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Early View

Original research article

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Exhaled volatile organic compounds and lung microbiome in chronic obstructive pulmonary disease: A pilot randomized controlled trial

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Abstract

Background

Breath analyses in a burgeoning field, with interest in volatile organic compounds (VOCs) as a non-invasive diagnostic tool or an outcome measure, but no randomized clinical trials (RCT) have yet evaluated this technology in a clinical trial longitudinally. In a pilot RCT, our exploratory objectives were feasibility of measuring VOCs via multiple techniques, assessing relationships between VOCs and *Haemophilus* colonization, and whether CXCR2 antagonism with danirixin altered lung microbiome composition in individuals with chronic obstructive pulmonary disease (COPD).

Method

43 participants had VOCs and sputum biomarkers evaluated. VOCs and induced sputum were collected after 6 hours of fasting at screening, Days 1, 7 and 14. VOCs were analyzed via gas chromatography mass spectrometry (GCMS), field asymmetric ion mobility spectrometry and eNose. The primary outcome for these analyses was the relationship between VOCs and *Haemophilus* abundance determined by 16S rRNA sequencing.

Results

A joint effects model demonstrated a modest relationship between 4 exhaled VOCs and *Haemophilus* relative abundance (R^2 = 0.55) measured only by GCMS, but not as measured GC FAIMs or eNose. There was considerable variability in absolute quantities of individual VOCs longitudinally.

Conclusions

VOC measurement in clinical trials to identify subsets of COPD is feasible but assessment of new VOC technologies must include concurrent GCMS validation. Further work to standardize collection of VOCs and measuring a background or “housekeeper” VOC is required to understand and normalize individual VOC quantities.

Introduction

Breath analyses via measurement of volatile organic compounds (VOCs) is a burgeoning and emerging field, with interest in the use of VOCs as a non-invasive tool for patients diagnosis and stratification or even as an outcome measure in clinical trials, with one such example being fractional exhaled nitric oxide (FeNO) having been successfully adopted into clinical and research practice (1). Breath analyses have been shown to distinguish smokers from non-smokers (2), and COPD from non-COPD subjects (3), as well as relating to COPD severity (4) in cross-sectional sampling.

The lung microbiome may be an important disease modulator in chronic obstructive pulmonary disease (COPD), where *Haemophilus influenzae* colonization appears to increase neutrophilic inflammation including formation of neutrophil extracellular traps (NETs) (5). Although ordinarily a host defense mechanism (6), excessive NETs may cause host damage in airways disease (7), and may provide the mechanistic link between airway infection/colonization, airway inflammation and disease progression. Non-invasive diagnosis of airway infection and inflammation could enhance targeting of antibiotic or anti-inflammatory therapies such as CXCR2 antagonists, which have been shown to reduce NETs production *in vitro* (8, 9).

Breathomics provide one such opportunity for non-invasive diagnosis. Breath samples can be analyzed on a variety of platforms. Gas chromatography mass spectrometry (GC MS) is considered the gold standard and can distinguish individual VOCs. Field asymmetric ion mobility spectrometry (FAIMS), for example, via Lonestar (Owlstone Medical, Cambridge, UK), relies on VOCs from a breath sample traveling through a charged chamber, and the pattern of migration according to ionic charge allows distinguishing between individual compounds. Electronic nose (eNose) devices are electronic systems containing chemoresistor sensors that generate an electric signal upon encountering a gaseous mixture.

For example, the Cyranose (Sensigient, CA, USA) device has been used widely in the field, containing 32 composite polymer carbon black sensors that each generate a signal. By definition, eNose devices cannot distinguish individual VOCs but may be useful for pattern recognition. Whilst other platforms for breathomics also exist, FAIMS and eNose methods are currently being explored widely in the clinical research setting. In the case of eNoses, these are portable and therefore can ultimately form point of care tests. FAIMS technology also has the potential to be miniaturized for point of care use if a pattern of VOCs of interest is established.

