled breath condensate was also collected and analyzed with liquid chromatography—tandem mass spectrometry for compound identification. The details are described in the online supplementary file (E-Methods) is available online at stacks.iop.org/JBR/12/036013/mmedia.

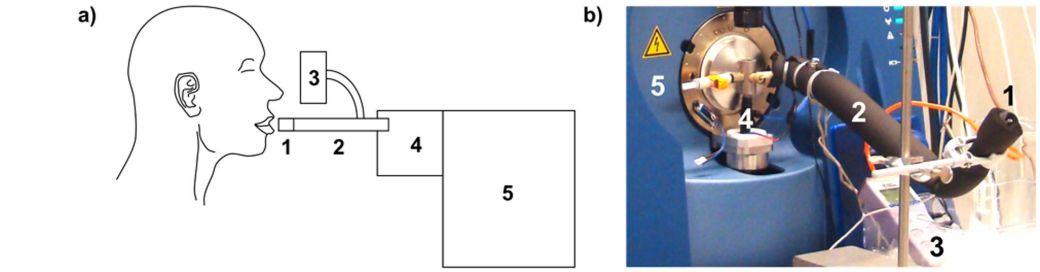

Figure 1. (a) Scheme and (b) illustration of the breath analysis setup consisting of the heated breath sampling tube (3) with disposable mouthpiece (with integrated saliva trap/1) and manometer (3), the SESI ionization source (4) and the high-resolution mass spectrometer (5).

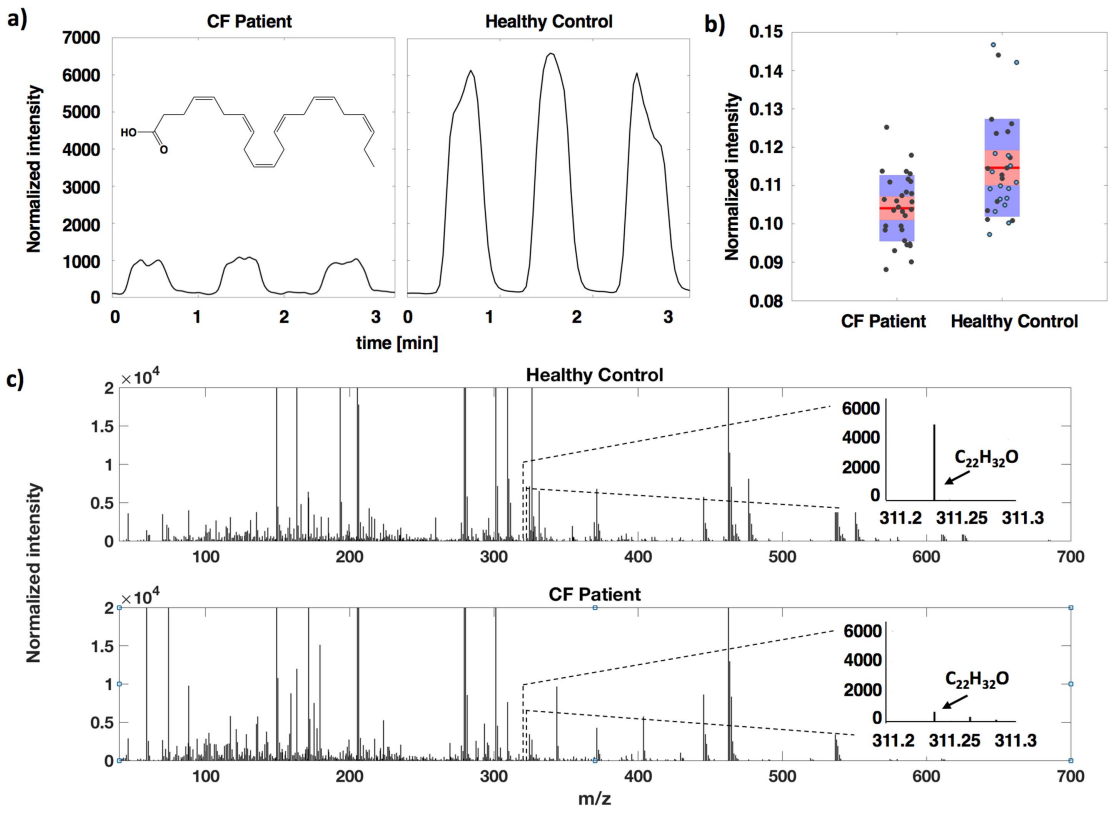

Figure 2. SESI-HRMS analysis of exhaled breath: (a) extracted time traces from docosahexaenoic acid ($C_{22}H_{32}O_2$ or DHA) from a CF patient and a healthy control. The compound concentration in the patient is decreased in comparison to the control (b) the plot shows for DHA breath intensities (mean) for all study participants, showing a distinct difference between both groups ($q = 0.079$). Per group the mean breath signal (red, middle line) with 95% confidence interval (red, inner boxes) and one standard deviation (blue, outer boxes) are presented. (c) Comparison of two mass spectra from a healthy control and a CF patient from one exhalation each. The region of the *m/z*-value 311.2–311.3 is enlarged.

