**Exhaled volatile organic compounds during inhaled mannitol challenges in adults**

Wang R[1,2]*, Peel A[3]*, Ahmed W[1], White I, Wilkinson M, Loke YK[4,5], Wilson AM[4-6]* and Fowler SJ[1,2]*

*these authors contributed equally to this work

1. Division of Immunology, Immunity to infection & Respiratory Medicine, School of Biological Sciences, The University of Manchester. UK.

2. Manchester University NHS Foundation Trust, Manchester, UK

3

4 Norwich Medical School, University of East Anglia, Norwich, UK

5 Department of Respiratory Medicine, Norfolk and Norwich University Hospital NHS Foundation Trust, Norwich

6 Asthma UK Centre for Applied Research

**Corresponding author:**

Professor Stephen J Fowler

The University of Manchester

Education and Research Building, 2nd floor

Wythenshawe Hospital

Southmoor Road

Manchester M23 9LT, UK

**Email:** Stephen.fowler@manchester.ac.uk

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**ABSTRACT**

**Background:** Inhaled mannitol provokes bronchoconstriction *via* mediators released during osmotic degranulation of inflammatory cells in asthma, and hence represents a useful diagnostic test and model for acute attacks, especially in phenotypes that share this mechanism such as exercise induced asthma. We hypothesised that mannitol challenge would trigger changes in exhaled volatile organic compounds (VOCs), generating novel insights into the origins of such VOCs and identifying potential biomarkers for future investigation.

**Methods:** Participants with a doctor-confirmed diagnosis of asthma, or suspected asthma being investigated by mannitol challenge were recruited. Inhaled mannitol challenges were performed, and in participants with hyperresponsiveness, a sham challenge was performed at least 14 days later. VOCs were collected before and after challenges and analysed using gas chromatography-mass spectrometry. Duplicate breath samples were taken for reproducibility assessment using interclass correlation coefficient (ICC). Pre-defined VOCs were extracted from samples using a semi-targeted approach including some reported previously from asthma. Univariate and multivariate (sparse Partial Least Squared Discriminant Analysis) analyses were carried out.

**Results:** Forty-six patients (mean [SD] age 52 [16] years) completed mannitol challenge and significant bronchoconstriction occurred in 16 (35%), 15 of whom attended for a sham challenge. Fifty-nine previously reported key asthma VOCs were identified, 9 were excluded due to poor reproducibility (ICC <0.6). Levels of 16 VOCs changed ($p<0.05$) following mannitol challenge, of which 12 also contributed to the multivariate model, with a classification error rate of 13.5% and area under the receiver operating characteristic curve of 0.90. Levels of 6 of these 16 VOCs also changed following the sham challenge, along with 3 further VOCs. In patients who had positive mannitol challenges who subsequently had a sham challenge, distinct VOCs signatures were observed following active and sham challenges.

**Conclusion:** Distinct changes in exhaled VOCs during mannitol and sham challenges were identified. Those differentially expressed VOCs merit further investigation as potential biomarkers of airway inflammation and bronchoconstriction in asthma.

## Introduction

Asthma exacerbations drive the majority of disease-related morbidity, mortality and healthcare resource utilisation [1], and the pathophysiology is heterogeneous [2]. Volatile metabolites in exhaled breath can reflect changes during loss of control and exacerbations [3-5], and may be useful in predicting clinical deterioration and elucidating its underlying mechanism. Indirect bronchial challenge tests, such as using mannitol, provoke bronchospasm through inducing pro-inflammatory processes, including the release of mast cells mediators [6]. Mannitol challenge is often used to diagnose asthma, particularly for the exercise-induced phenotype [7]. Therefore, the differences in exhaled volatile organic compounds (VOCs) between patients with and without mannitol-induced bronchospasm may reveal useful biomarkers for mast cell mediated asthma phenotype. Any alterations in breath signatures induced by mannitol challenges may also provide mechanistic insights into asthma exacerbations mediated *via* these pathways.

