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Exploring exhaled breath volatile organic compounds in occupational asthma: a pilot cross-sectional study

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Abstract

Occupational asthma (OA) is divided into allergic asthma and irritant-induced asthma (IIA). IIA can be divided further into three different phenotypic subtypes. Volatile organic compounds (VOCs) in exhaled breath can reflect metabolic changes in the body, and a wide range of them have been associated with various diseases in the last two decades. This is the first known study to explore breath VOCs in subjects with OA, aimed to identify potential biomarkers to distinguish OA from healthy controls, as well as between different OA subgroups. In a cross-sectional investigation, exhaled breath from 40 patients with OA and 45 respiratory healthy healthcare workers were collected with ReCIVA® breath sampler. Samples were analyzed through an untargeted approach using thermal desorption-gas chromatography mass spectrometry, and VOCs were identified according to tier classification. The data underwent analysis using both non-parametric and parametric statistical methods. 536 VOCs were identified. Significance ($p < 0.05$) was observed in several emitted VOCs. Among these, compounds such as 1-hexadecanol, 2,3-butanediol, phenol, xylene, acetone, 3-methylhexane, methylcyclohexane, and isoprene have biological implications or are associated with exposures linked to OA. These VOCs may reflect metabolic changes in the body and the microbiome, as well as external exposures due to occupation. In particular, 1-hexadecanol, 2,3-butanediol, phenol and xylene are associated with reduced nicotinamide adenine dinucleotide and production of reactive oxygen species, mechanisms that can be linked to asthmatic diseases and therefore suggests its potential as biomarkers. This study demonstrates that VOCs detected in exhaled breath could serve as indicators of occupational exposure and enhance diagnostic accuracy for asthma.

Abbreviations

AA	Allergic asthma
BMI	Body mass index
FeNO	Fractional exhaled nitric oxide
HC	Healthy control
HRAM	High-resolution accurate mass
IIA	Irritant-induced asthma
MSI	Metabolomics standards initiative
NIST	National institute of standards and technology
NADH	Nicotinamide adenine dinucleotide
COPD	Obstructive pulmonary disease
ROC-AUC	Receiver operator characteristic area under the curve
RSD	Relative standard deviation
ROS	Reactive oxygen species
TD-GC-MS	Thermal desorption-gas chromatography mass spectrometry
VOCs	Volatile organic compounds

1. Introduction

World-wide prevalence of asthma was assessed as 358 million individuals or approximately 5% of the world population in 2015 [1]. Asthma is believed to be caused by a combination of genetic and environmental factors. The diagnosis of asthma is a clinical diagnosis that relies on pulmonary function, response to therapy, clinical symptoms, and medical history. Currently, there are several tests and guidelines that can aid and confirm the diagnosis of asthma, but no single test or gold standard that objectively can identify all the different types of asthma [2]. Medications can alleviate asthma, yet a permanent cure remains elusive. 16% of all adult-onset asthma cases have been attributed to occupational exposures [3]. Occupational asthma (OA) manifests as late-onset work-related asthma, which also includes preexisting asthma that is worsened by workplace exposures (i.e. work-exacerbated) [4]. Historically, OA was commonly associated with antigenic exposure leading to a Type 1 immunological response, namely IgE-induced allergic asthma (AA) or sensitizer-induced asthma. However, not all exposures could be explained by this mechanism [5]. In 1989, irritant-induced asthma (IIA) was found to be prevalent (1:3 compared to AA) in patients referred for occupational airway disease [6]. IIA can be further categorized into three different phenotypes: definite IIA is characterized by a single high-dose exposure and an acute-onset asthma resembling reactive airways dysfunction syndrome; probable IIA is characterized by multiple high-dose exposures and delayed onset of asthma; and possible IIA is characterized by chronic moderate to low-dose exposures with delayed onset of asthma [7]. Dusts, gases, acids, alkali, fumes, halogenated derivates, solvents and mixtures of chemicals in cleaning and disinfection agents have been involved in IIA [8, 9].

While the pathophysiology of IIA remains unclear, it is suggested to involve an initial bronchial epithelial damage followed by a proinflammatory response [10]. Pathological studies have indicated an acute phase resembling a toxic mechanism with neutrophil recruitment and a chronic phase resembling an immunologic mechanism with eosinophil recruitment and airway remodeling [7, 11]. Reactive oxygen species (ROS) play a role in causing airway injury, inflammation, and airway remodeling. They can originate from recruited neutrophils and also arise from secondary NADH-related mitochondrial dysfunction triggered by excessive exposure to airway pollutants [12]. The different mechanisms underlying acute and chronic phases suggest that there may be biomarkers worth exploring that can help differentiate the different IIA subtypes, in addition to biomarkers of OA.

Metabolites or compounds derived from the metabolic processes, such as volatile organic compounds (VOCs) offer a snapshot of the physiological state in the human body. These compounds can be detected in different biological metrices, including breath. In the last 20 yrs, scientific articles exploring breath VOCs as disease biomarkers have increased to more than 5000 yearly. These studies comprised approximately 52% in lung cancer, 13% in asthma, 8% in diabetes, and 6% in chronic obstructive pulmonary disease (COPD) [13]. A recent study on exhaled breath in acute severe asthma exacerbation identified a selective depletion of correlated aldehydes, such as nonanal, decanal and hexanal, in comparison with COPD and pneumonia, which may indicate an activity by immune cells [14]. In an asthma phenotype study in adults, nonanal was increased in neutrophilic asthma, compared with other phenotypes [15]. These studies highlight the complex mechanisms underlying asthma and emphasize the need for ongoing exploration of VOCs in exhaled breath to deepen our understanding of this condition. While sampling and analytical methodologies have varied across studies, ongoing joint efforts toward standardization are promising [16]. These efforts, along with the non-invasive nature and convenience of breath sampling methods, suggest the potential of breath VOCs to be applied as disease biomarkers in clinical settings.

