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PAPER

Assessing infection and inflammation in people with cystic fibrosis using their breath: a pilot longitudinal study

Antao Gao[1], Josephine Ackah-Cudjoe[2], Mostafa Hashemi[1], Samantha L Durfey[3], Edward F McKone[4], Brandie D Wagner[5], Pradeep K Singh[3,6] and Jane E Hill[1,2,*]

[1] Department of Chemical and Biological Engineering, University of British Columbia, Vancouver, BC, Canada
[2] School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada
[3] Department of Microbiology, University of Washington School of Medicine, Seattle, WA, United States of America
[4] St. Vincent's University Hospital, Dublin, Ireland
[5] Department of Biostatistics and Informatics, Colorado School of Public Health, University of Colorado, Aurora, CO, United States of America
[6] Department of Medicine, University of Washington School of Medicine, Seattle, WA, United States of America
[*] Author to whom any correspondence should be addressed.

E-mail: jane.hill@ubc.ca

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Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR) modulators have revolutionized cystic fibrosis (CF) management, but airway infections and inflammation persist. This study evaluates longitudinal exhaled breath profiles and identifies volatile organic compounds (VOCs) that correlate with sputum bacterial load and neutrophil elastase (NE) levels during ivacaftor and/or antibiotic treatment. Eight people with CF (pwCF) with a heterozygous R117H CFTR mutation and chronic lung infection with *P. aeruginosa* (*Pa*) and/or *S. aureus (Sa)* were enrolled in a longitudinal study at St. Vincent's University Hospital in Dublin, Ireland. Breath samples and sputum samples were collected at multiple time points. Correlation analyses were performed between VOC abundances and bacterial load or NE levels. We observed temporal changes in breath profile across the eight subjects during the ivacaftor and/or antibiotic treatment. Within *Pa*-infected subjects, 54 VOCs showed consistent directional Pearson correlation and at least moderate repeated measures correlation (rmcorr) coefficient (r_{rm}) with bacterial load, including the 12 VOCs previously reported as *Pa*-associated markers. Similarly, within *Sa*-infected subjects, we identified eight VOCs that showed consistent directional correlation and at least moderate r_{rm} with bacterial load, including four previously reported *Sa*-associated markers. Additionally, we identified 7 exploratory VOC candidates that consistently correlated with changes in NE levels, each demonstrating agenerally weak r_{rm} across eight subjects. This study demonstrates that breath profiles of pwCF change over time during ivacaftor and/or antibiotic treatment. Identifying specific breath VOCs whose levels correlate with *Pa* or *Sa* CFU/ml, as well as NE levels, supports further evaluation of exhaled breath VOCs as non-invasive markers for monitoring airway infection and inflammation in pwCF.

1. Introduction

The leading cause of morbidity and mortality in cystic fibrosis (CF) is pulmonary disease driven by chronic airway colonization and infection, inflammation, and mucus obstruction, which ultimately results in bronchiectasis and progressive lung damage [1–3]. The extensive use of cystic fibrosis transmembrane conductance regulator (CFTR) modulators has revolutionized CF management [4, 5]. Studies on the use of CFTR modulators report improved clinical measures [5]; however, several studies reported the persistence of airway infection and inflammation following ivacaftor and/or lumacaftor treatment [6–14], even with antibiotic treatment [12]. This underscores the need for continued monitoring of lung infection

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and inflammation in people with CF (pwCF) under CFTR modulator and/or antibiotic treatment.

The conventional method for detecting lung pathogens and tracking lung inflammation in pwCF relies on obtaining a lung sample, typically sputum collection [15–17]. However, with nearly 90% of pwCF now eligible for CFTR modulators [18], reduced sputum production has been noted among adults with CF following initiation of modulator therapy [19, 20]. Additionally, sputum expectoration in pediatric CF patients has always posed challenges, leading to the use of bronchoalveolar lavage fluid (BALF), an invasive alternative sample [21]. As more individuals in the CF population transition to CFTR modulators, there is a pressing need to explore non-sputum alternatives for monitoring pathogen presence and burden, as well as the concomitant inflammation in pwCF.

Using volatile organic compounds (VOCs) in exhaled breath to detect and monitor lung infections and inflammation is a promising option for patients who produce little or no sputum. Breath studies on lung infections in pwCF were mostly conducted before the widespread use of CFTR modulators. In those studies, signatures to detect key CF pathogens, including *Pseudomonas aeruginosa* (*Pa*) [22–29], *Staphylococcus aureus* (*Sa*) [30, 31], and *Aspergillus fumigatus* [31–33], were reported. In pwCF receiving CFTR modulator therapy, studies exploring specific breath VOCs are limited. However, some have tracked changes in breath VOC profiles over time and observed significant differences before and after CFTR modulator initiation [30, 34, 35].

In this observational pilot study, we tracked the exhaled breath VOCs of a cohort of eight pwCF [12] through the initiation of ivacaftor treatment, intensive antibiotic therapy, and follow-up for over a year. We investigated the longitudinal dynamics of VOCs in relation to changes in sputum bacterial load of *Pa* and *Sa*, as well as changes in sputum neutrophil elastase (NE) level over time, to explore their potential as non-invasive markers for longitudinal monitoring of infection and inflammation.

2. Materials and methods

2.1. Study design and subjects

This was a longitudinal observational study performed as part of the clinical trial of CFTR modulators plus antibiotics (titled 'Combined Effect of CFTR Modifiers and Intensive Antibiotic Treatment') conducted at the National Referral Center for Adult CF at St. Vincent's University Hospital, Dublin, Ireland, in 2016. The study protocol was approved by both St. Vincent's Hospital (Protocol number: ARIS2) and Dartmouth College (CPHS number: STUDY00028248). Written informed consent was obtained from all subjects before enrollment.

Subject recruitment and sample collection occurred from November 2016 to December 2017. The study included pwCF aged 25–64, diagnosed with heterozygous CFTR-R117H mutations, and presenting with either chronic *P. aeruginosa* (*Pa*), *S. aureus* (*Sa*), or co-infections, which were confirmed by microbial culture [12]. All subjects received ivacaftor therapy throughout the study. One week after starting ivacaftor treatment, pathogen-specific antibiotics were administered for 3.5 months (figure 1). During the treatment period, physical examinations and pulmonary testing were performed. Respiratory samples were also collected, specifically sputum, throat swabs, and breath samples (figure 1).

2.2. CFU and NE

Sputum specimens were homogenized with cold 0.1% dithiothreitol. One aliquot of homogenized sputum was serially diluted and plated on MacConkey agar (Difco) and mannitol salt agar (Difco) to quantify *Pa* and *Sa* concentrations, respectively. Homogenized sputum was processed according to the CF Therapeutics Development Network Coordinating Center Standard Operating Procedure. Free NE activity was analyzed using a spectrophotometric assay (Sigma Diagnostics, St. Louis, MO) [12].