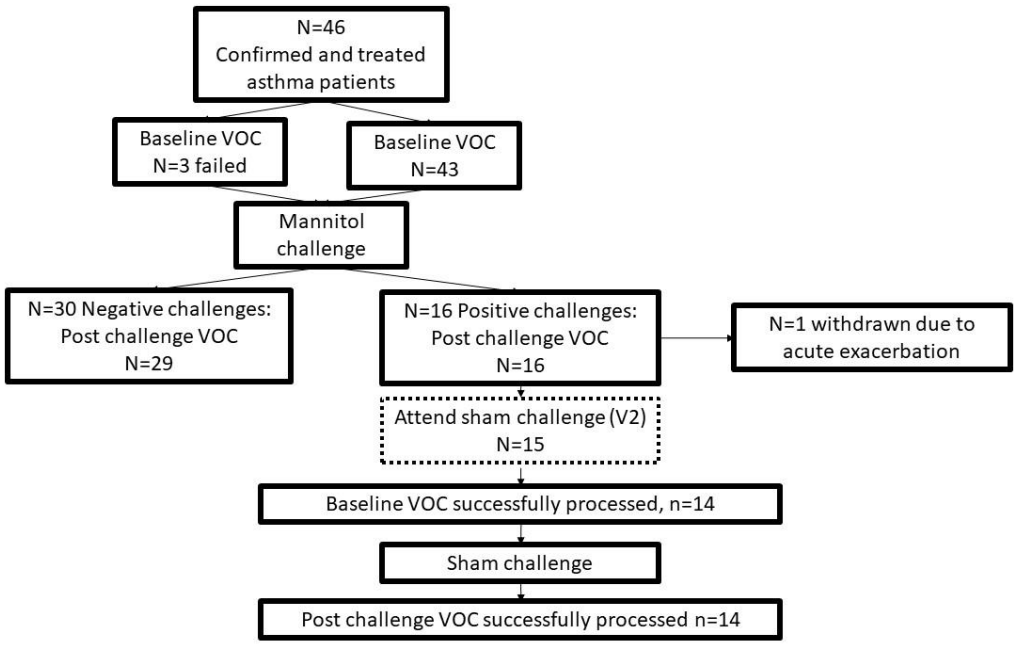
A previous study has demonstrated that acute bronchoconstriction induced by methacholine challenge (that acts by directly activating bronchial smooth muscle) did not affect the breath VOC profile as detected by electronic nose (eNose) [8]. However, the challenge procedure itself, using methacholine or normal saline (as a sham challenge), altered the breathomic profile relative to baseline, highlighting the necessity of sham challenges in the design of such studies. The use of direct bronchial challenge does not allow assessment of the impact of airway inflammation and the use of the eNose precluded the ability to identify individual analytes, unlike gas chromatography-mass spectrometry (GC-MS) which is considered the gold-standard for exhaled breath biomarker studies [9]. Therefore, using a sham-controlled study design, we aimed to investigate the changes in VOCs during mannitol challenges in patients with and without mannitol-induced bronchospasm using GC-MS.

## Methods

### Clinical trial design

This was a two-visit sham-controlled study (Figure 1) (NCT03575663). Participants with doctor-diagnosed asthma who are taking British Thoracic Society step 1-4 treatment, or with suspected asthma being investigated by mannitol challenge were recruited. Current smokers or ex-smokers with smoking history of >10 pack years, those with acute respiratory tract infection, asthma exacerbation or change in treatment within the previous four weeks, those taking long-term oral corticosteroids, and those with other major cardiorespiratory diseases were excluded.

*Figure 1. Study flow chart*




A clinical history was taken and asthma control assessed using the Asthma Control Questionnaire (ACQ) [10]. Patients underwent spirometry assessment before baseline VOCs were collected (ReCIVA, Owlstone, Cambridge, UK). Fractional exhaled nitric oxide (FeNO) (NIOX VERO, Aerocrine, Chicago, USA) was measured and blood collected for serum eosinophil levels. A mannitol challenge (Osmohale Pharmaxis, Sydney, Australia) was performed in accordance with the published guidelines [11] before post-challenge VOCs were sampled. A fall in the forced expiratory volume within 1 second ($FEV_1$) by 15% at any dose step constitutes a positive result. Participants with a positive mannitol challenge were invited to return for a sham bronchial challenge, during which further breath sampling before and after the challenge test was carried out.

The sham challenges, using empty capsules (Osmohale delivery device, 0mg, Pharmaxis, Sydney, Australia), were performed in an identical manner with the same number of maximum inhalations and spirometric manoeuvres as during the participant's mannitol challenge. The study visit interval was at least 14 days in order to avoid carrying over the effect of the challenge test. Study visits were postponed for participants reporting a recent asthma exacerbation, respiratory tract infection,

change in treatment or in those with a significant change in either asthma control, FeNO or $FEV_1$ within the previous four weeks.

The trial was approved by Research Ethics Committee (17/EE/0430). All participants gave informed written consent.

**Breath sample collection**

Exhaled breath VOCs were collected using a ReCIVA sampling device used in conjunction with a CASPER clean air filter (Owlstone Medical, Cambridge, UK) [12] onto inert-coated stainless steel tubes packed with Tenax GR sorbent material (Markes International, Bridgend, UK) according to the European Respiratory Society technical standards [13]. A total volume of 500mL of exhaled breath was sampled selectively during late phase expiratory breath. Silicone masks were conditioned before use.

Duplicate patient breath samples were taken for reproducibility assessment. Filtered air was sampled (ambient air samples) by attaching the CASPER filtered air outflow to the ReCIVA and mask which were fitted to a glass head.

