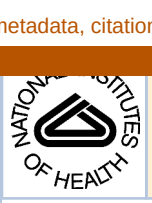



Published in final edited form as:

*Respir Med*. 2010 January ; 104(1): 29. doi:10.1016/j.rmed.2009.07.002.

# Neutrophilic inflammation is associated with altered airway hydration in stable asthmatics

**Ceila E. Loughlin, M.D.**[a], **Charles .R. Esther Jr, M.D., Ph.D.**[a], **Eduardo.R. Lazarowski, Ph.D.**[c], **Neil E. Alexis, Ph.D.**[b,d], and **David B. Peden, M.D.**[b,d]

[a]Department of Pediatrics, Divisions of Pediatric Pulmonology, The School of Medicine University of North Carolina, Chapel Hill, North Carolina

[b]Pediatric Allergy, Immunology, Rheumatology and Infectious Diseases, The School of Medicine University of North Carolina, Chapel Hill, North Carolina

[c]Cystic Fibrosis/Pulmonary Research and Treatment Center, The School of Medicine University of North Carolina, Chapel Hill, North Carolina

[d]Center for Environmental Medicine, Asthma and Lung Biology, The School of Medicine University of North Carolina, Chapel Hill, North Carolina

## Introduction

Acute airway dehydration is reported to be a trigger for bronchoconstriction in exercise induced asthma.[1, 2] Recent studies from both animal models[3] and human subjects [4] suggest that chronic changes in airway hydration may also play an important but under appreciated role in asthma pathophysiology. Chronic airway dehydration is known to contribute to defects in mucociliary clearance in cystic fibrosis, which is also characterized by increased neutrophilic inflammation.[5, 6]. Like cystic fibrosis, exacerbations of asthma are also associated with decreased mucociliary clearance7 and acute increases in neutrophilic inflammation.8 These observations suggest that airway neutrophilia may contribute to dehydration of the airway surface, which, in turn, may result in decreased mucociliary clearance. It has been previously shown that induced sputum selectively samples bronchial airway surface secretions[9] without dilution by fluids recovered from alveolar spaces, and that the percentage of sputum mass which is comprised of solid material (as determined by sputum weight before and after desiccation of the sample and reported as percent solids) is a marker of airway hydration.[4, 10, 11] As an initial exploration of the relationship between airway neutrophilia and dehydration in asthma, we compared percent solid content of induced sputum in a cohort of subjects with stable asthma with samples recovered from healthy controls. We further assessed the relationships between sputum percent solids and established markers of airways inflammation including granulocytes and cytokines characteristic of neutrophilic ($Th_1$) and allergic ($Th_2$) inflammation. In addition, we measured adenyl purines, including adenosine triphosphate (ATP) and adenosine (ADO) that act as signaling molecules for purinergic pathways that regulate both inflammatory



**Declaration of all sources of funding:** D. B. Peden has consulting arrangements with GlaxoSmithKline, Novartis, Funxtional Therapuetics, and Genentech; has received grant support from GlaxoSmithKline; The rest of the authors have no declarations or conflicts of interest.

responses by macrophages and neutrophils and hydration of airway secretions mediated by epithelial cells.[10, 12, 13, 14]

