Anaesthesia 2023, 78, 712–721 doi:10.1111/anae.15999

Original Article

Analysis of exhaled breath to identify critically ill patients with ventilator-associated pneumonia

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Summary

Ventilator-associated pneumonia commonly occurs in critically ill patients. Clinical suspicion results in overuse of antibiotics, which in turn promotes antimicrobial resistance. Detection of volatile organic compounds in the exhaled breath of critically ill patients might allow earlier detection of pneumonia and avoid unnecessary antibiotic prescription. We report a proof of concept study for non-invasive diagnosis of ventilator-associated pneumonia in intensive care (the BRAVo study). Mechanically ventilated critically ill patients commenced on antibiotics for clinical suspicion of ventilator-associated pneumonia were recruited within the first 24 h of treatment. Paired exhaled breath and respiratory tract samples were collected. Exhaled breath was captured on sorbent tubes and then analysed using thermal desorption gas chromatography–mass spectrometry to detect volatile organic compounds. Microbiological culture of a pathogenic bacteria in respiratory tract samples provided confirmation of ventilator-associated pneumonia. Univariable and multivariable analyses of volatile organic compounds were performed to identify potential biomarkers for a `rule-out´ test. Ninety-six participants were enrolled in the trial, with exhaled breath available from 92. Of all compounds tested, the four highest performing candidate biomarkers were benzene, cyclohexanone, pentanol and undecanal with area under the receiver operating characteristic curve ranging from 0.67 to 0.77 and negative predictive values from 85% to 88%. Identified volatile organic compounds in the exhaled breath of mechanically ventilated critically ill patients show promise as a useful non-invasive `rule-out´ test for ventilator-associated pneumonia.

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Accepted: 2 March 2023
Keywords: breath; diagnosis; ventilator-associated pneumonia
This article is accompanied by an editorial by A. Conway Morris and T. P. Hellyer, *Anaesthesia* 2023; **78**: 684–687.
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Introduction

Ventilator-associated pneumonia is the most common cause of nosocomial infection occurring in critically ill patients and is associated with significant morbidity, mortality and healthcare cost [1, 2]. Prompt use of broad-spectrum antimicrobial drugs is recommended due to the wide range of potential causative organisms [3, 4]. However, diagnosis of ventilator-associated pneumonia is complex and pneumonia is confirmed only in approximately one-third of patients suspected of ventilator-associated pneumonia [5]. Overuse of antimicrobial drugs is associated with drug-induced adverse events and drives emergence of antimicrobial resistance [6, 7]. Antimicrobial resistance is increasingly identified in critically ill patients with ventilator-associated pneumonia and is associated with poor clinical outcomes [8].

Antimicrobial stewardship aims to preserve the efficacy of antimicrobial drugs [9]. Improved diagnostic strategies are critical to an effective antimicrobial stewardship programme, allowing antibiotics to be stopped in patients without infection and narrowing the focus of antimicrobial therapy once a causative organism has been identified [10]. Soluble biomarker-led invasive diagnostic approaches to patients with suspected ventilator-associated pneumonia, collected via bronchoscopy, have been shown to outperform scoring systems as a `rule-out´ test, but have not led to a reduction in antibiotic use [11].

Measuring volatile organic compounds may offer non-invasive biomarkers that can be used to rule out ventilator-associated pneumonia without the need for bronchoscopy. Volatile organic compounds can be measured in human breath and diagnostic utility has previously been demonstrated in patients with asthma and chronic obstructive pulmonary disease [12, 13]. We have previously shown that volatile organic compound capture and off-line analysis of exhaled breath of critically ill patients is feasible [14], and further refined and validated the sampling method ex vivo and in vivo [15]. Here, we aim to establish `proof of concept´ for volatile organic compound capture and analysis as a potential `rule-out´ test for mechanically ventilated critically ill patients with ventilator-associated pneumonia. As secondary aims, we investigated the performance of individual volatile organic compounds and multivariable models in ruling out ventilator-associated pneumonia, and assessed changes in these volatile organic compounds following treatment.

