Extracellular Purines are Biomarkers of Neutrophilic Airway Inflammation

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Running Title: Purines as Biomarkers
Abstract

Purinergic signaling regulates airway defense mechanisms, suggesting that extracellular purines could serve as airway inflammation biomarkers in cystic fibrosis (CF).

The purines ATP, ADP, AMP, and adenosine were measured in sputum from 21 adults (spontaneously expectorated from 7 CF, induced from 14 healthy controls) to assess normal values and CF associated changes. Subsequently, purine levels were measured in bronchoalveolar lavage fluid (BALF) from 37 children (25 CF, 12 disease controls) and compared to neutrophilic counts, presence of airway infection, and lung function. To non-invasively assess airway purines, ATP levels were measured using luminometry in exhaled breath condensate (EBC) from 14 children with CF and 14 healthy controls, then 14 CF children during a pulmonary exacerbation.

Both ATP and AMP were elevated in sputum and BALF from CF subjects compared to controls. In BALF, ATP and AMP levels were inversely related to lung function and strongly correlated with neutrophil counts. In EBC, ATP levels were increased in CF relative to controls and decreased after treatment of CF pulmonary exacerbation.

The purines ATP and AMP are candidate biomarkers of neutrophilic airways inflammation. Measurement of purines in sputum or EBC may provide a relatively simple and non-invasive method to track this inflammation.

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Text Word Count: 2,998 words
Introduction

Extracellular adenyl purines, including the purine nucleotide ATP and its metabolites, are important signaling molecules on airway surfaces. These purines serve as agonists for purinergic signaling pathways that play critical roles in airways mucus clearance. Indeed, ATP mediated signaling regulates airway surface liquid (ASL) volume, ciliary function, and mucin secretion (1-3); each critical to control of mucus clearance rates.

Purines also mediate multiple components of inflammatory cell responses that are part of lung defense. For example, pro-inflammatory stimuli enhance release of ATP from epithelial (4-6) and inflammatory cells (7-10). In particular, stimulated neutrophils release ATP and accumulate both extracellular ATP and AMP (10, 11). Inflammation also impacts airway luminal extracellular purine metabolism, both by altering ectonucleotidase activity on epithelial cell surfaces (12) and by addition of ectonucleotidase activity associated with the accumulation of inflammatory cells (8). ATP released onto airway surfaces in response to inflammation also stimulates inflammatory cell responses, including chemotaxis and degranulation in neutrophils (10, 13-15), cytokine production and oxidative bursts in macrophages (16-18), and activation of lymphocytes and eosinophils (17). Adenosine formed in consequence to ATP release also acts as a signaling molecule with both pro-inflammatory and anti-inflammatory effects (19, 20).

The importance of purinergic signaling in lung inflammation has been demonstrated *in vivo*. For example, ATP receptor (P2Y<sub>1</sub>-R and P2Y<sub>2</sub>-R)-deficient mice exhibit impaired inflammatory responses and difficulty eradicating *Pseudomonas* from the lung (21). Similarly, mouse strains with modest increases in lung adenosine have enhanced IL-4 mediated inflammatory responses.
(22), and higher lung adenosine levels lead to airway inflammation and pulmonary fibrosis (23, 24). *In vivo* studies of airway purines in humans are limited, although increased levels of ATP have been observed in nasal lavage fluid (25) and blood (26) of subjects with CF, and adenosine levels in airway secretions are elevated in untreated asthma and correlate with disease state (27-29).

The role of purines as mediators of the inflammatory response suggests that they may also be markers of inflammation. However, the concentrations and pattern of extracellular adenyl purines in the normal and diseased human airways surface remain largely unexplored. The goal of this study was to measure the levels of purines in human airway secretions and evaluate their potential as biomarkers of airway inflammation, particularly in children with cystic fibrosis (CF). We first measured purine levels in sputum to establish normal values and assess changes associated with CF. In addition, we measured purine levels in secretions aspirated from CF lungs removed for transplantation. Next, we collected BALF from children undergoing clinically indicated bronchoscopy and sought correlations between purines and established markers of airways disease, including neutrophil counts, presence of infection, and lung function. Finally, we explored a simple and non-invasive method to measure airway purines in children by measuring ATP levels in exhaled breath condensate (EBC).

