

Circadian variation of sputum inflammatory cells in mild asthma

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Background: Asthma, like many conditions, demonstrates a circadian rhythm with a worsening of lung function in the early morning hours compared with in the late afternoon. **Objective:** Because eosinophilic airway inflammation is a proposed mechanism for worsening asthma, we characterized circadian variation in airway eosinophils and determined its relationship to variability in airway function. **Methods:** Pulmonary function testing, sputum induction, and phlebotomy were performed at 7 AM and 4 PM in 11 allergic subjects with mild asthma. Sputum was analyzed for cell viability, differential, and eosinophil-derived neurotoxin levels. IL-5 levels in serum were measured by means of ELISA. **Results:** Subjects had a significant decrease in FEV₁ (median [interquartile range] = 80% [70%-86%] vs 85% [82%-94%], $P = .009$) and a greater β -agonist reversibility (median [interquartile range] = 13% [7%-32%] vs 8% [5%-14%], $P = .024$) in the early morning compared with in the late afternoon. Sputum analysis showed an increase in early morning total sputum leukocytes (median [interquartile range] = 4.3×10^6 [2.3×10^6 to 6.1×10^6] vs 2.6×10^6 [1.7×10^6 to 3.6×10^6], $P = .044$) and eosinophils (median [interquartile range] = 7.0×10^4 [2.7×10^4 to 18.7×10^4] vs 3.6×10^4 [1.0×10^4 to 8.2×10^4], $P = .024$). Furthermore, sputum eosinophils correlated with β -agonist reversibility ($R_s = 0.665$, $P = .019$). Finally, levels of IL-5 in serum and eosinophil-derived neurotoxin in sputum were significantly increased at 7 AM. **Conclusion:** These data suggest that circadian variability in pulmonary function in asthma could be related to changes in airway eosinophil recruitment and activation. (*J Allergy Clin Immunol* 2003;111:308-12.)

Key words: Eosinophil, induced sputum, circadian variations

Airway function in asthma exhibits a well-known circadian rhythm, with an increase in symptoms and a decrease in lung function in the night and early morning.¹ Previous studies have shown that asthma is associated with increased diurnal variations in peak expiratory flow rate (PEFR),²⁻⁴ that these changes in PEFR are greater in patients with severe asthma than in those with mild dis-

Abbreviations used

BAL: Bronchoalveolar lavage
EDN: Eosinophil-derived neurotoxin
PEFR: Peak expiratory flow rate

ease,² and that this PEFR variability is related to diurnal changes of endogenous hormones.⁵ Therefore research into the mechanisms by which asthma symptoms worsen suggests a chronobiologic-based approach is needed.

Eosinophils are a key feature of airway inflammation that develops after allergen challenge. Eosinophil recruitment and activation has been linked to airway obstruction and hyperresponsiveness in asthma. Day-night variations in eosinophilic airway inflammation has been investigated in patients with asthma. Some of these studies have reported that patients with nocturnal asthma have increased numbers of eosinophils in the airway at night in bronchoalveolar lavage (BAL) fluid^{6,7} or in alveolar tissue obtained by means of transbronchial biopsy.⁸ However, other studies have not demonstrated a circadian variation in airway eosinophils by using BAL fluid or endobronchial biopsy specimens.⁹⁻¹⁴ Therefore the role of the eosinophil in the circadian variation of airway function in asthma remains controversial.

Although many studies of asthma pathogenesis have relied on invasive bronchoscopic techniques, noninvasive sputum induction has become an important research tool for clinical and pharmaceutical studies over the past decade.¹⁵⁻¹⁸ Induced sputum is a valuable and noninvasive technique to assess the relationship of the inflammatory process to airway obstruction in asthma. Using induced sputum, we recently showed that sputum eosinophils and sputum eosinophil-derived neurotoxin (EDN) levels are increased in patients with asthma after antigen challenge¹⁸ and are further augmented by psychologic stress.¹⁹ Additionally, messenger (m)RNA levels of IL-5, a key cytokine for eosinophil maturation and priming, have an increased expression in induced sputum from patients with asthma.²⁰ Levels of IL-5 in sputum supernatants are also increased after allergen exposure.^{21,22} Therefore induced sputum can be used to monitor airway eosinophilia, an important parameter of airway inflammation in asthma.

