It is noteworthy that this amyloid suppression of long-term potentiation appeared to be cytokine independent (data not shown).

Endothelial amyloids are heat stable, protease resistant, and RNase and DNase insensitive (1). They exhibit certain features of prion disease (10); for example, they are self-replicating and transmissible among cells (1). Here, we provide evidence that nosocomial pneumonia induces lung endothelial production of τ and Aβ oligomeric species that impair neurological information processing, supporting the hypothesis that these amyloids contribute to insidious end-organ dysfunction and suggesting the need for a larger cohort trial addressing this issue. In our studies, injurious amyloids were detected in the cerebrospinal fluid of infected, but not in uninfected, patients. However, our current sample size is small, and we have not determined whether other bacteria, viruses, fungi, or inflammatory conditions, such as the systemic inflammatory response syndrome, also elicit this endotheliopathy. Our studies focus on the acute consequences of infection-induced amyloids; it remains unknown as to whether the mechanisms tested here represent a cause of progressive and persistent memory loss in ICU patients. Indeed, in studies moving forward, it will be essential to determine the infection-induced τ and Aβ oligomer fate within the lung, blood, and other peripheral organs, including the brain. It will also be essential to determine whether infection-induced amyloid production and biodistribution contributes to cardiovascular disease, stroke, renal dysfunction, and pulmonary dysfunction in the aftermath of critical illness. ■

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## Time of Day Affects Eosinophil Biomarkers in Asthma: Implications for Diagnosis and Treatment

*To the Editor*:

Asthma is characterized by strong time-of-day rhythms: symptoms worsen around 04:00 (1) along with increased airway narrowing, as

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Author Contributions: H.J.D. conceived of the study, secured the funding, ran the study, analyzed the results, and prepared the manuscript. S.J.F. analyzed the results and prepared the manuscript. G.O.G.-T. analyzed the severe-asthma cohort data and prepared the manuscript. R.J.M. ran the statistical analysis, advised on the statistics used, and prepared the manuscript. K.K. performed eotaxin cytokine assays on serum and sputum samples, and analyzed the data. A.S.I.L. and J.F.B. prepared the manuscript. D.S. conceived the study, provided support to run the study, and prepared the manuscript. D.W.R. and A.S. conceived the study, analyzed the results, and prepared the manuscript.

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reflected by a reduced peak expiratory flow or $FEV_1$ (2). Clinically useful biomarkers in asthma include sputum and blood eosinophils (3), and results from several small studies looking at circadian variations in airway inflammation in asthma are conflicting (4–9). The sputum eosinophil percentage is used to guide management decisions in severe-asthma clinics (3), and therefore any diurnal variation in sputum eosinophilia could influence the management of patients.

We studied circadian variations in blood and sputum eosinophils in a cohort of patients with mild/moderate, atopic asthma compared with healthy control subjects. We then retrospectively compared sputum eosinophil counts from a severe-asthma clinic cohort in relation to the time of day of collection (morning vs. afternoon).

## Methods

Ten healthy volunteers and 10 adults with mild/moderate, atopic asthma (Asthma Control Questionnaire 7, 0.9 [0.8–1.1]) who were on regular inhaled corticosteroids (equivalent to beclomethasone dipropionate 400 μg [400–650 μg]) were recruited for this study. The study protocol was approved by the Health Research Authority, National Research Ethics Service Committee of 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 four visits, each 1 week apart, avoiding any potentiating effect of sputum induction on a subsequent sample. Visit 1 (V1) occurred at 16:00, V3 was at 10:00, and V4 was 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, a circadian analysis was performed retrospectively on sputum eosinophil counts obtained from patients with severe asthma attending either a morning or afternoon clinic.

The median (interquartile range) is reported. Wilcoxon's test, the Mann-Whitney *U* test, and two-way ANOVA were used to analyze the data. A *post hoc* power analysis performed on the sputum eosinophil data resulted in a power of 81.86% for our observed effect size (1.057), and 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 modeling was used to analyze the severe-asthma cohort data and potential confounders. Chi-squared Fisher's exact test and the Pearson correlation coefficient were also used for analyses. $P \leqslant 0.05$ was considered statistically significant.