Methods

We conducted a multicentre, prospective, observational, pragmatic cohort study in five UK ICUs between February 2016 and November 2018. Critically ill adults who had received invasive mechanical ventilation for at least 48 h, and in whom there was clinical suspicion of ventilator-associated pneumonia, were eligible for recruitment. Clinical suspicion of ventilator-associated pneumonia was defined as patients in whom antibiotic treatment was to be imminently commenced or those who had received antibiotic treatment for < 24 h where the primary antibiotic indication was healthcare acquired respiratory tract infection. Patients receiving end-of-life care, those with clinical suspicion of a highly infectious disease (such as novel coronaviruses, Ebola or resistant tuberculosis), and patients thought likely to poorly tolerate invasive airway sampling (hypoxia with a partial pressure of oxygen < 8 kPa on fraction of inspired oxygen > 0.7; positive end-expiratory pressure > 15 cmH_2O; peak airway pressure > 35 cmH_2O; heart rate > 140 bpm; mean arterial pressure < 65 mmHg; platelet count < $20 \times 10^9.l^{-1}$; international normalised ratio > 3, and intracranial pressure > 20 mmHg) were excluded, as all patients underwent invasive airway sampling to establish laboratory confirmation of ventilator-associated pneumonia.

Written informed consent was obtained from patients or from a legal representative if the patient was unable to provide consent for themselves due to illness or sedation for mechanical ventilation. The trial was conducted in accordance with the principles of ICH Good Clinical Practice guidelines and prospectively approved by the Greater Manchester South Research Ethics Committee. Clinical details including the participants' diagnoses, physiology,

thoracic radiology and laboratory data including microbiology were recorded.

Participants underwent duplicate breath and respiratory tract (broncho-alveolar lavage, non-directed lavage or tracheal aspirate) sampling. Breath sample collection, but not respiratory tract sampling, was repeated at 48–72 h in patients who remained invasively ventilated. Breath samples were collected using apparatus developed for the specific purpose of capturing volatile compounds from the expired air of mechanically ventilated patients [15]. Samples were collected by connecting the breath sampling system to a T-piece connected to the catheter mount of the ventilator circuit, which was in turn connected to a bacterial and hydrophobic filter (ref: 2000/05, Air Safety Limited, Lancashire, UK) and then to a stainless steel sampling tube packed with TenaxGR sorbent material (Markes International, Rhondda Cynon Taff, UK) for off-line analysis. Samples were drawn using a precision air sampling pump (Escort ELF Pump, Supelco, Dorset, UK) set at 500 ml.min^{-1}. Two consecutive samples of 1.2 l each were taken per patient per time-point. During sample collection, the inspired oxygen fraction was set at 1. If samples were not sent to the laboratory for immediate analysis, they were stored in a refrigerator at 4°C. Breath samples were collected by local investigators or research nurses. Bronchoalveolar lavage, non-directed lavage or tracheal aspirate were performed by either the clinical team and/or supported by a local clinical investigator as part of routine diagnostic investigations. Bronchoalveolar lavage was performed with the bronchoscope wedged in the most appropriate segment as identified from the chest radiograph [5]. A total of 120 ml of saline were instilled, aspirated and pooled during bronchoalveolar lavage. Non-directed lavage was performed using a total of 20 ml of saline blindly instilled, and aspirated via a suction catheter passed and wedged via the tracheal tube [16]. Tracheal aspiration was achieved by blind aspiration of tracheal contents through a suction catheter passed through, and extending just beyond, the tracheal tube [17].

The analytical method for breath samples has been described by van Oort et al. [15]. Briefly, sorbent tubes were conditioned at 330°C in nitrogen (OFN, BOC Ltd, Woking, UK; 50 ml.min^{-1}) using a sorbent tube conditioner (TC-20, Markes International, Bridgend, UK). After breath sample collection, samples were refrigerated at 4°C until analysis (median (range) storage time before analysis was 7 (0–42) days; volatile organic compounds have been shown to be stable within this range previously [18]). Samples were analysed by thermal desorption-gas chromatography–mass spectrometry using a thermal desorber coupled to an Agilent 7010 GC–MS (Agilent, Santa Clara, CA, USA). TenaxGR tubes are hydrophobic and in most cases were sufficiently dry purged during the pre-purge method in the thermal desorber (1 min at 50 ml.min^{-1} He flow; TD-100, Markes International, Bridgend, UK). Tubes were weighed before storage to check whether water had condensed within them (this was the case for 17 tubes, which were further dry purged with a counter flow of 50 ml.min^{-1} nitrogen for 4 min before tube storage). Analytes were initially desorbed at 280°C onto a general purpose focusing trap before a second desorption onto the GC column (DB-5 ms, 30 m × 0.25 mm, 25 µm film thickness, Agilent, Santa Clara, CA, USA). Analytes were ionised in an extractor high sensitivity EI source at 70 eV and mass spectra acquired in full scan mode with a range of m/z 40–500.

