Mass spectrometric analysis of biomarkers and dilution markers in exhaled breath condensate reveals elevated purines in asthma and cystic fibrosis

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Esther CR Jr, Boysen G, Olsen BM, Collins LB, Ghio AJ, Swenberg JW, Boucher RC. Mass spectrometric analysis of biomarkers and dilution markers in exhaled breath condensate reveals elevated purines in asthma and cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 296:L987–L993, 2009. First published March 20, 2009; doi:10.1152/ajplung.90512.2008.—Exhaled breath condensate (EBC) contains airway secretions that can be utilized to perform quantitative analyses of EBC biomarkers simultaneously with urea to control for dilutional variability. These studies focused on adenosine and AMP as biomarkers, adenylyl purines that are released onto the airway surfaces by resident airway cells and can act as signaling molecules to regulate host defenses. Several studies suggest that airway release of adenylyl purines is altered by disease, and elevated levels of adenylyl purines have been reported in subjects with asthma (6, 16). In addition, airway secretions from subjects with asthma exhibit elevated levels of ATP that are correlated with the severity of neutrophil inflammation (10). This correlation could reflect contribution of neutrophils to extracellular purine levels, since this inflammatory cell releases ATP, which is metabolized primarily to AMP (19, 20) as part of a purinergic signaling pathway critical for neutrophil chemotaxis (5). However, other mechanisms underlying the relationship between purines and neutrophils have not been excluded.

In this study, we assessed the utility of our mass spectrometric analysis of EBC purines and urea in two studies: 1) a cross-sectional study of healthy children compared with children with asthma or CF; and 2) a longitudinal study of CF subjects at the beginning and end of treatment of a pulmonary exacerbation. EBC was collected and analyzed using a recently described mass spectrometric method to measure the purines adenosine and AMP plus urea as a dilution marker. These studies provided insight into both the utility of the mass spectrometric approach to analysis of EBC purine biomarkers and the pathophysiological role of purinergic signaling in airways disease.

MATERIALS AND METHODS

Subjects. Subjects were children aged 4–19 yr who received care at North Carolina Children’s Hospital in 2006–2007 (Table 1). For the cross-sectional study, healthy controls were recruited from the University of North Carolina (UNC) General and Adolescent Pediatric Clinic, were free of known respiratory disease, and denied acute or chronic respiratory symptoms. Physician-diagnosed CF and asthma...
subjects were recruited from the Children’s Pulmonology Clinic at UNC during regularly scheduled visits. CF was diagnosed using Cystic Fibrosis Foundation criteria (25); asthma was a clinical diagnosis made by the subject’s pediatric pulmonologist. For the CF exacerbation study, CF subjects were recruited within 48 h of admission to the inpatient unit for intravenous antibiotics to treat a pulmonary exacerbation with treatment plan determined by the attending CF physician. EBC was collected at the time of recruitment and again within 48 h of discontinuation of antibiotics. Seventeen CF subjects were included in both the cross-sectional and CF exacerbation studies. All studies were approved by the institutional review board, and informed consent was obtained.

**Laboratory values.** Spirometry was obtained using a flow-based SensorMedics Vmax Spirometer (SensorMedics, Yorba Linda, CA), and percent predicted values were calculated using National Health and Nutrition Examination Survey (NHANES) III normative data (13). Serum blood urea nitrogen (BUN) was analyzed by the UNC Hospitals core laboratory using standard procedures.

**EBC collection.** EBC was collected using the RTube device (Respiratory Research, Charlottesville, VA). Subjects exhaled through the RTube for 7 min with the chiller sleeve at −80°C until analysis. Although salivary contamination was not specifically excluded, EBC collected using the RTube has been found to be free of salivary contamination when collected under supervised conditions such as those of this study (15).

**Table 1. Subject demographics**

<table>
<thead>
<tr>
<th>Study</th>
<th>Control</th>
<th>CF</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number enrolled</td>
<td>35</td>
<td>46</td>
<td>14</td>
</tr>
<tr>
<td>Not detected</td>
<td>7</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Number analyzed</td>
<td>28</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr</td>
<td>11.3±4.0</td>
<td>12.6±3.7</td>
<td>10.4±5.3</td>
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<tr>
<td>Sex, % male</td>
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<td>53</td>
<td>36</td>
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<tr>
<td>Race, % Caucasian</td>
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<td>88</td>
<td>36</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>79.1±20.1</td>
<td>91.2±18.0</td>
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<tr>
<td>Number mild disease</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Number prescribed ICS</td>
<td>16</td>
<td>18</td>
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</table>