If breathomics are to be used for patient selection in clinical studies, they first need evaluation for feasibility in randomized controlled trials (RCTs). The advent of sample collection via sorbent tubes with the ReCIVA device has only recently enabled centralized VOC analyses (10) via storage and shipping of breath samples at room temperature, but sparse longitudinal data to inform trial design has limited adoption, and to date no RCTs have evaluated VOCs longitudinally. Furthermore, no study has looked at *Haemophilus* breath signals specifically. Several studies have found VOCs or eNose signals associated with COPD exacerbations (11, 12) with three eNose studies specifically elucidating breath signals associated with bacterial/viral infection caused exacerbations (13-15). However, no study has focused on *Haemophilus* or microbiome colonization, and the eNose studies on viral/bacterial infections lacked the ability to determine specific VOCs.

We conducted pilot randomized control trial primarily to study the effects of a CXCR2 antagonist, danirixin, the primary results for which have been published (16). In this pilot RCT, our exploratory objectives were feasibility of measuring VOCs via multiple techniques, assessing relationships between VOCs and *Haemophilus* colonization, and whether CXCR2 antagonism with danirixin altered lung microbiome composition in individuals with COPD.

Methods

In a double-blind RCT (NCT03250689) (16), participants were randomized (3:1) to receive danirixin 35mg bid or placebo for 14 days (study details available in the online supplement).

Participants were included if they were aged between 50 and 75 years, with a clinical diagnosis of COPD with mild to moderate airflow obstruction (post-bronchodilator forced expiratory volume in one second (FEV1)/ Forced Vital Capacity (FVC) ratio <0.7 and FEV1% predicted (pred) ≥40% at screening), had elevated sputum neutrophil extracellular traps based on screening assay for histone elastase complexes of >0.5 units/ml sputum, and were current or former smokers with a minimum of 10 pack year history. Patients with lung diseases other than COPD or recent pneumonia were excluded, and patients on medication known to impact NETs formation were also excluded from the study, for example, use of phosphodiesterase-4 inhibitors (17): roflumilast, crisaborole and apremilast, broad spectrum phosphodiesterase inhibitors (e.g. theophylline), raloxifene and molecular weight heparin. Additionally, systemic immunosuppressive medication, including current oral corticosteroids at a dose >5 mg, concurrently or within 28 days preceding the screening visit, acute or chronic use of antibiotics, including macrolides for the prevention or treatment of COPD exacerbations were prohibited. Examples of chronic use include daily or two-three times per week for at least 3 months. Prohibited medications related to danirixin specifically were oral or injectable CYP3A4 or BCRP substrates with narrow therapeutic index.

Patients meeting inclusion criteria were randomized and underwent key assessments, including spirometry, VOC sampling via a ReCIVA device (Owlstone Medical, Cambridge, UK), induced sputum (via up to 4% nebulized saline) and venepuncture at screening, day 1, day 7 and day 14. Patients who failed screening still provided sputum and VOCs samples at the screening visit, though they were not

randomized to dosing groups for further visits and did not provide any other sample type. Sputum and breath samples from screen failures were included in analyses. VOC samples were taken after 6 hours of fasting on all visits; at the baseline visit subjects additionally provided an extra VOC sample 4 hours after dosing. Participants who were current smokers were asked to refrain from smoking for at least 4 hours prior to each visit. Sputum measurements included microbiome (profiled via 16S rRNA gene sequencing), NETs (immunoassays for histone-elastase and DNA-elastase complexes, confocal microscopy for sputum NET area), and sputum neutrophils using methods described previously (5). Sputum samples were additionally assessed for quality via percentage of squamous cell and viable leukocyte counts, and a primary completer population defined on the basis of having "good" or "acceptable" quality sputum at baseline and day 14; the primary completer population was used for NETs analyses although all sputum samples were used for the microbiome analyses. 4 pairs of VOC samples were taken at each measurement timepoint, and were subject to measurement via 3 techniques at a centralized laboratory; gas chromatography mass spectrometry (GC MS), field asymmetric ion mobility spectrometry (FAIMS) via Lonestar (Owlstone Medical, Cambridge, UK) and an electronic nose (eNose) device, Cyranose (Sensigient, CA, USA). The fourth VOC sample pair was analyzed by GC MS when possible (i.e., when the back-up was not needed due to failure of primary sample on any of the 3 techniques) to provide a replicate measurement to increase accuracy of VOC levels.