To date, breath VOC biomarkers in OA have not been explored. Although the proportion of IIA in OA population is unclear, it is substantial according to several studies [6, 8]. However, some studies still classify IIA as rare, and there is ongoing debate regarding the diagnosis of delayed onset IIA [17]. Over the past decade at our clinic, which covers approximately 2.5 million individuals, we have observed a significant change in referrals for OA, shifting from a primary focus on AA to a considerable majority (80%–90%) now categorized as IIA. The number of refereed OA has not changed, and it is plausible that occupational, safety and health (OSH) efforts in norway have caused the reduction of AA. The increase of IIA referrals, particularly of possible IIA, presents evaluation challenges due to the absence of biomarkers, exposure to low to moderate levels of airway irritants and limited clinical studies. Also, because of lack of biomarkers and causality, continuing occupational exposure to airway irritants will result in asthma exacerbations and a gradually more severe asthmatic disease. Having available biomarkers could optimize treatment of patients and thus reduce their symptoms.

Hence, this study aims to identify potential exhaled breath VOCs that can differentiate OA from respiratory healthy control (HC), as well as between different IIA subgroups, in patients exposed to organic and inorganic dust, acid and alkali, chemicals and biological agents.

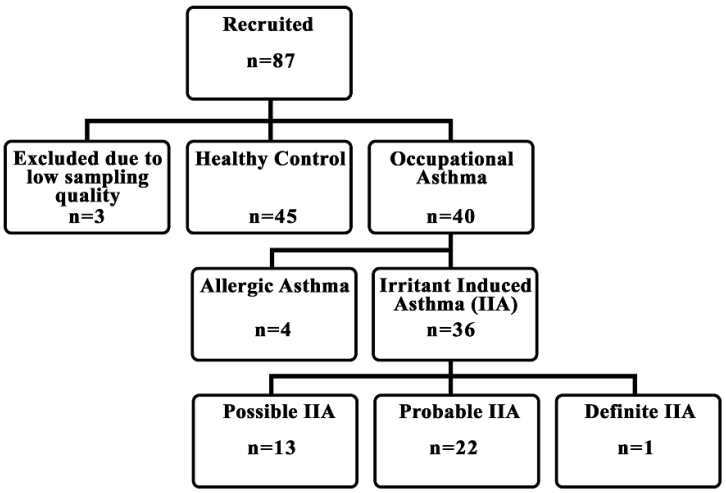

Figure 1. Overview of the study design.

2. Method

2.1. Study design

The overview of the study design is shown in figure 1. This is a single center cross-sectional study of 40 patients, diagnosed with asthma by a lung specialist, who were evaluated for OA. A control group of 45 respiratory healthy healthcare workers HC were recruited among hospital staff. Inclusion criteria were men and women above 18 yrs of age, capable of informed consent. All participants were recruited and sampled between October 2021 and March 2023. Accrual rate was above 90%. One participant in each group of OA and HC had been smoking 48 h and 11 h, respectively, before sampling, and both were included in the study. A fractional exhaled nitric oxide (FeNO) measurement was taken with Niox Vero® (NIOX Group PLC, UK) at the time of sampling. Type of and time since last relevant occupational exposure were assessed and recorded by an occupational hygienist, a specialist and a senior consultant in occupational medicine before breath sampling, by structural interviewing the participant according to the case report form. All samplings were performed at working daytime 09:00–15:00, mostly around noon. Brushing of teeth, type and time since last meal and drink, asthmatic medication, halitosis, sex, age and body mass index (BMI) were recorded and accounted for as confounders. A conclusive assessment of the asthma phenotype, before sample analysis, was conducted in agreement by the study occupational physicians, referencing information from the hospital records.

2.2. Sample collection

Breath samples, collected using the Breath Biopsy® Collection Station with ReCIVA® Breath Sampler and a CASPER® Portable Air Supply (Owlstone Medical Ltd, UK), were directed onto a Breath Biopsy Cartridge with four Tenax TA/Carbograph 5TD sorbent tubes (Markes International, UK) [18]. A pressure sensor enabled real-time monitoring of subjects' breathing duration, triggering pumps to collect subsequent portions of each exhaled breath based on pressure/flow parameters. Each pump drew breath through two sorbent tubes, yielding a collection of 1250 ml on each after about 10–15 min of sampling. The samples were stored in a dedicated refrigerator at 7 °C and collected within 5 d by DHL with overnight air delivery. Upon arrival, the samples were stored at 4 °C at Owlstone Medical, dry-purged within 14 d of collection, and kept at 4 °C until analysis.

2.3. Sample analysis

Samples were analyzed at the Breath Biopsy Laboratory (Owlstone Medical Ltd, UK) using the OMNI Breath Biopsy® Platform. Samples were dry purged with a TD100-xr thermal desorption autosampler (Markes International, UK) to remove excess water upon receipt. Thermal desorption on the TD100-xr autosampler, chromatographic separation using a programmed temperature ramp, and mass spectral data acquisition via electron impact ionization on a Q Exactive™ GC Orbitrap™ high-resolution accurate mass (HRAM) mass spectrometer (Thermo Scientific, USA) were conducted. For each sample, two sample tubes were desorbed into the thermal desorber cold trap. Carry-over was assessed during the validation process, with a quality control sample (sorbent tube spiked with a known chemical mixture) run after every six subject breath samples. To monitor background, a blank tube was run at the sequence's beginning and end, analyzing approximately 30 breath samples in between.

Additional column resolution checks were implemented to enhance the extraction of high-quality molecular features. Analytical variation associated with thermal desorption-gas chromatography mass spectrometry, analysis was assessed using the relative

standard deviation (RSD) of internal standards (eight compounds) monitoring platform performance. The observed 11.3%–16.4% RSD fell within Owlstone Medical's acceptable range (<20%).