## Methods

All subjects were non-smoking, 18-50 years of age. Atopic asthmatics (n=37) had physician diagnosed asthma and were skin test positive to a least one common aeroallergen in our standard skin test panel. The majority of subjects with asthma were in the NHLBI mild category based on $FEV_1$ (34 mild and 3 moderate persistent), and 14 were on inhaled corticosteroid therapy. Controls were healthy subjects (n=15) were free of disease. Lung function (mean % predicted FEV1) was modestly lower in subjects with asthma ($FEV_1$=98.7% with a range of 74-123%) versus controls ($FEV_1$= 109%, with a range of 91-121%, $p<0.05$). During a period of clinical stability, all subjects underwent pulmonary function testing, and sputum was obtained by induction with hypertonic saline as previously described.[15] In brief, sputum plug material was selected by visual inspection of the sample where identifiable plugs (more dense/opaque material vs. clear watery saliva) were manually retrieved using sterile pointed forceps. Sputum percent solids were assessed as a measure of airway hydration status by measurement of pre- and post- desiccation weights of selected sputum plugs (200-500mg), [4,11] and total and differential leukocyte counts on sputum were obtained from processing the remaining plug material.15 Sputum supernatants were obtained as previously described15 and analyzed for IL-1β, IL-8, IL-4, IL-5, and IL-13 in the asthma cohort (using Luminex based cytokine assays15)and for adenyl purines AMP, ATP, ADO, ADP (using etheno-derivatisation and HPLC16) in all subjects.

For statistical analysis, sputum measures did not follow a normal distribution. Therefore, all sputum measures were log transformed to normalize the distribution (transformed data passed KS normality test). Student's unpaired parametric t-test was utilized for between group comparisons and an overall level of $p \leq 0.05$ was considered to be statistically significant. All values were expressed as the mean ± SEM. Correlation analysis to test for the degree of a linear relationship between two variables was performed using Pearson's correlation coefficient (R), where $p<0.05$ was considered a statistically significant correlation. GraphPad Prism V.3.1 statistical software (GraphPad Software) was used for all statistical analyses.

## Results

We measured the percent solids content in sputum and observed it to be significantly elevated in asthmatics compared to sputum recovered from controls (Figure 1). To explore whether airway inflammation was related to airway hydration, we examined the correlations of sputum percent solids to inflammatory cell counts and fluid phase markers of inflammation (IL-1β, IL-8, IL-4, IL-5, and IL-13). These cytokines were chosen as representatives of the $Th_1$ and $Th_2$ pathways which are important in asthma. Sputum inflammatory cell counts were similar between groups with the exception of increased sputum eosinophils in subjects with atopic asthma (Table I). In asthmatics, sputum percent solids were significantly correlated with neutrophil counts and IL-8, markers of $Th_1$ inflammation (see Table II). Markers of $Th_2$ inflammation such as eosinophils, IL-4, IL-5 and IL-13 were not correlated with percent solids (Table III). These findings held true even when the asthma group was sub-divided into those on inhaled corticosteroid (ICS) therapy and those who were not taking ICS.

To determine the relationship between airway hydration and puringeric signaling, we measured adenyl purines in sputum supernatant. Consistent with previous studies,[17] we observed a significant increase in levels of sputum ADO in asthmatics compared to healthy controls (Figure 2). No other purines were statistically different in asthmatics relative to controls, though mean concentrations were higher in asthmatics. Furthermore, we did not observe a significant

difference between purine levels of subjects with asthma on inhaled corticosteroids (ICS) and those not in ICS therapy. Sputum percent solids were significantly correlated with AMP and to a lesser extent ADO (Table II). AMP was also correlated with markers of $Th_1$ inflammation including neutrophils (r=0.5, p=0.003), IL-8 (r=0.6, p=0.001), and IL-1b (r= 0.9, p=0.02) (Table II). In contrast, AMP levels did not correlate with markers of $Th_2$ inflammation including eosinophils (r=−0.15, p=0.43), IL-4 (r=−0.03, p=0.91), IL-5 (r=0.2, p =0.7), and IL-13 (r=0.3, p=0.4) (Table III).

