



Elevated Airway Purines in COPD

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Background: Adenosine and related purines have established roles in inflammation, and elevated airway concentrations are predicted in patients with COPD. However, accurate airway surface purine measurements can be confounded by stimulation of purine release during collection of typical respiratory samples.

Methods: Airway samples were collected noninvasively as exhaled breath condensate (EBC) from 36 healthy nonsmokers (NS group), 28 healthy smokers (S group), and 89 subjects with COPD (29 with GOLD [Global Initiative for Chronic Obstructive Lung Disease] stage II, 29 with GOLD stage III, and 31 with GOLD stage IV) and analyzed with mass spectrometry for adenosine, adenosine monophosphate (AMP), and phenylalanine, plus urea as a dilution marker. Variable dilution of airway secretions in EBC was controlled using ratios to urea, and airway surface concentrations were calculated using EBC to serum urea-based dilution factors.

Results: EBC adenosine to urea ratios were similar in NS (0.20 ± 0.21) and S (0.22 ± 0.20) groups but elevated in those with COPD (0.32 ± 0.30 , $P < .01$ vs NS). Adenosine to urea ratios were highest in the most severely affected cohort (GOLD IV, 0.35 ± 0.34 , $P < .01$ vs NS) and negatively correlated with FEV₁ ($r = -0.27$, $P < .01$). Elevated AMP to urea ratios were also observed in the COPD group (0.58 ± 0.97 COPD, 0.29 ± 0.35 NS, $P < .02$), but phenylalanine to urea ratios were similar in all groups. Airway surface adenosine concentrations calculated in a subset of subjects were $3.2 \pm 2.7 \mu\text{M}$ in those with COPD ($n = 28$) relative to $1.7 \pm 1.5 \mu\text{M}$ in the NS group ($n = 16$, $P < .05$).

Conclusions: Airway purines are present on airway surfaces at physiologically significant concentrations, are elevated in COPD, and correlate with markers of COPD severity. Purinergic signaling pathways are potential therapeutic targets in COPD, and EBC purines are potential noninvasive biomarkers.

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Abbreviations: AMP = adenosine monophosphate; ANOVA = analysis of variance; EBC = exhaled breath condensate; ECLIPSE = Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points; GOLD = Global Initiative for Chronic Obstructive Lung Disease; NS = nonsmoker; S = smoker

Purinergic signaling pathways regulate airways defense mechanisms, including mucociliary clearance and inflammatory responses. These pathways are activated by extracellular purines, which include adenosine triphosphate (ATP) and its metabolites adenosine diphosphate, adenosine monophosphate (AMP), and adenosine. Adenosine, in particular, has been linked to inflammatory airways diseases,¹ including twofold to fourfold elevations in blood^{2,3} and BAL fluid⁴ of subjects with asthma. Similarly, adenosine concentrations in exhaled breath condensate (EBC) are elevated in subjects with stable asthma,^{5,6} increased with asthma exacerbations,⁷ and decreased with successful treatment. Findings from animal models, particularly the adenosine deaminase knockout mouse,^{8,9} further support a link between airway inflammation and lung adenosine.

These data suggest that purinergic signaling pathways may be active in inflammatory lung diseases such as COPD and may be targets for therapeutic intervention.¹⁰ Indeed, elevated airway adenosine concentrations in COPD have been inferred from studies of human airway adenosine receptors,¹¹ and several reviews have touted potential benefits of pharmacologic blockade of adenosine receptors.^{10,12} Although these benefits are a reasonable extrapolation from the animal^{8,13} and human studies,^{4,5} a recent investigation suggested that airway adenosine concentrations (as measured in sputum) may actually be lower in COPD.¹⁴ However, adenosine concentrations in respiratory samples such as sputum or BAL can be altered unpredictably by extracellular metabolism during processing or by mechanical and osmotic forces that trigger purine release during collection.