For the VOC samples, a quality control (QC) sample was run between every 4 patient samples, and background monitoring was carried out with a blank tube run after every 4 patient samples and after every QC sample. Blank tubes were clean tubes used to monitor potential carry-over from one sample to the next or incomplete desorption; neither was found to be an issue. VOC levels were corrected for

analytical variation/instrument drift by normalization to the average drift in intensity of a mixture of external standards, i.e. the QC samples. This method was found to be superior to normalization methods using the breath samples themselves, such as scaling to total signal intensity, in reducing analytical variation. The QC samples were made by spiking on a fixed volume of a QC solution onto a clean sorbent tube and briefly purging with high-purity nitrogen. The QC solution was composed of a selection of chemicals meant to reflect classes of VOCs commonly found in breath all at fixed concentrations. Ambient background controls were not collected or used for background subtraction.

TD-GC-MS chromatogram was converted into a features list and automatic mapping was applied to identify a unique set of characteristics with subsequent visual inspection to check peak shape and retention time, and specificity of ions. All molecular features of interest (MFs) were run against NIST standard database; matches between library and compound was >70%, MF was given a tentative ID.

The primary endpoint for the study was change from baseline in sputum NETs as measured via histone elastase immunoassays, and although sample size was based on feasibility, we powered the study for a 70% probability of detecting a true reduction of 30% reduction in NETs. Changes in lung microbiome and VOCs were exploratory endpoints.

To test relationships between *Haemophilus* and VOCs, PCA, single effects models, and joint effects models were done, respectively, with the sklearn, statsmodels, and cvglmnet packages in Python v3.6 in June 2019. All analyses focused on samples taken at the screening visit and were cross-sectional across the entire patient population regardless of being screening pass/fail. The effect of screening pass/fail on subsequent findings was assessed by confounder analysis and was not found to have any significant

effect. Outlier capping was performed independently on each molecular feature using Tukey Fences prior to the single and joint effect analyses. In cases where patient provided two screening breath samples that were successfully analyzed by GC MS, single effect regression models used a Huber sandwich variance estimator to allow for the inclusion of multiple samples per patient. Permutation testing was used to adjust MF p values for multiple testing (18). Joint effects regression models were built on all MFs with unadjusted p value < 0.2 from the single effects analysis using least absolute shrinkage and selection operator (LASSO) regression. The shrinkage (lambda) parameter was estimated using leave-group-out cross-validation, each group being all samples from a single patient. Relative abundance of the *Haemophilus* genus was used as a continuous outcome variable. 16S rRNA gene PCR products were analyzed using the QIIME pipeline (version 1.9.1) (19) and taxonomies were assigned using a closed reference alignment to the Greengenes 16S rRNA database (version 13_8). If identification was not possible at the genus level, the operational taxonomic unites (OTUs) were classified at a higher taxonomic level. OTUs with a maximum representation in a sample of 0.5% were excluded.

All participants provided written, informed consent. The East of Scotland Research Ethics Service 1 (Reference: 17/SS/0111) provided ethical approval for the study, which was carried out in accordance with the Declaration of Helsinki.

Results

Baseline Characteristics

43 participants were screened, 19 randomized (14 danirixin: 5 placebo), out of a planned 32 (Figure 1); the study was terminated early due to cessation of the danirixin development program. Both treatment groups were similar in terms of age and baseline FEV_1 (Table 1), although there was a greater proportion of current smokers in the placebo group (43%) in comparison to the danirixin group (20%).

VOC measures

For participants who failed screening, the VOC samples and sputum samples for the microbiome were included in the analyses for exploring the relationship between *Haemophilus* abundance and VOCs at the screening visit. There were 41 participants who provided VOC samples at the screening visit; only 1 participant was unable to provide sputum at screening.