## Discussion

Acute dehydration of airway mucus during exercise has been identified as an important aspect of exercise-induced bronchospasm[1, 2] and has been reported in acute exacerbations of asthma and mortality of asthma.[11] In this study, we found that airway hydration was also reduced in clinically stable asthmatics at baseline. While the differences were modest, these results demonstrate that neutrophilic inflammation is associated with airway dehydration in asthmatics. We hypothesize that in asthmatics confronted with stimuli which are established causes of asthma exacerbation and are also associated with acute neutrophil influx, such as viral infection, pollutant exposures and even exercise, there will be even greater dehydration with loss of mucociliary clearance. While bronchoconstriction is the most immediate feature of airway pathophysiology in asthma, increased mucus secretion and loss of mucociliary clearance are also central features of asthma exacerbation.[7] We hypothesize that airway dehydration will result in both bronchoconstriction and decreased mucociliary clearance

This hypothesis is supported by recent experimental observations both in animals and humans which demonstrate that chronic changes in airway hydration can worsen bronchoconstrictive responses. Deletion of the aquaporin 5 water channel gene in mice results in reduced airway hydration and a greater sensitivity to bronchoconstrictor challenge agents.[3] A recent study in humans demonstrated a negative correlation between fluid secretion rates in tears and sweat[18] and sensitivity to methacholine induced bronchoconstriction. These data suggested that individuals who have difficulty rehydrating airway secretions after a dehydrating exercise challenge may be at greater risk for subsequent bronchoconstriction. Though we did not assess fluid secretion rates in this study, our finding of a significant decrease in airway hydration at baseline is consistent with this hypothesis.

While neutrophils counts did not differ significantly between asthmatic and control volunteers, there were significant correlations between percent solids of induced sputum and neutrophil counts and with other markers associated with $Th_1$ inflammation, including IL-8. Interestingly, the purine with the strongest correlation with sputum percent solids was AMP. This purine has been shown to correlate with neutrophilic or $Th_1$ inflammation[16, 19] and activated neutrophils are known to accumulate both extracellular ATP and AMP.[13, 14] Thus all of the measured markers that correlate with sputum percent solids are related to $Th_1$ inflammatory pathways. In other respiratory diseases such as cystic fibrosis, neutrophilic inflammation has been shown to strongly correlate with ATP and AMP levels in bronchioalveolar lavage fluid of these patients.[16]

In contrast, sputum percent solids was not correlated with any markers of $Th_2$ inflammation despite the fact that increased $Th_2$ inflammation is thought to be involved in the majority of individuals with asthma and sputum eosinophils were elevated in our asthmatic cohort compared to controls. These findings suggest that airway dehydration may predominantly affect asthmatics with more neutrophilic inflammation which may relate to the fact that neutrophilic asthmatics generally have more severe disease.[20] Elevated percent solids content in sputum has been reported in other respiratory diseases like cystic fibrosis, [5, 10, 12] bronchiectasis, [21] and primary ciliary dyskinesia[22] where neutrophils play a dominant role in

the airway. Our study suggests that in the airway of subjects with asthma, even modest variations in neutrophilic inflammation can influence airway hydration and play a more important role in asthma pathophysiology than previously suspected.

Although we observed an increase in adenosine in the asthmatic subjects, as previously reported in bronchioalveolar lavage fluid, [17] the correlation between adenosine and airway hydration was relatively weak. This lack of correlation is not surprising however, as purinergic signaling via ADO increases chloride secretion to the apical surface of the epithelium and would be expected to increase hydration of mucus on airways surface. Adenosine may form part of a feedback regulatory loop, where hypertonic conditions trigger the release of ATP and its subsequent metabolism to adenosine improve airways hydration.

The importance of airway dehydration in disease exacerbation in asthma is suggested by studies in which administration of nebulized hypertonic solutions that affect the osmolarity of airway surface liquid (and increase airway surface hydration) are shown to improve mucociliary clearance in asthma. Daviskas et al [4, 23] showed that subjects with asthma have improved mucociliary clearance after treatment with hypertonic saline and mannitol. We have likewise found that mucociliary clearance is improved in asthmatics after use of hypertonic saline to facilitate mucus clearance from the airway.24 These studies demonstrate that targeting hydration may be beneficial to asthmatics and build a case for its clinical relevance. Certainly hydration therapies have proven successful in other respiratory diseases. They are currently being used in patients with cystic fibrosis and investigated in patients with COPD. Our data suggests this may be important in asthma as well.