To assess airway purine concentrations noninvasively in COPD, we applied a previously reported mass spectrometric method^{6,15} to measure adenosine, AMP, and phenylalanine, plus urea as a dilution marker, in EBC collected from participants in two multicenter observational studies. Variable dilution of airway secretions within EBC was controlled by assessing biomarkers as ratios to urea or using serum to EBC urea-based dilution factors.¹⁶ Data were analyzed relative to GOLD (Global Initiative for Chronic Obstructive Lung Disease) stage¹⁷ and lung function (FEV₁ % predicted) to provide insights into the role of purinergic signaling in COPD disease progression.

MATERIALS AND METHODS

Study Subjects

Subjects were participants in one of two observational studies: RES19044 (A Multi-center Cohort Study to Evaluate Radiological, Physiological and Biochemical Biomarkers in Patients with Chronic Obstructive Pulmonary Disease and Age and Gender Matched Controls)¹⁸ or ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points) (clinicaltrials.gov; Identifier: NCT00292552).¹⁹ RES19044 was approved by Institutional Review Boards at St. Elizabeth's Medical Center (00195) and the University of Pennsylvania (801096), and details for ECLIPSE are as previously published.¹⁹ Subjects were diagnosed based on GOLD criteria (using postbronchodilator FEV₁ and FVC) and assigned to one of five cohorts at screening: healthy nonsmokers (NS group), healthy current or former smokers (S group), or one of three COPD cohorts (GOLD stages II, III, or IV). Patients with known respiratory disorders other than COPD were excluded.

Study Design

In both studies, EBC was collected by having subjects exhale through an RTube (Respiratory Research, Inc; Charlottesville, Virginia) using tidal breathing for 10 min, with the chiller held at -70°C until immediately prior to collection. Smokers refrained from smoking ≥ 3 h prior to collection. Samples were stored at -80°C and shipped on dry ice to the University of North Carolina at Chapel Hill for analysis. Samples for RES19044 were collected from 2005 to 2006 and were analyzed in late 2007.

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Samples for ECLIPSE were collected in 2006 and were analyzed in mid-2009. Demographic characteristics of the subjects in whom EBC biomarkers were successfully measured are in Table 1. Pulmonary function tests were performed as previously described.¹⁹ In RES19044, BUN was measured in serum obtained on the day of EBC collection. The samples from ECLIPSE represented a subset of that larger study chosen such that the subject demographics matched those of RES19044.

Mass Spectrometric Analysis

Sixty-one samples from RES19044 and 102 from ECLIPSE were analyzed using a previously published method^{6,15} (Fig 1A) modified to account for variable volumes. As previously described,¹⁵ 500 μ L of EBC (total volume if < 500 μ L) plus a 1:20 ratio of internal standard solution (50 nM [¹³C, ¹⁵N] adenosine, 250 nM [¹⁵N] AMP, and 50 μ M [¹³C] plus isotopically labeled amino acids [NSK-A; Cambridge Isotope Laboratories, Inc; Andover, Massachusetts]) were lyophilized, resuspended, and subjected to mass spectrometric analysis of urea, adenosine, and AMP. A second aliquot of up to 500 μ L was processed as described here for replicate analysis if sufficient volume was available. Detection of phenylalanine (166 \rightarrow 120) and isotopically labeled internal standard (172 \rightarrow 126) was added during the course of the study. Peak areas for biomarkers and internal standards were determined via an automated processing program (Xcalibur; ThermoFinnigan; San Jose, California) and concentrations calculated from standard curves run in parallel. The average of duplicate measures was used when available.

Ten samples (three from RES19044, seven from ECLIPSE) were excluded from further analysis: three likely contaminants (adenosine and AMP levels > 3 orders of magnitude above the mean); seven with urea concentrations below reliable quantification threshold of 1 μ M as determined in a previous study.¹⁵ Samples with subthreshold urea measurements were excluded since we could not determine the extent of dilution of airway secretions within the condensate. Two samples with AMP concentrations below quantifiable limits were assigned a value of one-half of the lowest measured concentration.