2.4. Data preparation

These data are produced through an untargeted feature extraction workflow. The identities of VOCs were classified into confidence tiers following the guidelines of the metabolomics standards initiative [19]. Tier 1 IDs (validated identifications) indicate the highest confidence level. Tier 2 IDs (putative identifications) were assigned by comparing spectral data to Owlstone Medical's internal HRAM library, while Tier 3 IDs (tentative identifications) were assigned based on spectral data comparisons to either Owlstone Medical's HRAM library or the National Institute of Standards and Technology (NIST) library. To ensure sufficient data quality, VOCs that were detected in fewer than 80% of the sample size of the possible IIA group ($n = 13$) were excluded from the analysis. The remaining missing values were imputed at 80% of the minimum observed intensity for that VOC across all cohorts. This imputation assumes that the reason for the missing value is that the true value lies below the limit of detection. As the breath samples were run across three analytical sequences, an internal standard-based normalization method was applied, which aims to correct for both between-sequence and within-sequence variability.

2.5. Statistical analysis

Post-hoc power analyses determined detectable effect sizes for group comparisons. The G*Power software, version 3.1.9.7, was used to calculate the power of two group comparisons. The two-sample t-test and Mann–Whitney U test uses Cohen's d as the effect size. Based on benchmarks suggested by Cohen, effect sizes can be interpreted as small ($d = 0.2$), medium ($d = 0.5$), and large ($d = 0.8$) [20]. With a power of 0.8 and a p-value threshold of 0.05, the two-sample t-test with 40 OA and 45 HC has a detectable effect size of 0.616. The Mann–Whitney U test with 22 probable IIA and 13 possible IIA has a detectable effect size of 0.769.

Data were analyzed using the python programming language (Python Software foundation) with pandas package and visualized with R scripts and GraphPad Prism. For initial data inspection, Principal component analysis (PCA) was performed to reduce dimensionality. The supervised method of principal components regression was used in a logistic regression model. Linear regression was used to model the relationship between a dependent variable and independent variable(s). Linear regression slope values (regression coefficients), i.e. the change of y for a one unit increase in x, were used in volcano plots and as overlay in heat map. Univariate analyses of VOCs was used in a model with demographic variables that were found to be significantly different between the compared groups. The recorded confounders mentioned in 2.1 Study design were analyzed for significant difference between the groups and adjusted for in the linear regression analysis. Only age, sex and BMI was shown to be significant different and adjusted for. The Benjamini–Hochberg method corrected P-values with a false discovery rate (FDR) of 0.1. An uncorrected P-value < 0.05 was deemed significant to avoid excluding potentially relevant VOCs. The Mann–Whitney U test compared VOC abundances between possible IIA and probable IIA groups without covariate adjustment due to sample size. A statistical enrichment approach of chemical classes at FDR $p < 0.05$ was performed with the ChemRICH package in R 4.3.1 [21]. Multivariate models and random forest, an ensemble learning model, were used for receiver operator characteristic area under the curve (ROC-AUC) performance classification.

3. Results

3.1. Subject characteristics

The demographic characteristics of the sample population are presented in table 1. For comparisons between HC and OA, storage time, BMI and sex were found to be significantly different and were adjusted for in linear regression analysis. For comparison between possible IIA and probable IIA, sex was found to be significantly different between the two groups. As expected, FeNO was significantly increased in OA, all IIA, probable IIA, possible IIA and definite IIA, compared to HC, but not in AA, likely because of sample size. Table of exhaled breath VOCs associated with FeNO is shown in supplement S1. Asthma medications were categorized into drug classes and analyzed for exhaled breath VOCs (see supplement S2). However, the sample size was deemed insufficient for further analysis.

3.2. Description of dataset and ROC-AUC performance

The final dataset consisted of 974 molecular features, of which 536 tier 1–3 distinct VOCs were identified. PCA of the VOCs in exhaled breath displayed imperceptible differences between the groups (supplement S3). Among chemical classes with significant difference between HC and OA, diazines were reduced and aldehydes, benzene derivates, ketones, phenols and sulphur compounds were increased in OA (supplement S4). Regression coefficient analysis of VOCs exhibiting significant differences between HC and OA, revealed 76 distinct tier 1–3 VOCs, illustrated as orange dots in the volcano plot presented in figure 2(A). Multivariate models were built utilizing these 76 VOCs to predict the performance classification of breath samples between HC and OA. As shown in figure 2(B), the principal component regression

Table 1. Demographic characteristics of the sample population.

				Irritant induced asthma			
	HC	OA	AA	All	Possible	Probable	Definite
Participants *n*	45	40	4	36	13	22	1
Age years (AD)	44.0 (9.0)	49.5 (11.0)	43.5 (6.0)	51.0 (12.0)	52.0* (7.0)	46.5 (13.5)	60.0[B] (0.0)
Male *n* (%)	13 (30)	26* (60)	3 (80)	23* (60)	4 (31)	18[A] (80)	1 (100%)
BMI kg m^{-2} (AD)	23.4 (2.0)	27.3* (3.6)	22.4 (1.1)	27.6* (2.9)	29.3 (2.9)	27.5 (2.1)	22.4 (0.0)
FeNO ppb (AD)	10.0 (6.0)	14.0* (6.0)	8.0 (0.0)	14.5* (6.5)	14.0* (7.0)	14.5* (5.5)	41.0 (0.0)
Time since exposure:							
<6 d		16	0	16	6	9	1
<1–4 weeks		4	1	3	1	2	0
<1–6 months		2	0	2	0	2	0
>6 months		18	3	15	6	9	0
Storage time days *n* (AD)	508.0 (58.0)	263.0* (120.5)	317.5 (154.0)	263.0* (105.0)	264.0 (54.0)	291.5 (141.0)	100.0 (0.0)
Tube thermal days *n* (AD)	6.0 (4.0)	4.0 (2.0)	7.5 (3.0)	4.0 (2.0)	3.0 (2.0)	4.0 (2.0)	27.0 (0.0)

*p-value < 0.05 vs HC.