In summary we report an elevated percent solids in clinically stable asthma compared to controls. Further investigation revealed an intriguing relationship between airway hydration and neutrophilic inflammation in the airways of stable asthmatics. Although our data do not necessarily indicate a causal relationship, they highlight the role of neutrophilic inflammation in mediating hydration of asthmatic airways. We suggest that future studies of experimental or naturally occurring asthma exacerbation should account for changes in airway hydration and the role that acute inflammation may have in this process.

## Acknowledgments

This work was funded by NIH grants T32HL007106, RO1-HL08033, P50-HL0849347 and U19AI077437. We would like to thank Martha Almond, Fernando Dimeo, Margaret Herbst, Lynn Newlin-Clapp, Carole Robinette, and Heather Wells, (University of North Carolina Center for Environmental Medicine, Asthma and Lung Biology) for their technical and clinical assistance.

## References

1. Anderson SD, Kippelen P. Airway injury as a mechanism for exercise-induced bronchoconstriction in elite athletes. J Allergy Clin Immunol 2008:122:225–235.
2. Anderson SD. How does exercise cause asthma attacks? Curr Opin Allergy Clin Immunol 2006;6:37–42. [PubMed: 16505610]
3. Krane CM, Fortner CN, Hand AR, McGraw DW, Lorenz JN, Wert SE, et al. Aquaporin 5-deficient mouse lungs are hyperresponsive to cholinergic stimulation. PNAS 2001;98:14114–14119. [PubMed: 11707570]
4. Daviskas E, Anderson SD, Young I. Inhaled mannitol changes the sputum properties of asthmatics with mucus hypersecretion. Respirology 2007:12:683–691.
5. Boucher RC. Evidence for airway surface dehydration as the initiating event in CF airway disease. J Intern Med 2007;261:5–16. [PubMed: 17222164]
6. Mall MA. Role of cilia, mucs and airway surface liquid in mucociliary dysfunction: lessons from the mouse models. JAerosol Med Pulm Drug Deliv 2008;21:13–24. [PubMed: 18518828]