**Gas chromatography-mass spectrometry analysis**

Breath VOC samples were dry purged with $N_2$ (50mL $min^{-1}$ at room temperature) to remove excess water and refrigerated for up to 2 weeks before analysis. During analysis, tubes were spiked with an internal standard (1ppmV p-bromofluorobenzene in $N_2$) then desorbed at 320 °C (TD-100, Markes International, Bridgend UK) and transferred onto a cold trap (kept at 0 °C) for cryo-focussing. The trap was then flash heated to 330 °C and a flow of helium carried VOCs into the GC column (DB-5 ms Ultra Inert column, length 30 m × internal diameter 0.25 mm, film thickness 25 µm, (5%-Phenyl)-methylpolysiloxane, Agilent Technologies, Santa Clara, USA) housed in a GC oven (7890B, Agilent Technologies, Santa Clara, USA). The column was subjected to the following temperature programme with a constant pressure method (69 kPa): ramped from 40 °C to 170 °C (at 6 °C $min^{-1}$), then ramped to 190 °C (at 5 °C $min^{-1}$), total GC cycle time of 23 min. After GC separation, VOCs were fragmented using electron ionisation (70eV) and detected in full scan mode between 40-500 Da (4 Hz) within the mass spectrometer (7010, Agilent Technologies, Santa Clara, USA).

**Data pre-processing**

Chromatograms were screened for inclusion in the final dataset by manual appraisal and all samples deconvolved using Masshunter Quantitative analysis (version B.07.00, Agilent Technologies, Santa Clara, USA). The software was used to build a search method using pre-defined quantifier ions and

retention times of target VOCs (MSI Level 2 identification). Putative compound identification was performed using the NIST 14 library with a mass spectral match score of >70.

**Data treatment and statistical analysis**

All statistical analysis were performed in R (version 4.1.1). Peak intensities were normalised to the internal standard and log-transformed. Reproducibility of VOCs was assessed between replicate breath samples using intra-class correlation coefficient (ICC) (irr R package). VOCs with poor reproducibility (ICC <0.6) in samples taken in parallel are unlikely to represent clinically useful biomarker candidates, and therefore were excluded from further analysis. The mean of log-transformed VOC peak intensities where the replicate breath samples had ICC of ≥ 0.6 were then used for further analysis.

For univariate analysis, Mann-Whitney U tests or Wilcoxon Rank Test were used for unpaired and paired data as appropriate to investigate changes between independent and related sample groups, respectively. Significant compounds with raw p-values (<0.05) and FDR adjusted (Benjamini-Hochberg method) q-values (<0.05) are reported.

For multivariate analysis, missing values were imputed using Random Forest proximity method and scaled [14]. Sparse Partial Least Squared Discriminant Analysis (sPLS-DA) was applied to the treated dataset. Multilevel models were used for paired samples collected before and after mannitol challenge. Data were not split into training and validation sets due to limited sample size. Five-fold cross validation (100 repeats) was used for model optimisation (mixOmics R package). VOCs with VIP>1 and stability >0.4 in the final model are reported. The mean and standard deviation (SD) of the area under the receiver operating characteristic curve (AUC) was calculated from training-cross validation sets.

**Results**

**Participants**

Forty-six patients (mean [SD] age 52 [16] years) completed mannitol challenge and 16 (34.8%) had a fall in $FEV_1$ by 15% and therefore a positive challenge (Table 1, Figures 1 and E1). Of these, 15 attended for further sham challenges and one was withdrawn due to asthma exacerbation. Of those who underwent sham challenge, VOCs samples were able to be processed in 14 patients both at baseline and post challenge (Figure 1). The median (IQR) time between mannitol and sham challenges was 39 (31-71) days but there were no significant changes in asthma control, FeNO, blood

eosinophils or $FEV_1$ between visits. No participant had a positive sham challenge (median [IQR] of change in $FEV_1$: -0.9 [-2.4, 1.7] %).

| *Table 1. Baseline characteristics of participants with positive mannitol challenge* | | | |
|---|---|---|---|
| | **Positive mannitol (n=16)** | **Negative mannitol (n=30)** | **p-value** |
| **Age , yrs** Mean (SD) | 50.5 (15.8) | 52.4 (17.0) | 0.70 |
| **Gender, male** N (%) | 6 (37.5) | 12 (40) | 0.93 |
| **BMI, kg/m$^2$** Median (IQR) | 25.2 (23.5, 31.5) | 26.4 (23.8, 32) | 0.80 |
| **Smoking history** N (%) | 5 (31.3) | 8 (25.8) | 0.71 |
| **BTS treatment step,** n (%) | | | |
| **Step 0** | 2 (13.3) | 4 (12.5) | 0.01 |
| **Step 1** | 8 (50.0) | 9 (30.0) | |
| **Step 2** | 4 (25.0) | 10 (33.3) | |
| **Step 3** | 1 (6.3) | 6 (20) | |
| **Step 4** | 1 (6.3) | 1 (3.3) | |
| **$FEV_1$, L** Median (IQR) | 2.58 (2.13, 3.40) | 2.8 (2.33, 3.58) | 0.41 |
| **$FEV_1$, % pred** Mean (SD) | 93.3 (16.5) | 102 (12.1) | 0.08 |
| **FVC, L** Median (IQR) | 3.74 (3.32, 4.31) | 3.83 (3.40, 4.64) | 0.64 |
| **FVC, % pred** Mean (SD) | 112 (15.3) | 114 (15.0) | 0.61 |
| **FEV1/FVC, %** Mean (SD) | 72 (63, 77) | 74 (70, 79) | 0.30 |
| **ACQ-6** Median (IQR) | 0.3 (0.2, 1.0) | 0.3 (0-0.7) | 0.24 |
| **Blood eosinophils, x10$^9$ cells/L** Median (IQR) | 0.24 (0.10, 0.41) | 0.19 (0.12, 0.34) | 0.73 |
| **FeNO, ppb** Median (IQR) | 21.5 (17, 55) | 20 (16, 30.8) | 0.41 |
| **Mannitol challenge % fall in $FEV_1$ at final dose** Median (IQR) | -17.8 (-20.3, -16) | -5.3 (-9, -2.7) | <0.001 |
| Abbreviations: BTS:British Thoracic Society; FEV1:Forced expiratory volume within 1 second; FVC: forced vital capacity; ACQ: Asthma Control Questionnaire; FeNO: fractional exhaled nitric oxide; | | | |