Subsequent to screening, a primary completer population for the study was identified on the basis of acceptable sputum quality at both baseline and day 14. This resulted in 3 patients in the placebo arm and 8 in the danirixin arm being part of the primary completer population for the purposes of measuring sputum NETs.

176 VOC samples were collected from 41 patients but 68 samples were excluded at QC stage (57 rejected due to detector saturation (thermal desorption), 7 rejected due to tube leak (thermal desorption), 1 rejected as poor-quality in lab (machine maintenance), 1 rejected due to low volume at collection) and 6 samples did not have matching microbiome analysis at screening. From the screening visit, this resulted in 31 samples from 22 patients. MFs or VOCs showed considerable variability in absolute levels longitudinally.

Microbiome

There were no statistically significant differences between treatment groups in microbiome alpha diversity, total bacterial load or relative *Haemophilus* abundance (Figure 2). Lung microbiome composition appeared broadly similar to that seen in other COPD cohorts (Figure 2), but abundance of *Haemophilus* was lower than that observed in COPD cohorts enriched for frequently exacerbating participants (20, 21).

*Relationship between individual VOCs and Haemophilus, sputum neutrophils & NETs*Cross-sectional correlations between VOC levels and other factors were assessed using samples from the screening visit. GC-MS identified 105 MFs; a single effects-model for individual MFs identified 4 VOCs with significant correlations (R~0.15) with *Haemophilus* abundance. A joint-effects model with 8 VOCs gave a modest correlation with *Haemophilus* (R^2 of 0.55) (Figure 3). FAIMS identified 55 MFs, a single-effects model for *Haemophilus* identified one significant MF with poor correlation, and a joint-effects model could not be properly evaluated.

There was no overlap between VOCs that had the highest correlations with *haemophilus* abundance, sputum neutrophils or NETs (Figure 4); no significant correlation between individual VOCs and sputum neutrophils and NETs as measured via GCMS or GCFAIMS was observed but the small number of paired samples available for these analyses limited definitive conclusions. VOCs or molecular features (MF) that correlated most strongly with H*aemophilus* were different to those that correlated most strongly sputum NETs or neutrophils, suggesting distinct biological pathways and/or origins for these VOCs.

For Cyranose, 8.5% of samples could not be analyzed as sensor data were abnormally low. Sensors displayed time trends unrelated to subject, treatment, or visit; after July 2018 there was a noticeable decrease in mean and variance for all sensors suggesting sensor drift. No relationship was observed between sensor signals and *Haemophilus* abundance across the population at baseline sputum neutrophils or NETs.

Discussion

Measuring exhaled VOCs is feasible in RCTs, however backup samples should be taken along with stringent instrument monitoring due to the potential for QC failure. Individual VOCs may relate to *Haemophilus* colonization, and a join-effects model found a modest correlation between VOCs and Haemophilus relative abundance but this relationship was only apparent via GC-MS analyses.

Three of the VOCs were tentatively identified as methylated hydrocarbons of similar chemical functionality to those previously associated with inflammatory conditions in human subjects, although different to hexane, nonanal and 1-propranolol recently identified as being related to eosinophilic asthma, and undecane, indicative of a pauci-granulocytic sputum phenotype (22). Identified VOCs from our clinical, in vivo samples were distinct from those reported in literature to be released *in vitro* by *H.influenzae* (23) however it is possible that some of the unidentified hydrocarbons may prove a match; also the *in vitro* versus *in vivo* VOC profile may differ since in vivo profiles will be modulated by other microbiota components in addition to other factors such as diet or airway inflammation. There is a paucity in data for disease-specific VOCs across literature however, with Christiansen *et al* noting that no

candidate breath biomarkers in COPD were detectable in all the studies in their literature review, and only three biomarkers being reported in more than one study (24) . Thus, our present data adds to the growing library of compounds that may be important in COPD and airways disease.