Statistical Analysis

EBC and demographic data are reported as mean \pm SD. Log transformation of EBC biomarkers was used to yield normal distributions by D'Agostino-Pearson test. To adjust for differences between studies, a two-way analysis of variance (ANOVA) model was used including study and cohort as factors, using the standard F-test to assess cohort differences. For significant results, pairwise differences were investigated with the Tukey-Kramer post-test adjustment. Linear contrasts were used to assess linear trends across cohorts as well as to test various group differences.

RESULTS

Adenosine and Other Biomarkers Measured in EBC

Airway concentrations of purines and other metabolites were successfully measured in EBC from 153 subjects: 36 healthy nonsmokers (NS group), 28 healthy smokers (S group), and 89 subjects with COPD (GOLD stage II, 29; GOLD stage III, 29; and GOLD stage IV, 31). Age and gender distribution was generally similar among cohorts, whereas FEV₁ was decreased significantly in COPD (Table 1). Use of inhaled corticosteroids was common in the COPD

Table 1—Subject Demographics

Demographic	Control Subjects		Subjects With COPD		
	NS	S	II	III	IV
No.	36	28	29	29	31
Age, y	60.9 ± 9.4	62.5 ± 9.1	60.8 ± 8.2	61 ± 7.5	62 ± 7.6
Gender, male (female)	18 (18)	19 (9)	14 (15)	18 (11)	16 (15)
FEV ₁ , % predicted	108.9 ± 12.6	107.9 ± 12.9	61.9 ± 10.5 ^a	39.8 ± 8.5 ^a	24.1 ± 4.1 ^a
ICS, %	3	0.0	69	97	81
BUN ^b	16.4 ± 6.5	18.8 ± 12.4	16.1 ± 4.1	16.8 ± 5.6	17.4 ± 8.6

II = GOLD stage II; III = GOLD stage III; IV = GOLD stage IV; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroids; NS = healthy nonsmoker; S = healthy smoker.

^a*P* < .01 vs all other cohorts.

^bBUN was assessed in a subset of subjects (16 NS, nine S, 10 GOLD II, seven GOLD III, 11 GOLD IV).

cohorts (Table 1), as was use of inhaled long-acting β agonists (73% of all subjects with COPD) and inhaled anticholinergics (67%). EBC urea correlated significantly with EBC adenosine and AMP ($r = 0.39, 0.41$, respectively, by Spearman, $P < .001$ for both), consistent with urea as a dilution marker. Within-sample reproducibility (accuracy) was assessed through replicate analyses of a subset with sufficient volume ($n = 17$, 11 control subjects, six subjects with COPD), with data analyzed as ratios to urea.⁶ Strong correlations were observed between replicate analyses ($r = 0.96$ for adenosine to urea, $r = 0.93$ for AMP to urea), with good agreement by Bland-Altman analysis (Fig 1B).

EBC Adenosine Is Elevated in COPD and Correlates With Disease Severity

Comparing EBC adenosine to urea ratios in the NS, S, and COPD groups revealed that the ratios differed among the groups ($P < .01$ by ANOVA), with

posttest analyses demonstrating elevations in COPD (0.32 ± 0.30) relative to NS groups (0.20 ± 0.21 , $P < .01$) with a trend toward statistical difference from the S group (0.22 ± 0.20 , $P = .09$) (Fig 2A). In contrast, EBC phenylalanine to urea ratios were similar among the cohorts (Fig 2B). Analysis with COPD groups stratified by GOLD stage revealed that EBC adenosine to urea ratios differed among the cohorts ($P < .03$ by ANOVA), with the highest ratios present in GOLD stage IV (0.35 ± 0.34 , $P < .02$ vs NS) (Fig 2C) and an increasing linear trend between EBC adenosine to urea ratios and cohort ($P < .01$). Smoking status was not independently predictive of EBC adenosine to urea ratio (not shown). Consistent with these observations, a modest but statistically significant negative correlation between EBC adenosine to urea ratio and percent predicted FEV₁ was observed ($r = -0.27$, $P < .01$) (Fig 2D).