Statistical analysis

The SESI-HRMS data was mass calibrated and normalized for further statistical analysis. Important breath features between patients and controls were assessed by a nonparametric Mann-Whitney-U-test ($p < 0.05$). False-discovery rate corrected p values ($q > 0.01$) were calculated and used to filter significant compounds [28]. Prediction power of the metabolites found was evaluated within a leave-one-out cross validation based on two features selected by a support vector machine algorithm. Correlations between breath signals and *Stenotrophomonas maltophilia* were calculated using linear fit models with bisquare weighting. Significant correlations were filtered by false-discovery rate corrected p values ($q < 0.04$) and Pearson correlation coefficients ($r^2 > 0.4$). (See online supplementary file for more details.) Results are presented as mean (+/− standard deviation) or in case of a skewness greater than 0.05 as median (interquartile range).

Results

Patient characteristics

A total of 12 adolescents and 48 adults participated in this study. All subjects were of Caucasian ethnicity and

Table 1. Baseline characteristics of participants.

	CF, $n = 30$	Controls, $n = 30$	p-value
Age, years	26.0 (±13)	27.9 (±14)	0.84
Male, %	77%	77%	—
Non-smoker, %	80%	80%	—
Smoker, %	3%	3%	—
Ex-smoker, %	17%	17%	—
Pack years (smokers only), PY	6.2 (±3.0)	5.5 (±3.0)	0.89
FEV1, % pred[a]	78.0 (54.5–97.8)	101.5 (93.8–106.5)	<0.05
FVC, % pred[a]	92.5 (69.8–105.5)	99.1 (90–105)	<0.05
BMI	21.6 (±3.6)	21.5 (±3.1)	0.91

Variables displayed as mean ± standard deviation or median (interquartile range) as appropriate. BMI, body mass index. FEV1, forced expiration in 1 s. FVC, forced vital capacity. PY, pack years of smoking.

[a] Due to the non-parametric distribution in patients with CF [3], both groups are presented as median (interquartile range).

none of the participants were biologically related. The age of patients and controls averaged 26.0 (±13) and 27.9 (±14) years, respectively. Spirometry revealed a varying degree of obstructive airways disease in the CF group (median forced expiratory volume in 1 s was 78.0% [IQR range 54.5–97.8]), but was within normal limits in all healthy control subjects. Apart from the matching variables, patients with CF did not differ from healthy controls in terms of BMI and pack years of smoking. Participant characteristics are summarized in table 1 and exemplary ion-chromatograms, breath intensities, and mass spectra are presented in figure 2.

The relevant mutation for each patient is shown in the supplementary material in eTable 1. Among the patients, 63% ($n = 19$) had at some point been admitted to the hospital as in-patient due to respiratory problems (last admission 6 ± 5 years ago). 24 out of 30 patients reported frequent use (>1/day) of bronchodilators and steroids. Additional blood markers, demographics and medication are summarized in the supplementary file (eTables 2–4).

CF specific breath patterns and disease prediction

SESI-HRMS analysis was able to detect 3273 features in exhaled breath from CF patients and healthy controls. A between-group comparison resulted in 49 significant features (p-value < 0.05 and q-value < 0.1). From these 49 features, 15 were enhanced and 34 decreased in CF patients' breath, respectively. No feature was exclusively present in only one group; all 49 features are compiled in table 2(A). We correlated the raw signal intensities of each feature with FEV1 (litre) using a linear fit model with bisquare weighting, in order to rule out any potential confounding bias in the CF cohort. There was no significant correlation detected (false discovery rate corrected p values/q-values < 0.05 nor q-value < 0.1) in the data set.

The prediction performance of the data set was evaluated by a leave-one-out cross validation based on two features (table 2(B)) selected by a support-vector-machine algorithm. The corresponding receiver operating characteristics show an accuracy of 77.1% (CI 62.2%–87.8%), specificity of 80.0%, sensitivity of 63.3%, positive predictive value of 76.0%, and a negative predictive value of 68.6%. The area under the curve (figure 3) covers 77.1% (confidence interval 62.2%–87.8%).