Several quality assurance steps were implemented to measure reproducibility and assist with data processing. These included assessing instrument background samples (blanks), injecting samples with a gaseous calibration standard (100 µl of 1 ppmV, 4-bromofluorobenzene in nitrogen, Thames Restek, High Wycombe, UK) immediately before primary desorption, and running external standard mixtures alongside breath samples [15]. Masshunter Quantitative Analysis software (version B.07.00, Agilent, Santa Clara, CA, USA) was used to extract and integrate peaks of compounds identified to metabolomics standards initiative level 1 (using the external standard mixture, mass spectral library search and retention indices) or metabolomics standards initiative level 2 (using mass spectral library search and retention indices only) [19]. Investigators performing breath sample analysis were blinded to the results of microbiological analysis.

Microbiological samples, as part of routine care, were inoculated onto a range of selective agar media and processed using standard laboratory techniques [20] by staff who were blinded to the results of breath sample analysis. A standardised method was used to give semi-quantitative bacterial counts for the bronchoalveolar and non-directed lavage samples, which were regarded as positive if the cultures exceeded 10^4 and 10^5 colony forming units.ml^{-1}, respectively. A pure (or predominant) heavy growth of a respiratory pathogen known to be associated with ventilator-associated pneumonia was regarded as a significant positive result for endotracheal aspirate samples. No significant growth was reported where no growth occurred following 48 h incubation. Laboratory confirmed ventilator-associated pneumonia was defined by a positive microbiological sample in a patient who was clinically suspected of having acquired a

lower respiratory tract infection after having been intubated for > 48 h.

A review of the literature was conducted to identify volatile organic compounds that may show utility as a biomarker for ventilator-associated pneumonia for inclusion in a targeted analysis. Volatile organic compounds were classified in hierarchical groups: volatile organic compounds identified from clinical studies of patients with ventilator-associated pneumonia; volatile organic compounds emitted from pathogens associated with ventilator-associated pneumonia; volatile organic compounds known to be associated with airway inflammation, and volatile organic compounds identified in exhaled breath or from air sampled from a mechanical ventilator. The full list is shown in online Supporting Information Table S1.

All statistical analyses were performed in R (v. 3.6 and 4.1., R Foundation, Vienna, Austria). Volatile organic compound abundances were $\log_{10}$-transformed for analysis where appropriate. Reproducibility was assessed by analysis of variance of each volatile organic compound across the three consecutively collected breath samples, and the intra-class correlation coefficient estimated. Volatile organic compounds which did not show good reproducibility (intra-class correlation coefficient < 0.6) were excluded from further analysis. Correlations between the relative abundance of each volatile organic compound were visualised as a heatmap of Spearman correlation coefficients with the volatile organic compound ordered according to assignments from a hierarchical clustering method.

The abundance of each of the volatile organic compounds in the group of patients with confirmed ventilator-associated pneumonia was compared with the group of patients in whom laboratory confirmed ventilator-associated pneumonia was excluded, using Mann–Whitney U-tests. For the volatile organic compounds identified from clinical studies of patients with ventilator-associated pneumonia, confirmation of association was defined as $p < 0.05$ with no multiplicity adjustment. For all the other volatile organic compounds, a false discovery rate adjusted significance level of $p < 0.05$ was used [21]. Longitudinal changes in volatile organic compounds demonstrating a statistical difference between patients with and without ventilator-associated pneumonia were investigated further using a false discovery rate-corrected Wilcoxon (paired) test between baseline and second sampling at 48–72 h. The difference in the change over time between ventilator-associated pneumonia and non-ventilator-associated pneumonia patients (interaction test) utilised false discovery rate-corrected Mann–Whitney U-test of this change. The intra-class correlation coefficient for inter-individual differences was computed from a linear model on log-transformed concentrations allowing for ventilator-associated pneumonia status, time and their interaction.