**Methods**

**Study Subjects**

Subject demographics are outlined in Table 1. Control populations were healthy individuals, except in the BALF study, which included the following disease controls: 2 subjects with
primary ciliary dyskinesia and 10 subjects with recurrent cough or wheeze, all of whom were clinically stable at the time of bronchoscopy. All human subjects were studied at the University of North Carolina at Chapel Hill, and studies were approved by the Institutional Review Board.

**Study design**

Differences between purine levels in airway secretions were assessed by comparison of CF to control populations. The relationship between purine levels and other markers of disease were assessed by regression analysis.

**Methods**

*Sputum* from healthy controls was collected and processed using induction using previously described methodology (30). Briefly, mucus plugs were selected, weighed, and incubated with 0.1% dithiotreitol to solubilize mucus. 0.32% sodium citrate was added to the sample buffers to limit hydrolysis of purines. Samples were then washed in Dulbecco's PBS, filtered, and analyzed for cell viability, cell counts, and differential. Sputum from CF subjects was collected by spontaneous expectoration, but was processed identically to induced samples.

*Supernatant of Mucopurulent Material (SMM)* was recovered from the airway lumens of 13 excised human CF lungs at the time of transplant (31). This material was centrifuged at 440,000 x g for 60 min at 4 °C, and the supernatant filtered through a 0.2-µm filter and frozen at −80 °C.

*BALF* was obtained from clinically indicated bronchoscopy. Aliquots were placed on ice, centrifuged at 11,000 g x 5 minutes at 4 °C to remove cells and bacteria, and the supernatant
immediately frozen and stored at -80 °C. Separate aliquots were processed for cell differential and quantitative microbiological culture.

*EBC* was collected using the RTube device from Respiratory Research, Inc. (Charlottesville, VA). The chiller tube was held at -10 °C until immediately before the collection, and the subject exhaled through the device during 7 minutes of tidal breathing. No nose clips were used. EBC was recovered from the RTube and frozen at -80 °C until analysis.

**Purine analysis.** Adenyl purines were measured in airway secretions using etheno-derivitization and HPLC (32). Samples were boiled 2 minutes prior to analysis to inactivate nucleotidases.

**Luminometry.** The luciferin–luciferase assay was a modification of a previously described protocol (33). In brief, 100 µl aliquots from each sample were analyzed in the light chamber of an LB953 AutoLumat luminometer (Berthold GmbH) after injection of a 100 µl of a luciferin–luciferase cocktail (luciferin 160 µg/ml and luciferase 8 µM, 100 µl/assay). Luminescence was recorded for 10 seconds and compared with an ATP calibration curve performed in parallel.

**Analysis.** All data are expressed as mean ± standard error, except demographic information, reported as mean ± standard deviation. Data which did not follow a normal distribution by D'Agostino-Pearson tests were log transformed prior to analysis, including all purine measurements and neutrophil counts. Comparisons between groups were performed using Student’s T-test and correlations performed using Pearson’s correlation.

**Results**
Purine levels in normal induced sputum and CF sputum

To establish normal purine levels and assess whether these were altered in CF, we measured purines in sputum collected by induction from 14 healthy adults and spontaneously expectorated from 7 adult CF subjects. Given the relationship between purines and inflammation, we hypothesized that sputum purines would be elevated in subjects with CF, a disease characterized by high levels of airway inflammation. Purines were measured using etheno-derivatization and HPLC, a technique which has been successfully utilized to measure purines from a variety of biological fluids (32). Assessment of recovery and metabolism of purines in sputum revealed that purines could be readily recovered and measured in both control and CF sputa (recovery rates 89% control, 81% CF), and that purine metabolism during processing could be limited by addition of sodium citrate to the sample (see Supplemental data).

Analysis of sputa revealed the expected increase in neutrophilic inflammation in the CF sputa (14,700 neutrophils/mg sputum CF, 240 neutrophils/mg sputum control, p=0.01). Differences in the pattern and concentration of purines were also observed. Analysis of purines in normal sputa revealed a pattern of ADO ≈ AMP > ADP > ATP. In contrast, CF sputa contained increased levels of both ATP and AMP, but not ADP or ADO, relative to controls (Figure 1A). Indeed, the ATP to adenosine ratio was elevated in sputa from CF subjects compared to control (CF 33.0 ± 6.6, Control 0.99 ± 0.2, p=0.003), as was the AMP to adenosine ratio (CF 1.85 ± 0.4, Control 0.08 ± 0.0, p=0.007). To ensure that induction procedure in controls did not impact the results, we examined purine levels in paired spontaneously expectorated and induced sputum from three individuals (1 CF, 2 COPD). Purine levels were actually modestly higher (~2-fold) in the
induced samples, suggesting that the differences between CF and control could not be attributed to use of induction in the control population (data not shown).