[A] p-value < 0.05 vs Possible IIA.

[B] p-value < 0.05 vs HC; Ap-value < 0.05 vs Possible IIA. B = anonymized 5-year interval. For continuous and discrete variables, the median value is shown, with the median absolute deviation (AD) provided in parentheses. Healthy Control (HC). Occupational Asthma (OA). Allergic Asthma (AA).

model yielded a ROC-AUC of 0.94, whereas the random forest model achieved a ROC-AUC of 0.90. Because tier 1 VOCs provide the highest confidence in compound identities, we focused on the top ten significant tier 1 VOCs listed in table 2 and performed classification between HC and OA. The result of principal component regression model generated a ROC-AUC of 0.75, while the random forest model had a ROC-AUC of 0.78 (figure 2(C)).

3.3. Breath exhaled significant tier 1 VOCs in occupational and IIA

All significant tier 1 VOCs are detailed in table 2 for both HC vs OA and possible IIA vs probable IIA. The most significant VOCs regardless of tier are shown in supplements S5 and S6. VOCs displayed in figures 3 and 4, were selected because of biological implications or exposures that show relevance to OA. In figure 3, box plots illustrate tier 1 VOCs that displayed significant differences between HC and OA, including 1-hexadecanol, phenol, 2,3-butanediol, o-xylene, acetone and isopropyl alcohol. Additionally for OA, recent relevant occupational exposure in the past 4 weeks, and late relevant occupational exposure for more than 4 weeks ago are displayed. No significant differences were noted for time since exposure among the VOCs. Figure 4 shows box plots for tier 1 VOCs with significant differences in possible IIA vs probable IIA, including 3-methylhexane, methylcyclohexane and isoprene. No significant differences were noted for time since exposure for neither possible IIA nor probable IIA among the VOCs.

3.4. Heat map of exhaled breath VOCs and occupational exposures

Figure 5 illustrates tier 1 VOCs that exhibit significance in occupational exposure groups compared to HC. Specifically, 1-hexadecanol showed associations with exposures of low molecular weight chemicals, acid or alkali, and chemical gas, smoke, or vapor. Acetone was associated with acid and alkali exposure, although this was a small group. Regarding chemical classes, aldehydes were associated with organic dust, low molecular weight chemicals and acid or alkali. Ketones were associated with inorganic and organic dust. Monoterpenes were associated with organic dust. Volcano plots of exposure groups are shown in supplement S7 and number of exposed in each group are shown in Supplement S8.

3.5. Classification of exhaled VOCs and a biological model of OA

Utilizing the VOCs that exhibited significant differences between groups, a categorization based on their origins is depicted in venn diagrams in figure 6(A). Additionally, a specific biological hypothesis tailored to OA is proposed in figure 6(B).

4. Discussion

This is the first study to explore breath VOCs in OA that can be potentially utilized as biomarkers to distinguish OA from HC or between different IIA subtypes. The untargeted approach selected allows

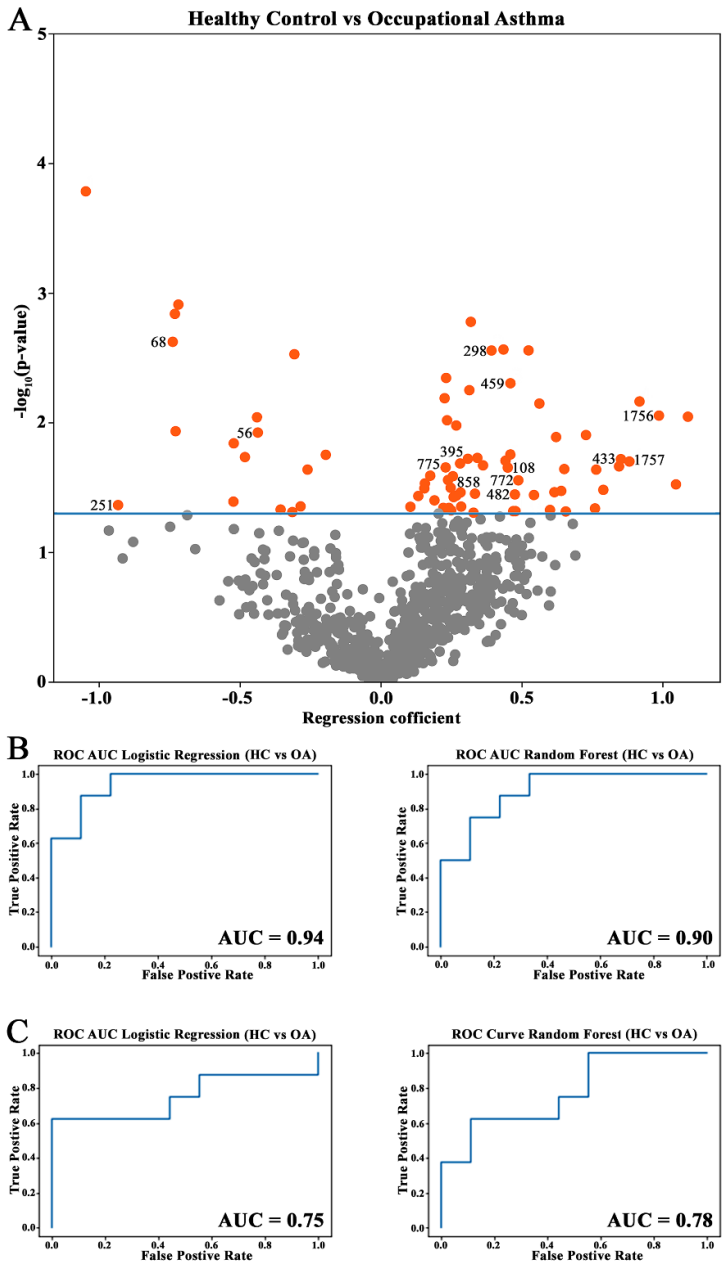

Figure 2. Volcano plot and ROC-AUC of significant VOCs in occupational asthma. (A) Volcano plot: orange dots denote 76 tier 1–3 VOCs that exhibited significant differences. The numbers correspond to 14 IDs described in table 2. The regression coefficient shows the unit change of an increase of 1 in the variable. (B) Receiver operating characteristic area under the curve (ROC-AUC) of 76 tier 1–3 VOCs showing significant differences. Left: logistic regression model. Right: random forest model. (C) ROC-AUC of the top ten significant tier 1 VOCs. Left: logistic regression model. Right: random forest model.