Association with airway bacteria

Breath signals from 28 patients with CF were correlated with six bacterial strains (*Pseudomonas aeruginosa, Staphylococcus aureus, Stenotrophomonas maltophilia, Haemophilus parainfluenzae, Haemophilus influenza, and Haemophilus parahaemolyticus*) which are associated with inflammatory processes. We found 11 features correlating (all with a q-value < 0.05; and r from 0.63 to 0.74) with *Stenotrophomonas maltophilia* colonialisation (present in 12 patients) while colonialisation with other strains yielded no significant results (see supplementary eTable 5 for the detailed list of markers and the corresponding values).

Compound identification

We attempted to identify all 49 significant breath features with the exact mass from the exhaled breath SESI-HRMS measurements and the aid of liquid chromatography MS/MS analysis of exhaled breath condensate. This approach is described in more detail in the supplementary material and as well as in a previous publication [29]. An initial search was performed using the Chemspider and Metlin databases. Where possible, reference substances were purchased and measured by UHPLC-MS//MS (retention time and MS/MS fragments). Otherwise, database fragment spectra or *in silico* generated fragment spectra were used. Furthermore, real-time SESI-HRMS/MS measurements in exhaled breath were performed to generate fragment ion spectra. In contrast to UHPLC-MS/MS fragment spectra, SESI-MS/MS fragment ion spectra should be only

Table 2. (A) CF-specific features. 49 features were significantly altered in patients with CF when compared to a corresponding matched control subject. A negative sign means that the *m/z*-value (mass-to-charge ratio) of the molecular ion was measured in negative ion mode. ^{13}C isotopes are removed. (B) Two features selected by support vector machine classification and tested in a leave-one-out-cross-validation ($n = 60$). Negative sign means that the *m/z*-value of the molecular ion was measured in negative ion mode. The ion state of each identified compound is described in eTable 6 in the supplementary file.

Breath signal features		Between-groups changes ($n = 60$)			
m/z measured ion	Tentative ID	*p*	*q*	Patient/control mean ratio	95% CI
95.0397	—	4.2E-04	0.079	0.88	0.56/0.93
96.0236	$C_5H_{10}N_2O$	4.2E-04	0.079	0.90	0.65/0.94
100.9789	—	1.3E-04	0.074	0.86	0.5/0.92
106.0077	—	1.1E-03	0.079	0.92	0.74/0.96
111.0528	—	5.6E-04	0.079	1.05	1.03/1.16
118.0055	Pyridine	1.0E-03	0.079	0.90	0.63/0.94
118.9900	—	6.9E-04	0.079	0.92	0.73/0.96
134.9556	—	5.6E-04	0.079	0.90	0.66/0.94
140.9714	—	3.4E-04	0.079	0.85	0.47/0.92
152.9647	—	3.4E-05	0.051	0.90	0.63/0.94
157.0476	$C_5H_{10}O_4$	4.1E-05	0.051	1.09	1.06/1.31
158.0512	—	1.2E-04	0.074	1.07	1.04/1.22
158.0574	—	1.7E-03	0.087	1.04	1.03/1.15
162.9496	—	2.5E-04	0.079	0.86	0.49/0.92
168.0491	Benzothiazole	8.1E-04	0.079	0.94	0.8/0.97
168.9664	—	1.1E-03	0.079	0.87	0.55/0.93
180.9597	—	4.7E-04	0.079	0.91	0.67/0.95
181.0847	$C_{10}H_{12}O_3$	1.7E-03	0.087	1.04	1.02/1.13
190.9444	—	1.9E-03	0.093	0.87	0.56/0.93
193.0843	$C_{11}H_{12}O_3$	1.2E-03	0.081	1.05	1.03/1.16
193.1212	—	1.5E-04	0.074	1.05	1.03/1.16
194.0573	$C_8H_{13}NO_2$	7.3E-04	0.079	1.06	1.03/1.19
199.0738	Hydroxyoctanoic acid	7.7E-04	0.079	1.04	1.02/1.12
201.0727	$C_{13}H_{13}S$	1.2E-03	0.083	1.08	1.06/1.28
203.0839	$C_{11}H_{16}O$	1.1E-03	0.079	1.05	1.03/1.15
205.9600	—	6.9E-04	0.079	0.94	0.78/0.96
207.1371	$C_{13}H_{18}O_2$	1.6E-03	0.087	1.04	1.02/1.13
212.9270	—	5.9E-04	0.079	0.87	0.55/0.93
214.9175	—	1.8E-03	0.090	0.88	0.59/0.93
224.8931	—	7.3E-04	0.079	0.91	0.67/0.95
230.9369	—	1.3E-03	0.083	0.86	0.52/0.93
231.1366	—	7.7E-04	0.079	1.05	1.03/1.16
233.1530	$C_{15}H_{20}O_2$	2.0E-03	0.094	1.03	1.02/1.11
244.0273	$C_9H_9NO_5S$	9.0E-04	0.079	0.93	0.75/0.96
246.0924	—	8.6E-04	0.079	1.07	1.04/1.23
247.1312	—	7.7E-04	0.079	1.04	1.03/1.15
264.9101	—	7.7E-04	0.079	0.90	0.65/0.94
266.0068	$C_9H_9NO_6$	1.4E-03	0.084	0.92	0.72/0.95
280.9138	—	1.4E-03	0.084	0.91	0.7/0.95
311.2389	Docosahexanoic acid	1.0E-03	0.079	0.93	0.76/0.96
348.9505	—	1.7E-03	0.087	0.92	0.72/0.95
408.9197	—	1.4E-03	0.083	0.89	0.62/0.94
412.8755	—	1.1E-03	0.079	0.86	0.51/0.93
−63.7675	—	1.3E-03	0.083	0.85	0.47/0.92
−64.9624	—	2.0E-03	0.094	0.85	0.45/0.92
−89.0064	C_3H_6OS	1.4E-03	0.083	1.08	1.05/1.27
−95.9522	—	9.5E-04	0.079	0.85	0.46/0.92
−99.0815	$C_6H_{12}O$	9.0E-04	0.079	1.03	1.02/1.10
−189.0768	Oxohexanoic acid	2.4E-04	0.079	0.86	0.51/0.93