## Results

***Mild/moderate asthma.*** Healthy and asthma groups were matched for age ($P = 0.63$) and body mass index ($P = 0.9$). $FEV_1$% predicted was lower in the asthma group (82.3% [73.0–89.0%]) than in the healthy group (97.7% [91.7–105.3%]; $P < 0.001$), with more reversibility in the asthma group (255 ml [172.5–355 ml]) than in the healthy group (35 ml [−15 to 122.5 ml]; $P < 0.01$). There was a nocturnal dip in $FEV_1$ in the asthma group ($P < 0.0001$) at 04:00.

For patients with mild/moderate asthma and control subjects, 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.

Among patients with mild/moderate asthma, the sputum eosinophil percentages were significantly higher at 04:00 than at 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 percentages were significantly increased in the asthma group compared with the healthy group ($P < 0.01$). Sputum eotaxin was significantly increased at 04:00 compared with 16:00 in the asthma group (62.9 [28.2–120.4] vs. 33.0 [14.5–73] pg/ml; $P < 0.05$), but not in the healthy group (29.7 [16.2–74.2] vs. 37.0 [25.5–54.5] pg/ml).

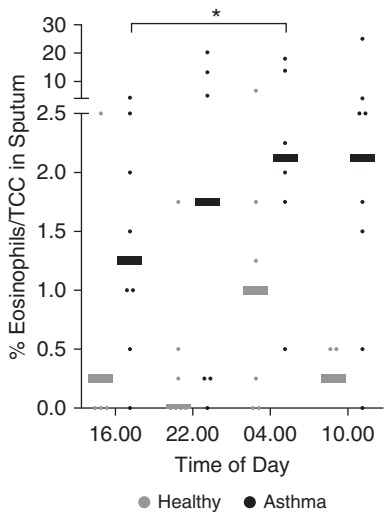


**Figure 1.** Diurnal variation in blood and sputum eosinophils. Sputum eosinophil percentages increased significantly at 04:00 compared with 16:00 in patients with asthma (*$P < 0.05$, Mann-Whitney *U* test). This was not seen in the healthy group ($P = 0.63$). Sputum eosinophil percentages were significantly higher in patients with asthma compared with healthy control subjects ($P < 0.01$, two-way ANOVA). Shown are individual sputum eosinophil percentages (dots) and medians (bars) for healthy control subjects (gray) and patients with asthma (black). TCC = total cell count.

***Severe asthma.*** A total of 131 patients attended the morning clinic and 193 attended the afternoon clinic. Groups were well matched for age ($P = 0.11$), body mass index ($P = 0.25$), $FEV_1$% predicted ($P = 0.85$), smoking status ($P = 0.3$), serum total IgE ($P = 0.23$), fractional exhaled nitric oxide ($P = 0.58$), blood eosinophil count ($P = 0.58$), and treatment (intramuscular triamcinolone [$P = 0.71$], oral prednisolone [$P = 0.31$], or daily inhaled corticosteroids [beclomethasone dipropionate equivalent]; $P = 0.31$). Significantly more subjects in the morning group produced sputum spontaneously than by induction (77.1% vs. 62.1%; $P < 0.005$).

An 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 than that produced in the afternoon clinic (morning sputum eosinophil percentage 1.25% [0.00–8.75%] vs. afternoon 0.5% [0.00–2.25%]; $P = 0.008$). A general linear modeling analysis showed that the time-of-day effect persisted even if patients were on high-dose steroids ($P = 0.41$) and was not affected by the type of sputum (spontaneous vs. induced; $P = 0.54$).

A significantly higher proportion of patients with severe asthma attending the morning clinic had positive sputum eosinophil counts (⩾3%) compared with 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 (⩾3% eosinophils) between spontaneously produced sputum and induced sputum (morning $P = 0.6$, afternoon $P = 1$).

