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Early View

Research letter

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CIRCADIAN RHYTHM OF EXHALED BIOMARKERS IN HEALTH AND ASTHMA

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Take home message:

Exhaled Volatile chemicals and fractional exhaled nitric oxide oscillate over 24 hours, highlighting the importance of time of day in diagnostic sampling and suggesting potential applications for chronotyping.

ABSTRACT

Question addressed: Circadian rhythms control many biological processes in the body in both health and disease. Greater understanding of diurnal variability in disease related biomarkers is crucial for their application in clinical practice and biomarkers of circadian rhythm are required to facilitate further research into disturbed chronicity. To determine if fractional exhaled nitric oxide and breath volatile biomarkers vary rhythmically during the day in healthy and asthmatic individuals.

Methods: Ten individuals with moderate, atopic asthma (on regular inhaled corticosteroids) and 10 healthy volunteers (all non-smokers) completed an overnight visit where their exhaled breath volatiles and forced exhaled nitric oxide levels were collected every 6 hours. Breath volatiles were analysed using gas chromatography mass spectrometry, after trapping these volatiles on sorbent materials for thermal desorption.

Results: Nine breath volatiles (including acetone and isoprene) exhibit diurnal variation across all individuals. Furthermore the circadian pattern of several VOCs is altered in individuals with asthma and fractional exhaled nitric oxide is rhythmic in asthma but not in healthy controls.

Conclusions: Markers of circadian rhythm can be identified in breath and may offer insight into circadian profiling to help treat disease. Additionally this work suggests that time of day must be controlled when designing future biomarker discovery studies. Further work is required with larger cohorts to validate and extend these findings.

INTRODUCTION

Circadian rhythms regulate and reflect many biological processes. Investigating circadian variability in biomarkers is important since the diurnal variability of any potential biomarker must be quantified and controlled in research and clinical practice. Time of day is particularly important in inflammatory diseases such as asthma, which are linked to exaggerated circadian rhythms. Airway narrowing in asthma is greatest at around 04:00 and coincides with an increase in symptoms; asthma deaths are also more likely to occur at this time (1,2). Likewise eosinophilic airway inflammation peaks in the morning, with clinical implications for biomarker-guided steroid therapy (3).

As asthma is a circadian disease we expected to observe newly rhythmic volatile organic compounds (VOCs) in breath when compared to a healthy population. We therefore investigated how exhaled VOCs and fractional exhaled nitric oxide (FeNO) vary over the 24-hour cycle in healthy individuals and in those with asthma.

METHODS

Study design

During an overnight visit to the research unit, exhaled breath was collected and FeNO measured at 16:00, 22:00, 04:00 and 10:00. Participants took standardised meals at regular intervals and kept their usual bedtime. Inhaled corticosteroids (ICS) were omitted 12 hours prior to measurements. The study protocol received ethical approval (ref: 14/NW/1352) and participants provided written informed consent.

Measurements

FeNO measurements were performed (NIOX Vero Aerocrine, Solna, Sweden) prior to VOC collection and spirometry as per manufacturer's recommendations. For VOC analyses 1 L of breath was collected across sorbent tubes packed with Carbograph 1TD/Carbograph 5TD

(Markes International, Llantrisant, UK), at a flow rate of 500 mL min^{-1} using an in-house sampler described elsewhere (4). A background air sample was taken at every time point by strapping the mask to a glass head and sampling 1 L of filtered air. Sorbent tubes were sealed and refrigerated immediately after sampling and analysed within one month. The TD-GC-MS protocol has been published previously (5).

Data analysis

All VOC data files were converted to the open mzXML format prior to pre-processing. Chromatograms were screened for inclusion in the final dataset by manual appraisal and all samples were deconvolved and aligned using eRah. A hierarchical Gaussian process model was used to detect oscillating VOCs. Data were *z*-normalised on individual patients and compounds, and modelled as Gaussian processes (GPs) with exponential covariance functions. The mean function of these GPs was then modelled using another GP, shared across patients, with zero mean and a periodic covariance (period = 24 h). This enables the model to account for inter-compound and inter-patient variation separately. The model was fitted using Hamiltonian Monte Carlo. Empirical *p*-values were obtained using Monte Carlo simulation from a null distribution of simulated non-rhythmic data and false discovery rates calculated. Analyses were implemented in R and Stan.