**CF exacerbation**

<table>
<thead>
<tr>
<th>Study</th>
<th>Control</th>
<th>CF</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number enrolled</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>3</td>
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</tr>
<tr>
<td>Not detected</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number analyzed</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>12.1±4.1</td>
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<tr>
<td>Sex, % male</td>
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<tr>
<td>Race, % Caucasian</td>
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<tr>
<td>Pretreatment FEV1, % predicted</td>
<td>69.8±24.0</td>
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<td></td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>11.0±3.2</td>
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</tbody>
</table>

Values are means ± SD. *Mild disease defined as percent predicted forced expiratory volume in 1 s (FEV1) >80% for cystic fibrosis (CF) by National Heart, Lung and Blood Institute guidelines for mild persistent disease for asthma. The 2 asthma subjects who did not have mild disease were classified as having moderate persistent asthma. †ICS, inhaled corticosteroids, either fluticasone (dose range 176 –500 µg daily) or budesonide (dose range 0.25–0.5 mg daily). Two subjects with CF and 1 subject with asthma had received oral corticosteroids (10-20 mg prednisone daily) before the study visit. BUN, blood urea nitrogen.

**Statistical analysis.** Purine concentrations from EBC studies were analyzed as ratios to urea or through calculation of dilution factors as described in the text. Because these data did not follow a normal distribution (P < 0.05 by D’Agostino and Pearson omnibus normality test), they were analyzed using nonparametric procedures: Mann-Whitney for comparison between groups, Wilcoxon matched pairs for paired data comparisons, Spearman coefficients for correlations, and Kruskal-Wallis for ANOVA with Dunn’s multiple comparison for posttest comparisons. All demographic measures in Table 1 are presented as means ± SD, and all EBC measures are graphed as medians ± interquartile range (IQR).

**RESULTS**

**EBC purines are elevated in CF: the cross-sectional study.** To determine whether airway purines were altered in respiratory disease, we measured EBC purines and urea using a highly sensitive and reliable LC-MS/MS-based protocol (Fig. 1; Ref. 11). EBC was collected from 34 healthy controls, 45 children with CF, and 14 children with asthma as disease controls during regular clinic visits (cross-sectional study, Table 1). Purines and urea successfully measured from a total of 28 healthy control, 40 CF, and 11 asthma EBCs (Table 1).

As expected from previous studies of EBC dilution, we observed a wide variability in purines and urea concentrations in these samples (Fig. 2A). The EBC concentrations of the dilution marker urea were significantly different among the groups and highly correlated with both adenosine and AMP (r = 0.65 and 0.66, respectively; P < 0.05), suggesting that much of the measured variability in purine concentrations resulted from variable dilution of airways secretions within the condensate. Therefore, we examined ratios of EBC constituents as an established method to control for dilution (15, 23). Using ratios (nanomolar purine to micromolar urea), we found that the AMP-to-urea ratio was significantly elevated in CF (median 1.3, IQR 0.7–2.0) compared with healthy control (median 0.75, IQR 0.3–1.4; Fig. 2B). In addition, we observed an elevation in the EBC adenosine-to-urea ratio in subjects with asthma (median 1.5, IQR 0.9–2.9) compared with control (median 0.4, IQR 0.2–1.6), in agreement with previous studies.
in this disease (6, 16). The adenosine-to-urea ratio in CF subjects (median 1.0, IQR 0.5–1.8) displayed a trend toward a modest increase ($P \approx 0.10$) relative to control. There were no significant differences between asthma and CF in either ratio ($P > 0.1$). In the CF subjects, we did not observe any relationship between the adenosine-to-urea or the AMP-to-urea ratio and either the presence of pathogens on culture or with lung function [percent predicted forced expiratory volume in 1 s (FEV1)] obtained at the time of the EBC.