Circadian changes in airway inflammation as reflected in induced sputum in asthma have not been evaluated. The following study was conducted to investigate whether factors that play an important role in allergic inflammation (eosinophils, IL-5, and EDN) exhibit a cir-

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cadian variation that corresponds to the circadian variations seen in airway obstruction in asthma. For this study, pulmonary function testing, sputum induction, and phlebotomy were performed at 7 AM and 4 PM in 11 subjects with asthma. Induced sputum was analyzed for cell viability, differential, and EDN levels. Serum was analyzed for circulating IL-5 levels.

METHODS

Subject selection and study design

All 11 nonsmoking allergic asthmatic subjects (age 21–40 years) were in good health other than having mild asthma. Each subject had physician-diagnosed asthma, with documented reversibility with β -agonists ($>12\%$ increase in FEV_1) or airway hyperresponsiveness (methacholine $PC_{20} <8$ mg/mL). All subjects were atopic, with a positive skin prick test response to at least one aeroallergen. None of the subjects were taking inhaled steroids, theophylline, long-acting β -agonists, or any other medicines that might interfere with the study outcome. All subjects were free of viral infections or asthma exacerbations for at least 4 weeks before and during the study period.

After screening, subjects were randomized to undergo sputum induction at either 7 AM or 4 PM. The 7 AM and 4 PM sputum inductions were performed no less than 2 days and no more than 2 weeks apart. During each 7 AM and 4 PM study visit, pulmonary function testing and blood draws were done, followed by sputum induction. Spirometry was performed immediately before and repeated 15 minutes after albuterol inhalation. Informed consent was obtained from each subject before participation. The study was approved by the University of Wisconsin–Madison Center for Health Sciences Human Subjects Committee.

Sputum induction

Sputum induction was performed after premedication with albuterol administered by means of metered-dose inhaler (180 μ g). Subjects then inhaled nebulized 3% hypertonic saline generated by using the DeVilbiss Ultra-Neb 99 ultrasonic nebulizer (DeVilbiss Co, Somerset, Pa) over 20 minutes, as previously described.¹⁸ Hypertonic saline inhalation was stopped at 10, 15, and 20 minutes after inhalation to collect sputum samples in a sterile container.

Sputum analysis

Sputum was processed as described previously.¹⁸ Briefly, after adding 0.05% dithiothreitol (Sputolysin Reagent; Calbiochem, San Diego, Calif), samples were placed in a shaking 37°C water bath for 15 minutes and then centrifuged, filtered, and washed. Sputum supernatant was removed and stored at -80°C to be used later for analysis of EDN. A total cell count of sputum leukocytes was done with a hemacytometer by using Turk's crystal violet solution. Leukocyte viability was assessed by means of Trypan Blue exclusion. Only sputum leukocytes were counted; squamous and epithelial cells were excluded from the total cell count and viability determination. Sputum leukocytes are expressed in terms of millions of viable cells. Cytospin preparations were fixed and stained with a Giemsa-based Diff-Quick solution (Baxter Scientific, McGaw, Ill). Slides were read by an observer blinded to subject and study phase (4 PM or 7 AM).

Measurement of cytokines and EDN

IL-5 levels in serum were measured by using a 2-step sandwich ELISA assay, as previously described.²³ The coating and biotinylated antibodies for the IL-5 ELISA were purchased from PharMingen (San Diego, Calif). EDN levels in sputum supernatants were measured by using a double-antibody competitive RIA.²⁴

Statistical analysis

Because some of the data were not normally distributed, we expressed all variables as medians with 25% and 75% interquartiles. Statistical analyses were performed by using Jandel Sigma-Stat software (SASS Inc, Chicago, Ill). Values from the 2 study phases (7 AM and 4 PM) were compared as appropriate for their distribution by using a paired *t* test for normally distributed data and a Wilcoxon signed-rank test for nonnormally distributed data.²⁵ Correlations were determined by using a Spearman rank order correlation test. A *P* value of less than .05 was considered to be statistically significant.

RESULTS

Subject characteristics

Subjects enrolled in this study (*n* = 11) had mild asthma, with documented airway obstruction, reversibility with β -agonist, and airway hyperresponsiveness (Table I). All subjects completed both phases of the study. There were no complications associated with study procedures. The time interval between sputum inductions was 7 ± 1 days (mean $\pm$ SE).

