# Circadian variability in breath volatiles is affected by the timing of inhaled corticosteroids in asthma



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*To the Editor:*

Many studies of exhaled volatile organic compounds (VOCs) have demonstrated changes associated with asthma severity and phenotypes [1, 2], but as yet, true external validation of any VOC biomarker is lacking. We recently highlighted a number of critical roadblocks to validation and clinical translation, including the lack of understanding and control for physiological and biological variability [3].

Asthma demonstrates diurnal variation in symptoms and pathophysiology [4]. Some VOCs exhibit diurnal rhythmicity in inhaled corticosteroid (ICS)-treated individuals [5]. Timing of ICS administration affects clinical efficacy [6], but its effects on the rhythmicity of VOCs are not known. We investigated how changing the timing of ICS dosing affected VOC signatures in asthma.

We conducted a randomised, three-way crossover trial of beclomethasone dipropionate (Clenil Modulite, *via* pressurised metered-dose inhaler) 400 µg daily dose in the morning (08:00 h ($OD_{AM}$)) *versus* mid-afternoon (16:00 h ($OD_{PM}$)), *versus* 200 µg twice a day (08:00 and 20:00 h (BD)), in participants with mild to moderate asthma [6]. Briefly, adults between the ages of 18 and 65 years were recruited with a history of physician-diagnosed asthma, allergy to at least one common inhaled allergen and evidence of at least one of the following: 1) bronchial hyperresponsiveness to methacholine (defined as a ⩾20% fall in forced expiratory volume within 1 s ($FEV_1$) at ⩽1 mg in dose or ⩽16 mg·mL$^{-1}$ in concentration); or 2) ⩾12% or ⩾200-mL improvement in $FEV_1$ following 400 µg inhaled salbutamol *via* a spacer. All participants stopped asthma maintenance therapies for a 14–21-day run-in period before the baseline visit. During treatment periods, adherence to ICS dosing regimens was recorded using diary cards and was excellent (>96%) [6]. At baseline, participants had exhaled VOCs collected (ReCIVA; Owlstone Medical, Cambridge, UK) 6-hourly (04:00, 10:00, 16:00 and 22:00 h). The baseline visit was followed by three 4-week treatment periods in random order with a washout period (14–21 days) in between. 6-hourly exhaled VOCs were collected over 24 h at the end of each treatment period; background room air samples with the mask attached were also collected at this time. Participants were given meals at set times during each overnight stay, with an evening meal provided between 17:00 and 18:00 h, a snack between 20:30 and 21:30 h, fasted overnight, and a breakfast given at 08:00 h and finished promptly by 08:30 h. This study was approved by the Research Ethics Committee (North West Greater Manchester South, 20/NW/0011) and registered at clinicaltrialsregister.eu as EudraCT 2019-004309-28. All participants provided written informed consent.

A total of 500 mL of the lower expiratory portion of breath was sampled through multibed sorbent tubes (Tenax TA and Carbograph 5TD; Markes International, Bridgend, UK). Samples were then analysed by thermal desorption–gas chromatography–mass spectrometry as previously described [7]. VOC peak intensities for 313 compounds were aligned and integrated (Masshunter Quantitative analysis; Agilent technologies, Cheadle, UK), with their identification confirmed using external chemical standards (n=60) or a combination of mass spectral library matches and retention index scores (n=253).

Time and dosing-regimen effects on VOC levels were analysed by repeated-measures ANOVA, modelling time using harmonic regression with individual participant identifier (ID) as a variable. This was performed first for all the VOCs together and then for individual VOCs. Tukey *post hoc* analysis was used

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**Dosage timings of inhaled corticosteroids and time of day are both important confounders for exhaled VOC levels in asthma. These factors must be appropriately accounted for at the biomarker discovery stage, allowing for external validation of findings.** https://bit.ly/435BQtt

