Validation of volatile metabolites of pulmonary oxidative injury: a bench to bedside study

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Exhaled breath is an exciting prospect in pulmonary disease diagnostics, yet despite a comprehensive translational approach, the validation of volatile biomarkers of oxidative stress was not possible in patients at risk of pulmonary oxidative injury https://bit.ly/3XDK5JM

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Abstract

Background Changes in exhaled volatile organic compounds (VOCs) can be used to discriminate between respiratory diseases, and increased concentrations of hydrocarbons are commonly linked to oxidative stress. However, the VOCs identified are inconsistent between studies, and translational studies are lacking.

Methods In this bench to bedside study, we captured VOCs in the headspace of A549 epithelial cells after exposure to hydrogen peroxide (H_2O_2), to induce oxidative stress, using high-capacity polydimethylsiloxane sorbent fibres. Exposed and unexposed cells were compared using targeted and untargeted analysis. Breath samples of invasively ventilated intensive care unit patients (n=489) were collected on sorbent tubes and associated with the inspiratory oxygen fraction (F_{IO_2}) to reflect pulmonary oxidative stress. Headspace samples and breath samples were analysed using gas chromatography and mass spectrometry.

Results In the cell, headspace octane concentration was decreased after oxidative stress (p=0.0013), while the other VOCs were not affected. 2-ethyl-1-hexanol showed an increased concentration in the headspace of cells undergoing oxidative stress in untargeted analysis (p=0.00014). None of the VOCs that were linked to oxidative stress showed a significant correlation with F_{IO_2} (R_s range: −0.015 to −0.065) or discriminated between patients with $F_{IO_2} \geqslant 0.6$ or below (area under the curve range: 0.48 to 0.55).

Conclusion Despite a comprehensive translational approach, validation of known and novel volatile biomarkers of oxidative stress was not possible in patients at risk of pulmonary oxidative injury. The inconsistencies observed highlight the difficulties faced in VOC biomarker validation, and that caution is warranted in the interpretation of the pathophysiological origin of discovered exhaled breath biomarkers.

Introduction

Oxidative stress is a pathological process due to a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences resulting in tissue damage [1, 2]. ROS mediate pulmonary injury through direct DNA damage, protein oxidation or lipid peroxidation [1, 3, 4] and have been implicated in the pathogenesis of multiple pulmonary disorders: asthma, cystic fibrosis, lung cancer,

acute respiratory distress syndrome (ARDS) and hyperoxic acute lung injury (HALI) [1, 2, 5]. Biomarkers of oxidative stress may therefore enable early detection and monitoring of pulmonary injury.

The analysis of volatile organic compounds (VOCs) found in exhaled breath is an appealing approach that is noninvasive and can provide results almost instantaneously [6]. Since the late 1980s, a particular focus has been the lipid peroxidation of polyunsaturated fatty acids (PUFAs). Found in cellular membranes, the oxidative degradation of these lipids has been hypothesised to lead to the release of VOCs detectable in breath that may reflect the presence of oxidative stress [7–9]. A plethora of identified VOC biomarkers for oxidative stress have been proposed [8, 10–15], yet concerns regarding study inconsistences, pathophysiological origin, reproducibility and subsequent clinical translation remain [4, 16, 17]. Consequently, a targeted translational approach combining *in vitro* and *in vivo* studies is an important next step to aid biomarker validation [18, 19] and is the goal of this study.

Here, we paired gas chromatography–mass spectrometry (GC-MS) with *in vitro* studies to investigate whether oxidative stress-derived volatile metabolites, both those reported in the literature and in particular those identified in the presented *in vitro* experiments, are also found in higher concentrations in the breath of patients exposed to high fractions of oxygen, a known cause of pulmonary oxidative stress [20].

Materials and methods

Cellular experiment

Cell line and cultivation

Immortalised human alveolar basal epithelial (A549) cells (CCL-185) were used for the *in vitro* component of this study. Cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, Penicillin-Streptomycin (5 mL containing 10 000 units per mL penicillin, 10 000 $\mu g \cdot mL^{-1}$ streptomycin, Gibco), L-glutamine, gentamicin and amphotericin. Cells were cultivated in 7-cm^2 cell culture flasks and incubated at 37°C in 5% CO_2. Cells were passaged every 3–4 days once ≈90% confluent. A similar passage was used for each experimental replicate. Prior to treatment with chemical oxidative reagents, the medium was removed, and the cells were washed with phosphate buffered saline. The cells were detached from the culture flask using 0.05% Trypsin-EDTA and counted in a haemocytometer before being resuspended in supplemented RPMI-1640.