**Semi-targeted analysis of exhaled breath VOCs**

Fifty-nine compounds were identified through manual inspection of chromatograms (Table E1). The reproducibility between duplicated samples was excellent for the majority of VOCs (median [IQR] ICC of 0.82 [0.71-0.91]) (Table E1, Figure E2). Nine VOCs were excluded from further analysis due to poor reproducibility (ICC<0.6). Key asthma VOCs such as acetone, isoprene, decane, octanal, nonanal, decanal, 2-methylfuran, pentane, heptane and 5, 9-undecadien-2-one, 6, 10-dimethyl-, (E)-, that has been previously reported in publications were identified [3, 15-23] and showed good reproducibility between the replicate samples. Correlations between identified compounds are shown in Figure E3.

*Effect of mannitol and sham challenges on exhaled breath VOCs*

Univariate analysis identified 16 VOC with changed levels ($p<0.05$) following mannitol challenge, seven with FDR <0.05 (Table 3). The majority of these VOCs were also identified in multivariate analysis with VIP>1 and good stability (Table 3, Figures 2 and 3); Two-component multilevel sPLS-DA model gave a classification error rate of 11.4% and mean (SD) AUC of 0.92 (0.03).

Levels of nine VOCs changed ($p<0.05$) following sham challenges, of which six were also observed following mannitol challenges (Table 3). These VOCs were also identified in the multivariate models with VIP>1 (Figure 2). However, no VOCs were significant after FDR adjustment. Two component multilevel sPLS-DA model gave a classification error rate of 11.0% and AUC of 0.92 (0.078).

Most of the identified VOCs had higher abundance in the breath samples compared to ambient air samples at baseline (Figure E4); trichloroethylene and heptane, 2,2,4,6,6-pentamethyl had higher levels ($p<0.05$) in the background compared to baseline breaths samples.

Table 3. Significantly altered VOCs before and after mannitol and sham challenges

| **Before *versus* after mannitol challenge in all patients N=40 patients∞** | | | | |
|---|---|---|---|---|
| | **Univariate analysis †** | | | **Multivariate analysis** |
| **Compounds** | **Raw p-value†** | **FDR** | **Direction of change** | **Stability of features (with VIP>1) in sPLSDA model** |
| Trichloroethylene∞ | **<0.001** | **0.003** | ↓ | 1 |
| 3, 5-dihydroxybenzamide | **0.001** | **0.026** | ↑ | 1 |

| | | | | |
|---|---|---|---|---|
| Oxime-, methoxy-phenyl- | **0.002** | **0.026** | ↑ | 0.99 |
| Benzophenone | **0.002** | **0.029** | ↑ | 1 |
| Acetophenone | **0.003** | **0.034** | ↑ | 1 |
| Benzofuran | **0.006** | **0.044** | ↑ | - |
| Phenylethyne | **0.006** | **0.044** | ↑ | 1 |
| 1,2 Benzenedicarboxylic acid | **0.009** | 0.059 | ↓ | 1 |
| Decane | **0.011** | 0.059 | ↓ | 1 |
| Methanesulfonyl acetic acid | **0.012** | 0.059 | ↑ | 0.99 |
| Heptane, 2,2,4,6,6-pentamethyl$^{\infty}$ | **0.022** | 0.107 | ↓ | 0.99 |
| Tetrachloroethylene | **0.030** | 0.117 | ↓ | 0.88 |
| Toluene | **0.032** | 0.117 | ↓ | 1 |
| β-Methylhistamine | **0.033** | 0.117 | ↑ | 0.91 |
| Octanal | **0.034** | 0.117 | ↑ | 0.98 |
| D-Limonene | **0.041** | 0.130 | ↓ | 0.97 |
| 5,9.Undecadien-2-one-6,10,dimethyl | 0.064 | - | - | 0.95 |
| Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl- | 0.084 | - | - | 0.99 |
| Acetone | 0.084 | - | | 0.99 |
| Ammonium Acetate | 0.087 | - | - | 0.99 |
| Styrene | 0.103 | - | - | 0.96 |
| **Before *versus* after sham challenge in participants who had positive mannitol challenges N=12 patients$^{\infty}$** | | | | |
| Heptane, 2,2,4,6,6-pentamethyl*$^{\infty}$ | **0.001** | 0.062 | ↓ | 1 |
| Acetone | **0.002** | 0.062 | ↓ | 1 |
| D-Limonene* | **0.005** | 0.083 | ↓ | 1 |
| Decane* | **0.007** | 0.087 | ↓ | 1 |
| Tetrachloroethylene* | **0.017** | 0.137 | ↓ | 1 |
| Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl- * | **0.017** | 0.137 | ↓ | 1 |
| Oxime-, methoxy-phenyl-* | **0.020** | 0.152 | ↑ | 0.98 |
| Dimethyl selenide | **0.027** | 0.152 | ↓ | - |
| 3-Carene | **0.027** | 0.152 | ↓ | 0.89 |