A significant fraction of the CF population was prescribed inhaled corticosteroids (ICS), a treatment that has been shown to influence adenosine in EBC (16). Therefore, we examined EBC purine ratios in the CF subjects relative to treatment with ICS (Fig. 2C). Within the CF subjects prescribed ICS, the EBC adenosine-to-urea ratio (median 1.1, IQR 0.88–2.2) was elevated relative to healthy controls ($P < 0.03$). Within the CF subjects not prescribed ICS, the EBC adenosine-to-urea ratio (median 0.70, IQR 0.41–1.4) was not significantly different from either healthy controls ($P = 0.13$) or CF subjects prescribed ICS ($P = 0.08$). In contrast, EBC AMP-to-urea values were similar between both CF groups (median 1.3, IQR 0.7–2.9 prescribed ICS, median 1.3, IQR 1.3–2.1 not prescribed ICS; $P = 0.95$), and AMP-to-urea remained elevated relative to control even after excluding all CF subjects prescribed ICS ($P < 0.02$).

The data set contained several elevated values that could be considered outliers based on visual examination of the data or statistical considerations (values >4 SD from the mean). Exclusion of these outliers (3 control, 3 CF, 2 asthma) did not significantly alter our findings, since we continued to observe

Fig. 1. Detection of urea, adenosine, and AMP in exhaled breath condensate (EBC) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). EBC samples were analyzed using a previously described LC-MS/MS method (10). A sample chromatogram from an EBC collected from a cystic fibrosis (CF) subject is shown, demonstrating specific detection of urea, adenosine, AMP, and their stable isotopically labeled internal standards.

Fig. 2. EBC purines are elevated in children with CF after controlling for dilution. A: concentrations of EBC urea, adenosine (Ado), and AMP were measured by LC-MS/MS from control ($n = 28$, white symbols), CF ($n = 40$, gray symbols), and asthmatic ($n = 11$, black symbols) children. The wide range of urea values suggested significant dilutional variability. Posttest analysis revealed significant differences between CF and asthma urea concentrations, although interpretation is limited by the relatively small number of subjects in the asthma group. Measured values after lyophilization are reported; native EBC values would be 20-fold lower. Reported concentrations are micromolar for urea and nanomolar for purines.

B: analysis of ratios of EBC urea, adenosine, and AMP (nanomolar purine to micromolar urea) to control for dilution revealed within group differences in adenosine-to-urea and AMP-to-urea ratios. Posttest analyses showed that EBC adenosine-to-urea ratio was elevated in both asthma relative to control and that EBC AMP-to-urea ratio was elevated in CF relative to control. *$P < 0.05$ for group by 1-way ANOVA, †$P < 0.05$ vs. control by Dunn’s multiple comparison test.

C: subgroup analyses of the CF subjects treated with inhaled corticosteroids (ICS+, $n = 16$) or not treated with ICS (ICS−, $n = 22$). The adenosine-to-urea ratios were higher in subjects receiving ICS relative to healthy controls ($P < 0.05$) and exhibited a trend toward statistical significance relative to CF subjects not treated with ICS ($P = 0.08$). The AMP-to-urea ratios were similar in both ICS-treated and untreated CF groups, and this ratio remained elevated relative to control after exclusion of CF subjects treated with ICS ($P < 0.02$). Two subjects receiving oral corticosteroids were excluded from this analysis. All data are shown as medians ± interquartile range (IQR).
significant differences in the adenosine-to-urea ratios between control and asthma (median 0.30, IQR 0.15–0.90 control; median 1.4, IQR 0.85–1.6 asthma; \( P < 0.05 \)) and in the AMP-to-urea ratios between control and CF (median 0.60, IQR 0.25–1.2 control; median 1.2, IQR 0.68–1.9 CF; \( P < 0.05 \)).

**EBC purine**s in the **CF** exaggeration group. Since treatment of CF exacerbations reduces neutrophilic airway inflammation (24), we hypothesized that treatment of exacerbations would reduce EBC AMP-to-urea ratios. We tested this hypothesis by enrolling CF subjects in the CF exaggeration study, from which EBC purines and urea were successfully measured in 26 EBC collection pairs. For this group, treatment averaged 20.1 ± 8.2 days, and lung function increased significantly after treatment (percent predicted FEV\(_1\) 69.8 ± 24.0 start antibiotics, 80.2 ± 22.3 end antibiotics; \( P < 0.001 \)).

Contrary to our predictions, neither the AMP-to-urea nor any other ratio changed significantly after treatment (Fig. 3A). However, we noted that the treatment response, as assessed by changes in lung function, varied considerably among subjects. Therefore, we examined the relationship between variability in treatment response (as indexed by changes in lung function) and changes in EBC purine ratios. These studies revealed a significant negative correlation between changes in percent predicted FEV\(_1\) and changes in the AMP-to-urea ratio over the treatment interval (Fig. 3B) and a similar correlation between change in percent predicted FEV\(_1\) and the adenosine-to-urea ratio (Fig. 3C). Overall, these data suggest that EBC purine ratios track the therapeutic response to treatment in CF.