Extracellular adenosine and other purines can be derived from metabolism of extracellular ATP,²⁰

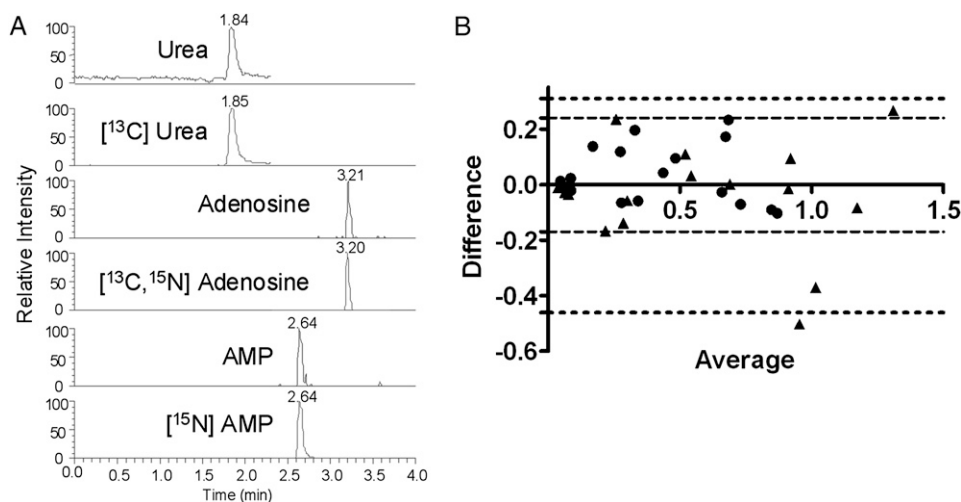


FIGURE 1. A, Chromatogram demonstrating detection of urea, adenosine, AMP, and isotopically labeled internal standards within an exhaled breath condensate (EBC) from a subject with COPD. B, Bland-Altman plot of replicate analysis of 17 samples demonstrated that most replicates fell within the 95% limits of agreement (circles and dotted lines represent adenosine to urea ratio; triangles and dashed lines represent AMP to urea ratio). AMP = adenosine monophosphate.

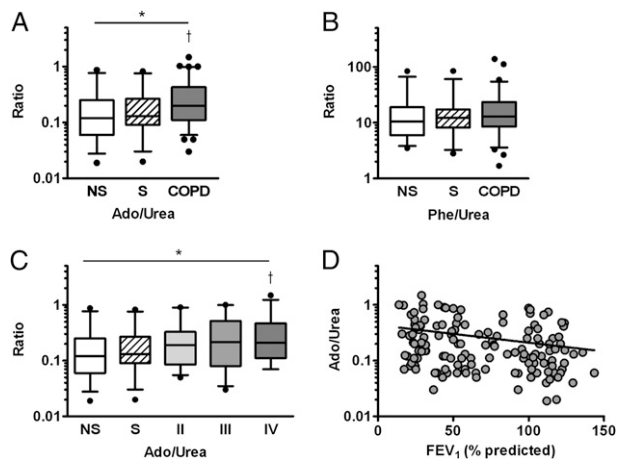


FIGURE 2. EBC Ado was successfully measured in 36 healthy NS subjects (NS group), 28 healthy S subjects (S group), and 89 subjects with COPD (29 GOLD II, 29 GOLD III, 31 GOLD IV) and reported as ratios of Ado (nM) to urea (μM) to control for variable dilution of airway secretions. A, Ado to urea ratios differed among the cohorts and were elevated in subjects with COPD relative to NS. B, EBC Phe was measured from EBC from 26 NS group subjects, 29 S group subjects, and 78 subjects with COPD; the Phe to urea ratio (μM Phe to μM urea) was similar among all cohorts. C, Analysis of Ado to urea ratio with subjects with COPD divided by GOLD stage; cohorts differed by analysis of variance (ANOVA) with Ado to urea ratio elevated in GOLD III and GOLD IV subjects relative to NS group subjects. D, A modest correlation ($r = -0.27$, $P = .002$) was noted between EBC Ado to urea ratio and FEV_1 % predicted. * $P < .05$ by ANOVA, † $P < .05$ vs NS by linear contrast with Tukey-Kramer adjustment. Ado = adenosine; NS = Global Initiative for Chronic Obstructive Lung Disease; NS = nonsmoker; Phe = phenylalanine; S = smoker. See Figure 1 legend for expansion of other abbreviation.