Multivariable prediction (Lasso and Ridge regression; alternative approaches to adjusting for the collinearity in the data) models were fitted using all available volatile organic compound data [22]. Both methods attempt to account for the overfitting due to the large number of predictors and small number of samples, but nevertheless cannot be expected to give a reliable predictive model given the numbers of samples in this dataset, and the models are presented as illustrative rather than definitive. A bootstrap method was used to assess the stability of the Lasso model, noting the number of times each compound was selected in the 1000 bootstrap samples.

The area under the receiver-operator curve (AUROC), specificity, likelihood ratio for a negative test and negative predictive values for cut-offs which ensured minimum 95% sensitivity were used as a measure of model performance. The observed prevalence of ventilator-associated pneumonia was used for the negative predictive value estimation. For comparison, the AUROC was computed for the clinical pulmonary infection score [23].

Finally, we generated a list of volatile organic compounds suitable for further investigation and validation based on performance, potential biological relevance and results of the correlation analysis.

Results

Ninety-six patients from five ICUs in the north-west of England were recruited (Table 1). Breath samples were available for analysis from 92 patients; samples were discarded from four patients due to failures of collection or storage. Collection of breath samples was feasible and safe with no adverse events occurring during collection. Median time from tracheal intubation and mechanical ventilation to the first day of sampling was 7 days. Seventy (76%) of these patients provided a second set of breath samples. Microbiological samples were collected by tracheal aspiration for 47 patients, non-directed lavage for 29 patients and bronchoalveolar lavage for 20 patients. Ventilator-associated pneumonia was identified in 40 patients due to 50 causative pathogens. *Staphylococcus aureus* (36%) and Enterobacteriaceae (28%) were the most commonly identified organisms (Table 2). Overall, 22/86 (26%) of patients died within 30 days of suspicion of ventilator-associated pneumonia (data not available for 10 patients). Thirty-day mortality was 28% in patients with confirmed ventilator-associated pneumonia and 22% in

Table 1 Patient characteristics and presenting conditions in patients with confirmed ventilator-associated pneumonia (VAP) and with ventilator-associated pneumonia excluded. Values are number (proportion) or median (IQR [range]).

	All recruited n = 96	Included n = 92	Confirmed VAP n = 40	VAP excluded n = 52
Male	64 (67%)	62 (67%)	28 (70%)	34 (65%)
Age, y	60 (47–70 [17–85])	60 (46–70 [17–85])	58 (44–66 [17–84])	61 (49–70 [23–85])
BMI, $kg.m^{-2}$	26.3 (22.0–30.7 [16.2–48.6])	26.3 (22.0–30.8 [16.2–48.6])	27.2 (22.2–31.2 [19.0–48.6])	26.2 (22.0–30.4 [16.2–37.5])
APACHE-2 score	15.0 (12.0–20.0 [3.0–29.0])	15.0 (11.5–20.0 [3.0–29.0])	13.5 (10.0–17.0 [5.0–26.0])	17.0 (13.0–21.0 [3.0–29.0])
Clinical pneumonia infection score	5 (4–6 [2–10])	5 (4–6 [2–10])	5 (5–6 [2–10])	5 (4–6 [2–8])
Time from intubation to VAP diagnosis, d	7 (5–12 [2–60])	7 (4–12 [2–60])	7 (5–10 [3–60])	7 (4–13 [2–31])
ICU mortality	17/90 (19%)	16/86 (19%)	5/37 (14%)	11/49 (22%)
30-day mortality	22/86 (26%)	20/82 (24%)	10/36 (28%)	10/46 (22%)
Admission type – medical	49/95 (52%)	47/91 (52%)	17/40 (42%)	30/51 (59%)
Admission type – surgical	46/95 (48%)	44/91 (48%)	23/40 (58%)	21/51 (41%)
Neurological	29/92 (32%)	29/88 (33%)	15/37 (41%)	14/51 (27%)
Trauma	24/93 (26%)	24/89 (27%)	16/38 (42%)	8/51 (16%)
Sepsis	14/92 (15%)	14/88 (16%)	2/37 (5%)	12/51 (24%)
Postoperative	35/92 (38%)	32/88 (36%)	15/37 (41%)	17/51 (33%)

patients in whom ventilator-associated pneumonia was excluded.