One reason for the differing VOC profiles across literature may be the variability in absolute levels of compounds, which we observed in our own study, and has been noted even for established biomarkers. Fractional exhaled nitrous oxide (FeNO) is an example of an exhaled compound that has successfully been implemented into clinical practice, and can used both clinically and in trials to identify patients with eosinophilic asthma. Despite becoming an established biomarker, FeNO still demonstrates considerable intra-day, intra-patient variability in terms of absolute levels (25) . Therefore the longitudinal variability points to the need for standardized sampling protocols, since it appears that a period of fasting alone may not be enough. There is also an urgent need for identifying background VOCs for use as “housekeepers” to normalize levels of compounds against. Taking ambient background samples may also help to eliminate some sources of variation. Our work with the Cyranose eNose device also points to the need for considering calibration and drift, especially if considering use at the bedside for diagnosis.

Although sampling was acceptable to patients and site staff in our study, with overall high compliance with sampling, there was a notable rate of QC failure at the analysis stage. Since our study, further work has suggested that it may be acceptable to freeze breath samples, which may allow for backup samples to be taken and can mitigate failure at the analyses stage, although further validation work is required in this regard. Coupled with the high QC failure rate for the VOC samples and early trial termination, the

limitation in paired sputum and VOC samples limited our ability to measure longitudinal relationships between Haemophilus abundance, sputum neutrophils or NETs and VOCs.

Study results were inconclusive in determining whether CXCR2 antagonism altered lung microbiome composition in COPD due to the early termination of the study, however the two week treatment period was likely too short to expect changes in microbiome composition. Furthermore, we sampled induced sputum for microbiome and subtle changes in the lower airway may be obscured by the high biomass from oral microbiome. The lower than anticipated sample size, high VOC sample failure rate and the lower than expected *Haemophilus* relative abundance limited the ability to detect a relationship between VOCs and *Haemophilus* at screening. Whilst we included individuals with elevated sputum NETs, which may correlate with *Haemophilus* abundance (5), our trial participants had higher FEV_1 and were not enriched for frequent exacerbations (20, 21) , which could explain the lower *Haemophilus* predominance in our study.

One limitation of our study was the imbalance between placebo and danirixin groups in baseline smoking status, which could lead to differences in both NETs formation and VOCs. Participants were asked to refrain from smoking for at least 4 hours prior to each visit, however that time limit may be of insufficient duration to impact VOCs. Furthermore, ongoing systemic inflammation from smoking between visits may impact NETs production. Only 1 subject in the danirixin group was on systemic steroids and anti-infectives during the study, therefore this is unlikely to impact our overall results and conclusions. Additionally we note that there is a lack of robust evidence that steroids impact NET production.

In conclusion, measuring exhaled VOCs is feasible in RCTs, and our results suggest that VOCs may relate to *Haemophilus* abundance. Several challenges remain for implementing breath analyses into RCTs, especially the longitudinal variability in individual VOC abundance. We recommend that GC-MS form part of any VOC evaluation, and that backup samples are taken in further exploration of the utility of VOCs as a diagnostic tool.

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Data Sharing statement: Upon publication, anonymized individual participant data and study documents can be requested for further research from www.clinicalstudydatarequest.com

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Table 1 Baseline Demographics of trial participants

		Placebo (n=5)	Danirixin (n=14)
Age (years)		62 (6)	65 (7)
Sex (Male/Female)		2/3	6/8
Race (n) **White/Caucasian/European**		5	14
Current smoker (%)		2043	4320
Smoking Pack Year History		48 (13)	44 (19)
Body Mass Index (kg/m^2)		30.9	27.1
FEV1 (L)		2.49 (0.64)	1.94 (0.71)
FEV$_{1\%predicted}$		79.1 (7.5)	69.5 (18.4)
FVC (L)		4.07 (1.16)	3.34 (1.17)
FEV1/FVC		0.62 (0.08)	0.59 (0.08)
CAT score		17.0 (1.00)	17.3 (5.97)
Medications, n (%)			
Long-acting cholinergic		3 (60)	9 (64)
Short-acting beta-2 agonist		4 (80)	8 (57)
Inhaled corticosteroid		3 (60)	6 (43)
Long-acting beta-2 agonist		3 (60)	9 (64)
Corticosteroid – systemic		0	1 (7)
Anti-infectives		0	1 (7)

Results presented as mean (standard deviation).