| | | | | |
|---|---|---|---|---|
| β-Methylhistamine * | 0.077 | - | - | 0.82 |
| 2-methylfuran | 0.339 | - | - | 0.88 |
| Tetradecane | 0.233 | - | - | 0.83 |
| Isoprene | 0.791 | - | - | 0.86 |
| Toluene | 0.791 | - | - | 0.81 |
| † Wilcoxon Rank test paired. ᵒᵒ only patients with paired pre/post-challenge VOCs were included in the analysis; *significant ($p<0.05$) levels were also observed following mannitol challenges. ᵒᵒ Higher in quantities in matched ambient air samples compared to breaths samples collected pre-challenges. | | | | |

**Figure 2. Variable Importance in the Projection (VIP) of VOCs in the final 2-component multilevel sPLS-DA models.**

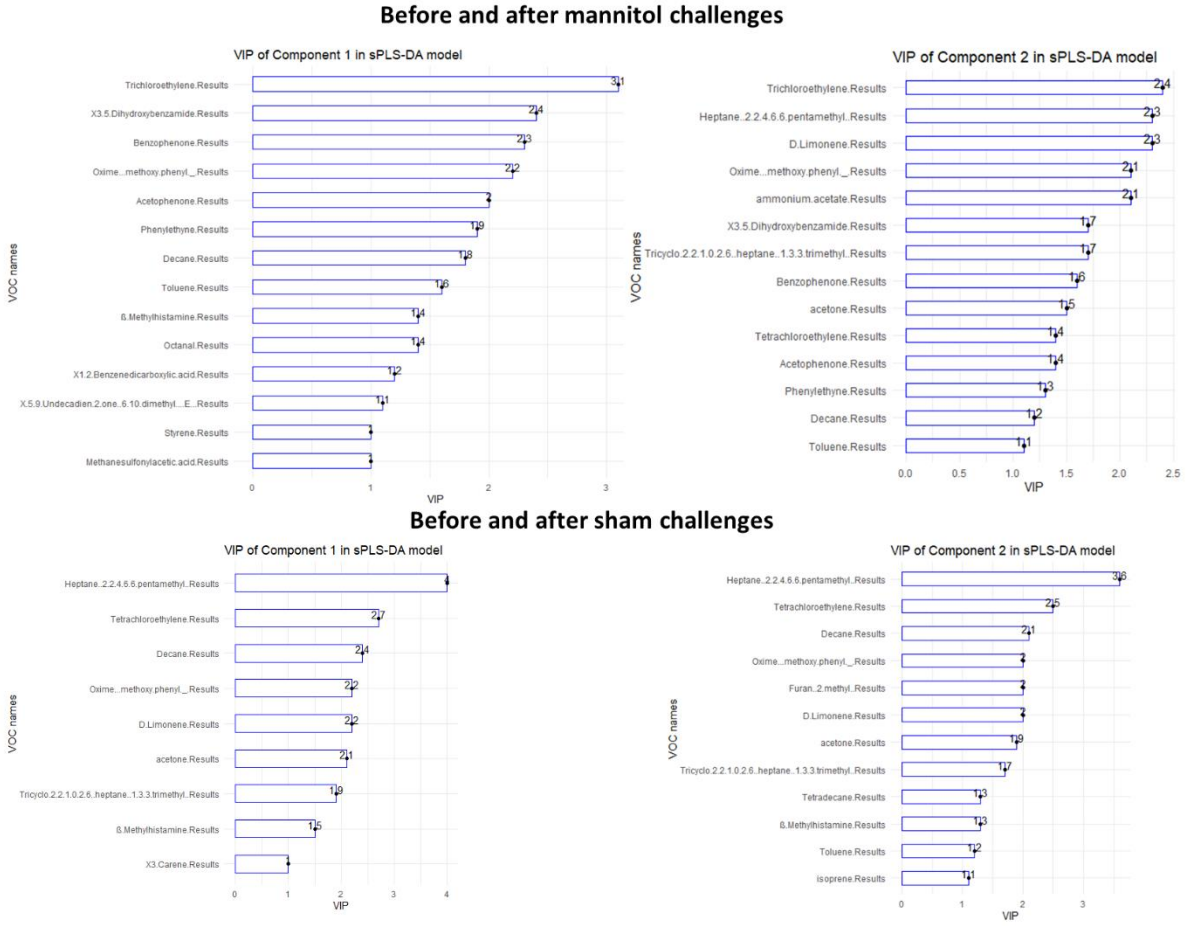
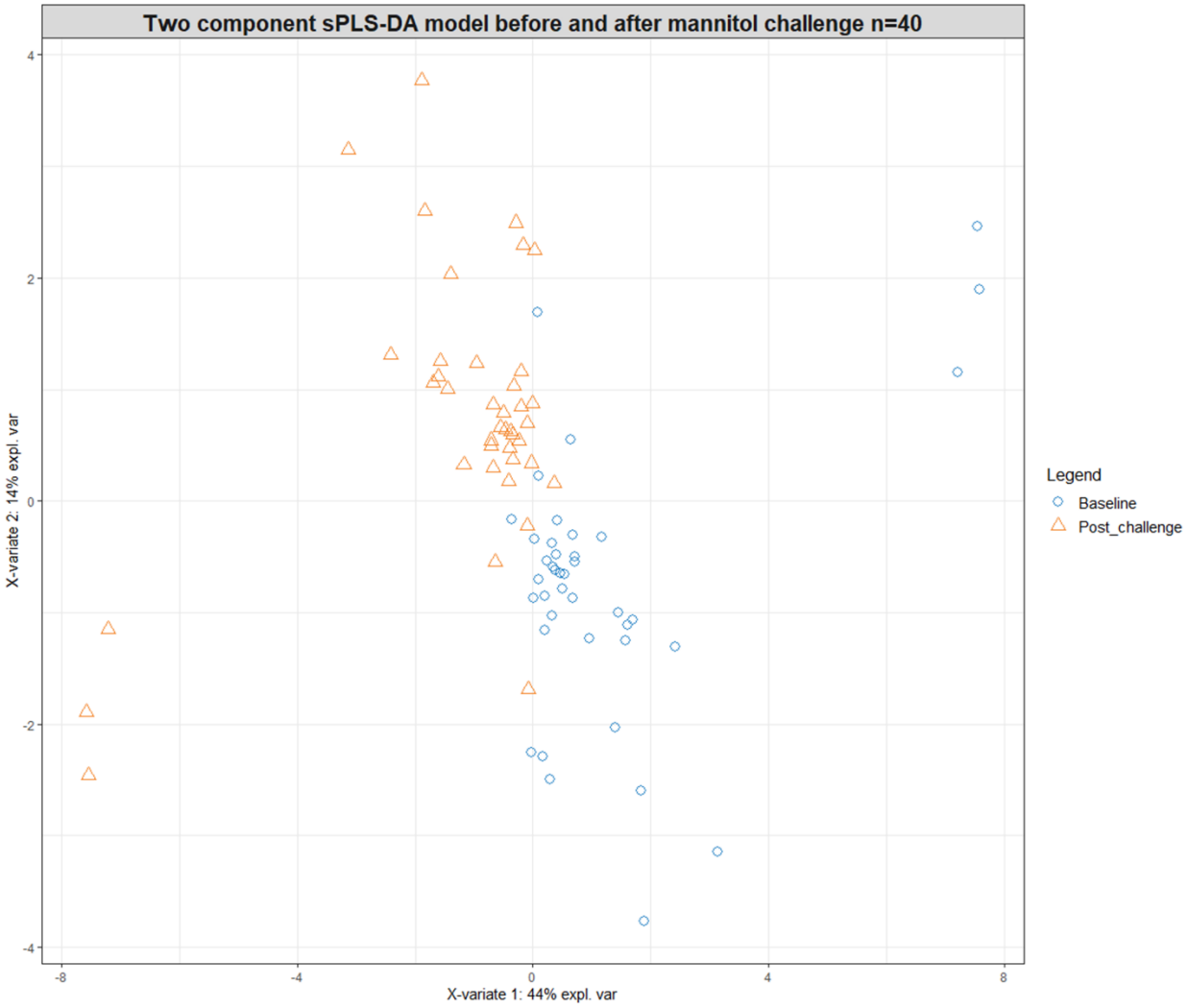
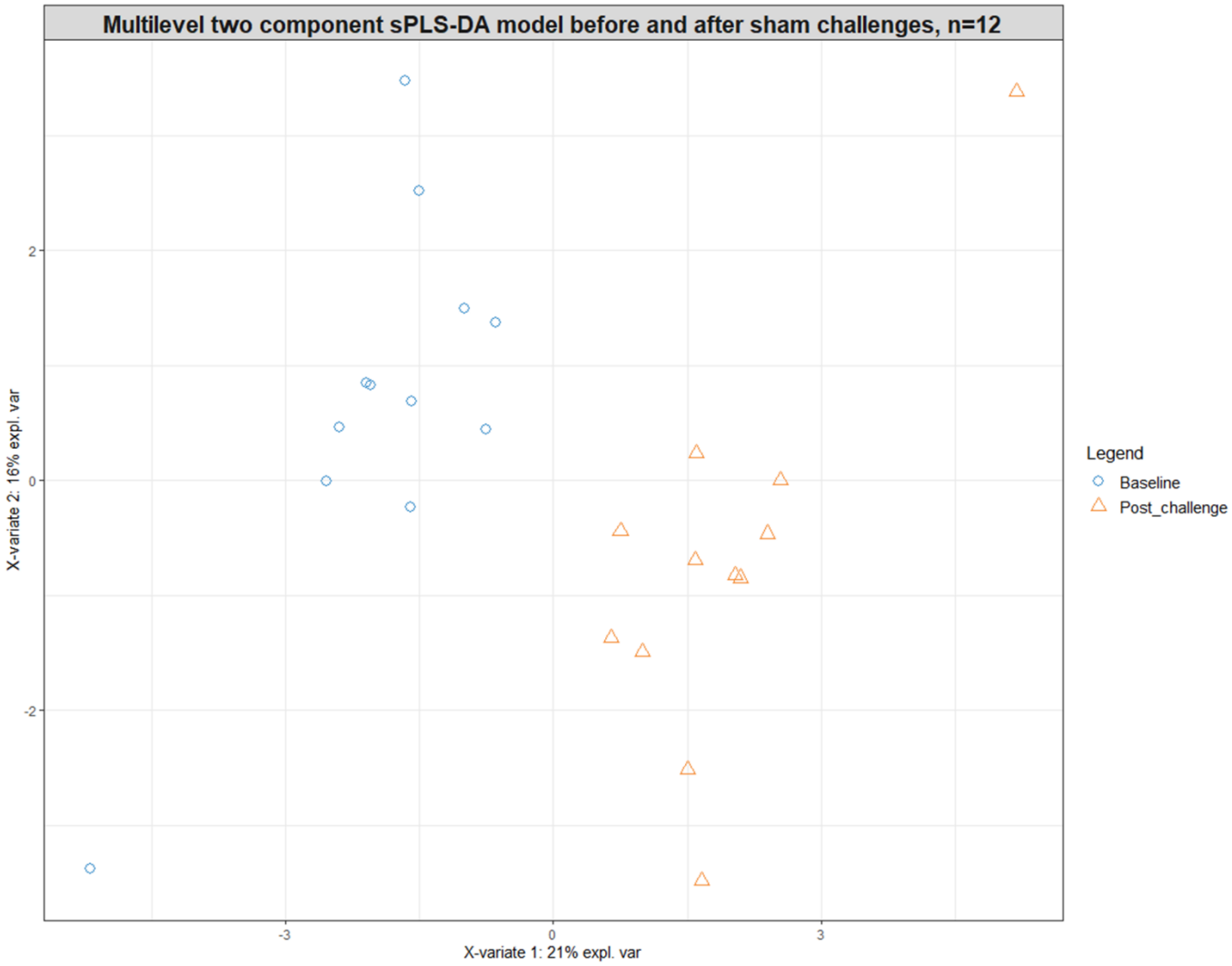