**Alternate methods to control for EBC dilution.** Although use of purine-to-urea ratios to control for dilution has the advantage of simplicity, this method does not account for variations in airway urea levels between subjects. These variations can theoretically be controlled using measures of serum urea, which are believed to provide a reasonable estimate of airway urea levels (7). We obtained serum urea values measured on admission in all subjects in the CF exacerbation study (Table 1), which were then used to calculate dilution factors. As expected, we observed variation in serum urea values, with a coefficient of variation of 29%. The calculated dilution factors were similar at the start and end of antibiotic treatment (25,300 ± 5,000 start, 26,500 ± 5,000 end; \( P = 0.8 \)), and the magnitude of dilution was consistent with previous reports (9). Using dilution factor corrected AMP concentrations, we continued to observe a correlation between changes in EBC AMP and changes in percent predicted FEV\(_1\) in the CF exacerbation study (Fig. 4A). Interestingly, the measured coefficients of variation were slightly higher for dilution factor-corrected purine levels for purine-to-urea ratios (coefficients of variation 1.25 corrected adenosine, 1.08 adenosine/urea; 1.24 corrected AMP, 0.94 AMP/urea). Thus, despite our predictions, the use of corrected dilution factors did not reduce the data variability.

The validity of urea as a dilution marker assumes that airway urea values are not significantly affected by disease pathology, which has been demonstrated in COPD (9) but not CF. To determine whether urea levels were altered in CF, we compared EBC urea concentrations to EBC concentrations of the electrolytes sodium plus potassium as an independent dilution marker. We hypothesized that CF-induced variations in airway urea values would be reflected in altered EBC electrolyte-to-urea ratios since serum electrolytes show little variation (coefficient of variation of sodium plus potassium 1.3% in the longitudinal study), and airway electrolyte concentrations are similar in CF and healthy airways (17). Sufficient volume for EBC electrolyte analysis was available in a subset of samples (\( n = 9 \) control and 8 CF from the cross-sectional study and 9 paired samples from the CF exacerbation study). In these samples, EBC urea and EBC electrolyte levels were significantly correlated (Fig. 4B), consistent with the hypothesis that both assess dilution of airway secretions. Furthermore, the electrolyte-to-urea ratios in control EBCs were similar to those in CF EBCs from both the cross-sectional and CF exacerbation studies (Fig. 4C), suggesting that airway urea levels were not significantly altered in CF. Assuming an airway fluid isosmotic to normal plasma, we calculated that electrolyte-based dilution factors were 18,000 ± 1,600, similar to the dilution factors previously assessed using urea. Using these factors,
increase in EBC AMP-to-urea in samples from CF relative to airway secretions within EBC. Our primary finding was an increase in EBC AMP-to-urea in samples from CF relative to control subjects (plus potassium) were measured as an alternative dilution marker in 35 samples from control subjects (10). The reasons for this discrepancy are not clear. It is possible that continued purine release and metabolism during isolation and processing of sputum or lavage fluid may have exaggerated differences between groups. Alternatively, the airway secretions contained within EBC may differ from those represented in sputum or BALF. The source of airway secretions in EBC is not well-understood but has been postulated to represent entraining of airway surface liquid microdroplets generated from turbulent airway flow (15). This mechanism would suggest a large number of patent airways throughout the lung contribute to droplet formation, whereas sputum and lavage fluid are generally derived from the most diseased airways. Furthermore, it is possible that microdroplet formation is reduced in the most diseased airways such that these are underrepresented within the condensate, a hypothesis consistent with our observation that the EBC urea concentrations are lower in subjects with asthma and CF, respectively (10).

We also observed an increase in the EBC adenosine-to-urea ratio in subjects with asthma. This finding is not unexpected since elevated levels of airway adenosine in asthma have been previously reported (6, 16). Interestingly, we observed a trend toward increased EBC adenosine-to-urea within our CF cohort, although previous studies had suggested that adenosine was not elevated in CF (10). It is possible that the EBC adenosine-to-urea ratio is affected by the subset of CF subjects with asthma, a relatively common comorbidity in CF populations (1). Indeed, we found that the EBC adenosine-to-urea ratio was elevated in the subset of CF subjects prescribed ICS, suggestive of a physician diagnosis of cormorbid asthma. Although further investigation is needed, these findings support previous studies suggesting that adenosine and AMP track different aspects of airway inflammation found in asthma and CF, respectively (10).

