

**Time of day affects eosinophil biomarkers in asthma: Implications for diagnosis and treatment.**

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### **Author's Contributions**

HJD conceived of the study, secured the funding, ran the study, analysed the results and prepared the manuscript.

SF analysed the results and prepared the manuscript.

GG-T analysed the severe asthma cohort data and prepared the manuscript.

RJM ran statistical analysis and advised on the statistics used and prepared the manuscript.

KK performed eotaxin cytokine assays on serum and sputum samples and analysed the data.

ASIL and JFB prepared the manuscript.

DS conceived the study, provided support to run the study and prepared the manuscript.

DWR and AS conceived the study, analysed the results and prepared the manuscript.

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Asthma is characterised by strong time of day rhythms; symptoms worsen around 04:00 (1), coincident with increased airway narrowing, reflected by a reduced peak expiratory flow (PEF) or Forced Expiratory Volume in 1 second (FEV<sub>1</sub>) (2). Clinically useful biomarkers in asthma include sputum and blood eosinophils (3) and results from several small studies looking at circadian variation in airway inflammation in asthma are conflicting (4-9). Sputum eosinophil percentage is used to guide management decisions in severe asthma clinics (3); any diurnal variation in sputum eosinophilia could therefore influence the management of patients.

We studied circadian variation of blood and sputum eosinophils within a mild/moderate, atopic asthma cohort compared to healthy controls. We then retrospectively compared sputum eosinophil counts from a severe asthma clinic cohort in relation to time of day of collection (morning *versus* afternoon).

## Methods

10 adults with mild/moderate, atopic asthma (ACQ-7, 0.9 (0.8-1.1), on regular inhaled corticosteroids (ICS) equivalent to beclomethasone dipropionate (BDP) 400µg (400-650) and 10 healthy volunteers were recruited. The study protocol was approved by the Health Research Authority, National Research Ethics Service Committee North West- Greater Manchester Central (REC 14/NW/1352)). Written informed consent was obtained from all participants. Spirometry, blood eosinophils, induced sputum and serum were collected during 4 visits, each 1 week apart, avoiding any potentiating effect of a sputum induction on a subsequent sample. Visit 1 (V1) occurred at 16:00, V3 at 10:00 and V4 at 22:00. V2 involved an overnight stay, with sampling occurring at 16:00, 22:00, 04:00 and 10:00 the following morning.

Next, circadian analysis was performed retrospectively on sputum eosinophil counts from severe asthma patients attending either morning or afternoon clinic.

Median  $\pm$  Interquartile range (IQR) are reported. Wilcoxon's test, Mann Whitney u and a two way analysis of variance (ANOVA) were employed to analyse the data. Post hoc power analysis performed on the sputum eosinophil data resulted in power of 81.86% for our observed effect size (1.057); the minimum detectable effect size was 1.037 given 80% power. Power calculations were based on a Wilcoxon signed-rank test to detect the time of day difference in asthma patients. General linear modelling analysed the severe asthma cohort data and potential confounders. Chi squared Fisher Exact test and Pearson correlation coefficient were also used for analyses.  $P \leq 0.05$  considered statistically significant.

## Results

**Mild/moderate asthma:** Healthy and asthma groups were matched for age ( $p=0.63$ ) and BMI ( $p=0.9$ ). FEV<sub>1</sub>% predicted was lower in the asthma group, 82.3% (73.0-89.0) versus healthy, 97.7% (91.7-105.3)  $p<0.001$  with more reversibility in the asthma group 255mls (172.5-355) versus 35 (-15-122.5)  $p<0.01$ .

There was a nocturnal dip in FEV<sub>1</sub> in asthma ( $p<0.0001$ ) at 04:00.

For mild/moderate asthma and controls, the number of blood eosinophils showed a time of day difference, peaking at 04:00 ( $p<0.01$ ), with no difference between groups. Serum eotaxin did not vary by time of day or between groups.

Amongst patients with mild/moderate asthma, sputum eosinophil percentage was significantly higher at 04:00 compared to 16:00 ( $p<0.05$ , Figure 1). There was no significant

time of day variation in sputum eosinophils in the healthy group ( $p=0.63$ ). Sputum eosinophil percentage was significantly increased in the asthma group compared to healthy ( $p<0.01$ ). Sputum eotaxin was significantly increased at 04:00 compared to 16:00 in the asthma group (62.9 (28.2-120.4pg/ml) versus 33.0 (14.5-73pg/ml),  $p<0.05$ , but not in healthy (29.7 (16.2-74.2pg/ml) versus 37.0 (25.5-54.5pg/ml).

**Severe Asthma:** 131 patients attended morning clinic; 193 afternoon clinic. Groups were well matched for age ( $p=0.11$ ), BMI ( $p=0.25$ ), FEV1%predicted ( $p=0.85$ ), smoking status ( $p=0.3$ ), serum total IgE ( $p=0.23$ ), FeNO ( $p=0.58$ ), blood eosinophil count ( $p=0.58$ ) and also for treatment (intramuscular triamcinolone ( $p=0.71$ ), oral prednisolone ( $p=0.31$ ), daily ICS (BDP equivalent) ( $p=0.31$ ). Significantly more of the morning group produced sputum spontaneously (77.1% versus 62.1%,  $p<0.005$ ), rather than induced.

Analysis of the severe asthma clinic cohort data revealed a significant time of day effect; sputum produced in the morning clinic contained a significantly higher percentage of sputum eosinophils compared to those produced in the afternoon clinic (morning sputum eosinophil percentage 1.25 (0.00-8.75) versus afternoon 0.5 (0.00-2.25),  $p=0.008$ ). General linear modelling analysis showed that the time of day effect persisted even if patients were on high dose steroids ( $p=0.41$ ), nor was it affected by the type of sputum (spontaneous versus induced)  $p=0.54$ .