7. Messina MS, O'Riordan TG, Smaldone GC. Changes in mucociliary clearance during acute exacerbations of o asthma. Am Rev Respir Dis 1991;143:993–7. [PubMed: 2024856]
8. Dougherty RH, Fahy JV. Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. Clin Exp Allergy 2009;39:193–202. [PubMed: 19187331]
9. Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from central airways confirmation using a radiolabeled aerosol bolus delivery technique. Am J Respir Crit Care Med 2001;164:1964–70. [PubMed: 11734453]
10. Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. Annu Rev Med 2007;58:157–170. [PubMed: 17217330]
11. Rubin BK, Romkiewicz R, Fahy JV, Green FHY. Histopathology of Fatal Asthma: Drowning in Mucus. Pediatr Pulm 2001;23(Supplement):88–89.
12. Tarran R, Button B, Picher M, Paradiso A, Ribeiro C, Lazarowski E, et al. Normal and cystic fibrosis airway surface liquid homeostasis: the effects of phasic shear stress and viral infections. J. Biol. Chem 2005;280:35751–35759. [PubMed: 16087672]
13. Chen Y, Corriden R, Inoue Y, et al. ATP release guides neutrophil chemotaxis *via* P2Y2 and A3 receptors. Science 2006;314:1792–1795. [PubMed: 17170310]
14. Lennon PF, Taylor CT, Stahl GL, Colgan SP. Neutrophil-derived 5'-adenosine monophosphate promotes endothelial barrier function *via* CD73-mediated conversion to adenosine and endothelial A2B receptor activation. J Exp Med 1998;188:1433–1443. [PubMed: 9782120]
15. Alexis NE, Lay JC, Haczku A, Gong H, Linn W, Hazucha MJ, et al. Fluticasone propionate protects against ozone-induced airway inflammation and modified immune cell activation markers in healthy volunteers. Environ Health Perspect 2008;116:799–805. [PubMed: 18560537]
16. Esther CR, Alexis NE, Clas ML, Lazarowski ER, Donaldson SH, Pedrosa R, Moore CG, et al. Extracellular purines are biomarkers of neutrophilic airway inflammation. Eur Respir J 2008;31:949–956. [PubMed: 18256064]
17. Driver AG, Kukoly CA, Ali S, Mustafa SJ. Adenosine in Bronchoalveolar Lavage Fluid in Asthma. Am Rev Respir Dis 1993;148:91–97. [PubMed: 8317821]
18. Park C, Stafford C, Lockette W. Exercise-induced asthma may be associated with diminished sweat secretion rates in humans. Chest 2008;134:552–558. [PubMed: 18641089]
19. Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC. Nucleotide release provides a mechanism for airway surface liquid homeostasis. J Biol Chem 2004;279:36855–36864. [PubMed: 15210701]
20. MacDowell AL, Peters SP. Neutrophils in Asthma. Curr Allergy Asthma Rep 2007;7:464–468. [PubMed: 17986378]
21. Daviskas E, Anderson SD, Gomes K, Briffa P, Cochrane B, Chan HK, et al. Inhaled mannitol the treatment of mucociliary dysfunction in patients with bronchiectasis: effect on lung function, health status and sputum. Respirology 2005;10:46–56. [PubMed: 15691238]
22. Bush A, Payne D, Pike S, Jenkins G, Henke MO, Rubin B. Mucus properties in children with primary ciliary dyskinesia; comparison with cystic fibrosis. Chest 2006;129:118–123. [PubMed: 16424421]
23. Daviskas E, Anderson SD, Gonda I, Eberl S, Meikle S, Seale JP, et al. Inhalation of hypertonic saline aerosol enhances mucociliary clearance in asthmatic and healthy subjects. Eur. Respir. J 1996;9:725–732. [PubMed: 8726937]
24. Lay JC, Alexis NE, Zeman KL, Peden DB, Bennett WD. Mild asthmatics demonstrate enhanced in vivo uptake of inhaled particles by airway phagocytes compared to normal volunteers. Thorax 2009;64:313–20. [PubMed: 19052052]