to better understand the potential multiple mechanisms contributing to a wide array of asthma phenotypes. This pilot study identified previously reported exhaled VOCs in asthma and discovered several novel VOCs in exhaled breath that may contribute to asthma development and could signal occupational exposure. The VOCs were classified into three groups: human metabolism, microbial metabolism and occupational exposure, as shown in figure 6(A).

1-hexadecanol, p-xylene, and phenol have previously been identified as potential breath biomarkers in asthma. 1-hexadecanol was reduced and p-xylene increased in a study of acute breathlessness, including asthma [14]. In a study to find VOCs that could discriminate asthmatics from healthy, phenol was reduced [22].

In this study, 1-hexadecanol, 2,3-butanediol, phenol and o-/p-xylene were all significantly elevated in OA. There appears to be a potential connection among them through a pathway involving reduced nicotinamide adenine dinucleotide (NADH), supported by associations in the search tool for interactions of chemicals database [23]. NADH is crucial in various processes, including quinone metabolism, and it plays a significant role in airway irritant metabolism, potentially contributing to asthma

Table 2. Significant tier 1 VOCs in exhaled breath in occupational and irritant-induced asthma subgroups.

	Healthy control vs occupational asthma				Possible vs probable irritant induced asthma		
VOC	ID	*p*-value	ROC-AUC	Ratio	VOC	*p*-value	Ratio
Isopropyl alcohol	68	0.002	0.74	0.40	3-Methylhexane	<0.001	3.28
Thiazole	298	0.002	0.71	1.53	p-Menthone	0.003	0.38
3-Methylpyridine	459	0.005	0.73	1.57	Methylcyclohexane	0.006	3.97
1-Hexadecanol	1756	0.007	0.68	2.43	4-Heptanone	0.007	0.37
Acetone	56	0.012	0.68	0.70	Wine lactone	0.010	0.26
2,3-Dihydrofuran	108	0.020	0.68	1.60	Isoprene	0.010	1.90
2,3-Butanediol	433	0.020	0.66	7.44	Cyclohexasiloxane	0.012	0.81
2-Hydroxybenzaldehyde	775	0.026	0.66	1.18	Dimethyl sulfide	0.028	0.37
2-Methylpyridine	395	0.026	0.69	1.21	Dodecane	0.030	0.86
Phenol	772	0.028	0.65	1.96	Methylcyclopentane	0.030	1.99
o-Xylene	482	0.036	0.64	1.57	Heptane	0.030	3.28
1-(Methylthio)-propane	251	0.043	0.67	0.82	1-Pentanol	0.033	0.38
o-Cresol	858	0.047	0.65	1.27	Pentane	0.039	1.32
p-Xylene	448	0.048	0.63	1.61	2,2-Dimethylpentane	0.049	1.72

IDs correspond to the row identities within the dataset, as depicted in figure 1(A)'s Volcano plot.