2.3. Breath sample collection

Breath samples were collected across seven time points at day 0, 2, 7, 21, 196, 217, and 385 over the 13 month duration of the study. Breath samples collected on Day 0 were obtained prior to the first dose of ivacaftor, and samples collected on Day 7 were obtained prior to the initiation of antibiotic therapy.

Breath sampling was performed during scheduled clinic visits, typically in the morning. Subjects were not required to fast. Inhaled medications were administered as part of routine clinical care. Whole breath samples were collected by having subjects exhale through a mouthpiece connected to a disposable filter (CareFusion, CA, United States) into a preconditioned 5 L Tedlar® bag (SKC Inc., PA, United States). Following normal inhalation, subjects were instructed to exhale a small volume, pause briefly, and then exhale the remainder of their breath into the bag. Two technical replicates (2 L each) were collected from each Tedlar bag by transferring the breath sample into thermal desorption tubes (TDTs, Carbopack *X*, *Y*, and Carboxen 1000, Gerstel, MD, USA) via a programmed vacuum pump. Paired room air samples were collected alongside the breath samples by drawing ambient room air directly onto TDTs using the same programmed vacuum pump. TDTs were shipped to the Hill Lab and stored at 4 °C until instrumental analysis. Prior to shipment, TDTs were stored at St. Vincent's Hospital for less than one month. Shipping duration was less than 72 h, and samples were kept at about 4 °C during shipment. Upon

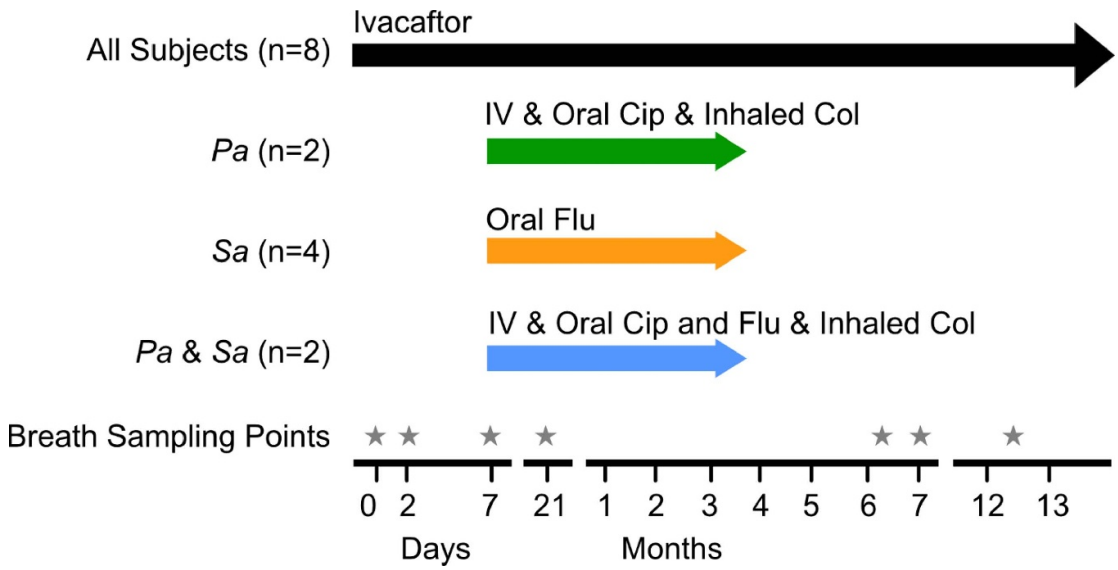


Figure 1. Study Design. All subjects received ivacaftor, while subjects infected with *P. aeruginosa* (*Pa*), *S. aureus* (*Sa*), or both, initiated intravenous (IV), oral, or inhaled antibiotics, depending on their infection status [12], for 3.5 months. The ⋆ represents breath collection. Antibiotic abbreviations: Flu, flucloxacillin; Cip, ciprofloxacin; Col, Colomycin.

arrival at the Hill Lab, TDTs were stored for 10 days to nine months before analysis.

2.4. Analytical instrument

Samples were analyzed within three analytical batches based on a specific time range of collection (table S1). Breath molecules collected on TDTs were desorbed at 330 °C into a cryogenically cooled (−120 °C) inlet liner (silanized glass wool with notch) of a two-dimensional gas chromatography time-of-flight mass spectrometer (GC × GC-TOFMS) analytical system (LECO Corporation, MI, USA) with an Agilent 6890 GC (Agilent Technologies, CA, USA) equipped with a thermal desorption unit, cooled injection system, and a multi-purpose sampler autosampler (Gerstel, MD, USA). After desorption, the inlet was heated from −120 to 275 °C at 12 °C s^{-1}, releasing analytes into a column set comprising a Rxi-624Sil primary column and a Stabilwax secondary column (Restek, PA, USA). The primary oven was initially held at 35 °C for 0.5 min, then ramped to 230 °C at 7 °C s^{-1}, and held for 5 min. The secondary oven (Stabilwax column) and quad-jet modulator were heated in sync with the primary oven, with temperature offsets of + 5 °C and + 30 °C, respectively. A modulation period of 2 s (alternating 0.5 s hot and 0.5 s cold pulses) was used. The helium carrier gas flow rate was 2 mL min^{-1}. The time-of-flight mass spectrometer collected spectra over the range of 30–500 m z^{-1} at a rate of 200 Hz.

2.5. Chromatographic data analysis

Chromatographic data from each batch were pre-processed and aligned with ChromaTOF software, version 4.7 (LECO Corporation, MI, USA). The maximum retention time deviations of 6 s and 0.15 s were set for the first and second dimensions, respectively. Peaks were identified by a forward search of the National Institute of Standards and Technology 2011 library. A signal-to-noise cutoff of 50:1 was implemented during data processing. Aligned data from different batches were merged by comparing retention time and mass spectra. The Metabolomics Standards Initiative (MSI) framework was adopted for compound identification. Putative compound identification (MSI level 2) required a library match score of ⩾750 and at least four library hits corresponding to the same compound name. When these criteria were not met, VOCs were assigned a chemical formula if a minimum match score of ⩾700 was achieved and at least five library hits gave the same chemical formula. Chemical class (MSI level 3) was assigned if a minimum match score of ⩾600 was achieved and the top five library hits belonged to the same chemical class.

2.6. Statistical data analysis

Following alignment, statistical analysis and machine learning were conducted in *R* version 4.2.3 (*R* Core Team, Vienna, Austria). Before statistical analysis, artifacts and environmental contaminants (e.g. silane, phthalate, sarcosine, nitrous oxide, etc) were removed from the data [36]. Data from two technical replicates for each sample were averaged and then normalized using probabilistic quotient normalization [37], followed by $\log_{10}$ transformation and auto-scaling. Heatmaps were used to visualize temporal changes in VOCs based on normalized peak

areas, with VOCs ordered by increasing repeated-measures correlation coefficients.