Plastic culture vessels, typically used for cell cultivation, are now widely recognised as a source of volatile contaminates that can lead to misinterpretation of *in vitro* VOC analysis. [21, 22]. In an attempt to eliminate this, cells were seeded ($\approx 1.5 \times 10^5$) in 1 mL of supplemented RPMI-1640 in 20 mL glass headspace vials (Markes International, Cincinnati, OH, USA) and incubated at 37°C in 5% CO_2 for 22–24 h. Adjustments were made in line with previous studies to optimise cell growth within the 24-h window prior to treatment [10].

Induction of oxidative stress in epithelium

Unlike other studies, where exposure optimisation is often performed in high-throughput plastic culture plates, H_2O_2 concentration for this study was optimised in the glass vials used for headspace sampling. 1 mM H_2O_2 was selected to induce oxidative stress in A549 cells for further experiments; see online supplementary material for detailed rationale of concentration chosen (supplementary methods and figure S1).

On the day of the experiment once the cells were 80–90% confluent, the medium was removed and replenished with either 200 µL RPMI-1640 (control) or 200 µL RPMI-1640 plus 1 mM H_2O_2 (treatment). The vials were then sealed for headspace sampling using polytetrafluoroethylene (PTFE) crimp top-caps and crimping tool (Markes International). All vials were incubated for 24 h in a purpose-built HiSorb agitator: T37C, RPM 200 (Markes International) [23].

Assessment of cell death and inflammatory response

Cell cytotoxicity was assessed using lactate dehydrogenase (LDH) quantification and proinflammatory response by measuring interleukin (IL)-8 concentration in cell supernatant. The culture medium was removed and the supernatant collected (1×1000 G, 5 min, 4°C) from the headspace vials prior to the treatment phase (T0) and following 24 h treatment (T24). The supernatant collected was stored at −80°C, and sample analysis was performed within 1 month of sample collection. The supernatant was analysed for LDH release as previously described by Zuurbier *et al.* [24]. IL-8 was measured using enzyme-linked immunosorbent assay (ELISA from R&D Systems Inc., Bio-Techne, Minneapolis, MN, USA) according to

the manufacturer's protocols. Both LDH and IL-8 release was expressed relative to the untreated control cells.

Headspace sampling

VOC extraction was performed for 2 h following 22 h of incubation using high-capacity PDMS sorbent fibres (HiSorbs; Markes International) (supplementary figure S1). HiSorbs represent an alternative to solid-phase microextraction (SPME) fibres with an increased absorptive capacity [25]. Similar to SPME they rely upon the formation of an equilibrium in a closed system, rather than requiring a constant airflow and have increased sensitivity compared to methods used for breath sampling. This is important since the expected volume of gas is much smaller for *in vitro* experiments than for clinical samples (several millilitres *versus* several litres). At the end of VOC capture, the HiSorbs were removed and cleaned using dampened sterile gauze before being transferred into empty sorbent tubes (Markes International). All samples underwent GC-MS analysis within 2 weeks of collection.

Clinical study

Patient recruitment and consent

This is a study performed within the DARTS ("Diagnosis of Acute Respiratory Disease Syndrome" – study, ID: Trial register NL8226). This was a prospective multicentre, observational cohort study performed in two Dutch academic intensive care units that included consecutive adult patients undergoing invasive ventilation for an expected duration of >24 h [26]. Subjects were excluded if they: 1) had received invasive ventilation for >48 h in the 7 days preceding inclusion; 2) were tracheotomised; and 3) were deemed clinically inappropriate to collect samples from or if consent was withdrawn. Written informed consent at the time of inclusion was taken from either the patient or patients' representative for the use of data for clinical research, as previously described [26].

Patient sample collection

Breath was collected on two consecutive days within 48 h after start of invasive ventilation [26]. Breath sampling was performed as previously described [26, 27]. In short, a sampler with a regulated flow (200 mL·min^{-1}) drew exhaled breath over a sorbent tube, filled with Tenax GR, *via* a PTFE side stream connection, distal from the heat-moisture exchange filter. Sorbent tubes were stored at 4°C prior to GC-MS analysis. Clinical parameters, including ventilation settings and inspiratory oxygen fraction (F_{IO_2}), were recorded during study assessment. Patient ROS exposure and subsequent pulmonary oxidative injury, for the current study, was defined by F_{IO_2} with a $F_{IO_2} \geqslant 0.6$ used to signal patients at high risk of developing pulmonary oxidative stress and HALI [20].

VOC analysis of samples from cells and breath

Samples collected from cells, stored on HiSorbs, and patients' breath, stored on sorbent tubes, were desorbed using the Markes TD100 autosampler and desorber and analysed by means of GC-MS as previously described [26]. HiSorbs and Sorbent tubes were heated to 250°C for 5 min with a flow of 30 mL·min^{-1}. VOCs were captured on a cold trap at 25°C and re-injected by rapidly heating the trap to 280°C for 1 min. VOCs were injected splitless through a transfer line at 180°C onto an Inertcap 5MS/Sil GC column (30 m, ID 0.25 mm, film thickness 1 µm, 1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane (Restek, Breda, the Netherlands)) with a flow of 1.2 mL·min^{-1}. Oven temperature was kept isothermal at 40°C for 5 min, then increased to 280°C at 10°C·min^{-1} and kept isothermal at 280°C for 5 min. Molecules were ionised using electron ionisation (70 eV), and the fragment ions were detected using a quadrupole mass spectrometer (GCMS–GP2010; Shimadzu, Den Bosch, the Netherlands) with a scan range of 37–300 Da.