A significantly higher proportion of severe asthma patients attending the morning clinic had positive sputum eosinophil counts ( $\geq 3\%$ ) compared to those attending the afternoon clinic (37.4% vs 21.6%,  $p=0.002$ ) Figure 2.

We found no difference in the proportion of cell counts that were classed as eosinophilic ( $\geq 3\%$  eosinophils) between those that were spontaneous and those that were induced (morning  $p=0.6$ , afternoon  $p=1$ ).

## Discussion

This is the most comprehensive circadian study of biomarkers in asthma to date. We report, for the first time, a circadian variation in sputum eotaxin, peaking at 04:00. We confirmed that airway eosinophils are significantly circadian rhythmic in induced sputum from mild/moderate asthmatics with a peak influx at 04:00 coinciding with peak sputum eotaxin concentration suggesting a chemotactic mechanism. We demonstrated that this was in anti-phase with FEV<sub>1</sub>. In contrast to airway eosinophils, blood eosinophils oscillated diurnally in both health and asthma, suggesting the physiological mechanism controlling circadian variation in blood eosinophils is not up-regulated in asthma. In support of this there was no difference in serum eotaxin levels between groups.

We noticed a nadir in sputum eosinophils at 4pm and although not statistically significant, sputum eosinophils appeared higher at 10am, hinting at a possible time of day effect, relevant within the clinical working day. We postulated that a patient attending morning clinic may produce a sputum sample with higher eosinophils than if attending an afternoon clinic. In our retrospective evaluation, we identified that severe asthma patients attending morning clinic were almost twice as likely (37.4% vs 21.6%) to have sputum eosinophilia ( $\geq 3\%$ ) compared to patients attending afternoon clinic. These findings need confirmation in a prospective study; however, the implications are clinically important. In severe asthma, having raised sputum eosinophils is an indicator for treatment escalation (3); we propose



that based on our results different clinical decisions could be made based on whether the patient is allocated a morning or afternoon appointment.

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## Figure Legends

### **Figure 1 Diurnal variation in blood and sputum eosinophils.**

Sputum percentage eosinophilia increased significantly at 04:00 compared to 16:00 in asthma,  $p < 0.05$  (Mann Whitney U test). This was not seen in the healthy group ( $p = 0.63$ ). Sputum eosinophil percentages were significantly higher in asthma compared to healthy ( $p < 0.01$ , 2 way ANOVA). Individual sputum eosinophil percentages for healthy (grey dots) and asthma participants (black dots), median shown (solid bar).

### **Figure 2 Sputum analysis from patients attending morning and afternoon severe asthma clinic**

Percentage of positive (black) and negative (grey) sputum samples from severe asthma patients attending either morning or afternoon clinic. 37.4% of severe asthma patients had positive sputum eosinophil counts ( $\geq 3\%$ ) in the morning clinic compared to 21.6% in the afternoon clinic ( $p = 0.002$ , Chi squared, fishers exact test).

### Figure 1

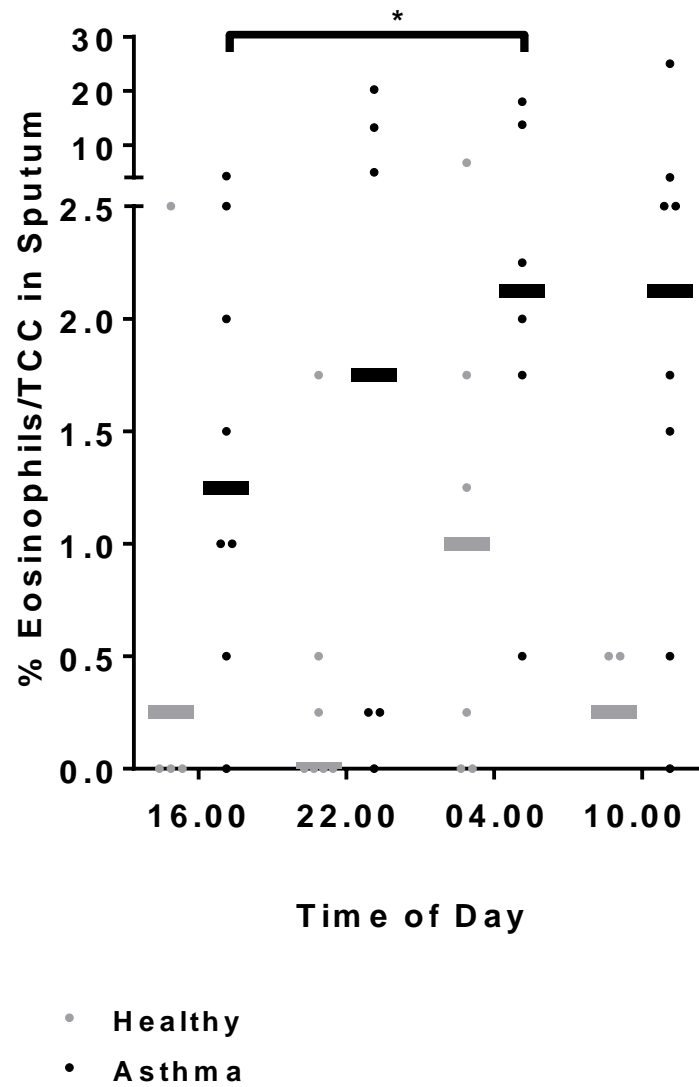


Figure 2