A principal component analysis and PERMANOVA were applied to evaluate the presence of batch effects (figure S1). To assess whether prolonged TDT storage was associated with systematic changes in the measured VOC profiles, samples were grouped according to storage duration ($\leqslant$1 month versus $\geqslant$6 months) (table S1). The total number of detected chromatographic peaks and the total peak area per sample were compared between the two storage-duration groups using Wilcoxon rank-sum test. No significant differences were observed (figure S2).

Associations between longitudinal changes in VOC abundance and changes in sputum CFU/ml or NE were assessed in two sequential steps (figure S3). Step 1—Within-subject directional screening. For each VOC and each subject, a Pearson correlation [38] was calculated between the normalized VOC peak area and the corresponding $\log_{10}$-transformed sputum colony-forming units per milliliter (CFU/ml) or NE levels. These per-subject coefficients were only used to assess directional consistency. A VOC was carried forward only if the sign of the within-subject Pearson coefficient was the same in every subject in that group. Step 2—Pooled repeated-measures correlation. For VOCs passing Step 1, a repeated-measures correlation (rmcorr) was calculated across all participants within the group using the *rmcorr* R package [39], with participant ID as the repeated-measure factor. Observations contributing to each rmcorr analysis were summarized in table S2. This yields one common coefficient (r_{rm}). The analysis was performed using days when both sputum CFU/ml or NE level and breath samples were available, and NA values in CFU/ml or NE were excluded. Correlation strengths were categorized as weak ($0.2 \leqslant |r_{rm}| < 0.4$), moderate ($0.4 \leqslant |r_{rm}| < 0.6$), and strong ($|r_{rm}| \geqslant 0.6$) [40]. Raw p-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) method. Sensitivity to analytical batch and storage time by refitting each rmcorr model with these terms as additional fixed effects (VOC $\sim$ subject + analytical batch + storage time + biomarker). The biomarker coefficient from each adjusted model was converted to a partial correlation coefficient for comparability with r_{rm}.

3. Results and discussion

3.1. Clinical course

A total of eight pwCF (three females) were recruited during routine clinic visits. Participants had a mean age of 41.4 $\pm$ 7.7 years and a mean body mass index (BMI) of 24.3 $\pm$ 4.2. All subjects had heterozygous CFTR-R117H mutations, with five subjects carrying R117H/ΔF508 mutations. At enrollment, two subjects had chronic *Pa* infection, four had chronic *Sa* infection, and two were co-infected with *Pa* and *Sa*. All subjects initiated ivacaftor therapy at the start of the study. One week later, antibiotic treatment was administered and continued for 3.5 months. Participants' demographics, clinical characteristics, and treatment regimens are summarized in table 1. The CFU counting, NE levels, and breath collection history are summarized in tables S1 and S3–5.

3.2. Volatile profiles change in pwCF receiving ivacaftor and/or antibiotic treatment

We aimed to characterize the breath profile of all pwCF by (1) exploring the chemical composition of pwCF's breath, and (2) examining the temporal changes in pwCF's breath profile during ivacaftor and antibiotic treatment.

We identified 536 unique VOC features in breath samples collected from all eight subjects. The chemical composition of all breath samples across these pwCF was mainly composed of aliphatic hydrocarbons (54% of the total 536 peaks), carbonyl compounds (15%), aromatic compounds (9.3%), followed by halogenated compounds, alcohols, ethers, and others. Exploratory PLS-DA visualization suggested possible time-related structure in the breath VOC profiles across sampling days (figure 2). Given the small cohort and high dimensionality, this pattern should be interpreted cautiously and does not represent a validated temporal classification. We observed temporal changes in each subject's breath profile from day 0 to day 385 in the heatmap, with each subject generally exhibiting a subject-specific pattern (figure S4).

Although not in breath, several studies have explored chemical classifications of VOCs in BALF and sputum headspace. Nasir and colleagues [41] reported hydrocarbons as the most abundant class in both *Pa* ($n = 11$) and *Sa* ($n = 9$) chronic infections, followed by ketones and alcohols, indirectly supporting our finding. Hahn and colleagues [42] reported that alcohols dominated the sputum headspace VOCs of a school-aged pwCF with multiple lung infections (including *Pa*), differing from our results. Studies have reported changes in breath VOC profiles following modulator therapy and observed differences in these profiles before and after treatment with Trikafta or Orkambi [30, 34]. Consistent with these findings, we observed temporal changes across all post-ivacaftor therapy samples relative to pre-therapy samples.

These overall VOC profile changes prompted us to examine whether specific VOCs were associated with longitudinal changes in clinical measurements. We therefore examined whether individual VOCs

Table 1. Study population characteristics.

Subject ID	Genotype R117H/	Age at entry (years)	Sex at birth	BMI	Pathogen cultured at entry	Oral antibiotics	Inhaled antibiotics	IV antibiotics
SA1	ΔF508	45	Male	25.2	*Sa+*	Flu	—	—
SA2	ΔF508	40	Male	24.2	*Sa+*	Flu	—	—
SA3	ΔF508	41	Female	32.5	*Sa+*	Flu	—	—
SA4	2622 + 1 G → A	25	Male	21.1	*Sa+*	Flu	—	—
PA1	M156R	42	Female	18.5	*Pa+*	Cip	Col	Mer, Col
PA2	ΔF508	46	Female	25.1	*Pa+*	Cip	Col	Cef, Tob
PS1	ΔF508	52	Male	26.1	*Pa+&Sa+*	Flu, Cip	Col	Cef, Tob
PS2	M156R	40	Male	21.4	*Pa+&Sa+*	Flu, Cip	Col	Cef, Col

Sa+: S. aureus culture positive; *Pa+: P. aeruginosa* culture positive; *Pa+&Sa+*: Co-infection of *P. aeruginosa* & *S. aureus; Pa-&Sa-*: Negative culture for both *P. aeruginosa and S. aureus*. Subject IDs: SA1–4, subjects infected with *S. aureus*; PA1–2, subjects infected with *P. aeruginosa*; PS1–2, co-infection with *P. aeruginosa and S. aureus*. Antibiotic abbreviations: Flu, Flucloxacillin; Cip, Ciprofloxacin; Col, Colomycin; Cef, Ceftazidime; Mer, Meropenem; Tob, Tobramycin; Azt, Aztreonam.