Statistical analysis and data processing

Statistical analysis was performed in R (version 3.6.1) through the R studio interface. Differences in cell death and chemotactic response were tested using Mann–Whitney U test. Prior to statistical analysis of VOCs, raw GC-MS spectra were imported and underwent: de-noising, peak detection and alignment, using the R "xcms" package (Scripps Center for Metabolomics, La Jolla, CA, USA) as previously described [28]. Data were visually inspected and excluded if evidence of failed chromatography runs or technical errors before a three-dimensional data matrix was generated including sample metadata, retention time (rt) and mass-to-charge ratio (m/z). The subsequent peak table was then log10 transformed prior to further analysis. For the *in vitro* headspace analysis, differences in VOC expression between cells undergoing oxidative stress and the control were assessed using a targeted and untargeted approach. For the targeted analysis, literature-reported VOCs associated with oxidative stress were sought and evaluated in relation to cellular exposure to H_2O_2. To ensure accurate detection, compounds were only selected for the targeted analysis if they could be qualified using an external gas standard (Massachusetts APH Mix, Supelco®;

Supelco Inc., Bellefonte, PA, USA), thus allowing for appropriate compound qualifier ion selection, and retention time windows (see supplementary table S1). The Mann–Whitney U test was used to evaluate differences in identified compound expression. For the untargeted analysis, individual volatile metabolites were identified using a differential expression analysis performed by *limma* package adjusting for experimental day differences and visualised using a volcano plot. A fold change threshold ⩾1 with $p<0.05$ was chosen to ensure biologically meaningful differential VOC expression and limit false discovery. Compounds carried forward were then identified using the GC-MS solutions (Shimadzu, Den Bosch, the Netherlands) platform incorporating the National institute and Technology library (NIST), adhering to the metabolomics standards initiative [29].

To investigate whether *in vitro* identified or literature-reported VOCs were associated with patient's increased oxygen exposure, indicative of likely pulmonary oxidative stress, Spearman rank correlation was used to assess the relationship between F_{IO_2} and compound intensity, while an area under the receiver operating characteristics curve (AUROCC) was calculated to assess the diagnostic potential of individual metabolites using a F_{IO_2} threshold of ⩾0.6 to define oxygen toxicity. Furthermore, as a sensitivity test, the diagnostic value of the identified VOCs was also evaluated using: 1) different diagnostic F_{IO_2} thresholds; and 2) an extended oxygen toxicity of >24 h.

Results

Cellular experiment

Following 24 h exposure to 1 mM hydrogen peroxide (H_2O_2), A549 cells (n=23) showed a significant degree of oxidative stress as evidenced by an increased fold change in both LDH and IL-8 production compared to the control (n=22) (figure 1a, b). 357 VOCs were detected in the headspace.

Targeted analysis

Despite evidence of cellular stress following H_2O_2 exposure, literature-reported VOCs associated with oxidative stress showed there was no significant difference between A549 cells exposed to H_2O_2 and those not (figure 2a–d, f). This was true for all but one compound, octane, which showed a significant lower concentration for cells undergoing oxidative stress (p=0.0013, figure 2e).

Untargeted analysis

An untargeted discovery approach was therefore adopted to see if alternative VOCs were released by A549 cells undergoing oxidative stress. The comparison between H_2O_2 exposed cells and control is shown in