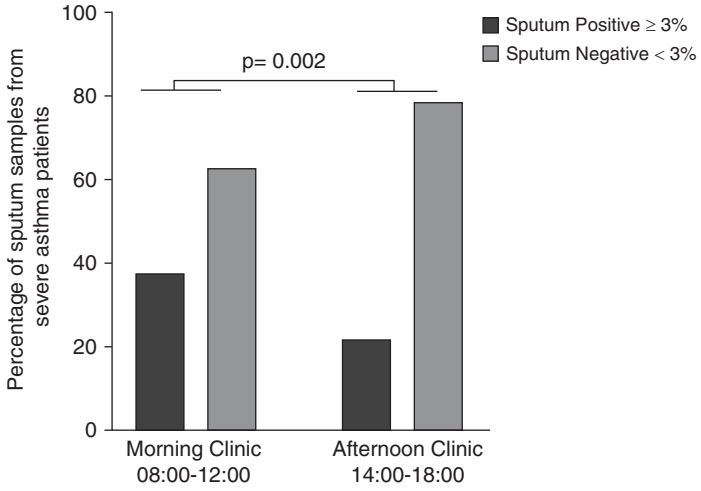


**Figure 2.** Analysis of sputum from patients attending morning and afternoon severe-asthma clinics. The figure shows the percentage of positive (black) and negative (gray) sputum samples from patients with severe asthma attending a morning or afternoon clinic: 37.4% of patients with severe asthma had positive sputum eosinophil counts (≥3%) in the morning clinic, compared with 21.6% in the afternoon clinic ($P = 0.002$, chi-squared, Fisher's exact test).

## 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 showed a significant circadian rhythm in induced sputum from patients with mild/moderate asthma, with a peak influx at 04:00 coinciding with peak sputum eotaxin concentrations suggesting a chemotactic mechanism. We demonstrated that this was in antiphase with $FEV_1$. In contrast to airway eosinophils, blood eosinophils oscillated diurnally in both healthy subjects and patients with asthma, suggesting that the physiological mechanism that controls the circadian variation in blood eosinophils is not upregulated in asthma. In support of this, there was no difference in serum eotaxin levels between the groups.

We noticed a nadir in sputum eosinophil levels at 16:00, and although the difference was not statistically significant, sputum eosinophil levels appeared to be higher at 10:00, hinting at a possible time-of-day effect that would be relevant within a clinical working day. We postulated that a patient might produce a sputum sample with higher eosinophil counts while attending a morning clinic versus an afternoon clinic. In our retrospective evaluation, we identified that patients with severe asthma attending a morning clinic were almost twice as likely (37.4% vs. 21.6%) to have sputum eosinophilia (≥3%) than those attending an afternoon clinic. These findings require confirmation in a prospective study; however, the implications are clinically important. In patients with severe asthma, increased sputum eosinophil counts are an indicator for treatment escalation (3). Based on our results, we propose that different clinical decisions could be made based on whether the patient is allocated a morning or afternoon appointment. ■

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## The Y Chromosome Regulates BMPR2 Expression via SRY: A Possible Reason "Why" Fewer Males Develop Pulmonary Arterial Hypertension

*To the Editor*:

Reduced lung *BMPR2* (bone morphogenetic protein receptor type 2) expression and female predominance are two major features of most pulmonary arterial hypertension (PAH) subtypes (1). In addition, germline mutations in *BMPR2* are present in more than 75% of patients with heritable PAH, and about 20% of patients with idiopathic PAH (2). However, only 14% of males, compared with 42% of females, who harbor *BMPR2* mutations develop PAH (3).

There is a growing body of molecular and *in vivo* work supporting the concept that sex and *BMPR2* are intimately related to each other, to PAH pathogenesis, and perhaps to right ventricular adaptation. For example, estrogen receptor α binds to the *BMPR2* promoter in Cos-7 cells, leading to decreased expression and signaling of BMPR-II, whereas female human pulmonary artery smooth muscle cells exhibit estrogen-driven suppression of BMPR-II signaling (4, 5); however, estrogen signaling appears to support right ventricular response to stress (6). Although likely relevant, a focus only on sex hormones ignores an obvious difference between females and males: the sex chromosomes (XX vs. XY). Importantly, recently in the *Journal*, Umar and colleagues demonstrated a protective effect of the Y chromosome in murine hypoxia-induced pulmonary hypertension (7). We explored the hypothesis that the higher female incidence in PAH is driven in part by factors specific to the Y chromosome that enhance *BMPR2* expression, with a focus on the transcription factor *SRY* (sex-determining region Y).