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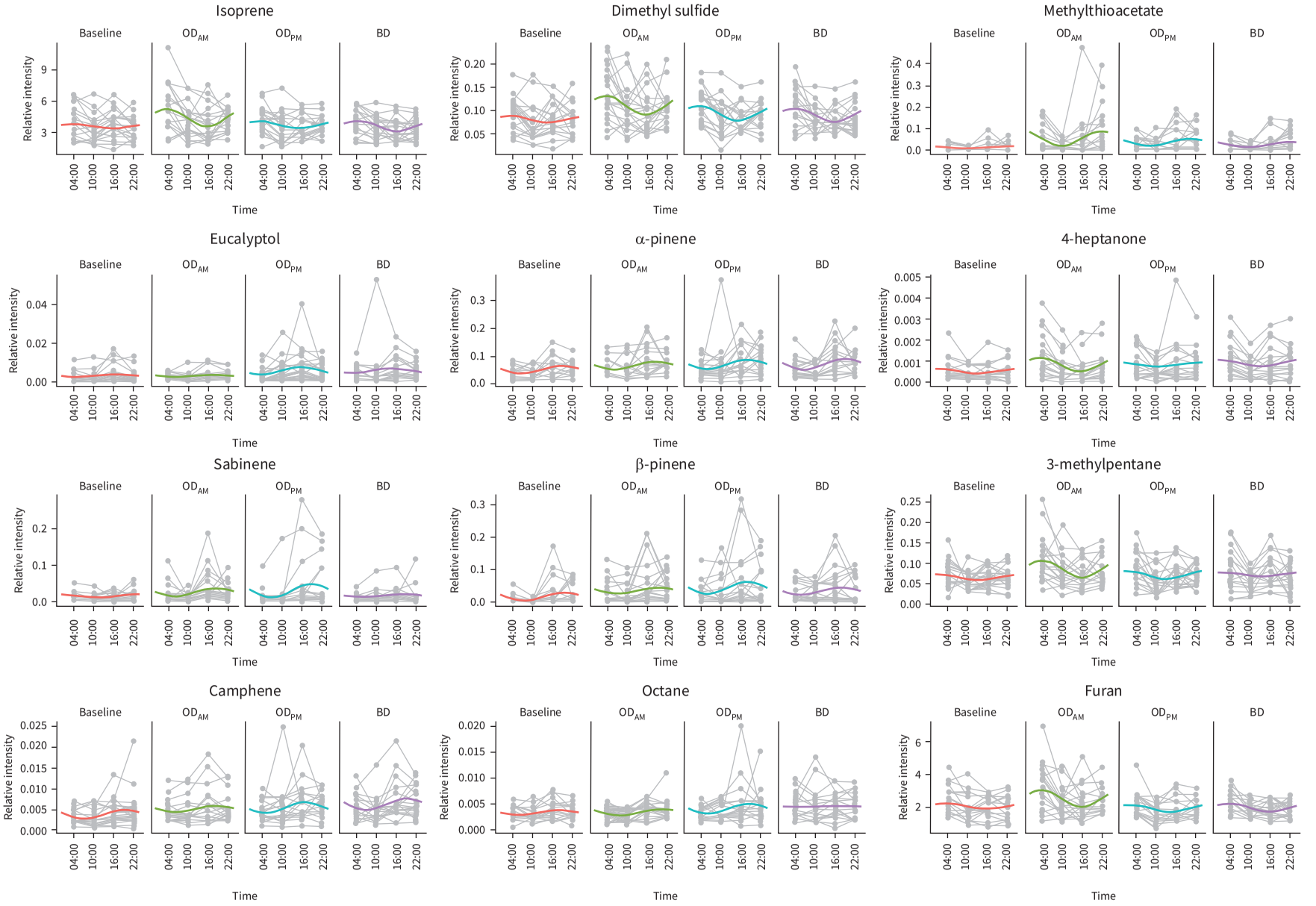


FIGURE 1 Volatile organic compound levels across time for the 12 compounds that changed significantly with both dosing pattern and time (adjusted p<0.05). Harmonic regression fitted line shown. OD$_{AM}$: 400 µg daily dose in the morning (08:00 h); OD$_{PM}$: 400 µg daily dose mid-afternoon (16:00 h); BD: 200 µg twice a day (08:00 and 20:00 h).

to investigate pairwise differences. Effect at individual timepoints (04:00, 10:00, 16:00 and 22:00 h) was analysed by repeated-measures ANOVA with participant ID as a variable. False discovery rate was used to adjust for multiple testing. A hierarchical Gaussian process model was used to detect oscillating (rhythmic) VOCs as outlined previously [5] and implemented in Stan (version 2.21.0). Missing data points (3.4%) were imputed using a random forest proximity method (missForest R package, version 1.4). All statistics were performed using R (version 4.0.2) in RStudio (version 2024.09.1).

21 participants (61.9% male, median (interquartile range (IQR)) age 40 (32–48) years, body mass index 26.2 (23.7–29.1) $kg \cdot m^{-2}$) completed all dosing regimens. The median (IQR) $FEV_1$ at baseline was 3.0 (2.4–3.6) L (86% (70–90%) predicted), with $FEV_1$/forced vital capacity ratio of 71.4% (63.5–78.6%), fractional exhaled nitric oxide 50 (24–70) ppb and blood eosinophil count 0.21 (0.17–0.35)$\times 10^9$ cells$\cdot L^{-1}$.

Both dose-timing and time-of-day of measurement were found to have an effect on VOC level (both $p<0.01$). Pairwise differences were found between all dosing regimens *versus* the baseline (adjusted $p<0.05$), except BD. 104 VOCs changed with dose-timing and 47 compounds changed with sampling-time (adjusted $p<0.05$). 12 compounds changed significantly with both dosing pattern and time (figure 1), none of which changed in the background room air control.