and concentrations of ATP have paralleled its metabolite AMP in previous studies.²¹ Therefore, we hypothesized that extracellular AMP concentrations would be elevated if ATP was the source of elevated purines (our assay was insufficiently sensitive to measure ATP directly). Consistent with this hypothesis, we observed increased EBC AMP to urea ratios in COPD relative to NS groups (0.58 ± 0.97 COPD, 0.29 ± 0.35 NS, $P < .01$). With the subjects with COPD stratified by GOLD stage, EBC AMP to urea ratio differed among the cohorts and was significantly elevated in GOLD stage IV (0.61 ± 0.85 GOLD IV, $P < .05$ vs NS) (Fig 3A), with an increasing linear trend between EBC AMP to urea ratio and cohort ($P < .02$). A weak but statistically significant correlation between EBC AMP to urea ratio and FEV_1 % predicted was also observed ($r = -0.17$, $P < .05$) (Fig 3C).

Airway Surface Concentrations and Dilution in EBC

To assess airway surface concentrations of adenosine, we used a previously described method to calculate dilution factors based on ratios of urea EBC to urea in serum,^{16,22} measured in a subset of subjects (16 NS group subjects, nine S group subjects, 10 with GOLD stage II, seven with GOLD stage III, and 11 with

GOLD stage IV). Serum urea did not vary significantly among cohorts (see Table 1). Airway surface adenosine concentrations differed among the groups and were elevated in COPD cohorts ($3.2 \pm 2.7 \mu\text{M}$ COPD, $1.7 \pm 1.5 \mu\text{M}$ NS, $P < .05$) (Fig 4A) and negatively correlated to FEV_1 % predicted ($r = -0.39$, $P < .01$) (Fig 4C). No statistically significant differences in corrected AMP concentrations were observed within this subset.

Calculated dilution factors within this subset varied widely among the samples and were modestly but not significantly higher in COPD (median, 5,200; interquartile range, [IQR], 3,000-9,000) relative to healthy control subjects (median, 4,300; IQR, 2,000-7,500 in NS plus S groups, $P = .33$). To explore dilution of airway secretions in EBC in all subjects, we examined EBC urea as a surrogate measure of dilution. Interestingly, we observed a modest trend toward decreased EBC urea in subjects with COPD ($P = .08$ by ANOVA), which became statistically significant with both healthy control groups considered together (median, 45 μM ; IQR, 25-83 μM urea healthy control subjects; median, 36 μM ; IQR, 21-55 μM urea COPD; $P < .05$ by Mann-Whitney) (Fig 4A).

DISCUSSION

This study demonstrated that purine concentrations on airway surfaces, as measured through analysis of