To assess whether these findings were reproducible in airway secretions not accessible to expectoration, we obtained supernatants of mucopurulent material (SMM) aspirated from the airways of explanted CF lungs at the time of transplantation. The pattern of purines in the SMM from thirteen explanted CF lungs was similar to that observed in CF sputum, with proportionally higher levels of AMP and relatively low levels of adenosine (Figure 1B). Thus, the pattern of purines in CF secretions appears to be independent of sample collection technique.

Airway purines are elevated in BALF from children with airways disease and correlate with neutrophilic bronchitis

We wished to determine if the relationship between purines and airways disease would be observed in children. Because children often cannot expectorate sputum, we obtained airway secretions using bronchoalveolar lavage. We prospectively collected and analyzed BALF from 25 children with CF and 12 non-CF subjects (disease controls [DC]). Similar to the previous data, we observed higher levels of both ATP and AMP in CF BALF, compared to DC BALF, with AMP present at the highest absolute concentration (Figure 2A). Neither ADP nor adenosine levels differed among the groups.

While we observed higher levels of ATP and AMP in BALF from CF subjects, we could not distinguish whether the differences were CF specific or reflected the higher levels of airway bacterial infection and/or neutrophilic bronchitis that characterize CF airways disease(34). To
determine the variables most closely correlated with purine levels, we utilized a multiple regression model to assess whether CF status, presence of airway infection (>50,000 pathogens/ml on BALF culture), or neutrophil cell count best predicted ATP or AMP levels in BALF. In the resulting model, only neutrophilic counts emerged as a significant predictor of ATP or AMP levels, with strong correlations to both ATP and AMP (Figure 2B). In contrast, neutrophil counts were not strongly correlated with either adenosine (r=-0.22, p=0.19) or ADP (r=0.25, p=0.14). Similar correlations were observed in the sputum samples between neutrophil counts and both ATP (r=0.75, p=0.0007) and AMP (r=0.81, p=0.0001), although the results are limited by the small number of subjects in whom accurate cell counts could be obtained (n=3 CF, 14 control). These data suggest that ATP and AMP are primarily biomarkers of neutrophilic inflammation.

Airway purines correlate with lung function

Because neutrophilic inflammation mediates many of the clinical manifestations of CF airways disease, including declines in lung function, we tested for correlations between purine levels in BALF and lung function. Although we observed significant correlations between raw ATP and AMP values with lung function (data not shown), we recognized that variable dilution of airway secretions in BALF could confound the relationship between BALF biomarkers and clinical outcomes. We attempted to control for dilution using the BALF to serum urea ratio, a widely used albeit problematic dilution marker (35). However, we observed a correlation trend between urea based dilution factors and neutrophil counts (R=-0.40, p=0.056), suggesting that BALF urea levels were artifactually increased in diseased airways, perhaps by increased efflux of urea through inflamed epithelia during lavage (35). Therefore, we examined an alternative method to
control for dilution using ratios of ATP or AMP to adenosine, since ratios are not affected by
dilution. This method is based on the observation that both ATP and AMP correlate with
markers of airway disease, whereas adenosine does not. As anticipated, we observed significant
correlations for both ATP/adenosine and AMP/adenosine ratios with the percentage of
neutrophils as dilution-independent markers of bronchitis (Figure 3A). Furthermore, we also
observed significant correlations between the purine ratios and percent predicted FEV₁ (Figure
3B). These data indicate that with appropriate methods to control for dilution, airway ATP and
AMP levels correlate with a clinically relevant index of disease severity as expected for a
biomarker of neutrophilic inflammation.

ATP in EBC is correlated with airway inflammation

While our data demonstrate that the purines ATP and AMP are potential biomarkers of
neutrophilic inflammation in children with CF, risks and technical challenges associated with
bronchoalveolar lavage limit clinical application. To determine if airway purines could be
measured using a simple and non-invasive method in children, we explored the feasibility of
detecting purines in exhaled breath condensate (EBC).