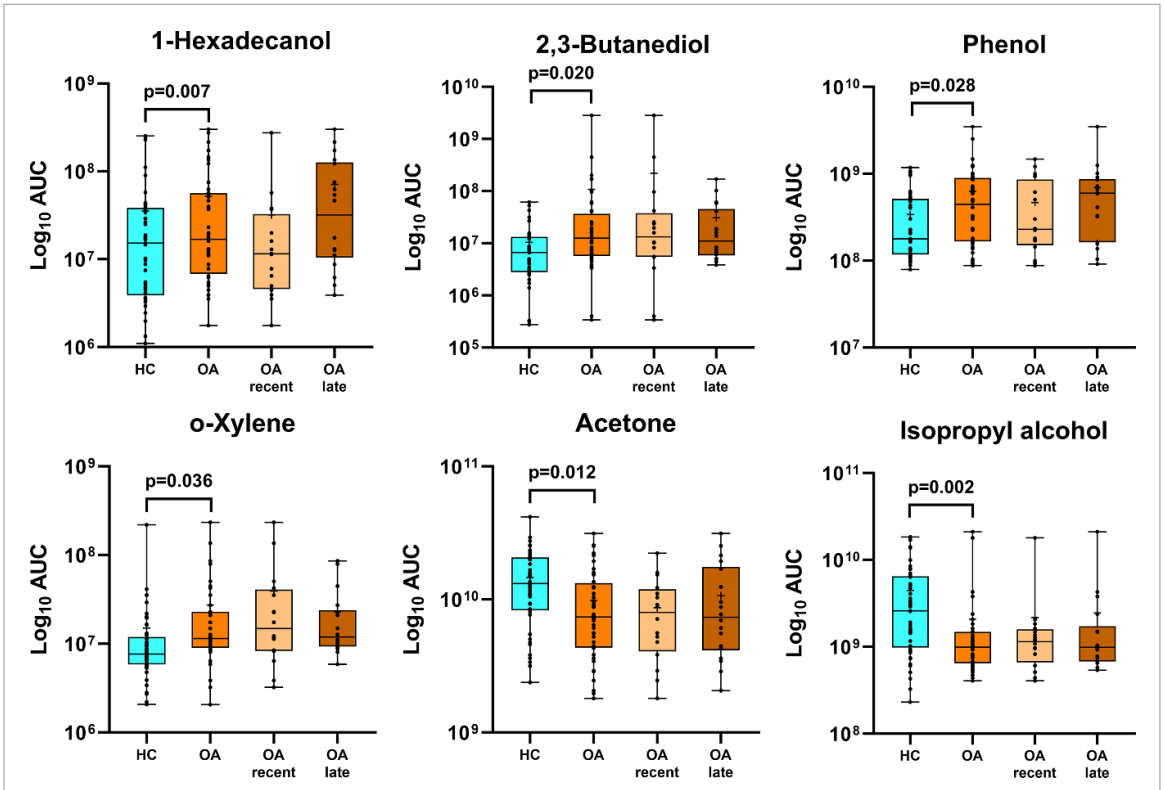

Figure 3. Significant tier 1 exhaled breath VOCs in occupational asthma. Box plots depicting VOCs exhibiting significant differences. Healthy control (HC). Occupational asthma (OA). Recent = occupational exposure in the past 4 weeks. Late = occupational exposure more than 4 weeks ago.

by promoting the generation of ROS and inflammation, as depicted in figure 6(B) [24, 25]. It is possible that these VOCs are related to a mitochondrial dysfunction generating ROS, as an effect of exposure to airway irritants or airway remodeling.

1-hexadecanol has been associated with nuclear factor NF-κB activation in rat lungs and aryl hydrocarbon receptor signaling pathways [26]. It is suggested to have anti-inflammatory properties [27, 28]. Interestingly in our study, 1-hexadecanol showed a more than 2-fold increase in exhaled breath and it was associated with occupational exposures of low molecular weight chemicals, acid or alkali, and chemical gas, smoke, or vapor. These findings suggest that 1-hexadecanol may serve as a potential marker for inflammation and airway injury in the context of occupational exposures.

Although the increase was not significant, 1-hexadecanol levels were higher during late occupational exposure compared to recent exposure. We speculate that this may indicate a late asthmatic effect, potentially involving inflammation and airway

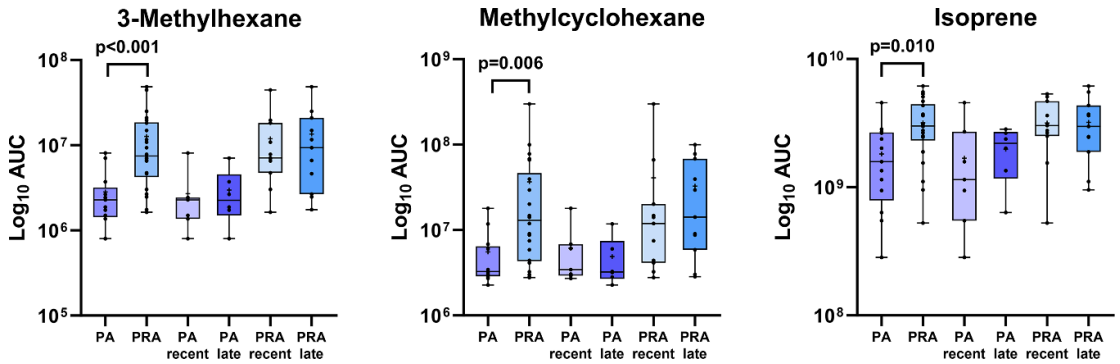

Figure 4. Significant tier 1 exhaled breath VOCs in irritant-induced asthma. Box plots depicting VOCs exhibiting significant differences. Healthy control (HC). Occupational asthma (OA). Possible irritant-induced asthma (PA). Probable irritant-induced asthma (PRA). Recent = occupational exposure in the past 4 weeks. Late = occupational exposure more than 4 weeks ago.

remodeling, although it could also be associated with mitochondrial dysfunction.

Remarkably, 2,3-butanediol, a pyruvate metabolite associated with microbial fermentation, exhibited a 7-to 8-fold increase in OA compared to HC. To our knowledge, this compound has never been reported in connection with asthma, which could make it a novel and intriguing biomarker in this field. 2,3-butanediol has demonstrated the ability to alleviate lipopolysaccharide-induced acute lung injury and influence lung microbiota in murine models [29, 30]. Additionally, the closely related 2,3-butanedione has been identified as a noteworthy molecule in a study investigating microbial fermentation within the airways of individuals with cystic fibrosis [31]. Phenol may originate from occupational exposure as a metabolite of benzene, in addition to human and microbial metabolism [32]. Phenol in exhaled breath has been associated with COPD [33]. Additionally, low-dose exposure to phenol has been reported to induce bronchoconstriction [34]. It is possible that 2,3-butanediol and phenol represents a specific microbiome profile in OA, mainly driven by IIA in our study. Colonization of bacteria in the respiratory tract can influence the phenotype of asthma and other inflammatory lung diseases. For example, bacterial colonization has been linked to prolonged asthma duration and more frequent exacerbations [35]. Thus, metabolites generated by bacteria, including 2,3-butanediol and phenol, may emerge as compelling candidates for potential diagnostic indicators of bacterial colonization and associated exacerbations.

We observed significantly higher levels of the isomers o-xylene and p-xylene in breath of OA compared to HC. This finding is in line with prior research linking xylene to increased asthma risk and airway inflammation [36, 37]. Xylene has previously been shown to increase ROS generation in human lymphocytes [38]. Xylene is commonly used as a solvent and may serve as both an exposure indicator and a risk factor for OA. In this study, xylene was increased, although not significantly, in occupational exposures within the last 4 weeks compared to later exposures, which may support xylene as an occupational exposure.