A detailed review of the literature (see online Supporting Information Table S1) identified 152 volatile organic compounds which were included in a semi-targeted analysis together with the internal standard. Of these, nine were identified from clinical studies of patients with ventilator-associated pneumonia; 60 were selected as they are emitted from pathogens associated with ventilator-associated pneumonia; a further 45 were added as they are known to be associated with airway inflammation and oxidative stress alongside 38 volatile organic compounds previously measured in exhaled breath or from air sampled from a mechanical ventilator. All 152 volatile organic compounds showed sufficient reproducibility to be included in the target analysis. The mean intra-class correlation coefficient was 0.89 across all volatile organic compounds. The lowest intra-class correlation coefficient was 0.65 (1-methyl-indole) with three other volatile organic compounds having an intra-class correlation coefficient < 0.7 (methyl-methacrylate, pyridine, decanoic acid). Correlations between the various compound abundances are represented in a heatmap (online Supporting Information Figure S1). Four of the nine volatile organic compounds previously associated with ventilator-associated pneumonia were confirmed to be associated with ventilator-associated pneumonia in this study (Table 3, Fig. 1a), with individual AUROCs between 0.66 and 0.71. Nonanal had the highest negative predictive value (0.83) at a 95% sensitivity threshold. Thirty-two additional volatile organic compounds demonstrated association with ventilator-associated pneumonia and had a false discovery rate below the 0.05 threshold (Table 3, Fig. 1b), with AUROCs ranging from 0.66 to 0.77. Benzene, cyclohexanone, pentanol and undecanal had the highest negative predictive value (0.88) with a 95% sensitivity. The AUROC for the clinical pulmonary infection score was 0.63, with a negative predictive value of 0.67 using a 95% sensitivity. Thirteen volatile organic compounds demonstrated changes over time in patients with ventilator-associated pneumonia compared with only one which changed over time in patients without ventilator-associated pneumonia (online Supporting Information Table S2). However, none of the volatile organic compounds demonstrated a differential change over time between ventilator-associated pneumonia and non-ventilator-associated pneumonia using a formal test of interaction. There was considerable inter-individual variation in volatile organic compound concentrations which persist over time with intra-class correlation coefficients for individual effects between 0.68 and 0.92.

Both the Lasso and the Ridge regression identified models with modest predictive ability (AUROC 0.79 and 0.81, respectively; the Lasso is shown in Fig. 2). The negative predictive value of the two models was 0.88 for the Lasso

Table 2 Pathogen frequency in patients with confirmed ventilator-associated pneumonia (VAP) and with VAP excluded. Values are number (proportion).

Organism	Confirmed VAP	VAP excluded*
Staphylococcus aureus	18 (36%)	11 (17%)
Staphylococcus epidermidis	0	1 (2%)
Moraxella catarrhalis	1 (2%)	0
Streptococcus pneumoniae	4 (8%)	0
Haemophilus influenzae	7 (14%)	0
Haemophilus parainfluenzae	0	1 (2%)
Acinetobacter spp.	1 (2%)	1 (2%)
Pseudomonas aeruginosa	4 (8%)	4 (6%)
Stenotrophomonas maltophilia	1 (2%)	3 (5%)
Escherichia coli	4 (8%)	4 (6%)
Klebsiella spp.	6 (12%)	3 (5%)
Proteus mirabilis	2 (4%)	0
Enterobacter spp.	0	1 (2%)
Serratia spp.	2 (4%)	1 (2%)
Candida spp.	0	32 (51%)
Aspergillus fumigatus	0	1 (2%)
Total	**50**	**63**

*Cultures falling below the thresholds defined as positive are reported here. Samples in this category were classified as not meeting the criteria for laboratory diagnosis of VAP.

model and 0.92 for the Ridge regression model using the 95% sensitivity cut-off. The Lasso model identified four volatile organic compounds as prognostic within the model: benzene, ethylfuran, pentanol and 2,4-dimethyl-1-heptene. Bootstrap re-sampling indicated that the Lasso model was unstable. Only three compounds were selected on more than 50% of samples, two in the original model (2,4-dimethyl-1-heptene and benzene) and one not included (dimethyltrisulfide). Two other compounds were selected 44% (pentanol) and 24% (ethylfuran) of times. When looking at the changes over 48–72 h following initiation of antibiotics, there were significant changes in the models using ventilator-associated pneumonia samples, but not in non-ventilator-associated pneumonia samples, and a significant interaction for ventilator-associated pneumonia vs. non-ventilator-associated pneumonia in the Lasso but not the Ridge models.