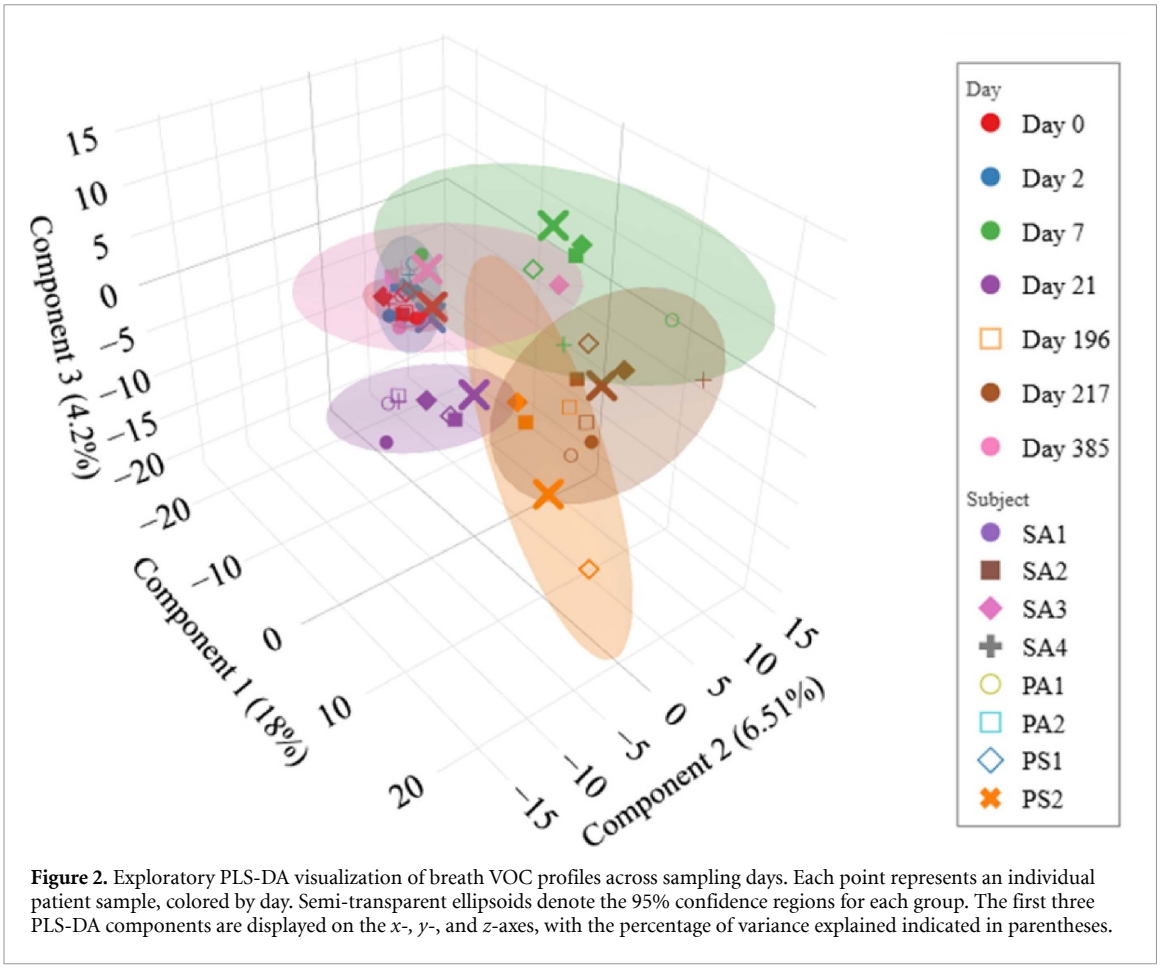


Figure 2. Exploratory PLS-DA visualization of breath VOC profiles across sampling days. Each point represents an individual patient sample, colored by day. Semi-transparent ellipsoids denote the 95% confidence regions for each group. The first three PLS-DA components are displayed on the *x*-, *y*-, and *z*-axes, with the percentage of variance explained indicated in parentheses.

were correlated with longitudinal changes in sputum bacterial load or NE level.

3.3. VOCs correlate with bacterial load

3.3.1. VOCs show consistent directional correlation with Pa sputum CFU/ml within two subjects with Pa infection

We suspected that a subset of VOCs in the breath of our pwCF with chronic *Pa* lung infection might correlate with temporal changes in sputum *Pa* CFU/ml, which, to our knowledge, has not been explored previously in *in vivo* samples.

From the breath samples of patients PA1 ($n = 6$) and PA2 ($n = 5$), we identified 54 VOCs of particular interest. Among these, 12 VOCs (table 2) had been previously reported among a list of 216 putative *Pa* volatile candidate markers from VOC studies on breath [23, 25, 28, 29, 43–52], sputum [27, 42, 53, 54], and BALF [41] in pwCF, and *in vitro* studies of *Pa* VOCs of clinical strains [55–60] (figure 3).