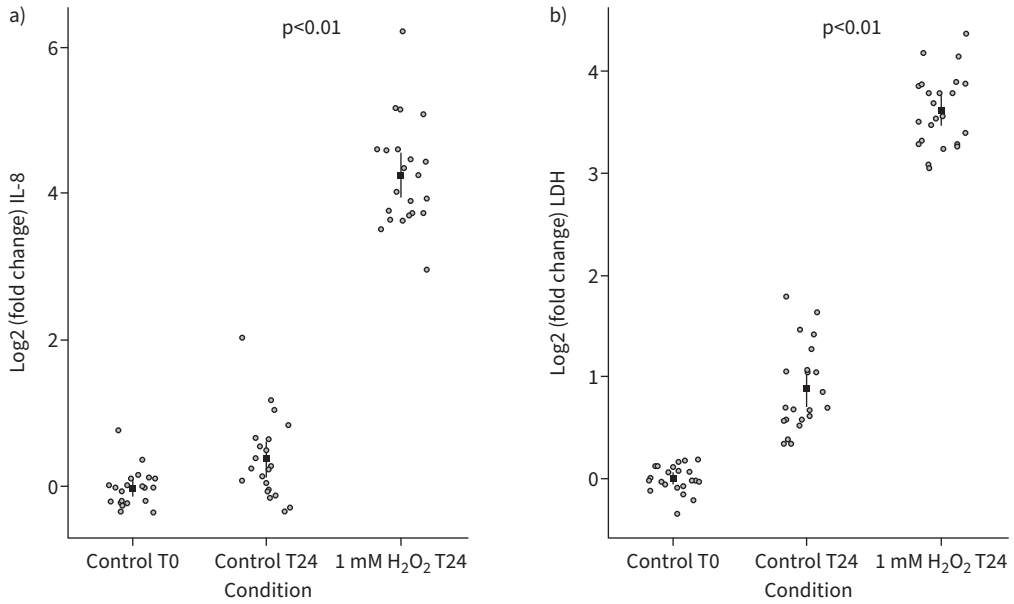

FIGURE 1 a) Interleukin (IL)-8 and b) lactate dehydrogenase (LDH) release shown for a549 cells treated for 24 h (T24) with or without 1 mM hydrogen peroxide (H_2O_2). Values expressed relative to untreated control cells (T0) and p-value calculated using Mann–Whitney U test.