The majority of our findings were partially associated with occupational exposures, as depicted in the graphical summary in figure 6(A). Notably, isopropyl alcohol was increased in HC, and it exhibited the highest significance among the tier 1 compounds that differed between HC and OA. It is plausible that isopropyl alcohol represents an occupational exposure among healthcare workers [39]. Acetone is highly expressed in breath, mainly related to ketone and lipid metabolism in humans [40]. The levels of acetone have been known to be influenced by several factors, including insulin resistance, diurnal fluctuations, lipolytic activity, diet, exercise, fasting status [41]. Individuals with a more pronounced asthma-COPD overlap syndrome have shown heightened acetone levels, contrary to our results showing a reduction in OA compared to HC [42]. In our study, the OA group had a significantly higher BMI compared to the HC group, which could potentially complicate the interpretation of acetone levels, although BMI were not correlated with exhaled breath acetone in one study [43].

Interestingly, acetone and isopropyl alcohol are related, as acetone can be converted to isopropyl alcohol in the body by hepatic alcohol dehydrogenase [44]. However, there were no indications that any individuals in the HC group were on a calorie-restricted diet or in a ketonemic state, which would be characterized by the smell of acetone. It is worth noting that increased acetone levels in healthcare worker environments has been reported [39].

As far as the comparison between IIA subtypes, 3-methylhexane, methylcyclohexane, and isoprene were elevated in probable IIA when compared with possible IIA. 3-methylhexane (an isomer of heptane) and methylcyclohexane, are indicative of occupational industrial exposures of solvents, lubricants and rubber [45]. It is worth noting that the possible IIA

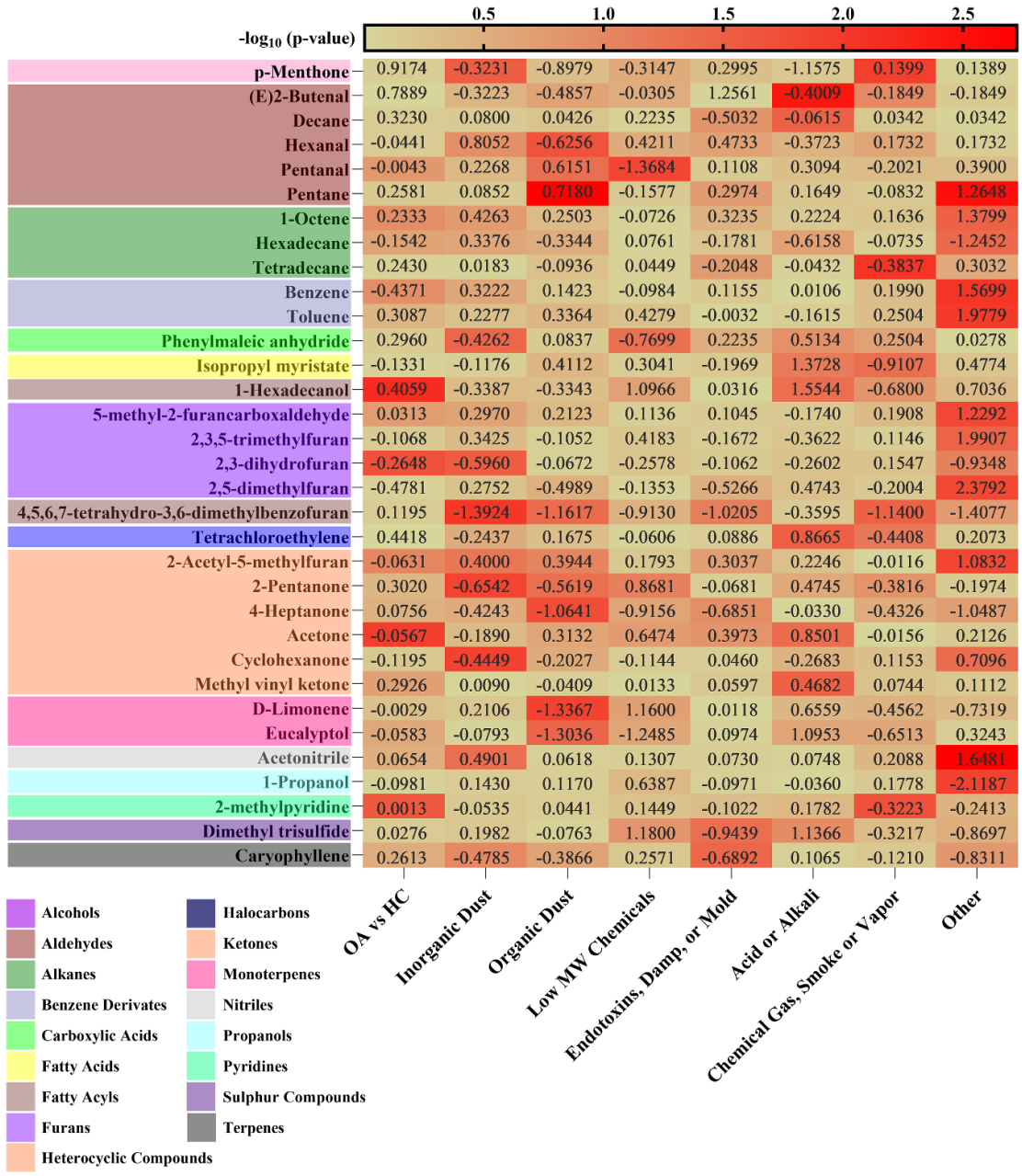

Figure 5. Exhaled breath VOCs associated with occupational exposures. Heat map depicting patients with occupational asthma (OA) categorized based on occupational exposures containing at least one VOC exhibiting a significant difference ($p < 0.05$, linear regression adjusted for BMI, sex, age, and storage time) compared to a healthy control (HC) group. *P*-values are illustrated as -$\log_{10}$, and the overlay of values reflects the regression coefficients. VOCs are further categorized into compound classes.

group typically experiences less exposure to industrial and manufactural settings [46]. Therefore, these VOCs could be potential markers to suggest temporal exposures, although there were no significant differences when comparing recent and late exposures, as shown in figure 4. The reason to this may be the sample size, time scale or non-linear metabolic clearance rate, although other causes than occupational exposures to the elevated levels cannot be excluded. Isoprene may be an occupational exposure during polymer production and environmentally through smoking [47]. Mainly, it seems to originate from human and less from microbial metabolism [48]. Isoprene has also been proposed as an oxidative stress biomarker in asthma [49, 50]. The findings regarding isoprene's role in asthma have been inconsistent and it should be further investigated in IIA.