FEV$_1$%predicted = Forced expiratory volume in 1 second % predicted

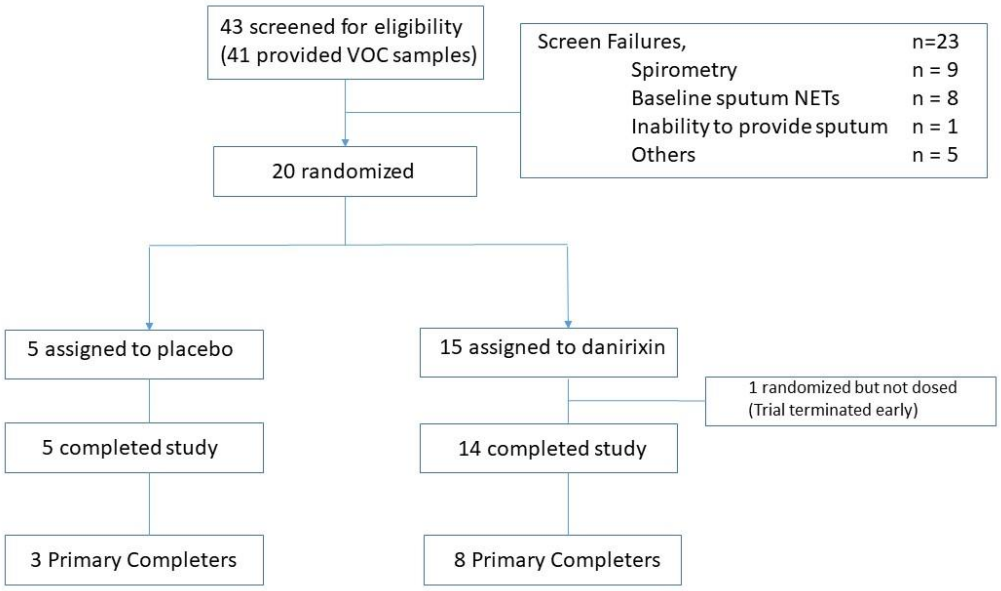

Figure 1 CONSORT diagram

CONSORT diagram for trial participants. The “Primary Completer” population was defined via subjects who provided “good” or “acceptable” quality sputum samples (based on % of squamous cells and viable leukocytes) at baseline and day 14. The primary completer population was used for sputum NETs analyses, but the entire study population was used for the microbiome analysis.