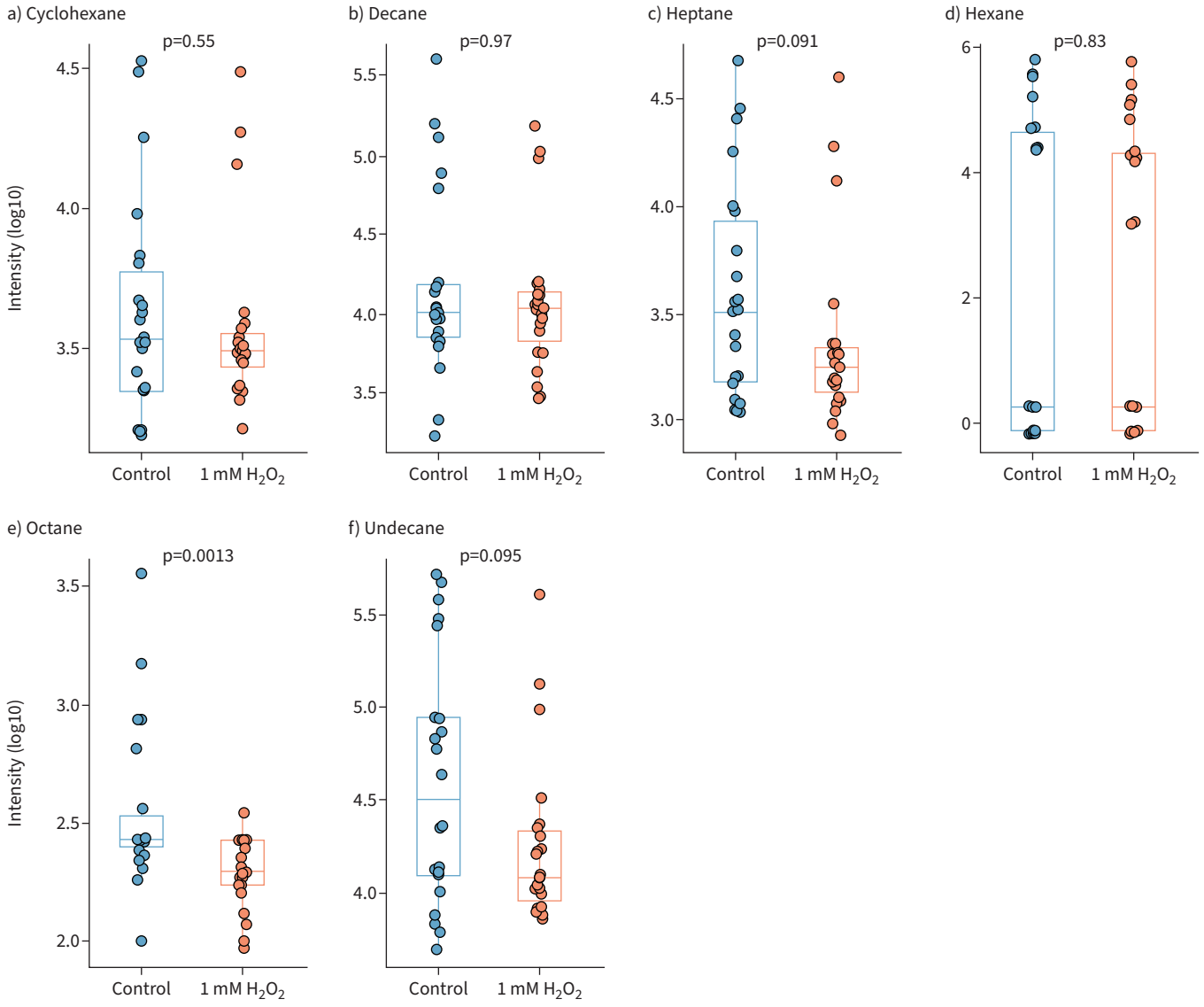

FIGURE 2 Previously identified *in vivo* volatile organic compounds observed in *in vitro* cellular headspace, split per treatment group, A549 cells in Roswell Park Memorial Institute (RPMI) media (n=22) and 1 mM hydrogen peroxide (H_2O_2)-treated a549 cells (n=23). Differences between experimental groups evaluated using Mann–Whitney U test. a) Cyclohexane; b) decane; c) heptane; d) hexane; e) octane; and f) undecane.

figure 3a. Of the 357 VOCs identified, only one showed differential expression between experimental groups as predefined with a fold change ⩾1 and p<0.05. The VOC was identified using the GC-MS platform as 2-ethyl-1-hexanol (CAS: CAS: 104–76–7) with an increased concentration in the headspace of cells exposed to H_2O_2 (figure 3b, p=0.00014).

Clinical validation of in vitro *results*

Patient characteristics

A total of 519 patients were included in the DARTS study. Exhaled breath samples and clinical information relating to oxygen exposure on the day of inclusion were available for 489 patients (94.2%). Of these 489 patients, 90 (18.4%) were identified as high risk for oxygen toxicity and pulmonary oxidative stress with a F_{IO_2} ⩾0.6. Patient characteristics are summarised in table 1.

Diagnostic performance of oxidative stress-derived VOCs in patient exhaled breath samples

The relationship between the identified compounds associated with oxidative stress and patient oxygen exposure was first evaluated using Spearman's rank correlation to assess whether these compounds were

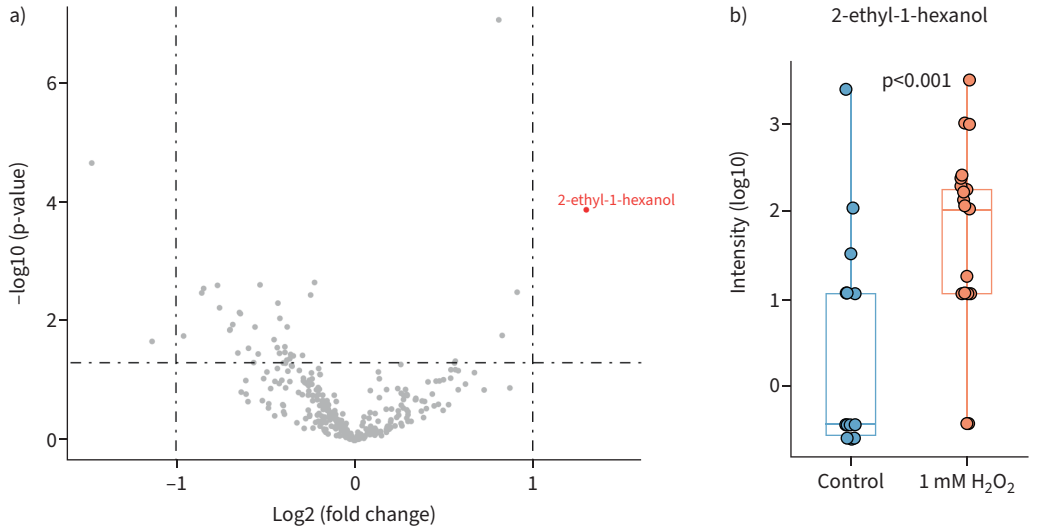

FIGURE 3 a) Volcano plot of all volatile organic compounds significantly released by a549 cells (fold change ⩾1, p<0.05) either treated or not treated with hydrogen peroxide (H_2O_2) (n=45). b) Differences in experimental groups for the identified volatile organic compound, 2-ethyl-1-hexanol (p<0.001).

found in higher concentrations in the breath of patients exposed to high F_{IO_2}, a known contributor of pulmonary oxidative stress. Only three of the VOCs of interest could be identified in the exhaled breath of patients at sufficient concentrations: decane, hexane and octane. The *in vitro* identified compound 2-ethyl-1-hexanol and cyclohexane, heptane and undecane were not found in the exhaled breath of patients. None of the three identified VOCs showed a significant correlation with patient oxygen exposure (figure 4a–c). The diagnostic performance for the identified literature-reported compounds was then evaluated using an AUROCC to assess the discriminatory potential of such metabolites for patients' risk of oxygen toxicity (F_{IO_2} ⩾0.6). All compounds showed poor discrimination for patients identified as high risk for oxygen toxicity (AUROCC range: 0.48 to 0.55, figure 4d). Additionally, a sensitivity analysis using the same molecules was conducted to firstly assess the influence of differing diagnostic F_{IO_2} thresholds, and secondly, to evaluate the impact of greater oxygen exposure on breath samples taken on day 2 with a recorded F_{IO_2} ⩾0.6 on 2 consecutive days. Despite differing F_{IO_2} thresholds or a prolonged exposure to injurious concentrations of oxygen, diagnostic performance showed little improvement for all compounds (see online supplementary material for further details).