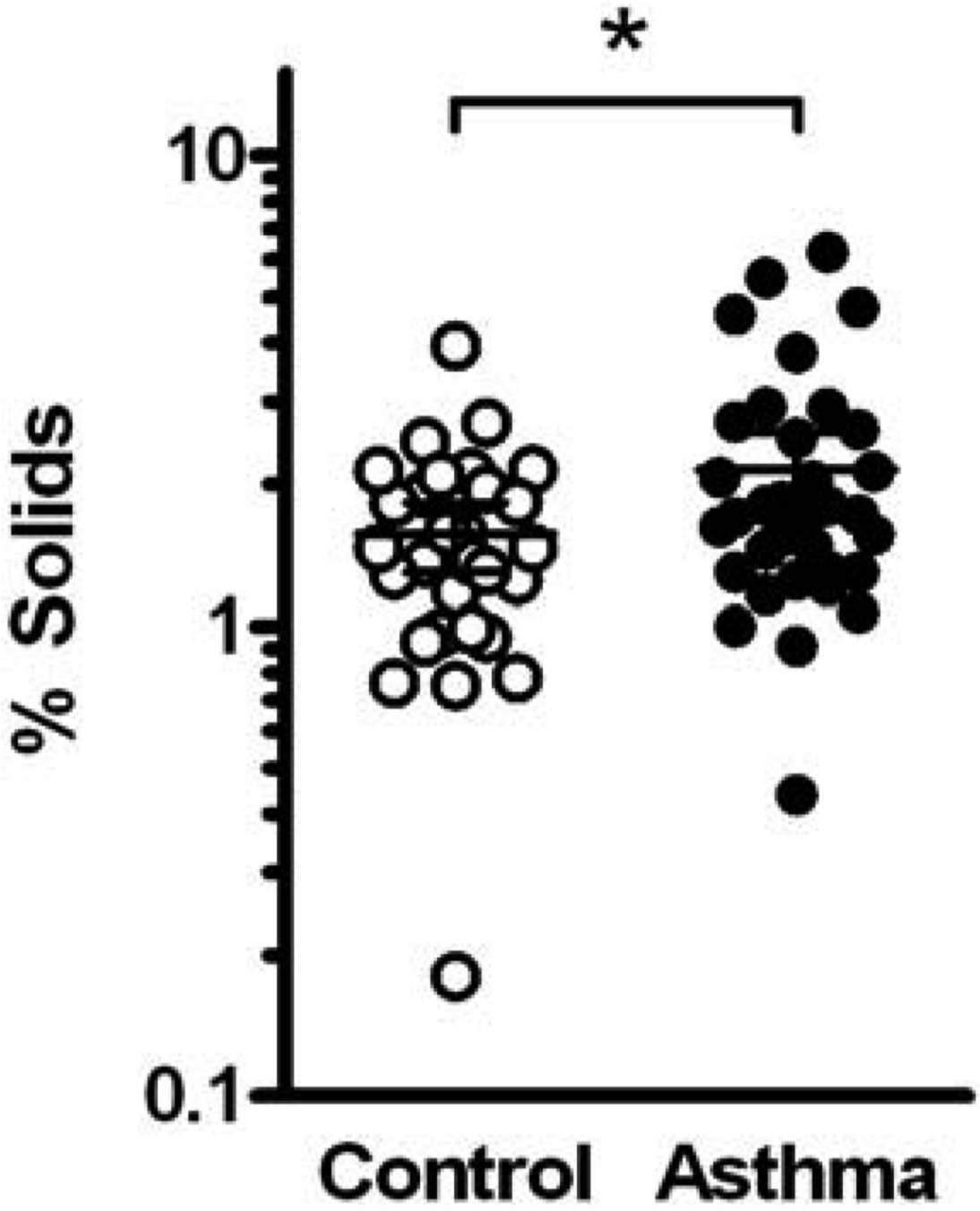


**Figure 1.**
The percent of total weight of sputum samples (95% confidence intervals) which is comprised of solid matter is increased in subjects with stable asthma (2.2%) as compared to normal subjects (1.6%).