Compounds of interest were putatively identified using the National Institute of Standards and Technology (NIST) library following MSI standards. VOCs were screened to remove common contaminants arising from the sampling equipment and any VOCs found to be rhythmic in the background samples.

RESULTS

Demographics

Data from one patient with asthma were excluded due to technical faults with the GC-MS, leaving complete datasets for ten healthy individuals and nine with asthma. The groups were matched for median (IQR) age [45.5 (27.5-49.3) *versus* 47.0 (26.0-49.5) years, p = 0.92], body mass index [27.1 (23.4-30.5) *versus* 26.9 (22.3-27.2) kg/m^2, p = 0.5] and gender ratio (7:3 *versus* 7:2 M:F, p = 1), and. All individuals with asthma were atopic, with significantly lower median (IQR) FEV_1 compared to healthy [82.3 (73.0-89.0) *versus* 97.7 (91.7-105.3) % predicted, p=0.02] and prescribed daily ICS (equivalent to beclomethasone dipropionate) 400 (400-500) µg, median (IQR).

Breath analysis

Of 76 breath samples collected (four time points per participant), six were removed from the analysis due to errors in sampling or analytical processing. Background samples were collected at 59 time-points immediately prior to breath sampling (15 background samples were excluded due to errors in sampling or analytical processing). A mean (SD) of 312 (45) compounds was detected the breath samples. Once aligned and quality checked to remove contaminant compounds and deconvolution artefacts 102 VOCs were included in the Gaussian process analysis.

Circadian rhythm in breath VOCs

In the combined dataset five VOCs were shown to be rhythmic [false discovery rate (FDR) p < 0.01]. Dimethoxymethane, chlorobenzene and an unidentified VOC (*m/z* 56) showed a nadir in the morning, whereas the opposite diurnal pattern was seen for isoprene and 1-butoxy, 2-propanol, (Figure 1).

Four VOCs were rhythmic with $p < 0.05$ but not significant after FDR correction; acetone and 1-butanol were highest at 10:00 and lowest at 16:00 while xylene and phenol showed the reverse pattern.

Asthma *versus* healthy individuals

A secondary analysis of the data was performed to investigate the rhythmic nature of VOCs associated with asthma. Camphene was annotated as being rhythmic only in asthma, $p < 0.05$, with a peak at 10:00 and a nadir at 16:00. Acetone and isoprene (both rhythmic in the combined analysis) were also found to be rhythmic in the asthma group alone. Two compounds were shown to be rhythmic in the healthy group but not the asthmatic group (xylene and isobutylacetate). Both demonstrated rhythms in anti-phase with VOCs found to be rhythmic in asthma only, namely a peak at 16:00 and a trough at 10:00.

FeNO measurements

A rhythmic cycle for FeNO, $p < 0.05$, was detected only in the asthmatic group, with a peak at 10:00 and nadir overnight. The median (IQR) FeNO was 37.5 (18.3 - 78.0) ppb at 10:00 and 25.5 (14.7 - 56.6) ppb at 04:00, (Figure 1).

DISCUSSION

We have demonstrated that there is rhythmic variability in a proportion of exhaled VOCs over 24 hours. Further, when comparing asthmatic to healthy breath differential patterns of VOC release were observed. Acetone is the most abundant VOC in breath and has been previously linked to asthma (6,7). Changes in the level of acetone overnight in this study replicate findings by King *et al* (8). Isoprene, the next most abundant VOC in breath, has also been linked to asthma (6,9,10). Similar to acetone the changes observed in the levels of isoprene agree with previous work (8,11). For both VOCs this study provides insight into the diurnal pattern of expression, adding to the nocturnal profiling detailed in the literature.

Camphene and xylene have been included in models to distinguish asthma from healthy controls (4,12) where they were shown to be reduced in the asthmatic cohort. Camphene has also been shown to inhibit the release of nitric oxide in stressed rat macrophages (13). All other VOCs shown to be rhythmic in this work have previously be found in breath and have been linked to a variety of diseases.

We have also shown that FeNO demonstrates a strong circadian rhythm in asthma with lower levels detected during the night than during the day. FeNO is used in diagnostic asthma algorithms with cut-offs varying between 25 ppb (14) and 35-40 ppb (15). We found the diurnal FeNO variability straddled these cut-offs and it is crucial that larger studies validate our findings, which may impact on diagnostic recommendations.