TABLE 1 Patient characteristics

	High risk	Low risk	p-value
Patients n	90	399	
Age years, mean±SD	63.2±12.6	61.5±15.2	0.30
Male, n (%)	63 (70)	269 (67.4)	0.73
Admission type, n (%)			0.30
Medical	72 (80)	287 (71.9)	
Planned surgical	8 (8.9)	49 (12.3)	
Emergency surgical	10 (11.1)	63 (15.8)	
APACHEII score, median (IQR)	20 (15–24)	20 (15–26)	0.13
MV duration in hours prior to inclusion, median (IQR)	18 (6–32)	21 (14–30)	0.10
F_{IO_2} %, median (IQR)	0.69 (0.60–0.74)	0.31 (0.25–0.40)	
P_{max} cmH_2O, median (IQR)	25 (21–30)	19.5 (16–25)	<0.001
PEEP cmH_2O, median (IQR)	10 (8–12)	8 (5–8.5)	<0.001
ICU LOS days, median (IQR)	9 (5–19)	6 (3–11.8)	0.001
ICU mortality, n (%)	39 (43.3)	124 (31.1)	0.067

MV: mechanical ventilation; F_{IO_2}: inspiratory oxygen fraction; P_{max}: maximum airway pressure; PEEP: positive end-expiratory pressure; ICU: intensive care unit; LOS: length of stay.

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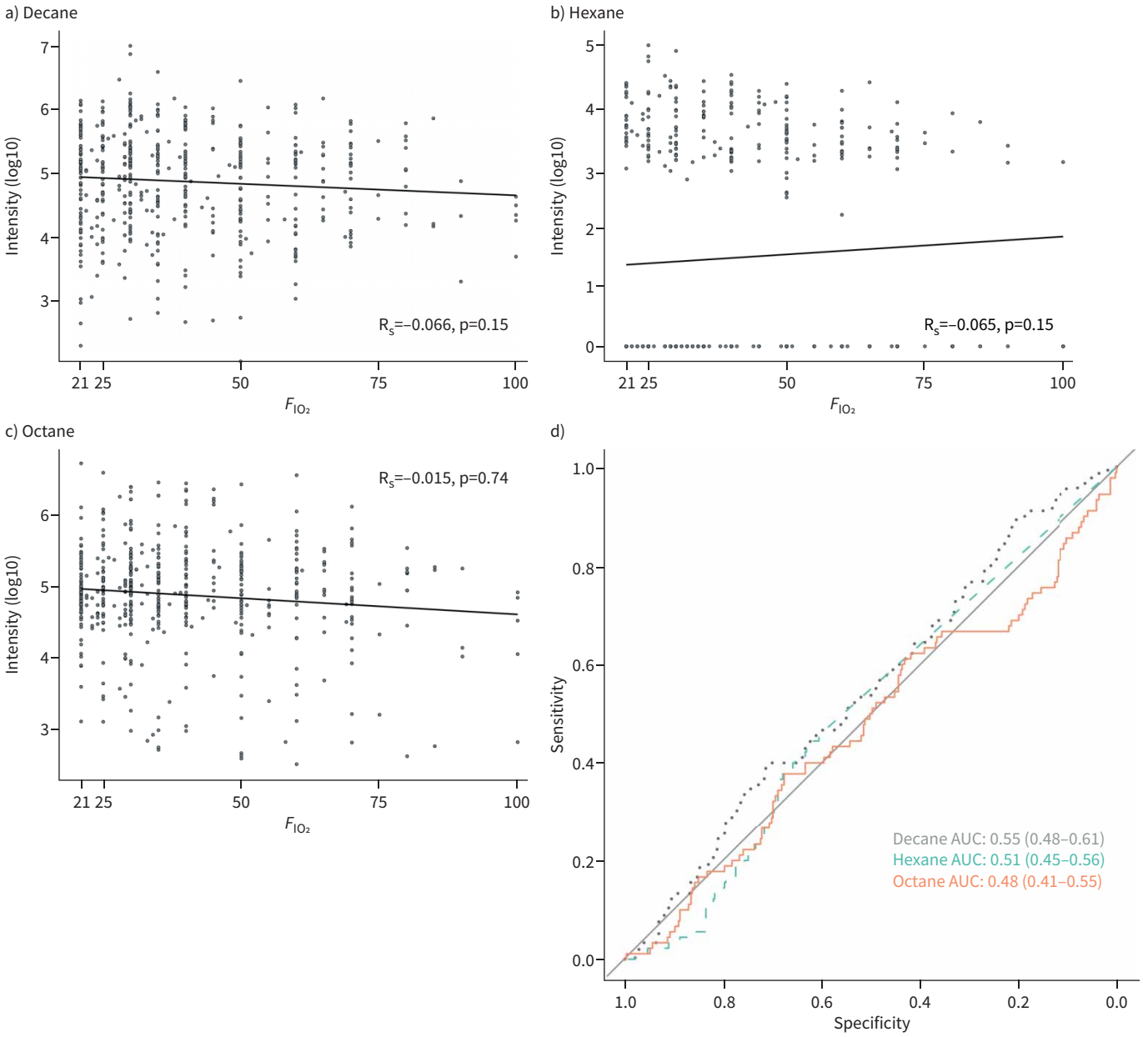