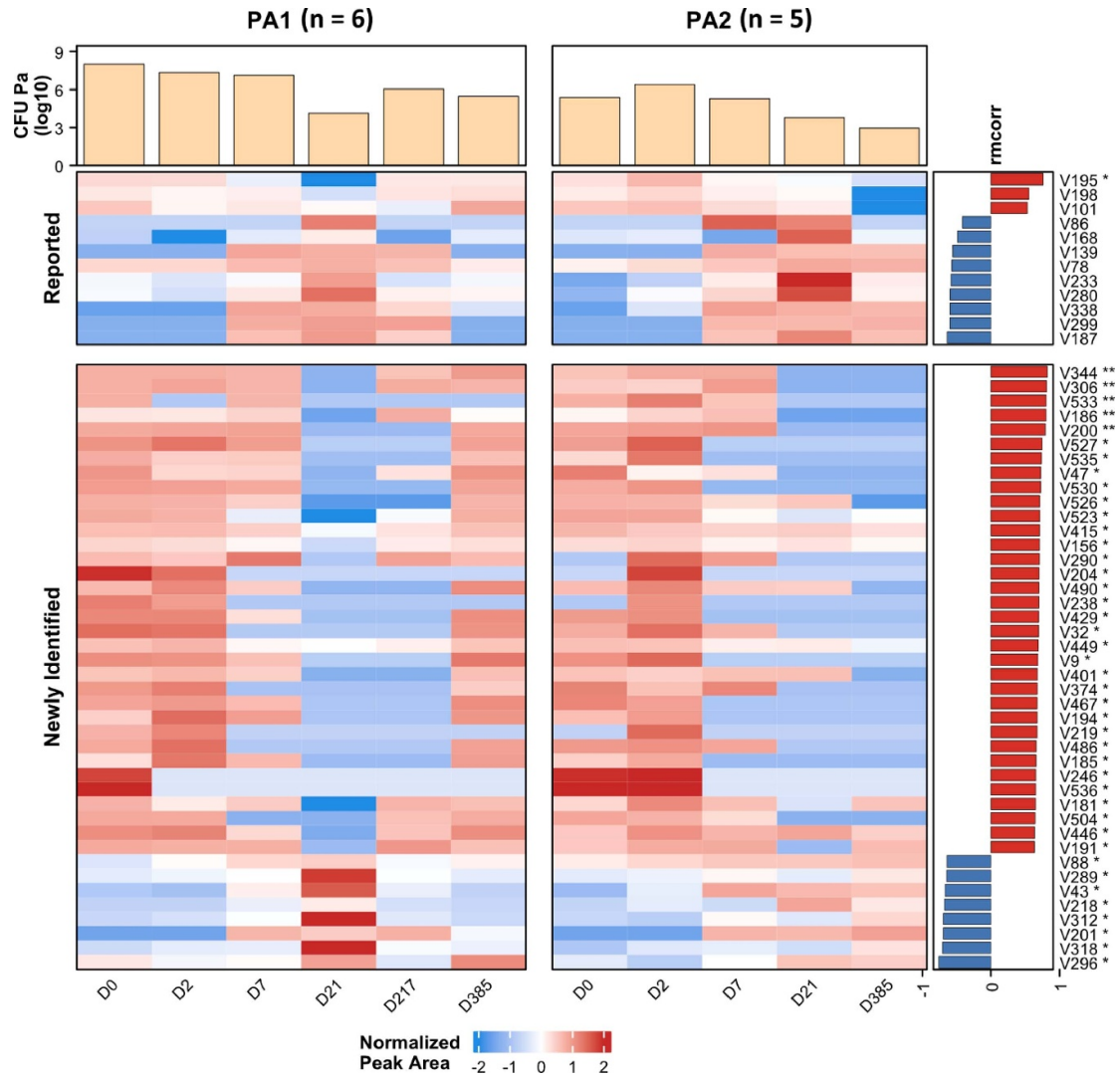


Figure 3. Previously reported *Pa* VOCs and new exploratory VOC candidates. Heatmap showing the normalized peak areas of previously reported and new exploratory VOC candidates. The labels at the bottom indicate the days since the treatment initiation. The top annotation of the heatmaps is a bar plot showing $\log_{10}$-transformed *Pa* CFU/ml in PA1 and PA2. Paired breath–CFU observations per participant are indicated by n; only paired time points were included. The right annotation shows repeated measures correlation coefficients (r_{rm}) between VOCs and *Pa* CFU/ml. VOC indices were annotated for each VOC and can be referenced in tables 2 and S5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (before FDR correction).

The remaining 42 (table S5) were new exploratory VOC candidates with strong correlation ($|r_{rm}| > 0.6$) and raw $p < 0.05$. Subject-specific Pearson correlation coefficients between *Pa* CFU/ml and these VOCs in PA1 and PA2 are provided in tables S6 and S7. Eighteen of the 54 VOCs were detected in at least one room air sample. When the same correlation analysis was applied to room air samples, five of these 18 VOCs showed correlations with direction and magnitude comparable to those observed in breath (table S13).

Among the 12 reported VOCs (table 2), eight VOCs showed negative correlations ($r_{rm} = -0.41$ to -0.59) with sputum *Pa* CFU/ml, whereas four VOCs showed positive correlations ($r_{rm} = 0.53$–0.76). Of the 42 newly identified VOCs (table S6), 34 VOCs were positively correlated with sputum *Pa* CFU/ml ($r_{rm} = 0.64$–0.83), including 22 hydrocarbons, three carbonyl compounds, 1-heptanol and one other alcohol, one ester, one ketone, and five VOCs that need further confirmation. The remaining eight newly identified VOCs were negatively correlated with sputum *Pa* CFU/ml ($r_{rm} = -0.76$ to -0.64), comprising four hydrocarbons, one carbonyl compound (putatively identified as 1-octanal), one halogenated compound, one heterocyclic compound, and one unconfirmed compound.

Although it is challenging to compare our longitudinal study with the published cross-sectional studies of breath and sputum in pwCF, we observed some encouraging similarities, as well as some differences, compared to those studies. Out of the 12 previously reported VOCs (figure 3), eight VOCs showed correlations with sputum *Pa* CFU/ml that align with findings in previous clinical or *in vitro* studies [27, 50, 54, 57, 59]. This observed alignment implies that VOCs

Table 2. Previously reported VOCs associated with *Pa* or *Sa*.

VOC Index	Putative VOC Name	r_{rm}	Raw p-value	FDR	MSI Level	Prior Evidence	Study Matrix	Group with *Pa* or *Sa* infection	Control Group
Previously Reported *Pa* VOCs									
V139	2-Pentanone	−0.55[a,c]	0.10	0.26	Level 2	Kos *et al* [50]	Breath	16 pwCF with *Pa* lung infection	28 pwCF who did not have a positive *Pa* sputum culture
V338	Phenol	−0.59[b]	0.07	0.26	Level 2				
V187	Hexanal	−0.63[b]	0.05	0.26	Level 2	Španěl *et al* [25]	Breath	20 pwCF with *Pa* lung infection	38 pwCF who had a negative *Pa* culture at the time of sampling
V195	2,4-Dimethyl-1-heptene	0.76[a,c]	0.01	0.26	Level 2	Savelev *et al* [27]	Sputum	Four pwCF and 18 people with non-CF bronchiectasis cultured positive for *Pa*	12 people with non-CF bronchiectasis cultured negative for *Pa*
V198	2,3-Dimethylheptane	0.55[a,c]	0.10	0.26	Level 2	Goeminne *et al* [54]	Sputum	19 pwCF or non-CF bronchiectasis harboring a chronic *Pa* lung infection	nine pwCF or non-CF bronchiectasis not infected with *Pa*
V86	2-Ethoxy-2-methylpropane	−0.41[a]	0.23	0.45	Level 2				
V338	Phenol	−0.59[a,c]	0.07	0.26	Level 2				
V168	Octane	−0.49[a]	0.15	0.32	Level 2				
V78	1-Propanol	−0.56[a]	0.09	0.26	Level 2				
V139	2-Pentanone	−0.55[a,c]	0.10	0.26	Level 2				
V195	2,4-Dimethyl-1-heptene	0.76[a,c]	0.01	0.26	Level 2				
V299	Limonene	−0.59[b]	0.07	0.26	Level 2				
V233	Heptanal	−0.58[b,d]	0.08		Level 2	Davis *et al* [57]	*Pa* culture	—	—
V101	Tetrahydrofuran	0.53[a]	0.11	0.27	Level 2				
V280	Benzaldehyde	−0.59[a,c]	0.07	0.26	Level 2	Boots *et al* [59]	*Pa* culture	—	—
Previously Reported *Sa* VOCs									
V30	Ethanol	0.44[a]	0.02	0.37	Level 2	Neerinx *et al* [78]	Breath	13 pwCF with a *Sa* lung infection	Five pwCF with a negative culture for *Sa*
V15	Butane	0.56[a]	0.07	0.32	Level 2	Filipiak *et al* [56]	*Sa* Culture	—	—
V30	Ethanol	0.44[a]	0.02	0.37	Level 2				
V280	Benzaldehyde	0.38[b]	0.23	0.38	Level 2				
V30	Ethanol	0.44[a]	0.02	0.37	Level 2	Zhu *et al* [58]	*Sa* Culture	—	—
V157	3-Methylheptane	−0.40[b,c]	0.07	0.32	Level 2	Karunarathne *et al*	Breath Condensate	24 pwCF with a *Sa* lung infection	—

r_{rm} denotes the rmcorr correlation coefficient.