Circadian variation in lung function

FEV_1 was significantly lower at 7 AM compared with values at 4 PM (median [interquartile range] = 80% [70%–86%] vs 85% [82%–94%], *P* = .009; Fig 1, A). Moreover, β -agonist reversibility was significantly greater at 7 AM compared with that at 4 PM (median [interquartile range] = 13% [7%–32%] vs 8% [5%–14%], *P* = .024; Fig 1, B).

Circadian variation in inflammation

Sputum leukocytes were significantly increased at 7 AM compared with at 4 PM (Table II). Sputum leukocyte viability was similar at both time points. There were no significant changes in the percentage or number of sputum macrophages, lymphocytes, or neutrophils. However, the number of sputum eosinophils was significantly greater at 7 AM versus at 4 PM (median [interquartile range] = 7.0×10^4 [2.7×10^4 to 18.7×10^4] vs 3.6×10^4 [1.0×10^4 to 8.2×10^4], *P* = .024; Fig 2, A). Furthermore, there was a significant increase in sputum EDN concentrations at 7 AM compared with at 4 PM (median [interquartile range] = 193 ng/mL [119–462 ng/mL] vs 83 ng/mL [68–290 ng/mL], *P* = .032; Fig 2, B). In the circulation IL-5 levels were significantly greater at 7 AM than at 4 PM (median [interquartile range] = 40 pg/mL [31–64 pg/mL] vs 37 pg/mL [27–54 pg/mL], *P* = .032; Fig 2, C).

Relationship among reversible airway obstruction, sputum eosinophils, and EDN

At 7 AM, the sputum eosinophil percentage positively correlated with β -agonist reversibility ($R_s = 0.665$, *P* = .019, *n* = 11; Fig 2, D). Sputum eosinophil numbers positively correlated with β -agonist reversibility ($R_s = 0.879$, *P* < .001, *n* = 10 after excluding one outlier; with the outlier included: $R_s = 0.573$, *P* = .06) and inversely correlated with FEV_1 at 7 AM ($R_s = -0.609$, *P* = .043). Furthermore, at 7 AM, sputum EDN correlated with eosinophil

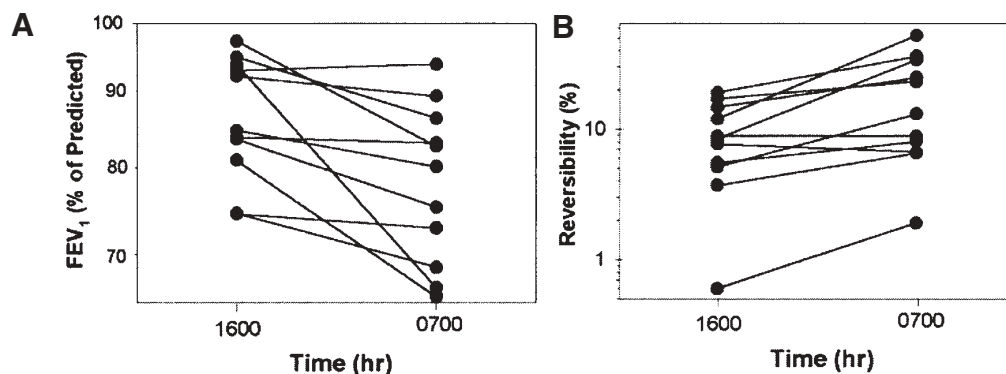


FIG 1. Circadian variation in pulmonary function. **A**, Spirometry was performed immediately before sputum induction at 4 PM and 7 AM. FEV₁ is presented as a percentage of predicted value ($n = 11$, $*P = .009$). **B**, Reversibility to inhaled β -agonist was determined at 4 PM and 7 AM ($n = 11$, $*P = .024$).