Discussion

Detection of volatile organic compounds in the exhaled breath of mechanically ventilated patients offers a novel, non-invasive approach to assist in managing critically ill patients with suspected ventilator-associated pneumonia [15]. Capture and analysis of volatile organic compounds appears safe. This study provides promising preliminary data to support ongoing development of a potential `rule-out´ test in the management of critically ill patients.

Our approach focused on confirmation of previously identified volatile organic compounds and identification of novel volatile organic compounds as single biomarkers and in combination. As the aim of the study was to identify biomarkers for a `rule-out´ test, the emphasis of the analysis was on the negative predictive value. The performance of the most promising volatile organic compounds was superior to conventional objective clinical scoring using the clinical pneumonia infection score (negative predictive value = 0.67). Of the previously described volatile organic compounds, nonanal had the highest negative predictive value (0.83). This study identified a number of new candidate biomarkers with four (benzene, cyclohexanone, pentanol and undecanal) performing better than nonanal with a negative predictive value of 0.88. The best performance overall was identified from the multivariate analysis with the ridge regression model generating a negative predictive value of 0.92.

Many of the compounds identified as potential biomarkers here can be linked to relevant biological processes. Three of the four significant volatile organic compounds that were reported in previous ventilator-associated pneumonia studies, and many more of the volatile organic compounds found in other studies (Table 3) were alkanes or aldehydes, which are potentially fatty acid breakdown products and markers of oxidative stress [24]. All the significant compounds were reduced in patients with ventilator-associated pneumonia. There are a number of potential explanations for this observation including: absorption of volatile organic compounds into inflamed lung tissue or the mucous lining of the airways; altered perfusion in the lung due to infection; or additional metabolism resulting in `consumption´ of volatile organic compounds in infected lung tissue. A concomitant increase in volatile metabolic products, providing evidence for the latter process, was not observed. Furthermore, partitioning related to absorption in tissue or altered perfusion would probably affect compounds according to their physicochemical and thermodynamic properties, which is not apparent in the hierarchical clustering presented in online Supporting Information Figure S1 or the volatile organic compounds listed in Table 3. This significant decrease in biomarker levels with ventilator-associated pneumonia is however consistent with findings from previous studies, e.g. dodecane and tetrahydrofuran [25], methyl isobutyl ketone [26] and acetone [25, 26]. In other

Table 3 Area under the receiver operating characteristic curve (AUROC) and negative predictive values (NPV) of volatile organic compounds identified as significant predictors of ventilator-associated pneumonia.

				95% Sensitivity		
	Compound	**AUROC**	**p**	**Specificity**	**NPV**	**LR−**
Volatile organic compounds identified from clinical studies of patients with ventilator-associated pneumonia	Heptane	0.71	0.0007	17%	82%	29%
	Tetradecane	0.70	0.0010	6%	60%	87%
	Dodecane	0.67	0.0045	10%	71%	52%
	Ethylbenzene	0.66	0.0072	12%	75%	43%
	Nonanal	0.60	0.11	19%	83%	26%
	3-carene	0.60	0.12	4%	50%	130%
	Methyl isobutyl ketone	0.58	0.18	10%	71%	52%
	Acetone	0.56	0.34	12%	75%	43%
	Tetrahydrofuran	0.52	0.76	6%	60%	87%
Other identified volatile organic compounds*	Pentanol	0.77	0.0011	27%	88%	19%
	2,4-dimethyl-1-heptene	0.76	0.0016	19%	83%	26%
	Ethylfuran	0.74	0.0038	23%	86%	22%
	Nonane	0.71	0.013	19%	83%	26%
	2,3-dimethylheptane	0.72	0.013	13%	78%	37%
	Dodecanal	0.70	0.021	10%	71%	52%
	Isoamyl alcohol	0.70	0.021	12%	75%	43%
	Octane	0.70	0.022	25%	87%	20%
	Butyl acetate	0.69	0.024	21%	85%	24%
	3-pentanone	0.69	0.024	19%	83%	26%
	2,4-dimethylheptane	0.69	0.024	10%	71%	52%
	Methylcyclohexane	0.69	0.024	12%	75%	43%
	Styrene	0.69	0.024	29%	88%	17%
	Benzene	0.69	0.024	23%	86%	22%
	2-ethyl-1-hexanol	0.68	0.028	6%	60%	87%
	Cyclohexane	0.68	0.028	27%	88%	19%
	Cyclohexanone	0.68	0.031	21%	85%	24%
	Hexanal	0.68	0.031	15%	80%	33%
	4-methyloctane	0.68	0.031	29%	88%	17%
	Undecanal	0.67	0.033	21%	85%	24%
	Benzaldehyde	0.67	0.033	15%	80%	33%
	4-methyldecane	0.67	0.033	15%	80%	33%
	2,2,4,4,6,8,8-heptamethyl-nonane	0.67	0.033	12%	75%	43%
	Benzonitrile	0.67	0.035	15%	80%	33%
	Pentadecane	0.67	0.038	12%	75%	43%
	4-ethyl-2,2-dimethylhexane	0.66	0.039	15%	80%	33%
	2,2,4,6,6-pentamethyl-heptane	0.66	0.039	17%	82%	29%
	Tridecane	0.66	0.039	8%	67%	65%
	3-methyl-1-pyrrole	0.66	0.039	21%	85%	24%
	(z)-3-octene	0.66	0.048	8%	67%	65%
	Phenylethyne	0.66	0.048	23%	86%	22%
	3-heptanol	0.66	0.048	13%	78%	37%