FIGURE 4 a–c) Spearman's rank correlation analysis showed no association between identified volatile organic compounds (VOCs) (decane, hexane and octane) in patients' exhaled breath (n=489) and the fraction of inspired oxygen (F_{IO_2}). d) Equally, compounds showed poor discriminative power for pulmonary oxidative injury when comparing individual VOCs as predicator variables for whether patients had a F_{IO_2} ⩾0.6 (n=489). AUC: area under the curve.

Discussion

We present a bench to bedside study to assess the validity of known and novel oxidative stress-derived volatile metabolites. The translational strategy presented here represents an unmet need for VOC biomarker verification and subsequent clinical application, as there is a replication crisis in exhaled breath metabolomics as in many other areas of research [30–32]. Despite extensive prior evidence, the release of volatile metabolites from human alveolar basal epithelial cells undergoing oxidative stress was limited in a contamination-free method, and neither previously suggested or novel biomarkers were different between patients who were and were not exposed to high F_{IO_2}, which is associated with pulmonary oxidative injury.

We discerned differences in VOC expression of A549 cells undergoing H_2O_2-induced oxidative stress using a targeted approach. While there was clear evidence of cellular stress following H_2O_2 treatment, metabolites previously reported as markers of oxidative injury were either not detectable or showed no differential expression between experimental groups. These findings differ from the current literature wherein the release of hydrocarbons, as sought in this study, have been linked to oxidative stress [8, 11, 12, 14, 15]. The evidence for these biomarkers thus resulted from correlation analysis in clinical studies, frequently with diseases or syndromes in which oxidative stress is only one of the pathophysiological mechanisms. Highly dimensional breath profiles resulting in multiple testing combined with complex, multifactorial reference diseases, might explain why studies frequently identified “oxidative stress related VOCs”.

The above-mentioned explanation is exemplified by the relations found between octane concentration and oxidative stress. It was identified by Phillips *et al*. [12] as an important biomarker of unstable angina, and they linked its origin to the ROS-mediated lipid peroxidation. Furthermore, it has been suggested as a biomarker of ARDS [14], a condition characterised by a high oxidative stress response [33]. Yet, when evaluated at the cellular level, the association between octane and oxidative stress was inversed with lower concentrations in the headspace of stressed cells, which is in line with our findings [22]. A suggested explanation for this discrepancy is the relative hyperoxic cell culture environment that was used in those studies, compared to the likely hypoxic condition *in vivo* [34]. The increased hypoxia *in vivo* is likely to alter cellular metabolism and increase oxidative stress [34]. Yet, in the present study, we found a lower concentration of octane in the headspace of cells exposed to oxidative stress despite an artificially enhanced oxidative state with H_2O_2.

While a link between several hydrocarbons and oxidative stress has been suggested [8, 11, 12, 14, 15], the endogenous biosynthetic pathway of PUFA lipid peroxidation of only a few, namely ethane and pentane, has been formally demonstrated [35]. These molecules, however, are difficult to capture on sorbent material due to their low molecular weight and could therefore not be measured reliably in the current study and are not evaluated in most of the clinical studies evaluating exhaled breath metabolites [36]. Alternative alkanes more readily detected in breath have been linked to oxidative stress with the hypothesis that they too are by-products of the same metabolic pathway. However, the metabolic pathways or enzymes linking their production or degradation to oxidative stress is not fully determined [36, 37] and may only partially account for their detection in exhaled breath [36, 37].

For the identification and detection of VOCs using an untargeted strategy, only one compound, the alcohol 2-ethyl-1-hexanol, was statistically increased in the headspace cells undergoing oxidative stress. This finding is consistent with previous *in vitro* studies that report an increased concentration secondary to excessive ROS exposure [38, 39]. A postulated metabolic pathway for such alcohols is linked to the oxidative stress-mediated lipid peroxidation of PUFAs [40]. This theoretically leads to an increased availability of alkanes that could undergo oxidation to alcohols *via* cytochrome p450 [40]. Yet, this remains speculative and relies on an increased formation and presence of alkanes following oxidative stress, a finding not reflected by cells exposed to H_2O_2 in the current study. Furthermore, not all available evidence points towards the same conclusion, and 2-ethyl-1-hexanol may instead be exogenous and originate from culture environment or medium [19, 36]. However, our model attempted to minimise the impact of such contaminates through the use of a sealed glass system with minimum media. Subsequently, the reduced number of significant compounds observed in the current study likely reflects this compared to other *in vitro* studies that typically used larger culture vessels and quantities of media, which may be prone to more experimental artefacts [10, 38, 39]. The forementioned inconsistencies emphasise why a translational, bottom-up approach employing optimal *in vitro* conditions remains critical to the development and validation of VOC biomarkers.