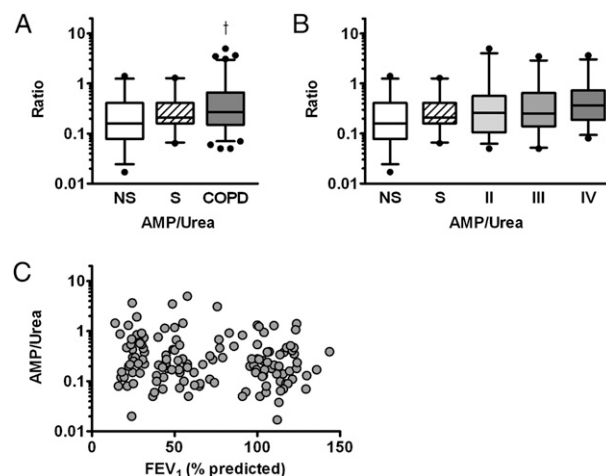


FIGURE 3. EBC AMP was measured and assessed as ratios of AMP (nM) to urea (μM). A, A trend toward group differences in NS, S, and COPD groups was observed ($P = .056$ by ANOVA), and EBC AMP to urea ratio was elevated in subjects with COPD vs NS group subjects. B, Analysis of AMP to urea ratio with subjects with COPD stratified by GOLD stage. Cohorts did not differ significantly by ANOVA ($P = .18$), but a linear trend was noted between EBC AMP to urea ratio and cohort ($r = 0.132$, $P < .05$). C, A weak but statistically significant correlation between EBC AMP to urea ratio and percent predicted FEV_1 was noted ($r = -0.17$, $P < .05$). † $P < .05$ vs NS by linear contrast with Tukey-Kramer adjustment. See Figure 1 and 2 legends for expansion of abbreviation.

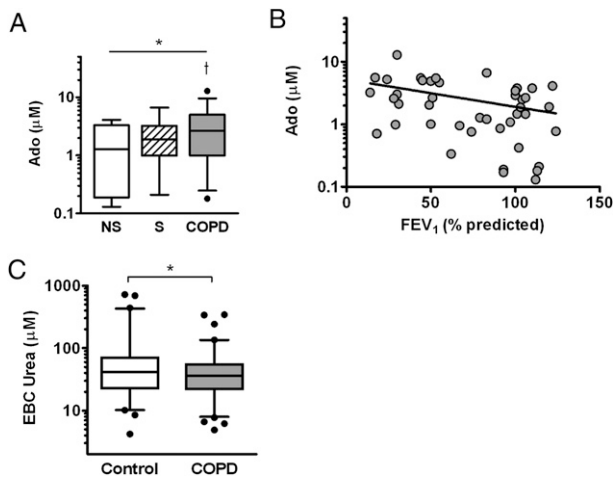


FIGURE 4. A, Concentrations of Ado in airway surface liquid were calculated using EBC to serum urea-based dilution factors. Airway surface Ado concentrations differed among the cohorts and were higher in subjects with COPD relative to NS group subjects. * $P < .05$ by analysis of variance, † $P < .05$ vs NS. B, Airway Ado concentrations correlated with FEV₁ % predicted ($r = -0.39$, $P < .01$). C, EBC urea concentrations (measured after lyophilization) were assessed as a surrogate marker of dilution available in the entire cohort. Values were highly variable but modestly lower in subjects with COPD relative to healthy control subjects. * $P < .05$ by Mann-Whitney. See Figure 1 and 2 legends for expansion of abbreviations.

EBC, are elevated in COPD and correlated with markers of disease severity. These results are consistent with previous human^{4,5} and animal studies^{9,13} that link adenosine and airway inflammation and suggest that this purine plays a pathophysiologic role in COPD.^{10,23,24} Although indirect evidence of elevated airway adenosine in COPD has previously been reported,¹¹ this study represents the first direct measurement of elevated airway adenosine in COPD.

Although the observed differences in airway purine concentrations were modest, they were similar in magnitude to those reported for EBC adenosine in asthma^{5,7} and for other EBC biomarkers in COPD.²⁵ Differences in adenosine may have been masked by treatment effects, since use of inhaled corticosteroids (common within our COPD cohorts) has been associated with reduced EBC adenosine.⁵ In addition, smoking has been shown to alter airway purines^{4,26} and may have made it more difficult to distinguish COPD from healthy smokers, although the strong relationship between COPD and smoking limited our ability to directly assess the impact of smoking in this study. Indeed, our findings support a model in which inflammatory responses to smoking result in increased airway purines (adenosine and AMP), which intensify as chronic inflammation develops and worsens in COPD. This mechanism is consistent with a recently published study demonstrating increased ATP in BAL fluid of smokers and subjects with COPD.²⁶