*p values are false discovery rate corrected. LR− negative likelihood ratio.

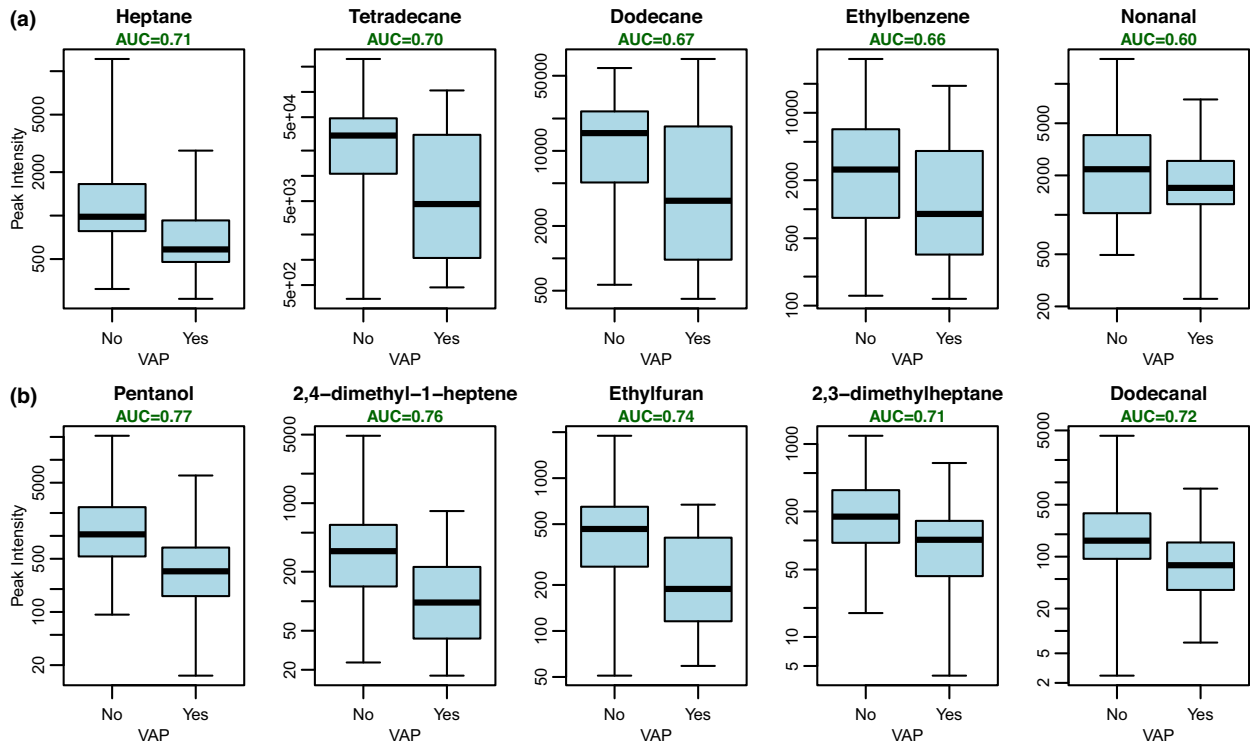

Figure 1 Box and whisker plots illustrating the distribution of the best five performing volatile organic compounds as previously identified in pneumonia studies (a) and in other studies (b). AUC, area under the ROC curve.