When looking for an effect of dosing regimens (compared to baseline) on VOC levels at individual timepoints, 31 VOCs showed a difference with treatment in at a least one timepoint (adjusted $p<0.05$). Of these, six VOCs showed a difference after $OD_{AM}$, 17 showed a difference after $OD_{PM}$ and nine after BD.

Four VOCs were significantly rhythmic in $OD_{AM}$ after adjusting for multiple testing (3,3- dimethylhexane, thymol, menthol and isocetane) using Gaussian process model, but none in other dosing patterns and at baseline.

We have demonstrated that ICS dosage-timing confounds exhaled VOC levels and patterns in a time-of-day dependent manner, underscoring the importance of understanding contributing factors for the biological and physiological variabilities in asthma. We confirmed that following ICS treatment, some VOCs become significantly rhythmic (such as isoprene, a commonly reported potential breath biomarker) [5]. Furthermore, we observed that certain chemical classes are significantly associated with ICS dosage timing including sulfur-containing compounds (methyl thioacetate and dimethyl disulfide) and monoterpenoids (α-pinene, β-pinene, sabinene, camphene and eucalyptol). Although both chemical classes are commonly linked to dietary metabolism and synthetic flavour or scent additives, breath concentrations have also been noted to be altered in patients with liver cirrhosis and respiratory infections [8, 9]. Notably, we have previously shown that levomenthol (a terpenoid) in exhaled breath could distinguish oral corticosteroid (OCS) use in patients with severe asthma [10]. Therefore, the metabolism of exogenous (ICS and OCS) or endogenous steroids (such as cortisol, associated with circadian rhythmicity) may contribute to exhaled monoterpenoids and their monomer isoprene [8–10]. In our study, the time interval between ICS administration and VOC collection varied depending on ICS dosing regimens, potentially confounding the different daily variations observed. Although we did not detect the propellant (norflurane) in breath samples, both the immediate effects of propellent and/or ICS on local VOC production, and any therapeutic effect [6], may have contributed to the variations observed. There is limited knowledge on the influence of timing of ICS administration before exhaled breaths collection; this may be important and should be accounted for in future studies.

We note that the rhythmic characteristics of some VOCs reported in the previous study [5] were not the same in the current study. This is likely due to differences in study population, asthma treatment, ICS dosing regimens and washout periods.

In summary, as dosage timings in ICS and time-of-day are important confounders for VOCs levels in asthma, these factors must be appropriately accounted for at the biomarker discovery stage. This is mandatory if we are to confirm reproducibility of results, external validation of findings and move towards clinical application.

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Data availability: The study protocol is available on the trial registration website. Individual participant data that underlie the results reported in this article after deidentification will be shared 3 years following article publication with investigators whose proposed use of data has been approved by the study sponsor. Proposal will be directed to pmoore@crosolutions.co.uk. To gain access, data requestors will need to sign a data access agreement.

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This observational study is registered at EudraCT with identifier number 2019-004309-28.

Ethics statement: This study was approved by the Research Ethics Committee (North West Greater Manchester South, 20/NW/0011) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines (registered with EudraCT: 2019-004309-28, https://www.clinicaltrialsregister.eu/ctr-search/trial/2019-004309-28/GB). All participants provided informed written consent.

Author contributions: H.J. Durrington, D. Ray, A. Loudon, A. Simpson, D. Singh and S.J. Fowler contributed to the conception of the study, planning, set up and reviewing of the submitted article. R. Wang and W. Ahmed contributed to the data acquisition, statistical analysis and writing of the article. R. Maidstone carried out the statistical analysis and contributed to the writing of the article. H.J. Durrington is responsible for the overall content as guarantor. The guarantor accepts full responsibility for the finished work and the conduct of the study.

Conflict of interest: D. Singh reports consultancy fees from Adovate, Aerogen, Almirall, Apogee, Arrowhead, AstraZeneca, Bial, Boehringer Ingelheim, Chiesi, Cipla, CONNECT Biopharm, Covis, CSL Behring, DevPro Biopharma LCC, Elpen, Empirico, EpiEndo, Genentech, Generate Biomedicines, GlaxoSmithKline, Glenmark, Kamada, Kinaset Therapeutics, Kymera, Menarini, MicroA, OM Pharma, Orion, Pieris Pharmaceuticals, Pulmatrix, Revolo, Roivant Sciences, Sanofi, Synairgen, Tetherex, Teva, Theravance Biopharma, Upstream and Verona Pharma. R. Wang, R. Maidstone, W. Ahmed, D. Ray, A. Loudon, A. Simpson, H.J. Durrington and S. J. Fowler report no conflict of interest.

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