Although the elevated EBC AMP-to-urea ratio in CF was consistent with our predictions, the ~2-fold differences between CF and control were much less than those previously observed in sputa or bronchoalveolar lavage fluid (BALF; Ref. 10). The reasons for this discrepancy are not clear. It is possible that continued purine release and metabolism during isolation and processing of sputum or lavage fluid may have exaggerated differences between groups. Alternatively, the airway secretions contained within EBC may differ from those represented in sputum or BALF. The source of airway secretions in EBC is not well-understood but has been postulated to represent entraining of airway surface liquid microdroplets generated from turbulent airway flow (15). This mechanism would suggest a large number of patent airways throughout the lung contribute to droplet formation, whereas sputum and lavage fluid are generally derived from the most diseased airways. Furthermore, it is possible that microdroplet formation is reduced in the most diseased airways such that these are underrepresented within the condensate, a hypothesis consistent with our observation that the EBC urea concentrations are lower in subjects with asthma and CF (see Fig. 2A). Increased metabolism of adenosine and AMP values in the CF exacerbation group were correlated with changes in lung function (1). Indeed, we found that the EBC adenosine-to-urea ratio is affected by the subset of CF subjects with asthma, a relatively common comorbidity in CF populations (1). Indeed, we found that the EBC adenosine-to-urea ratio was elevated in the subset of CF subjects prescribed ICS, suggestive of a physician diagnosis of cormorbid asthma. Although further investigation is needed, these findings support previous studies suggesting that adenosine and AMP track different aspects of airway inflammation found in asthma and CF, respectively (10).

DISCUSSION

Our results demonstrate successful application of the EBC biomarker approach to analysis of airways disease in children, using mass spectrometry to simultaneously measure purine biomarkers and urea to control for known variable dilution of airway secretions within EBC. Our primary finding was an increase in EBC AMP-to-urea in samples from CF relative to control and a relationship between EBC AMP-to-urea levels and treatment response to an acute exacerbation. These findings suggest that the EBC AMP-to-urea has potential to serve as a biomarker of CF airways disease.

The simplest interpretation of these results is that EBC AMP-to-urea is a biomarker of neutrophilic airway inflammation, which has been shown to be increased in CF compared with controls and decreased in CF subjects after effective treatment of an exacerbation (24). This explanation is consistent with the reported accumulation of extracellular AMP by activated neutrophils (19, 20) and the reported correlation between AMP and neutrophilic inflammation in other airway samples (10). However, since we did not directly assess an established marker of neutrophilic airway inflammation, we cannot rule out the possibility that the elevated EBC AMP-to-urea ratios in CF reflect some other aspect of CF airways disease. Furthermore, we cannot definitively exclude the possibility that salivary contamination influenced our results, although such contamination has not been previously observed with the RTube under supervised collections such as those of our study (15). Although the presence of such contamination could contribute to the outliers in our data set, we note that our significant findings for CF and asthma are not altered by exclusion of these outliers.

We also observed an increase in the EBC adenosine-to-urea ratio in subjects with asthma. This finding is not unexpected since elevated levels of airway adenosine in asthma have been previously reported (6, 16). Interestingly, we observed a trend toward increased EBC adenosine-to-urea within our CF cohort, although previous studies had suggested that adenosine is not elevated in CF (10). It is possible that the EBC adenosine-to-urea ratio is affected by the subset of CF subjects with asthma, a relatively common comorbidity in CF populations (1). Indeed, we found that the EBC adenosine-to-urea ratio was elevated in the subset of CF subjects prescribed ICS, suggestive of a physician diagnosis of cormorbid asthma. Although further investigation is needed, these findings support previous studies suggesting that adenosine and AMP track different aspects of airway inflammation found in asthma and CF, respectively (10).