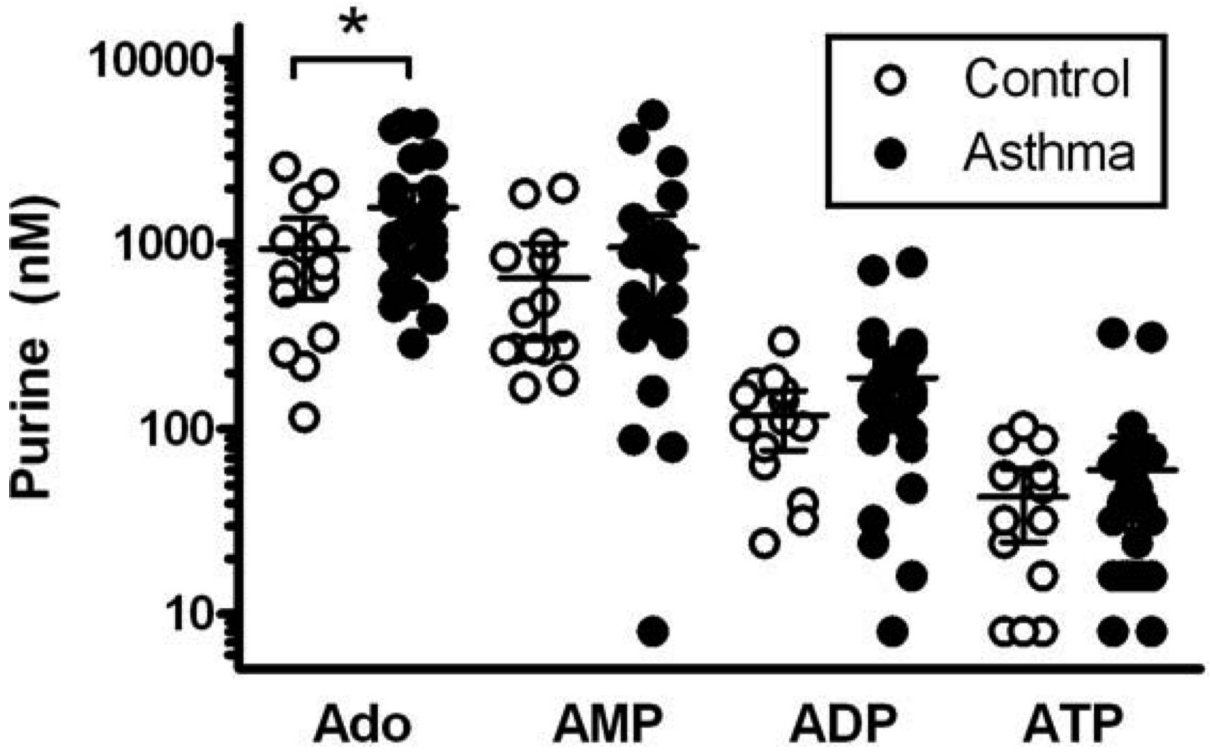


**Figure 2.**
Adenyl purine levels (95% confidence intervals) in sputum samples from asthmatic volunteers (solid bars) were elevated compared to those from normal volunteers (open bars), with this difference being significant for adenosine.

## Table I

Subject demographics and sputum characteristics

| | | Healthy Controls | Asthma |
|---|---|---|---|
| **Subject Demographics** | **n=** | **15** | **37** |
| | **Age** | 27±5.3 | **30±9.5** |
| | **$FEV_1$ (% predicted)** | 108.8 ± 9.4 | 98.7 ± 14.2* |
| **Sputum Cell Counts** | **n=** | **13** | **33** |
| | **TCC/mg** | 1052 ± 664 | 1389 ± 2638 |
| | **PMN/mg** | 236 ± 147 | 769 ± 2359 |
| | **Macrophages/mg** | 797 ± 565 | 603 ± 577 |
| | **Lymphocytes/mg** | 6 ± 5 | 13 ± 32 |
| | **Eosinophils/mg** | 1.2 ± 2 | 24 ± 62* |
| | **Epithelial cells/mg** | 11 ± 18 | 22 ± 29 |

## Table II

Correlations of percent solids with specific sputum endpoints in subjects with asthma.

| | Pearson's r value | P value |
|---|---|---|
| **PMN/mg (n=31)** | **0.5** | **0.001** |
| **TCC/mg (n= 32)** | **0.5** | **0.004** |
| **IL-8 (n=32)** | **0.7** | **<0.0001** |
| **IL-1b (n=7)** | 0.7 | 0.07 |
| **Adenosine (n= 27)** | 0.4 | 0.07 |
| **AMP (n= 27)** | **0.8** | **<0.0001** |
| **ADP (n= 27)** | 0.1 | 0.56 |
| **ATP (n= 27)** | 0.3 | 0.16 |

PMN: polymorphonuclear leukocytes; TCC: total cell count; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate

## Table III

Comparison of TH1 and TH2 correlations with percent solids.

| | Pearson's r | P value |
|---|---|---|
| **TH1 Inflammatory markers** | | |
| PMN/mg (n=31) | 0.5 | 0.001 |
| IL-8 (n=32) | 0.7 | <0.001 |
| **TH2 Inflammatory markers** | | |
| Eosinophils/mg (n=31) | 0.1 | 0.6 |
| IL-4 (n=15) | −0.4 | 0.1 |
| IL-5 (n=7) | 0.1 | ns |
| IL-13 (n=10) | 0.6 | ns |