[a] denotes VOCs identified in our analysis showing trends consistent with previous studies.

[b] denotes VOCs identified in our analysis showing trends that contrast with previous studies.

[c] denotes VOCs that correlated with *Pa* or *Sa* CFU/ml regardless of co-infection status.

[d] denotes VOCs whose abundance in room air samples showed correlations with CFU/ml comparable to those in breath.

positively correlated with sputum *Pa* CFU/ml in our analysis were previously reported at higher levels in *Pa*-positive than in *Pa*-negative samples, whereas VOCs negatively correlated with sputum *Pa* CFU/ml tended to be reported at lower levels in *Pa*-positive samples. Three VOCs, however, showed a correlation that contrasts with prior findings. Phenol displayed conflicting trends across one sputum and one breath study. A summary of these previously reported *Pa* VOCs and comparisons is listed in table 2.

The possible origins of these VOCs are elusive and understudied; thus, the links remain largely speculative [61, 62]. Based on their chemical classes, we proposed possible biochemical context for the 54 exploratory VOCs. The positive correlation between hydrocarbons and *Pa* CFU/ml might be attributed to elevated lipid peroxidation in host cells [63–65], a process in which fatty acids are degraded, producing straight-chained hydrocarbons [61, 66, 67] or possibly branched hydrocarbons [66–68] as end products. Lipid peroxidation might be, at least in part, triggered by increased oxidative stress [69] during *Pa* infection. The negative correlation might relate to the activity of alkane hydroxylases (*alk*B1 and *alk*B2) as reported by Beilen and colleagues [70], who demonstrated that *Pa* can consume C_5—C_{16} alkanes as an energy source in an *in-vitro* study.

The negative correlations between the three aldehydes, hexanal, heptanal, and octanal, and *Pa* CFU/ml may relate to the activity of aldehyde dehydrogenase reported in *Pa*. This process converts aldehydes to acids or acyl-CoA derivatives [71], likely as a function of detoxification and utilizing them as carbon sources in osmotic stress [72, 73]. Specifically, Nathan and colleagues [74] have shown in an *in vitro* study that aldehyde dehydrogenase purified from *Pa* can metabolize butanal, pentanal, hexanal, and decanal, and convert them to associated acids, potentially supporting our observation. Indeed, this metabolic pathway was also proposed by Filipiak and colleagues [56], who suggested that *Pa* may metabolize aldehydes as carbon and energy sources. One alcohol, 1-heptanol, showed a strong positive correlation with *Pa* CFU/ml, which might be linked to the α- or β-oxidation of fatty acid derivatives in *Pa*. For all the remaining VOCs, no specific metabolic pathways could be identified. However, these pathways remain largely speculative and require further investigation.

In addition to PA1 and PA2, two subjects in this study, PS1 and PS2, were chronically co-infected with both *Pa* and *Sa*. According to the literature, approximately 30% of pwCF are co-infected with *Pa* and *Sa* [75–77]. We sought to determine whether the *Pa*-associated VOCs had consistent correlations in the context of co-infection with *Sa*. We observed that seven previously reported and seven new VOC candidates (table 2, table S6) maintained the same directional correlation in PS1 and PS2 as in PA1 and PA2, providing preliminary supporting evidence for the consistency of the VOC–CFU/ml associations in a co-infection context.

3.3.2. VOCs exhibit the same directional correlation with Sa sputum CFU/ml within subjects with Sa infection

We suspected that a subset of VOCs in the breath of pwCF with chronic *Sa* infection may correlate with temporal changes in sputum *Sa* CFU/ml, showing consistent directional trends across pwCF with *Sa*. To the best of our knowledge, this is the first study to explore such a longitudinal correlation in breath.

From the breath samples of patients SA1—SA4, we identified a panel of nine VOCs that correlated with sputum *Sa* CFU/ml. Of these, four VOCs—ethanol, butane, 3-methyl-heptane, and benzaldehyde—had been reported previously among a list of 102 putative *Sa* VOC markers from eight VOC studies [41, 56, 58, 78–82] (figure 4, table 2). The remaining four were newly identified VOC candidates with $|r_{rm}| > 0.6$ and raw $p < 0.05$ (table S8), including 1-heptanol, one nitrogen-containing compound, one aromatic compound, and a compound requiring further confirmation. Subject-specific Pearson correlation coefficients between *Sa* CFU/ml and these VOCs in SA1-4 are provided in table S9. Three of the nine VOCs were detected in at least one room air sample, and none showed correlations with direction and magnitude comparable to those observed in breath (table S13).

Among the previously reported VOCs, ethanol and butane were positively correlated with sputum *Sa* CFU/ml, whereas 3-methylheptane and benzaldehyde were negatively correlated. Among the newly identified four VOCs, 1-heptanol, the nitrogen-containing compound, and the unidentified compound were positively correlated with sputum *Sa* CFU/ml, each showing a moderate correlation ($r_{rm} = 0.6$–0.7). The aromatic compound was negatively correlated with *Sa* CFU/ml ($r_{rm} = -0.64$).

Although direct comparison between our longitudinal study and previously published studies is challenging, we observed both similarities and differences by indirect comparisons. Two previously reported VOCs, butane and ethanol, showed correlations with sputum *Sa* CFU/ml that align with previous clinical or *in vitro* studies [56, 58, 78, 80]. In contrast, benzaldehyde and 3-methylheptane showed trends opposite to those reported previously.

We also speculated about the possible metabolic pathways associated with the eight VOCs. We suspect that the positive correlation between butane and *Sa* CFU/ml might reflect increased lipid peroxidation in host cells triggered by inflammation [83], in which polyunsaturated fatty acids are degraded to produce straight-chained hydrocarbons [61, 66, 67]. For the remaining seven VOCs, we were unable to relate their correlations with sputum *Sa* CFU/ml to any specific pathways.