This pilot study has some limitations. Although our study showed a medium effect size at 80% power, sample size constraints posed challenges in achieving significant outcomes with FDR. At 90% power and an effect size at 0.5, an estimated 170 patients would be needed. The use of healthcare workers as controls ensured consistency in occupational exposure profiles, but presented challenges in matching demographic factors such as age and sex. Among the IIA

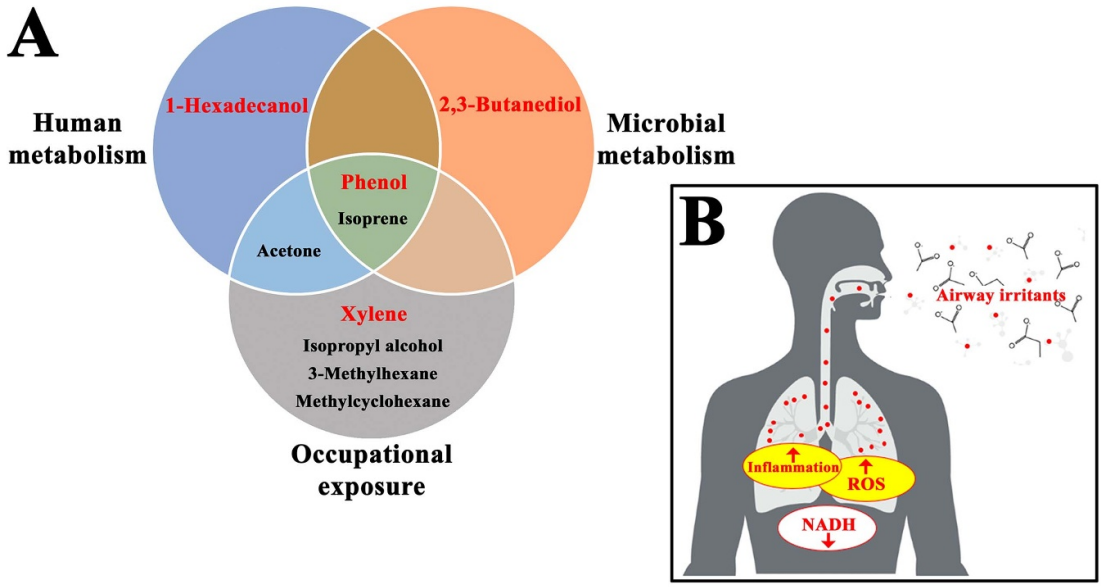

Figure 6. Exhaled breath VOCs associated with occupational asthma. (A). Categorization of exhaled breath volatile organic compounds. Highlighted in red 1-hexadecanol, 2,3-butanediol, phenol and xylene are increased in occupational asthma and connected to nicotinamide adenine dinucleotide (NADH). (B). Proposed biological mechanism of airway inflammation involves the generation of reactive oxygen species (ROS) through quinone metabolism by airway irritants, leading to the reduction of NADH.

subgroups, there was a notable imbalance in sex distribution, with 80% males in the probable IIA group compared to only 31% in the possible IIA group, reflecting the industrial and manufactural predominance among men. It is noteworthy that isopropyl alcohol and potentially acetone were identified as occupational exposures among the HC group. Although the number of AA subjects only comprised 10% of the OA group, it reflects the OSH efforts in norway of the last decades. Therefore, while AA was not the main focus of this study, including these subjects would help increase the chances of identified biomarkers in this study being translated into clinical applications, where OA patients will also be a mixed population.

While all 76 tier 1–3 compounds showing significant differences yielded a high ROC-AUC performance of 0.94, this rate decreased to a moderate 0.78 for the top ten significant tier 1 compounds, and below 0.70 for the single compounds of interest in OA. Looking ahead, we recommend developing a computational model that utilizes key VOCs to predict the onset of OA. This model could enhance diagnostic precision by forecasting the likelihood of OA onset. The VOCs would be needed to be validated in an independent cohort with larger sample size in a multicenter study.

5. Conclusion

The findings of this study are somewhat consistent with the proposed pathophysiology of IIA, which involves airway injury followed by a proinflammatory response. 1-hexadecanol, 2,3-butanediol, phenol and xylene association to NADH as a potential driver for ROS production and airway inflammation in OA should be further investigated. The complexity of OA extends to the occupational exposures of VOCs and their metabolites, an aspect that has been understudied in exhaled VOC research. Future investigations are expected to broaden our understanding and potentially identify a distinct set of VOCs capable of distinguishing between OA and its subgroups.

Data availability statement

The data cannot be made publicly available upon publication because they contain sensitive personal information. The data that support the findings of this study are available upon reasonable request from the authors.

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Conflict of interest

A C, L G, M T are employed by Owlstone Medical Ltd None of the authors have any other conflict of interest. The study was initiated and directed by Oslo University Hospital.

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Author contributions

BH originally planned the study. HH, TTH and BH performed the sampling and collected the data. AC, LG, MT and BH did the statistical analysis. All authors revised the drafted paper.

Ethics approval and consent to participate

All participants gave informed consent, and the study followed the Declaration of Helsinki. The study was approved by the regional ethics committee (Case No. 50249), the Hospital data protection officer (Case No. 19-28576) and registered at www.clinicaltrials.gov (Identifier: NCT04301674).

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