Figure 3. Scores plot for multilevel sPLS-DA model before and after mannitol and sham challenges.




Multilevel two component sPLS-DA model before and after sham challenges, n=12
X-variate 2: 16% expl. var
2
0
-2
-3
0
3
X-variate 1: 21% expl. var
Legend
Baseline
Post_challenge

*Differences in exhaled breath VOCs after mannitol* versus *sham challenge in patients with bronchial hyperresponsiveness to mannitol*

When post-mannitol challenge VOCs were compared to those of post-sham challenge in patients with positive challenges, 2-methylfuran, phenylamaleic anhydride, isoprene, 3, 5-dihydroxybenzamide and dimethyl selenide were significantly different ($p<0.05$), but none remained significant following FDR adjustment. Two-component multilevel sPLS-DA model gave a classification error rate of 12.1% and AUC of 0.93 (0.04) (Table 4). No VOCs were significantly different at baseline between mannitol and sham challenges.

Table 4. VOCs with altered levels between post- mannitol and post-sham challenges in patients with bronchial hyperresponsiveness to mannitol.

| Post-mannitol *vs.* post-sham challenge N=11 patients[∞] | | | |
|---|---|---|---|
| | **Univariate analysis** † | | **Multivariate analysis** |
| **Compounds** | **Raw p-value†** | **Direction of change**** | **Stability of features (with VIP>1) in sPLSDA model** |
| 2-methylfuran | **0.019** | ↑ | 0.97 |
| 3, 5-dihydroxybenzamide | **0.019** | ↓ | 1 |
| Phenylmaleic anhydride | **0.024** | ↓ | 1 |
| Isoprene | **0.024** | ↑ | 0.95 |
| Dimethyl selenide | **0.032** | ↑ | 0.97 |
| Acetophenone | 0.054 | - | 1 |
| Benzoic acid | 0.054 | - | 1 |
| Dimethylsulfoxonium Formylmethylide | 0.123 | - | 0.85 |
| Styrene | 0.054 | - | 0.88 |
| Sulfur dioxide | 0.054 | - | 0.84 |
| Benzonitrile | 0.054 | - | 0.79 |
| Tetradecane | 0.175 | - | 0.63 |
| † Wilcoxon Rank test paired. [∞] only patients with *paired* pre/post-challenge VOCs were included in the analysis; **↑ refers to compounds in higher abundance in post-sham challenge samples; ↓ refers to compounds in lower abundance in post-sham challenge samples | | | |

*Difference in baseline exhaled breath VOC levels between patients with positive and negative mannitol challenges*

No VOC was significantly ($p>0.05$) different between patients who subsequently had positive and negative challenges at baseline and post mannitol challenges. sPLS-DA model gave high error rates of 51% (mean [SD] AUC: 0.33 [0.08]) and 57% (AUC:0.34 [0.07]), respectively.