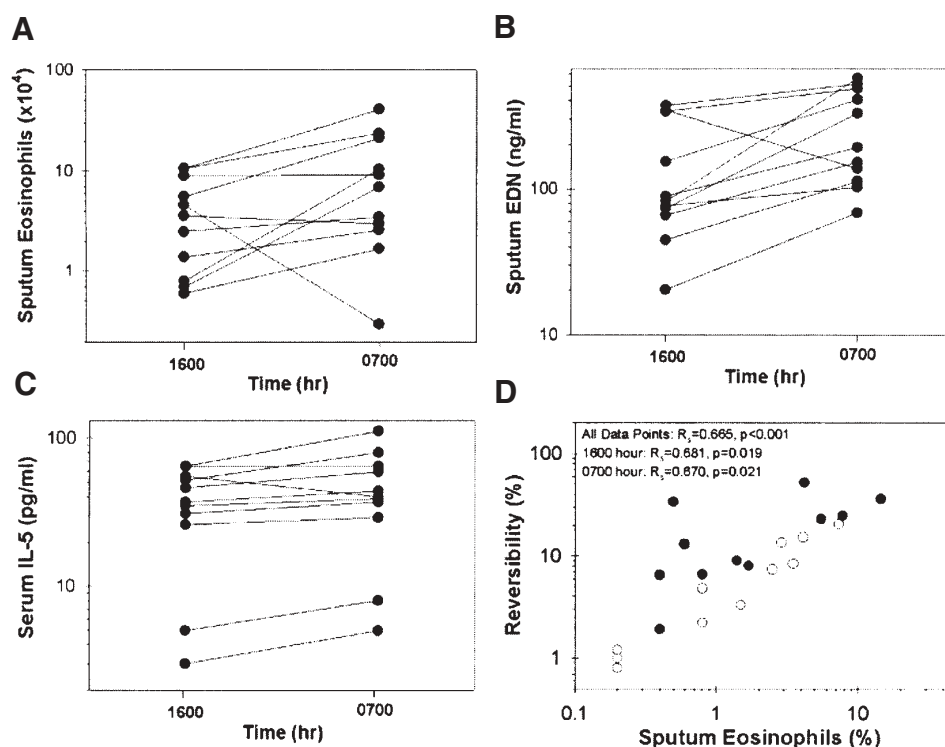


FIG 2. **A**, Circadian variation in sputum eosinophils. Sputum induction was performed at 4 PM and 7 AM ($n = 11$, $*P = .024$). **B**, Circadian variation in EDN levels in sputum ($n = 11$, $*P = .032$). **C**, Circadian variation in circulating IL-5 levels ($n = 11$, $*P = .032$). **D**, Correlation between reversible airway obstruction and airway eosinophils. Data represent values obtained from 11 subjects at 4 PM (open circles) and 7 AM (closed circles).

numbers ($R_s = 0.60$, $P = .05$), percentage ($R_s = 0.64$, $P = .03$), and β -agonist reversibility ($R_s = 0.59$, $P = .05$). At 4 PM, β -agonist reversibility correlated with sputum eosinophil percentage ($R_s = 0.861$, $P < .001$; Fig 2, *D*) and number ($R_s = 0.686$, $P < .001$).

DISCUSSION

This study demonstrates a circadian variation of airway eosinophil numbers, cellular activation, and circulating IL-5 in patients with mild asthma. Using induced

sputum, we observed a significant increase in the number of airway leukocytes and eosinophils at 7 AM compared with at 4 PM. In addition, there was an increase in sputum EDN and circulating IL-5 levels at 7 AM, which might reflect greater eosinophil activation at 7 AM compared with at 4 PM. The early morning increase in airway eosinophils correlated with enhanced airway obstruction and reversibility. Furthermore, levels of EDN correlated significantly with the degree of airway obstruction.

These findings suggest that the early morning increase in inflammation is associated with enhanced generation

of IL-5 in the circulation, which might lead to recruitment of eosinophils, activation of eosinophils, or both. In fact, the increased numbers of airway eosinophils present at 7 AM might not be as important to the degree of airway obstruction as the fact that they are more activated compared with late in the day. The release of eosinophil granule proteins supports the notion of a greater proinflammatory potential of eosinophils during the early morning hours. The correlation among sputum eosinophils, sputum EDN levels, and airway obstruction suggests that a greater level of eosinophil activation might contribute to circadian variations in airway obstruction.

The reproducibility of measurements in induced sputum has been disputed, with some studies showing low repeatability²⁶ and others showing reliability coefficients of greater than 0.6 for most parameters.²⁷⁻³¹ Because the significant difference in airway eosinophils we observed between 4 PM and 7 AM was also accompanied by an increase in EDN and IL-5 and a functional change in lung function (spirometry), the changes are less likely caused by random variations. We also demonstrated that EDN and spirometry correlated with airway eosinophils, all suggesting a biologic and physiologic relevance to these observations. Furthermore, this circadian pattern was not seen with other cell types (Table II).