In addition to the clinical implications, this work demonstrates that time of day is an important parameter to consider when undertaking VOC sampling, especially in untargeted hypothesis-generating studies.

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FIGURE LEGEND

Figure 1: (A) Circadian analysis of the pooled dataset of 19 participants for the compounds that had a false discovery rate less than 0.05 after the Gaussian process analysis was applied. (B) Compounds found to differentially rhythmic between the asthmatic and healthy cohorts. *Z*-score values are shown in light grey with the fitted rhythm overlaid. All compounds were identified as rhythmic with a *p*-value less than 0.05 using the Gaussian process analysis. All compounds denoted by * were identified to metabolomics standards initiative (MSI) level 1, otherwise compounds were identified to MSI level 2.

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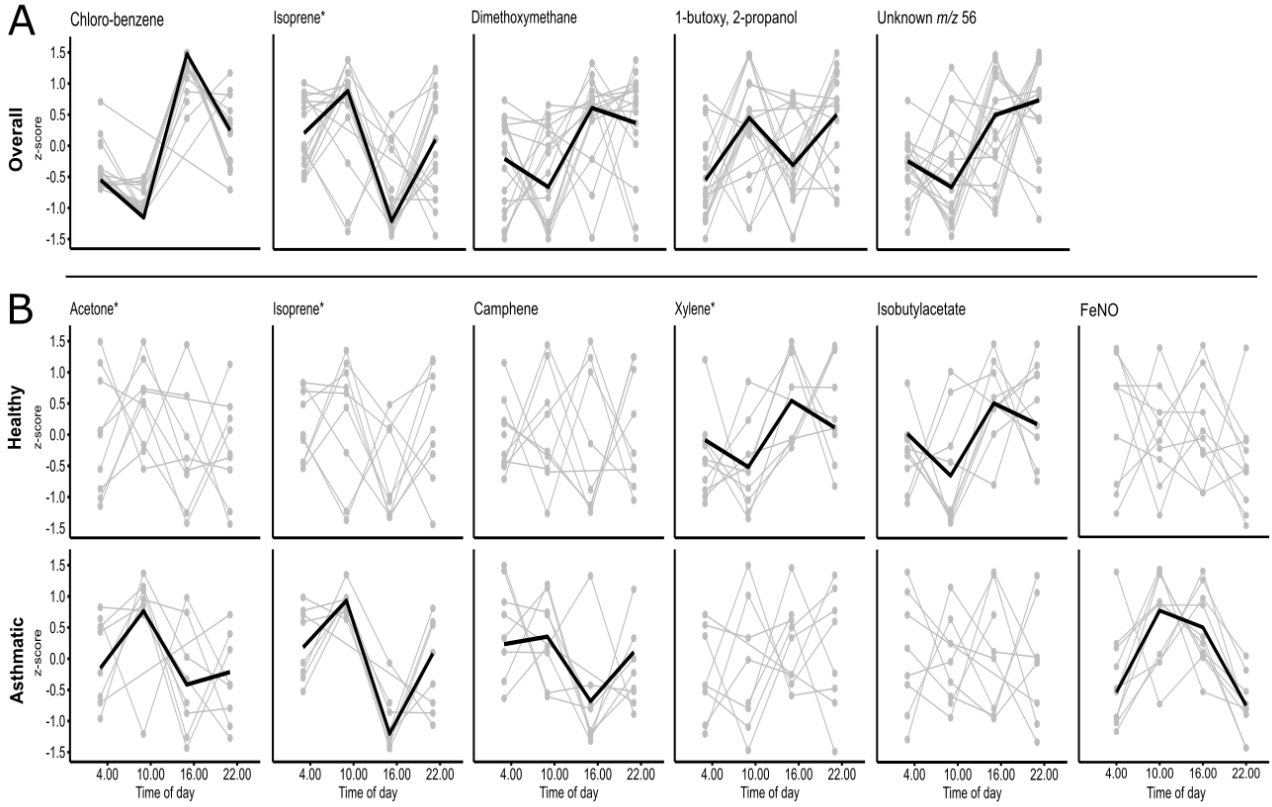
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