To start, we analyzed *BMPR2* proximal regulator sequences using the transcription element search system and transcription factor database (http://www.gene-regulation.com). These analyses predicted *BMPR2* upstream regulatory regions had at least five SRY binding sites. This suggested to us that SRY may regulate *BMPR2* mRNA expression.

We next evaluated *SRY* expression in different cell types and found that *SRY* expression was low in multiple different male lung vascular cell lines but high in dermal fibroblast cell lines from multiple control patients and patients with PAH (data not shown). We then sought to determine whether *SRY* regulates native *BMPR2* expression. We used RNA interference to knockdown *SRY* expression in fibroblasts from a male patient with PAH. We found that reducing *SRY* expression resulted in decreased *BMPR2* mRNA and protein expression (Figure 1A). Although Smad 1/5/8 protein expression was not demonstratively reduced (data not shown), the breadth of canonical and noncanonical BMPR2 signaling was not assessed. We then used a *SRY* expression construct to overexpress *SRY* in a female HEK293 cell line (which does not express *SRY*). We found that *SRY* overexpression resulted in ∼20% increased *BMPR2* expression compared with control (Figure 1B).

We next investigated whether *BMPR2* is regulated by *SRY* in a dose-dependent manner. A *BMPR2* promoter expression construct pGL3-BMPR2-Luciferase (containing the predicted *SRY* binding sites) was used in a cotransfection assay with a varying amount of *SRY* expression construct. We found that an increased

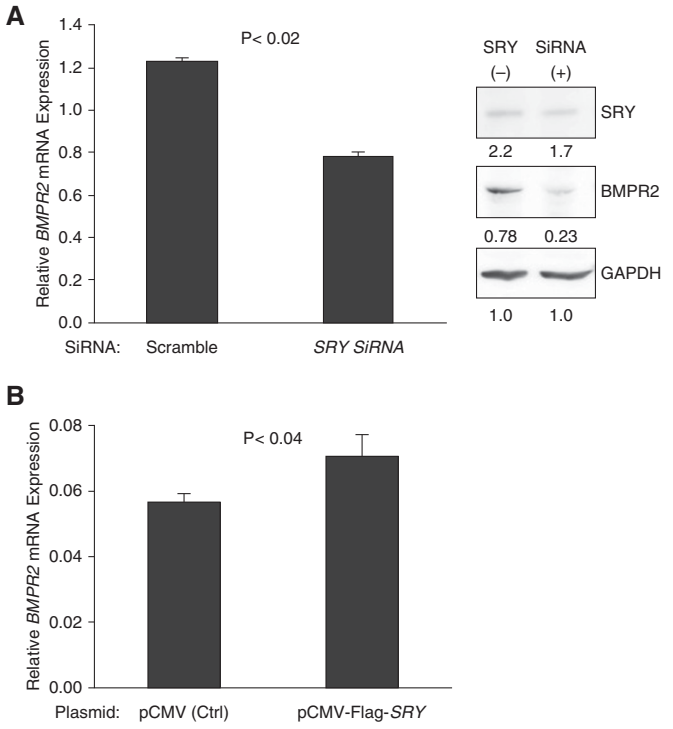


**Figure 1.** *SRY* (sex-determining region Y) positively regulates *BMPR2* (bone morphogenetic protein receptor type 2) expression in human cells. (*A*) *SRY* knockdown resulted in lower *BMPR2* mRNA and protein expression in male fibroblasts. The dermal fibroblasts, derived from a male patient with pulmonary arterial hypertension, were transfected with scrambled siRNA or *SRY* siRNA. *BMPR2* mRNA levels were quantified by real-time PCR, and protein levels by Western blot. (*B*) SRY overexpression resulted in increased BMPR2 mRNA expression in female HEK293 cells (which do not express native SRY). HEK293 cells were transfected with plasmids: control pCMV or pCMV-FLAG-SRY (which expressed SRY). BMPR2 mRNA levels were quantified by real-time PCR.

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Author Contribution: L.Y. conducted experiments, analyzed data, and wrote the manuscript; J.D.C. analyzed data; L.K.H. and B.N. conducted experiments; and L.Y., R.H., and E.D.A. conceived the study; designed the experiments; acquired, analyzed, and interpreted the data; and wrote and edited the manuscript.

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