Although the elevated EBC AMP-to-urea ratio in CF was consistent with our predictions, the ~2-fold differences between CF and control were much less than those previously observed in sputa or bronchoalveolar lavage fluid (BALF; Ref. 10). The reasons for this discrepancy are not clear. It is possible that continued purine release and metabolism during isolation and processing of sputum or lavage fluid may have exaggerated differences between groups. Alternatively, the airway secretions contained within EBC may differ from those represented in sputum or BALF. The source of airway secretions in EBC is not well-understood but has been postulated to represent entraining of airway surface liquid microdroplets generated from turbulent airway flow (15). This mechanism would suggest a large number of patent airways throughout the lung contribute to droplet formation, whereas sputum and lavage fluid are generally derived from the most diseased airways. Furthermore, it is possible that microdroplet formation is reduced in the most diseased airways such that these are underrepresented within the condensate, a hypothesis consistent with our observation that the EBC urea concentrations are lower in subjects with asthma and CF (see Fig. 2A). Increased metabolism of

Fig. 4. Alternate methods to control for EBC dilution. A: airway concentrations of adenosine and AMP values in the CF exacerbation group were estimated using serum and EBC urea values to calculate a dilution factor for airway secretions with EBC. Corrected AMP levels remained significantly correlated with changes in lung function (n = 25). B: EBC electrolytes (sodium plus potassium) were measured as an alternative dilution marker in 35 samples from control subjects (n = 9), CF subjects during the cross-sectional study (n = 8), and CF subjects during CF exacerbation study (n = 18). A significant correlation between EBC electrolytes (Na + K) and EBC urea was observed, suggesting that both urea and electrolytes were functioning as dilution markers. C: the ratio of electrolytes to urea measured in EBC from control subjects was similar to the ratio measured in EBC from CF subjects enrolled from the cross-sectional (Clinic), the CF exacerbation (ABX), and in all CF values combined (All), suggesting that airway urea levels are not significantly altered by CF lung disease.
purines in EBC could also account for the differences but seems unlikely since we have previously demonstrated that ATP is stable within EBC (10), and our use of isotopically labeled internal standards controlled for metabolism occurring during specimen processing. The discrepancies between purines levels in EBC, sputum, and BALF highlight the need to better understand the physiological mechanisms and sites of generation for each of these airway samples.

Consistent with many previous reports (2, 8, 9, 11), we found that the fraction of airway secretions contained within EBC was highly variable as assessed using either EBC urea or electrolytes as dilution markers. These findings strongly reinforce the need to control for dilution when reporting nonvolatile EBC biomarkers. Our methodology has the advantage of allowing simultaneous measurement of both a dilution marker (urea) and biomarkers (purines) within one relatively small sample volume. Although use of urea as a dilution marker has been a topic of controversy, much of this controversy stems from studies of BALF in which diffusion of urea into the lavage fluid during the procedure can significantly confound results (14). Since EBC collection does not modify airway surface conditions during collection, such limitations should not apply (7, 9). Our results provide further validation for urea as dilution marker in EBC and suggest that a simple ratio of biomarker to urea may suffice to control for dilution. Although use of a simple ratio does not control for potential variations in airway urea, we found that use of serum urea measurements to control for individual variations in airway urea did not alter our conclusions and actually increased variability within the data set. These data suggest that variations in airway concentrations of urea and other dilution markers are not driven primarily by variations in their serum concentrations but may reflect other influences that are not well understood. We conclude that a biomarker-to-urea ratio may be sufficient to control for vari- able dilution in EBC, allowing measurement of airway biomarkers controlled for dilution without need for even a minimally invasive blood draw.

Using the dilution factors calculated using electrolytes or urea, we estimated that the average adenosine and AMP concentrations on airway surfaces in healthy children are in the low (1–5) micromolar range. Interestingly, these estimates were significantly lower than the 60 μM airway adenosine concentrations derived from a previous study of BALF (6). The reasons for this discrepancy are not clear, although we note that the osmotic and mechanical forces generated during lavage could confound evaluation of purines and/or urea (14, 28). Our airway adenosine concentration estimated from EBC are similar to the reported EC50 values for many adenosine receptors (A1, A2A, and A3) found on resident airway and inflammatory cells (12, 21), suggesting that even the modest increases in adenosine concentration observed in this study could have physiological impact.

In summary, our data suggest that EBC purines are biomarkers of airway disease when controlled for variable dilution and more specifically that the EBC AMP-to-urea ratio may serve as a biomarker of airways disease in children with CF. With further investigation, this finding could form the basis for a simple and noninvasive method to assess the disease severity and the response to treatment. Furthermore, the use of a flexible mass spectrometry-based assay allowed for simultaneous measurement of a dilution marker and offers the potential to add other biomarkers to the EBC analysis in the future. However, our results also underscore the fact that additional investigation is needed to validate the EBC approach to assessing airway biomarkers in CF or other diseases.

GRANTS

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