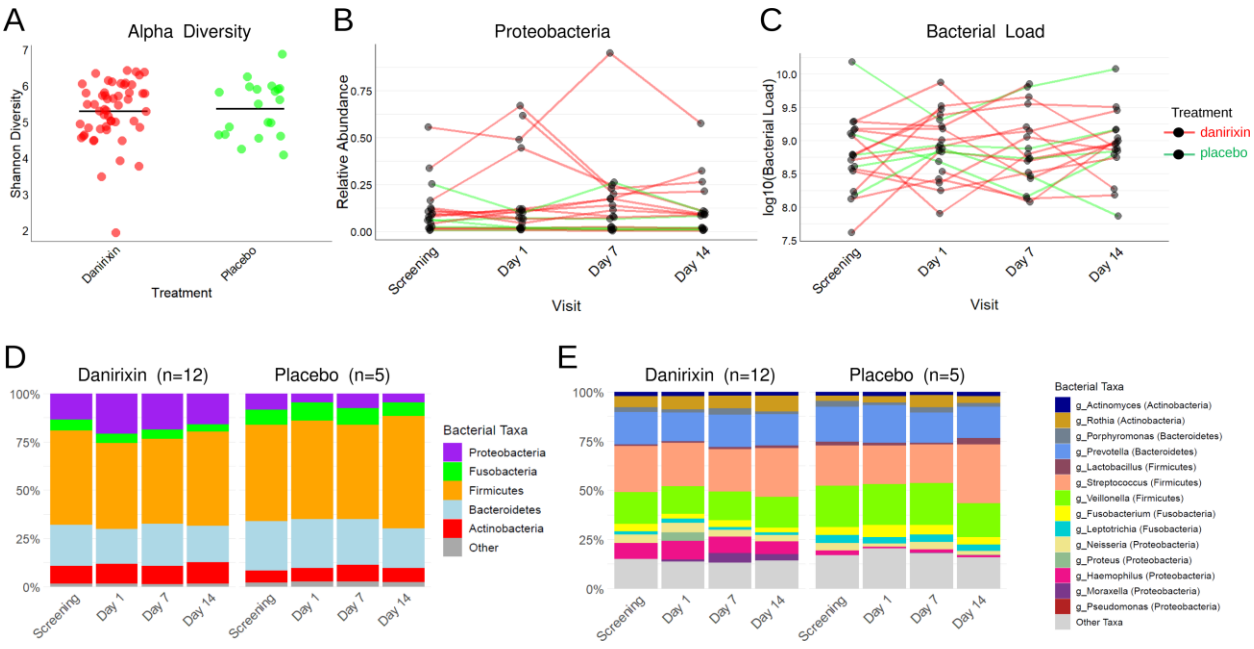

Figure 2 Changes in lung microbiome composition and bacterial load during study

Figure 1A: Alpha (Shannon) diversity showed no significant differences by treatment group (p=0.858, Wilcoxin rank-sum test) using pooled samples across visits between treatment groups 1B: Changes in relative abundance of Proteobacteria (including *Haemophilus*) during study. No significant differences in a linear mixed-effects model (using the patient as a random effect) were observed between danirixin (n=12) and placebo (n=5) groups (p=0.174). 1D: No significant differences in bacterial load as measured via 16S qPCR between danirixin and placebo groups was observed (p=0.8551, LME). 1D-E: Overall microbiome composition was similar between danirixin and placebo groups at the (D) phylum and (E) genus levels.