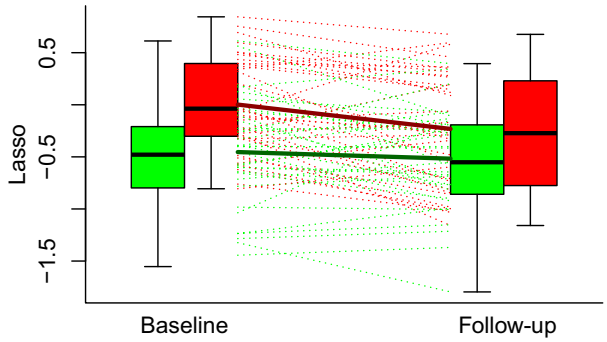

Figure 2 Results of the Lasso regression prediction model at the time of suspected ventilator-associated pneumonia (VAP; baseline) and showing change over time following treatment (follow-up). Performance of the model at baseline with 95% sensitivity: specificity 29%, negative likelihood ratio 17%, negative predictive value 88%. Whiskers on the boxplots here extend to the full range of the data. The broken lines join the values for individual patients over the time course and the solid lines denote the group means. Green, no VAP; red, VAP.

cases, the direction of volatile organic compound biomarkers with ventilator-associated pneumonia has been mixed, e.g. heptane [14, 26]. This observed duality may reflect the complex evolution of volatile organic compounds with the course of infection and requires further investigation in mechanistic studies. Whatever mechanisms are being suppressed due to the onset of ventilator-associated pneumonia, there is evidence they are returning to their pre-ventilator-associated pneumonia state following treatment, as can be seen in Figure 2.

Less than half (46%) of the significant volatile organic compounds reported in Table 3 were identified as potentially emitted from pathogens based on previous studies. On closer inspection, no clear emission pattern relating to specific species can be discerned but this is perhaps unsurprising given the wide variety of pathogens isolated, with very few replicates of each species. Furthermore, the apparent down-regulation of volatile organic compounds with infection implies that a more likely source of volatile organic compounds used to `rule-out´ ventilator-associated pneumonia would be the host lung (and systemic) inflammatory response. Pathogen-specific volatile organic compound profiles have been identified [27] and the proposed `rule-out´ test could further be enhanced in combination with a `rule-in´ test with high specificity for individual pathogens.

This clinical study was designed as proof-of-concept and limited by the sample size of 92 patients. Although the

concentrations of a number of volatile organic compounds changed over time, they did not reach statistical significance when assessed using a formal test of interaction. Additionally, the predictive models were fitted as exemplars and are not ready to be evaluated for clinical use without prospective validation. This is demonstrated by the instability of the bootstrap analysis of the Lasso model. Assessment of the predictive ability of both the single volatile organic compounds and the multivariate models may be further hampered by the potential poor performance of the disease definition for ventilator-associated pneumonia that was used as a reference standard, since this may produce either false-positive or false-negative classification of cases.

Overall, the study results demonstrate that a model can be developed utilising volatile organic compounds identified in exhaled breath of mechanically ventilated critically ill patients which could `rule out´ ventilator-associated pneumonia with clinically acceptable performance [11]. The `rule-out´ test would suggest avoiding or stopping antibiotics in approximately half the patients without ventilator-associated pneumonia, thus avoiding unnecessary antibiotic treatment in these patients.

Acknowledgements

This research was funded by the NIHR Invention for Innovation programme and supported by the NIHR Manchester Biomedical Research Centre, the NIHR Manchester Clinical Research Facility, the NIHR Centre for Precision Approaches to Combatting Antimicrobial Resistance and Manchester University NHS Foundation Trust Diagnostics and Technology Accelerator. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. No competing interests declared.

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Supporting Information

Additional supporting information may be found online via the journal website.

Figure S1. Correlations between the various organic compound abundances represented in a heatmap.

Table S1. Previously reported sources of volatile organic compounds included in the analysis.

Table S2. Changes in volatile organic compounds between baseline sampling and follow-up for patients with and without ventilator-associated pneumonia using Wilcoxon's test.