B

Breath signal features		
m/z	Tentative ID	Selection frequency
96.0236	$C_5H_{10}N_2O_3$	60/60
−189.0768	Oxohexanoic acid	59/60

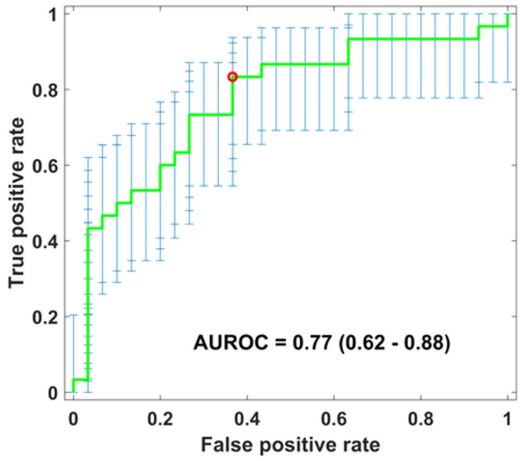

Figure 3. Plot of the receiver operating characteristics from a prediction with two features selected by a support vector machine model and tested within a leave-one-out cross validation (LOOCV). The area under the curve covers 77.1% (confidence interval 62.2%–87.8%).

used for tentative annotations because they contain fragment ions from different precursors on the same unit mass. In addition, compound classes were detected by classifying possible sum formula. Finally, 5 compounds were putatively annotated (tentative compound name) and 13 compounds were putatively characterized (tentative sum formula). For 31 compounds, it was not possible to find a rationale formula. The same issue was present in previous breath analysis studies [12, 13] and can possibly be explained by unusual fragment ions, elemental compositions other than the ones searched for, or influences of the ion source. A detailed list of the compound identification can be found in the supplementary material (eTable 6).

Discussion

In this study, we applied a novel real-time untargeted breath analysis by means of SESI-HRMS to discover metabolites that allow for the differentiation of patients with CF and healthy controls. With a total of >3000 detected features in 60 patients the dataset of this study is more comprehensive than most previous studies and represents a further step towards biomarker identification in patients with CF [15–20, 22].

Recently there has been an increasing interest in exhaled breath biomarkers for patients with CF. One exemplary study investigated CF patient's exhaled breath trapped in Tedlar bags by using gas chromatography—mass spectrometry [21]. It was possible to measure 6000 compound and approx. 1000 features were used for further analysis. In addition, it was possible to predict CF with an area under the ROC curve of 0.962. These results are excellent, however, besides the statistical imprecision there are several disadvantages of such methods: first of all, breath is not directly analyzed but stored in plastic bags. This leads to sample degeneration due to, e.g, adsorption of sticky molecules onto the plastic surface. In addition, the analysis does not take place in real-time, because of the chromatographic method. With the goal to overcome these pre-analytic problems, this is the first time that SESI-HRMS breath analysis was applied to patients with CF. In contrast to previous studies [15–20, 22], this untargeted approach enables the identification of disease-specific breath patterns including VOCs and fatty acids. This method allows for the simultaneous analysis of tiny concentrations of pathophysiological relevant markers.