**Discussion**

*Summary of results*

The levels of a number of exhaled volatile organic compounds changed following inhaled mannitol challenges, many of which were not changed by sham challenge. Whilst compounds such as toluene, octanal and 6, 10-dimethyl-5, 9-undecadien-2-one had been associated with asthma or acute exacerbations in the literature [3, 16, 21], to our knowledge, the majority of VOCs where levels fell or rose following mannitol challenges have not been previously reported. Some of these changes in VOC levels were also observed following the sham-challenges, indicating that these changes were likely due to the procedure itself rather than the physiological effects of mannitol powder. Notably, when investigating the changes of VOCs levels between mannitol and sham challenges in patients who had a positive mannitol challenges, differences in the levels of post-challenge compounds were found, including 2-methylfuran, which have been previously reported to predict asthma exacerbation in children [3]. Interestingly, the subgroup analysis did not demonstrate differences in post-challenge VOCs between patients with positive and negative mannitol challenges. This may be due to small sample size. It is also important to note that the fall in $FEV_1$ post-mannitol challenge is a continuous variable and the dichotomous clinical definition of “positive” and “negative” results may be somewhat arbitrary.

It is important to highlight that histamine, the central mediator released from mast cells following degranulation, plays an important role in airway smooth muscle contraction, bronchial secretion and airway mucosal oedema in asthma. Therefore, the increase in the levels of β-Methylhistamine in the exhaled breaths following the mannitol (and not sham) challenges can be explained by this known pathophysiological mechanism and may be a useful biomarker for airway inflammation. Whilst the endogenous production of many other altered VOCs have not been previously reported, there have been some studies demonstrating their effects in animal models. For example, tricholoroethylene, a widely used industrial solvent, evokes both acetylcholine-induced and high K+ -induced smooth muscle contraction in swine trachea, and a reduced acetylcholinesterase activity was also observed [24]. Interestingly, a *reduction* of the quantities of tricholoroethylene in the exhaled VOCs was

observed following mannitol (and not sham) challenges, of which the mechanism remains unclear and should be investigated in future studies. The synthetic acetophenone derivative has been studied in murine models and found to attenuate airway hyperresponsiveness (AHR) and inhibited airway remodelling in ovalbumin-sensitised mice [25]. Exogenous benzofuran derivatives exhibit potent anti-inflammatory effects in murine models [26, 27]. Using ovalbumin-induced allergic asthma model, Kwon et al demonstrated benzofuran derivatives significantly reduced the levels of IL-5, IL-13 and IgE production, eosinophils and macrophages influx, ICAM-1/MCP-1 expression, mucus secretion and airway hyperresponsiveness in mice, suggesting benzofuran exerts protective effect to airway inflammation. Similarly, methoxy-phenyl-oxime derivatives have demonstrated anti-inflammatory activities [28] and dimethyl selenide derivatives alters interleukin-4/13 signalling pathways in vitro [29]. Other previously unreported compounds should be subjected to further investigation into their potential roles in asthma exacerbation and airway inflammation.

Many identified VOCs were more abundant in the baseline breaths samples compared to matched background samples, suggesting that these are likely endogenous. Notably, nearly all significant VOCs decreased in quantities following the sham challenges and the majority (VOCs with FDR>0.05) increased after mannitol challenges. The reductions of VOC levels following sham challenges may be due to the wash-out effects of repeated and forced inhalation and exhalation during the challenge procedures, and highlights the importance of the sham-controlled trial design in such studies. As it is unlikely that the VOCs which increased in quantities following mannitol challenges are the breakdown products of mannitol powder, the observed changes are likely due to the pathophysiological effects of mannitol (i.e. airway inflammation and bronchoconstriction). Therefore these VOCs may be potential biomarkers and merit future validation.

*Limitations*

Our study is limited by small sample size and hence the absence of external validation. Although internal cross validation were performed for the multivariate analysis, this may be prone for overfitting and therefore an external validation in a larger cohort is mandatory. The identification of the compounds were based on GC-MS retention time and mass spectra only (MSI level 2), and not by external chemical standards.

**Conclusion**

We have identified distinct changes in exhaled volatile organic compounds signatures following mannitol and sham challenges. These results may be useful in elucidating the pathophysiological mechanism of mannitol-induced airway inflammation. However, further studies in larger cohorts are needed for external validation.

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