The time points used in this study differ slightly from the reported peak and nadir of pulmonary function, generally 4 PM and 4 AM, respectively. We used 2 time points, 7 AM and 4 PM, because we were also interested in how measurements of inflammation can be affected by circadian rhythm and how these variations might affect the outcome of clinical trials in which repeated measurements occur at different times during the day. Importantly, we did observe significant circadian variation in airway obstruction and parameters of inflammation at 7 AM versus 4 PM. Previous studies have also reported an increase in airway hyperresponsiveness at night in patients with asthma.³² Because the circadian changes are likely to be gradual, selecting the 2 time points that we did might actually underestimate the true fluctuations at the peak-trough. As D'Alonzo et al³³ showed in their study, comparisons of lung function measurements (FEV₁ and PEFR) taken on arising and retiring with those taken every 2 hours greatly underestimate the actual variability in airflow of patients with asthma.

Even though the subjects used in this study had mild disease, they demonstrated significant circadian variation in lung function. Avoiding patients with more severe disease allowed us to exclude subjects taking oral or inhaled steroids and those with more frequent exacerbations, both of which could potentially affect study outcome. We would not expect healthy (nonasthmatic) subjects to have significant or measurable levels of eosinophils or IL-5 or show circadian variations in these levels. As shown extensively in other studies, nonasthmatic subjects, as well as asthmatic subjects without nocturnal asthma, do not demonstrate circadian variations in inflammatory parameters.^{6,7,12,14,34,35} Therefore a separate control group was not included, and we elected to use each

TABLE I. Subject characteristics at screening

Subject characteristics (n = 11)	Median (interquartile range)
FEV ₁ (% predicted)	85 (77-96)
FVC (% predicted)	97 (92-103)
FEV ₁ /FVC (%)	84 (73-91)
FEF ₂₅₋₇₅ (% predicted)	60 (49-69)
β-Agonist reversibility (%)	13 (12-22)
Methacholine PC ₂₀ (mg/mL)	1.3 (0.5-3.3)

FVC, Forced vital capacity; FEF₂₅₋₇₅, forced expiratory flow at 25% to 75% of forced vital capacity.

TABLE II. Sputum analysis results*

Sputum analysis (n = 11)	4 PM	7 AM
Total sputum leukocytes (×10 ⁶)	2.6 (1.7-3.6)	4.3 (2.3-6.1)†
Sputum leukocyte viability (%)	69 (64-75)	76 (67-79)
Macrophages (%)	78 (67-85)	65 (53-78)
Total macrophages (×10 ⁴)	192 (143-214)	186 (153-331)
Lymphocytes (%)	2 (1-3)	2 (2-4)
Total lymphocytes (×10 ⁴)	5 (3-9)	10 (3-17)
Neutrophils (%)	18 (11-30)	25 (13-39)
Total neutrophils (×10 ⁴)	49 (19-99)	92 (34-304)
Eosinophils (%)	1.5 (0.4-3.4)	1.4 (0.5-5.2)
Total eosinophils (×10 ⁴)	3.6 (1.0-8.2)	7.0 (2.7-18.7)†

*Data are expressed as median (25%-75% interquartile range).

†P < .05.

patient as his or her own control subject.

Several studies have found increased numbers of eosinophils at night when using BAL or endobronchial or transbronchial biopsy to study nocturnal asthma.⁶⁻⁸ However, other studies have not shown a dramatic increase in the numbers of eosinophils at night when using BAL or endobronchial biopsy.⁹⁻¹⁴ In the current study we observed a significant increase in airway eosinophils, as measured in sputum obtained in the early morning. Eosinophil recruitment to the airway probably varies by time of day, but differences in sampling methods, timing, and subject selection can influence the ability to detect this change.

Although the mechanisms leading to circadian variations in eosinophil recruitment to the airway and their activation remain to be explained, we provide evidence that enhanced IL-5 generation might be involved in this process through an early morning increase in circulating IL-5 levels. Because of the limited ability to measure sputum IL-5 levels,³⁶ we chose instead to evaluate circulating IL-5 levels. Our finding of a circadian variation in circulating IL-5 levels is in contrast to the findings of a previous study, in which no circadian variation was observed in serum IL-5 levels.³⁷ This discrepancy could be related to differences in timing of blood sampling, disease severity, or methods of cytokine analysis.