Our findings suggest that purinergic signaling pathways play a role in the pathophysiology of COPD, differing from other reports suggesting that airway adenosine (measured in sputum)¹⁴ and ATP (measured in EBC)²⁷ are not elevated in this disease. These contradictory findings may reflect issues associated with different methods of collecting and analyzing airway secretions. For example, the sputum derived from the larger airways²⁸ may accumulate adenosine deaminase that lowers adenosine concentrations, whereas adenosine concentrations could remain elevated in the smaller airways from which airways secretions in EBC are generated.²⁹ Alternatively, sputum collection and processing may alter adenosine concentrations, perhaps by osmotic or mechanically stimulated purine release or cellular activity/necrosis during sample preparation.³⁰ Such issues may also affect purine measures in BAL.^{4,26}

Although EBC collection does not introduce chemical gradients or expose airway surfaces to mechanical forces,^{16,22} we cannot exclude the possibility that patient selection, storage time, or the particular EBC collection device used in this study influenced measured concentrations. Furthermore, the very low and highly variable fraction of airway secretions within the condensate posed considerable analytic challenges. We observed a nearly two orders of magnitude variation in dilution factors, consistent with several previous studies.^{27,31,32} Controlling for dilution using ratios to urea or dilution factors provided a more accurate assessment of airway surface concentrations. Furthermore, decreased EBC urea in subjects with COPD suggests that EBC from these subjects may contain a smaller fraction of airway secretions than normal, although these decreases were modest and may have been difficult to detect in smaller studies.²² Overall, these findings reinforce the need to control for dilution when reporting nonvolatile biomarker concentrations in EBC and complicate interpretation of studies in which EBC dilution was not measured, including the previous analysis of EBC ATP.²⁷ Our analyses suggest that a simple ratio to urea is sufficient to control for dilution, and our mass spectrometry method offers the simplicity of measuring urea simultaneously with several biomarkers with the potential of adding novel biomarkers.

Dilution factor measurements allowed us to estimate adenosine concentrations of ~ 1 to $2 \mu\text{M}$ in healthy subjects. These values are much lower than the $60\text{-}\mu\text{M}$ concentrations reported in BAL fluid,⁴ although not dissimilar to values measured in cultured airway epithelia³³ and our own previous studies.⁶ Given the likelihood that mechanical and osmotic forces may alter concentrations of purines measured in lavage or sputum,³⁰ estimates from EBC may more accurately reflect the unperturbed airway, although

potentially limited by experimental and treatment effects noted previously. Nevertheless, our measured concentrations remain above the submicromolar EC₅₀ values of the A₁, A_{2A}, and A₃ adenosine receptors found on airway epithelial and inflammatory cells.³⁴ Although signaling via these receptors triggers complex responses, activation of the A_{2A} receptor (and possibly the A₁ receptor) generally suppresses inflammatory responses.^{1,10} Thus, our results suggest that airway adenosine may be antiinflammatory at the concentrations observed in the healthy airway. In contrast, higher adenosine concentrations in COPD may increase A_{2B} receptor activation, which has a higher EC₅₀³⁴ and has been associated with more proinflammatory responses.¹ Although these hypotheses are appealing, the relationships reflect complex relationships between various receptor subtypes and inflammatory cascades. Furthermore, localized elevations of adenosine (eg, from metabolism of AMP at cell surfaces³⁵) could trigger signaling events without significant alteration of adenosine concentrations in airway secretions as measured by EBC or other methods.

In summary, our data indicate that airway purines are elevated in subjects with COPD and can be assessed simply and noninvasively by mass spectrometric analysis of EBC. These findings suggest that purinergic signaling pathways may be an appropriate target for therapeutic intervention. With further study to better define variability and reproducibility, evaluation of EBC purines (appropriately corrected for dilution) could serve as biomarkers of disease severity in COPD.

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Author contributions: Dr Esther had full access to the data and vouches for the integrity of the manuscript.

Dr Esther: contributed to performing mass spectrometry, data analyses, and wrote and critically reviewed the manuscript.

Dr Lazaar: contributed to providing samples and critically reviewed the manuscript.

Dr Bordonali: contributed to providing biostatistical support and critically reviewed the manuscript.

Dr Qaqish: contributed to providing biostatistical support and critically reviewed the manuscript.

Dr Boucher: contributed to supervising the project and critically reviewed the manuscript.

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