While our current experimental setting with an untargeted approach may be prone to false positive results, we aimed to identify corresponding molecules and thereby focusing on crucial pathophysiological aspects. For example, in our study diverse fatty acids and their analogous such as hydroxyoctanoic acid and oxohexanoic acid were found to be altered in exhaled breath, which confirms previous research [10]. Changes in the metabolism of fatty acids are known to be closely related to oxidative stress cascades and acidification of the airways, which is a known characteristic of CF and other inflammatory diseases [30, 31]. In CF, the oxidative degeneration of membrane lipids is a well-studied critical element which alters the fatty acid metabolism [32]. Another fatty acid, docosahexaenoic acid (DHA) was found to be significantly decreased in breath of patients with CF. Our data confirms the findings of Njoroge *et al* who showed that the levels of blood and tissue polyunsaturated fatty acids, such as DHA and lineolate, are decreased in patients with CF [33]. This metabolic alteration may be caused by the higher activity of fatty acid desaturases, which transform e.g. lineolate to arachidonate [33]. It was also shown that DHA could be converted back to eicosapentaeonate, which lowers the DHA concentration even more [33].

Moreover, the correlation of a bacterial strain to breath compounds is strengthened on a molecular level by the fact that we were able to measure elevated concentrations of compounds containing sulphur—which are indicative of bacterial aldehyde dehydrogenase [34].

We scanned databases for possible matches in terms of candidate biomarkers for CF resulting in no direct hit [15–20, 22]. Interestingly, one study suggested benzothiazoles as a potential biomarker for CF (stating $a > 10$ fold increase), however, we could not reproduce these indirect findings [21]. While we confirmed its presence in exhaled breath of patients with CF, benzothiazole levels were found to be approximately two fold decreased in exhaled breath from 30 patients with CF when compared with controls with no diagnostic value whatsoever. The CF specific pathophysiological difference of this molecule remains unknown and it is plausible, that environmental or

pre-analytical factors might have contributed to this difference.

Since only 9 out of 30 patients with CF (~30%) were treated with antibiotics at the time of measurement (eTable 3), it can be ruled out that their presence might have contributed to the discriminatory power of the study, since according to our algorithm, a feature needs to be present in at least 50% of one group (i.e. in a least 15 of 30 patients with CF).

It is important to note that the study design does not allow concluding a causal relationship between disease-specific markers and CF itself. Although we adhered to recommendations suggesting one hour fasting [27], we have not systematically assessed possible influences related to nutrition. Moreover, potential confounders (e.g. antibiotics, co-medication, diurnal effects etc) not related to the disease might have biased the results. To rule out possible confounders and type-I-errors, the disease-specific markers need to be prospectively validated in a larger set of well-characterized patients with CF (preferably in another CF-cohort). Additionally, the presented 'CF-specific' features in table 2(A) were crosschecked against already known features for COPD [12], salbutamol intake [35] and obstructive sleep apnea [13] and no overlap was detected. Within this study, this problem was tackled by using an internal leave-one-out cross validation with science-based mechanistic interpretation as a first step towards a validation study. Finally, the lack of a calibration device for the SESI-HRMS instrument is a limitation, which needs to be addressed in further studies.

Notwithstanding these limitations, the prospect of identifying CF specific markers and exhaled metabolites reflecting underlying pathophysiological mechanisms in real-time with high chemical selectivity is of general interest to clinicians. Currently, most treatment decisions in CF patients remain based on clinical judgment and secondary parameters derived from spirometry, radiology, or blood marker analysis [36]. Supplementary exhalome analysis might therefore prove as an attractive and valuable tool in everyday clinical practice [5]. Potential areas of interest of this novel technique include (subgroup) diagnostics, therapy guidance, and monitoring.

Conclusion

Real-time SESI-HRMS breath analysis allows identifying CF specific compounds. Certain compounds could be linked to already known pathophysiological aspects of CF, suggesting a biomarker potential. In particular, the colonization of bacterial strains may be traceable via real-time exhaled breath analysis. A validation study is warranted to put the currently retrieved dataset to a prospective test.

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Contributorship

Conception and design: MK, TG, LB, PMLS, RZ. Data acquisition: LB, TG, DGG and NS. Analysis and interpretation of data: LB, TG and MTG. Drafting the article: TG, LB, MK and RZ. Revising the article for important intellectual content and final approval: all authors.

Data access, responsibility, and analysis

TG and LB had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflit of interest

Dr Singer reports personal fees from Vertex, personal fees from Novartis, outside the submitted work.

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