Other studies have found nocturnal increases in levels of inflammatory mediators in the BAL fluid, such as eosinophil cationic protein,⁶ IL-1β,¹² and superoxide generation.¹¹ Additionally, we have reported an increase in the proportion of upregulated, hypodense eosinophils in peripheral blood at 4 AM compared with at 4 PM in subjects with nocturnal asthma.³⁸ In the current study we

demonstrated that increased EDN levels in sputum in the early morning correlated with the degree of airway obstruction. This suggests the eosinophils and their state of activation might contribute to airway obstruction, corroborating the findings of previous reports.^{7,11,14} Taken together, these data suggest that the circadian variation in pulmonary function in asthma is associated with enhanced eosinophilic airway inflammation.

In conclusion, we have demonstrated circadian variation in airway leukocytes and eosinophils by using induced sputum. Additionally, we have demonstrated circadian variation of eosinophil activation and levels of circulating inflammatory mediators. Furthermore, these inflammatory changes were shown to correlate with airway obstruction and reversibility. These data suggest that circadian variability in pulmonary function in asthma could be related to changes in airway eosinophil recruitment and activation.

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REFERENCES

- Martin RJ, Banks-Schlegel S. Chronobiology of asthma. *Am J Respir Crit Care Med* 1998;158:1002-7.
- Shamoo DA, Linn WS, Peng RC, Solomon JC, Webb TL, Hackney JD, et al. Time-activity patterns and diurnal variation of respiratory status in a panel of asthmatics: implications for short-term air pollution effects. *J Expo Anal Environ Epidemiol* 1994;4:133-48.
- Lebowitz MD, Krzyzanowski M, Quackenboss JJ, O'Rourke MK. Diurnal variation of PEF and its use in epidemiological studies. *Eur Respir J Suppl* 1997;24:49S-56S.
- Wijnhoven HA, Kriegsman DM, Hesselink AE, Penninx BW, de Haan M. Determinants of different dimensions of disease severity in asthma and COPD: pulmonary function and health-related quality of life. *Chest* 2001;119:1034-42.
- Kraft M, Pak J, Martin RJ. Serum cortisol in asthma: marker of nocturnal worsening of symptoms and lung function? *Chronobiol Int* 1998;15:85-92.
- Mackay TW, Wallace WA, Howie SE, Brwon PH, Greening AP, Church MK, et al. Role of inflammation in nocturnal asthma. *Thorax* 1994;49:257-62.
- Martin RJ, Cicutto LC, Smith HR, Ballard RD, Szeffler SJ. Airway inflammation in nocturnal asthma. *Am Rev Respir Dis* 1991;143:351-7.
- Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:1505-10.
- Oosterhoff Y, Timens W, Postma DS. The role of airway inflammation in the pathophysiology of nocturnal asthma. *Clin Exp Allergy* 1995;25:915-21.
- Oosterhoff Y, Kauffman HF, Rutgers B, Zijlstra FJ, Koeter GH, Postma DS. Inflammatory cell number and mediators in bronchoalveolar lavage fluid and peripheral blood in subjects with asthma with increased nocturnal airways narrowing. *J Allergy Clin Immunol* 1995;96:219-29.
- Jarjour NN, Busse WW, Calhoun WJ. Enhanced production of oxygen radicals in nocturnal asthma. *Am Rev Respir Dis* 1992;146:905-11.
- Jarjour NN, Busse WW. Cytokines in bronchoalveolar lavage fluid of patients with nocturnal asthma. *Am J Respir Crit Care Med* 1995;152:1474-7.
- ten Hacken NHT, Timens W, Smith M, Drok G, Kraan J, Postma DS. Increased peak expiratory flow variation in asthma: severe persistent increase but not nocturnal worsening of airway inflammation. *Eur Respir J* 1998;12:546-50.
- Kraft M, Martin RJ, Wilson S, Djukanovic R, Holgate S. Lymphocyte and eosinophil influx into alveolar tissue in nocturnal asthma. *Am J Respir Crit Care Med* 1999;159:228-34.
- Pin I, Freitag AP, O'Byrne PM, Girgis-Gabardo A, Watson RM, Dolovich J, et al. Changes in the cellular profile of induced sputum after allergen-induced asthmatic responses. *Am Rev Respir Dis* 1992;145:1265-9.