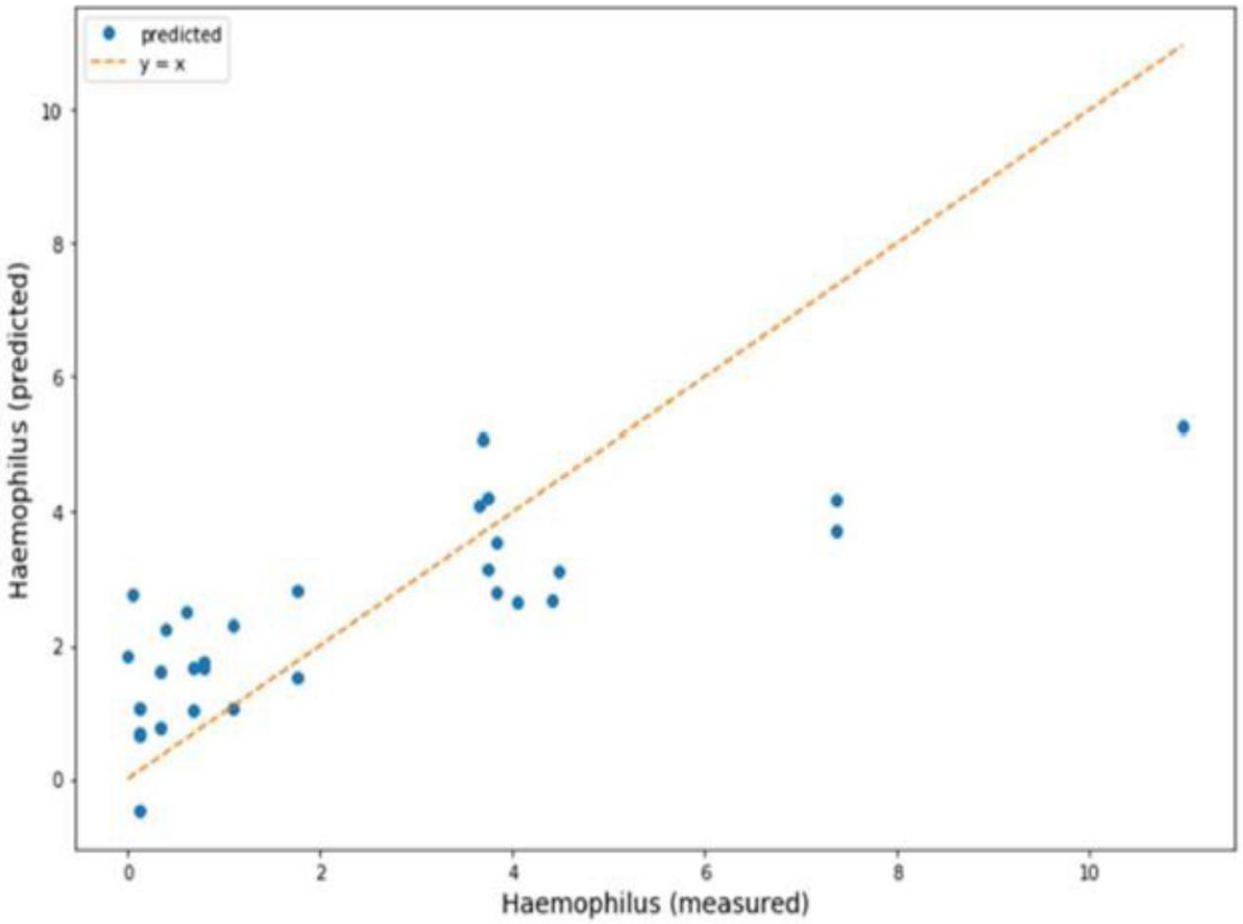

Figure 3 Joint-effects model to evaluate predictive ability of VOCs for *Haemophilus Influenzae* relative abundance

Plot of predicted values for joint-effects model for VOCs against the measured values of *Haemophilus* relative abundance. Each point represents the predicted and measured value for a single sample at the screening visit (n=31). The dashed orange line represents the lines for a perfect model.

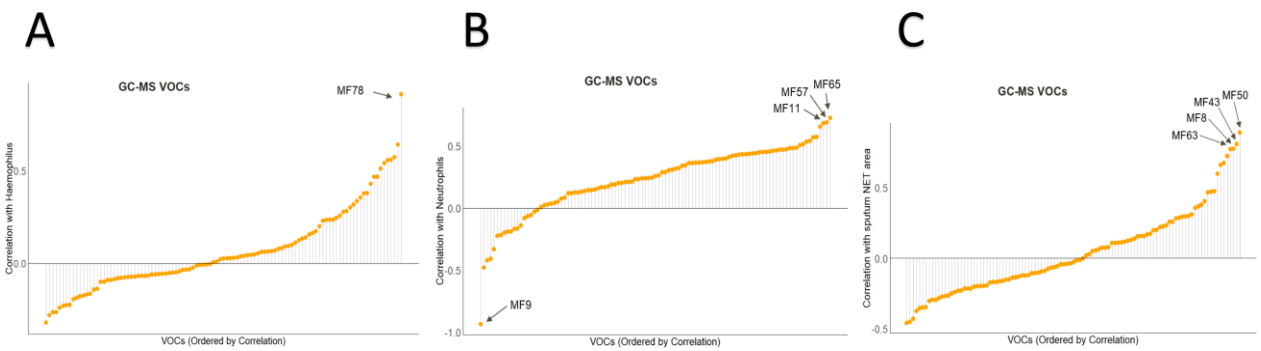

Figure 4 Individual VOCs ordered by correlation with *Haemophilus influenza* relative abundance, percent sputum neutrophils and sputum NET area as measured by GC-MS

Individual VOCs or molecular features (MFs), measured by GC-MS, ordered by correlation against a) *Haemophilus influenza* relative abundance b) percent sputum neutrophils and c) sputum NET area. MF 78 has strongest correlation with *Haemophilus influenza* relative abundance, MFs 65, 57 and 11 with percent sputum neutrophils and MFs 50, 43, 8 and 63 with sputum NET area, showing overall lack of overlap between VOCs that may relate to host microbiome, sputum neutrophils and sputum NETs.