Further *in vivo–in vitro* disparity was observed when attempting to translate the identified compounds to the exhaled breath of patients. Compounds were either not detectable or showed poor correlation with oxygen exposure, and limited discrimination for patients with pulmonary oxidative injury. Importantly,

2-ethyl-1-hexanol could not be found in the patients' exhaled breath. Its absence may therefore support the findings that it is an exogenous contaminate rather than potential biomarker of oxidative stress [19, 36]. This again emphasises the need for caution if novel VOC biomarkers are identified using only a clinical or pre-clinical study. Furthermore, the lack of correlation between *in vivo* reported compounds and oxygen exposure observed in the current study again suggests that previously assumed metabolic pathways may only partially explain the presence of these VOCs. This is particularly true given the work of Morita *et al*. [41] who demonstrated the endogenous release of pentane after as little as 30 min of high F_{IO_2}, let alone 24 h. Unfortunately, we were unable to replicate these findings due to the chromatographic method employed and early elution of pentane. However, a sensitivity analysis to capture the effect of prolonged oxygen exposure was performed and showed no improvement for individual compounds. Further studies that look to address this should consider incorporating isotope-labelled compounds as a means to better determine the biosynthetic pathways.

An attributable strength of this study was the incorporation of both glass culture vessels and a sealed system into the headspace sampling methodology. This optimised reproducibility with over 20 repeats per experimental group while also minimising unwanted contaminants. In addition, we performed a comprehensive multistep translational approach utilising a large multicentre, heterogeneous patient cohort, to our knowledge the largest to date, for thorough compound validation. Finally, *in vitro* and *in vivo* samples were all analysed using the same GC-MS method in the hope to aid translation and avoid compound misidentification. However, it is important to recognise that while this may improve translation, it could have also had a negative impact of limiting *in vitro* compound discovery. Equally, sampling from the ventilator circuit could also lead to the dilution and reduced recovery of VOCs [42]. However, the method used in the current study was optimised for breath collection for large-scale clinical studies, while minimising the risk to patients. An additional limitation is the choice of a two-dimensional cell culture model over more sophisticated *in vitro* models. While these models may better mimic the *in vivo* environment, the exclusion of plastics (often found in three-dimensional cultures) from our model was an active decision to optimise VOC capture while minimising contaminates that may lead to misinterpretation. Finally, the lack of a diagnostic gold standard and biological confirmation of pulmonary oxidative injury in our patient cohort may lead to the misdiagnosis of patients. A defined criteria was set according to patients' F_{IO_2} as suggested by the current literature [20]; however, without a comparative biological standard, it is difficult to say definitively that pulmonary oxidative injury was present. A sensitivity analysis was performed at different F_{IO_2} exposure for the compounds of interest to evaluate the influence of this difference but showed no significant improvement.

Even though oxidative stress is the most cited pathophysiological mechanism resulting in changed VOC concentrations in exhaled breath, we could not validate any of the suggested VOCs from bench to bedside. The findings have several important implications. First, we now better recognise that the impact of exogenous contaminates is important in *in vitro* and *in vivo* studies and can result in misinterpretation. Second, the lack of overlap between *in vivo* and *in vitro* studies suggests that we are still unfamiliar with most of the pathophysiological mechanisms resulting in VOC formation and that citing oxidative stress as the molecular mechanism resulting in their formation is probably false. Third, the challenges of contamination and untargeted analysis in small patient populations increase the risk of chance findings resulting in false discovery, which hampers replication. These challenges need to be tackled if exhaled breath is to become clinically applicable rather than remain a "promising tool". Efforts to address some of these, as highlighted by Kwak *et al*. [36], have been incorporated with increased success [18, 19], but further work is needed in order to bring exhaled breath one step closer to clinical practice.

In conclusion, the presented bench to bedside approach failed to validate any of the known volatile biomarkers of oxidative stress despite limiting the influence of contamination in the *in vitro* study and validation in one of the largest patient cohorts to date. These difficulties in translation and the inconsistencies observed remind us that caution is warranted in the interpretation of the pathophysiological origin of exhaled breath biomarkers and their clinical application. Emphasis should be towards the better understanding of compound biosynthetic origination and incorporation of translational models that minimise the impact of exogenous artefacts.

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