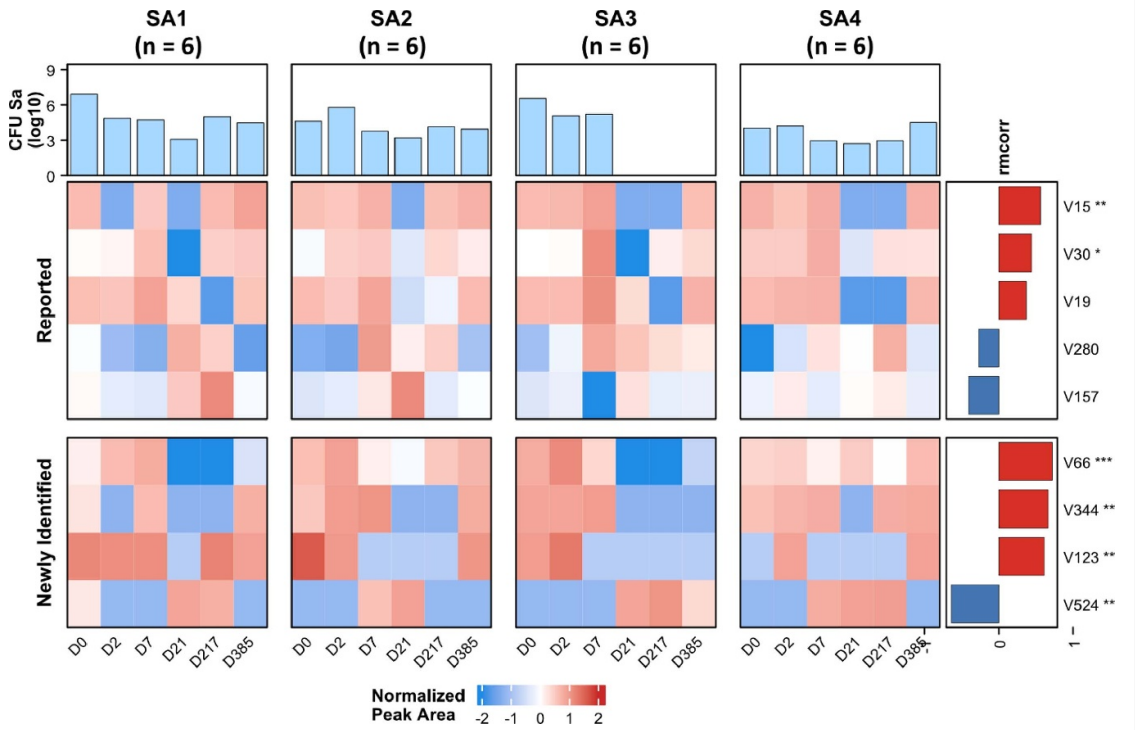


Figure 4. Previously reported *Sa* VOCs and new exploratory *Sa* VOC candidates. Heatmap showing the normalized peak areas of previously reported and new exploratory *Sa* VOC candidates. The labels at the bottom indicate the days since the treatment initiation. The top annotation of the heatmaps is a bar plot showing $\log_{10}$-transformed *Sa* CFU/ml in SA1-4. Paired breath–CFU observations per participant are indicated by n; only paired time points were included. The right annotation shows repeated measures correlation coefficients (r_{rm}) between VOCs and *Sa* CFU/ml. VOC indices were annotated for each VOC and can be referenced in tables 2 and S6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (before FDR correction).

We examined whether VOCs that correlated with *Sa* CFU/ml in subjects SA1-4 showed similar associations in the co-infected subjects PS1 and PS2. One new exploratory *Sa* VOC candidate, classified as an alcohol, maintained the same directional correlation in PS1 and PS2. While remaining speculative, this compound with concordant trends might represent a breath VOC candidate more specifically associated with *Sa*, independent of co-infection with *Pa*.

3.4. VOCs exhibit the same directional correlation with sputum NE level across eight subjects

Chronic infections in CF are associated with sustained inflammation, marked by elevated levels of inflammatory mediators such as NE [84, 85]. This inflammation may cause increased oxidative stress [86], which has been linked to lipid peroxidation and the production of potential inflammatory volatile markers in other non-CF lung diseases [87, 88]. In addition to serving as a marker of inflammation, NE is identified as an important predictor of future lung function decline in both CF and non-CF bronchiectasis [89]. We therefore explored whether a subset of VOCs in the breath of people in this study might correlate with the temporal changes in sputum NE levels. To our knowledge, no prior studies have explored this correlation in pwCF.

In breath samples from the eight subjects infected with *Pa, Sa*, or both, we detected 14 previously reported inflammation markers in our data. Although none demonstrated a consistent correlation trend across these subjects, two VOCs—octanal and heptanal—demonstrated a moderate r_{rm} ($p < 0.05$; before adjustment) (table S10).

Additionally, seven VOCs showed consistent directional correlation with sputum NE levels across PA1-2, SA1-4, and PS1-2, with a weak to moderate ($0.2 < |r_{rm}| < 0.5$) repeated-measures correlations (figure 5, table S11). Subject-specific Pearson correlation coefficients between NE level and these VOCs in all participants are provided in table S12. Of these seven VOCs, six exhibited a positive correlation with sputum NE levels, and one demonstrated a negative correlation. The six positively correlated VOCs were composed of five hydrocarbons and one VOC that needs further confirmation. The one negatively correlated VOC was an alcohol. Four of the seven VOCs were detected in at least one room air sample, and none showed correlations with direction and magnitude comparable to those observed in breath (table S13). As an exploratory comparison, we applied the same correlation analysis to sputum IL-8 and IL-1β, which were also measured in this study [12]. One VOC met the selection criteria for IL-8, whereas no VOCs met the criteria for IL-1β (table S11).

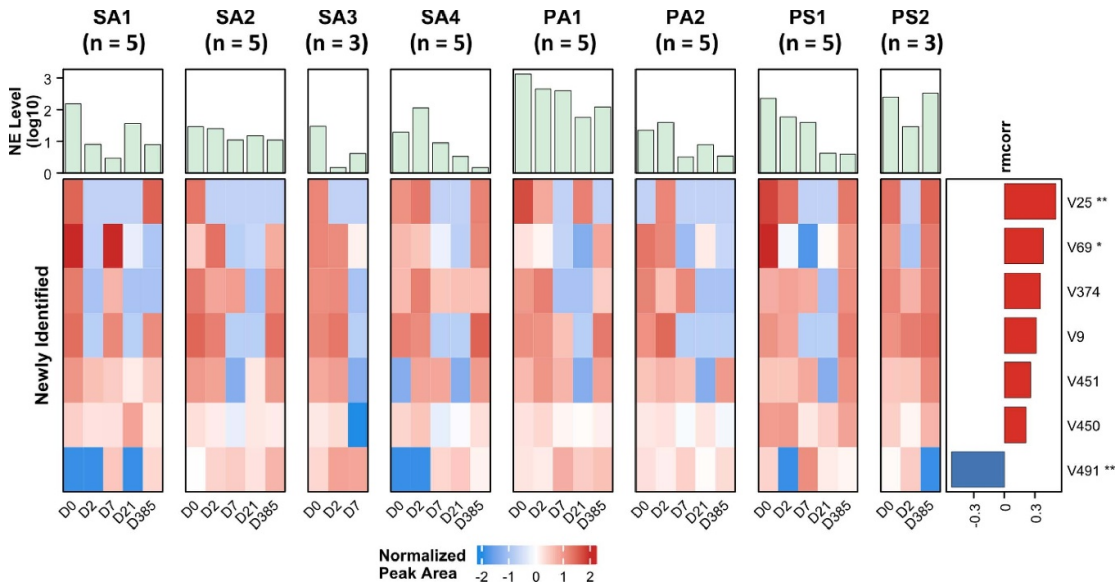


Figure 5. New exploratory VOC candidates correlated with NE level. Heatmap showing the normalized peak areas of new exploratory VOC candidates correlated with NE level. Paired breath–NE observations per participant are indicated by n; only paired time points were included. The labels at the bottom indicate the days since the treatment initiation. The top annotation of the heatmaps is a bar plot showing $\log_{10}$-transformed NE level. The right annotation shows repeated measures correlation coefficients (r_{rm}) between VOCs and NE level. VOC indices were annotated for each VOC and can be referenced in table S11.