- Fahy JV, Kim KW, Liu J, Boushey HA. Respiratory pathophysiologic responses. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843-52.
- Fahy JV, Liu J, Wong H, Boushey HA. Cellular and biochemical analysis of induced sputum from asthmatic and healthy subjects. *Am Rev Respir Dis* 1993;147:1126-31.
- Liu LY, Swenson CA, Kita H, Kelly EAB, Busse WW. The relationship of airway eosinophilia and sputum cell generation of IL-5 following antigen challenge. *J Allergy Clin Immunol* 2000;106:1063-9.
- Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, Busse WW. School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 2002;165:1062-7.
- Olivenstein R, Taha R, Minshall EM, Hamid QA. IL-4 and IL-5 mRNA expression in induced sputum of asthmatic subjects: comparison with bronchial wash. *J Allergy Clin Immunol* 1999;103:238-45.
- Keatings VM, O'Connor BJ, Wright LG, Huston DP, Corrigan CJ, Barnes PJ. Late response to allergen is associated with increased concentrations of tumor necrosis factor- α and IL-5 in induced sputum. *J Allergy Clin Immunol* 1997;99:693-8.
- Sulakvelidze I, Inman MD, Rerecich T, O'Byrne PM. Increases in airway eosinophils and interleukin-5 with minimal bronchoconstriction during repeated low-dose allergen challenge in atopic asthmatics. *Eur Respir J* 1998;11:821-7.
- Kelly EA, Rodriguez RR, Busse WW, Jarjour NN. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am J Respir Crit Care Med* 1997;156:1421-8.
- Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989;142:2393-400.
- Glantz SA. *Primer of bio-statistics*. 4th ed. New York: McGraw-Hill Companies, Inc; 1997.
- Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Scullace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308-17.
- Fahy JV, Boushey HA, Lazarus SC, Mauger EA, Cherniack RM, Chinchilli VM, et al. Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. *Am J Respir Crit Care Med* 2001;163:1470-5.
- Wilson NM, Bridge P, Spanevello A, Silverman M. Induced sputum in children: feasibility, repeatability, and relation of findings to asthma severity. *Thorax* 2000;55:768-74.
- Thomas PS, Yates DH, Barnes PJ. Sputum induction as a method of analyzing pulmonary cells: reproducibility and acceptability. *J Asthma* 1999;36:335-41.
- Spanevello A, Migliori GB, Sharara A, Ballardini L, Bridge P, Pisati P, et al. Induced sputum to assess airway inflammation: a study of reproducibility. *Clin Exp Allergy* 1997;27:1138-44.
- in't Veen JC, de Gouw HW, Smits HH, Sont JK, Hiemstra PS, Sterk PJ, et al. Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma. *Eur Respir J* 1996;9:2441-7.
- Martin RJ, Cicutto LC, Ballard RD. Factors related to the nocturnal worsening of asthma. *Am Rev Respir Dis* 1990;141:33-8.
- D'Alonzo GE, Steijnmans VW, Keller A. Measurements of morning and evening airflow grossly underestimate the circadian variability of FEV₁ and peak expiratory flow rate in asthma. *Am J Respir Crit Care Med* 1995;152:1097-9.
- Martin RJ. Small airway and alveolar tissue changes in nocturnal asthma. *Am J Respir Crit Care Med* 1998;157:S188-90.
- Postma DS, Oosterhoff Y, van Aaldren WM, Kauffman HF, Wempe JB, Koeter GH. Inflammation in nocturnal asthma. *Am J Respir Crit Care Med* 1994;150:S83-6.
- Kelly MM, Leigh R, Horsewood P, Gleich GJ, Cox G, Hargreave FE. Induced sputum: validity of fluid-phase IL-5 measurement. *J Allergy Clin Immunol* 2000;105:1162-8.
- ten Hacken NHT, Oosterhoff Y, Kauffman HF, Guevarra L, Satoh T, Tollerud DJ, et al. Elevated serum interferon- γ in atopic asthma correlates with increased airways responsiveness and circadian peak expiratory flow variation. *Eur Respir J* 1998;11:312-6.
- Calhoun WJ, Bates ME, Schrader L, Sedgwick JB, Busse WW. Characteristics of peripheral blood eosinophils in patients with nocturnal asthma. *Am Rev Respir Dis* 1992;145:577-81.