Given the generally weak correlations between these VOCs and sputum NE levels, it is challenging to definitively link them to specific metabolic pathways. However, we speculate that the positive correlation between the five hydrocarbons, which is the predominant class, and sputum NE level may be linked to an expected elevated lipid peroxidation in host cells during inflammation, a process producing straight-chained hydrocarbons or possibly branched hydrocarbons [61, 66–68] as end products. For the remaining VOCs belonging to other chemical classes, we were unable to link them to specific metabolic pathways.

We compared the 54 VOCs associated with *Pa*, the nine associated with *Sa*, and the seven associated with NE to evaluate overlap among these groups (figure S5). Benzaldehyde and 1-heptanol were shared between the *Pa*- and *Sa*-associated VOCs. In addition, two hydrocarbons were common to the *Pa*- and NE-associated sets, both of which were positively correlated. No VOCs were shared between the *Sa*- and NE-associated sets or across all three groups.

3.5. Exploring the impact of confounding factors on data analysis

Cross-sectionally, individual-level confounding factors present within each patient raise concerns about how best to account for them in the analysis. For example, the two subjects infected with *Pa* were given different IV antibiotic treatment regimens. Although statistical methods such as mixed-effects modeling could potentially assess whether differences in antibiotic treatment regimens significantly impact VOC selection, the small number of subjects per treatment combination limits the reliability and interpretability of such an approach in this dataset. Given these constraints, our approach of identifying VOCs with consistent directional correlation in combination with rmcorr analysis across time and subjects provides a practical alternative in the context of this study.

Longitudinally, antibiotic administration after sample collection on Day 7 until Day 138 introduced a potential confounding factor in the correlation analysis, raising uncertainty about whether the associations observed across the full time frame were influenced by this intervention. To evaluate its impact, a sensitivity analysis was performed by excluding samples from Day 21, (collected while on antibiotics) and comparing correlations with those from the full dataset. We found that 38 of 54 (including both previously reported and new exploratory) *Pa* VOCs retained the same directional correlation and maintained $|r_{rm}| > 0.4$. In contrast, two out of the nine identified *Sa* VOCs showed retained consistent directionality. These findings suggest that many of the *Pa* associations remained generally consistent with the inclusion of Day 21 measurements, whereas the *Sa* associations were less stable in this cohort, although this observation should be interpreted cautiously given the limited sample size and treatment heterogeneity. We further assessed sensitivity to analytical

batch and storage duration by refitting each correlation with both as covariates (section 2.5). 49 of 54 *Pa*-associated VOCs, all 9 *Sa*-associated VOCs, and all 7 NE-associated VOCs retained their direction of association, with 35, 4, and 7, respectively, retaining the r_{rm} threshold.

Although not the primary objective of this study, we explored the degree of change in the identified VOCs associated with *Pa* or *Sa* over the first seven days of ivacaftor treatment and the two weeks following antibiotic administration. We aimed to investigate whether the observed alterations in VOCs might be partially attributable to ivacaftor or antibiotic treatment. In PA1 and PA2, out of the 54 identified VOCs, six demonstrated a substantial ($|\log_2 FC| > 2$) change between Day 0 and 7, and six between Day 7 and Day 21 (table S14). Similarly, out of the eight VOCs identified in SA1-4, one demonstrated a substantial change between Day 0 and 7, and four between Day 7 and Day 21 (table S15).

4. Limitations and future directions

This proof-of-concept study provides insights into the longitudinal dynamics of exhaled breath VOCs, exploring their relationship with sputum bacterial load and NE levels for the first time.

However, several limitations should be acknowledged. This pilot study included only eight subjects, and pathogen-specific analyses were based on very small numbers of subjects (two for *Pa* and four for *Sa*), limiting statistical power and generalizability. The concurrent effects of ivacaftor and antibiotic treatment make it difficult to fully distinguish treatment-related temporal changes from direct associations with bacterial burden or inflammation. Analytical factors, including batch effects, sample storage time variability, and environmental background VOCs, were evaluated through additional analyses and room air controls; however, their contributions to VOC variability cannot be completely excluded. Finally, VOC identities were putatively assigned rather than confirmed with authentic standards. These findings should therefore be considered hypothesis-generating and require validation in larger, prospectively designed cohorts.

We recommend that future studies include a higher number of subjects that span a variety of CFTR mutations, lung infection histories, and current highly effective CFTR modulator therapies. Future studies should incorperate batch-correction strategies, dedicated quality-control samples, and authentic standard confirmation. Future studies would also benefit from more stringent control or systematic documentation of factors known to influence breath VOC profile (i.e. time since last meal, time of day, etc).

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Supplementary material available at https://doi.org/10.1088/1752-7163/aebd12/data1.

Conflict of interest

The authors declare a conflict of interest. Some specimen collection was funded by an unrestricted investigator-initiated research grant from Vertex, Inc. No sponsor (including Vertex) influenced the study design, data analysis or interpretation, decision to publish, manuscript content, or approved the manuscript.

Author contributions

Antao Gao 0000-0002-9073-9778
Data curation (equal), Formal analysis (lead), Visualization (lead), Writing – original draft (lead), Writing – review & editing (equal)

Josephine Ackah-Cudjoe 0009-0005-1422-7593
Data curation (equal), Formal analysis (supporting), Validation (equal), Visualization (supporting), Writing – original draft (supporting), Writing – review & editing (equal)

Mostafa Hashemi 0009-0003-0433-7778
Formal analysis (supporting), Writing – review & editing (supporting)

Samantha L Durfey
Writing – review & editing (supporting)

Edward F McKone
Writing – review & editing (supporting)

Brandie D Wagner
Formal analysis (supporting), Methodology (supporting), Validation (supporting), Writing – review & editing (supporting)

Pradeep K Singh
Funding acquisition (equal), Project administration (equal), Writing – review & editing (supporting)

Jane E Hill 0000-0003-3534-5765
Conceptualization (equal), Funding acquisition (equal), Investigation (lead), Methodology (lead), Project administration (lead), Supervision (lead), Writing – review & editing (equal)

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