In a pilot study, we obtained EBC from a small group of healthy and CF children (n=4 each
group). Initial analyses using etheno-derivitization and HPLC revealed that EBC purine
concentrations were below the etheno-derivitization detection threshold in most individuals (data
not shown). As a more sensitive method, we utilized the highly sensitive and specific
luminometry method, which is widely used to measure ATP levels in biological samples(25, 36,
37). With luminometry, we detected a signal above background in each of our pilot EBC
samples (840 ± 320 arbitrary light units [ALU], range 157 to 2428). Consistent with the signal being ATP-specific, the signal disappeared with sample pre-incubation for 30 min with 2 units/ml of the ATP degrading enzyme apyrase (post treatment 15 ± 5 ALU, Figure 4A), but not in time control incubations. Based on comparison to known standards, EBC ATP concentrations were in the low picomolar range, consistent with those measured in BALF and sputum after taking into account the $10^3$ to $10^4$ (or higher) dilution of airway secretions in EBC (38, 39). At this concentration range, reproducibility was modest, with within-sample coefficients of variation between 20 to 30%. For this reason, EBC ATP levels were reported as the average of duplicate measures.

To further assess EBC ATP levels with the luminometric method, the pilot study was expanded to include EBCs collected from a total of 18 children with CF and 14 healthy controls during regular clinic visits. Consistent with our previous studies, EBC ATP levels were elevated in children with CF compared to healthy controls (Figure 4B). Because treatment of a CF exacerbation reduces neutrophilic airway inflammation (40), we hypothesized that EBC ATP levels would drop during treatment. To explore this relationship, we collected EBC from 14 children with CF at the beginning and end of a course of intravenous antibiotics for a pulmonary exacerbation, including four CF subjects from the previous study. Treatment lasted an average of 21.6 + 3.3 days with antibiotics chosen by the treating physician. As predicted, lung function improved after antibiotic treatment (% predicted FEV₁ 60.1% + 20.1% pre antibiotics, 70.1% + 19.9% post antibiotics, p = <0.01). This improvement was associated with a 3-fold fall in EBC ATP levels (Figure 4C), consistent with previously observed changes in sputum markers of neutrophilic inflammation after treatment (40). Although these data are preliminary, they do
indicate that EBC ATP levels fit the pattern expected for a biomarker of neutrophilic inflammation.

**Discussion**

Extracellular purines are the signaling molecules of purinergic signaling pathways that regulate airway defenses, including mucociliary clearance and intraluminal inflammatory responses. In this study, we demonstrate that both the pattern and concentration of these purines have a strong relationship to CF airways inflammation. Analyses of secretions from CF subjects obtained as sputum, SMM, and BALF provided a remarkably consistent picture in which AMP was the dominant purine, and both ATP and AMP were elevated in CF compared to control (Table 2). This pattern of purines differs significantly from the relatively equal levels of AMP and adenosine observed in sputum from normal subjects, which in turn is similar to that reported in cultures of normal airway epithelia (32). Although we studied primarily subjects with CF, the data suggest that the relationship between purines and neutrophilic inflammation is not CF specific. However, further study is necessary before we can confidently generalize our findings to non-CF airways disease.

While we did not identify the source airway purines in this study, the strong correlation between purine levels and neutrophil counts suggests that neutrophils may be a major contributor to purine levels in CF airway secretions. This conclusion is consistent with previous studies showing that activated neutrophils exhibit increased release of ATP and accumulate AMP(41). However, purine release from necrotic or apoptotic cells cannot be excluded, nor can we rule out a significant contribution from inflamed airway epithelia or other inflammatory cells. Although
bacteria are another potential source of extracellular purines, it seems unlikely that bacteria significantly contributed to ATP release at numbers commonly found in CF sputum (~10^7/ml) (42). The accumulation of AMP suggests a relative absence of 5’ nucleotidase metabolism of AMP to adenosine, a pattern which has been observed in isolated neutrophils (which lack 5’ nucleotidase) but not airway epithelia (32, 43). Therefore, the high levels of AMP in samples obtained from diseased airways suggest that neutrophils dominate the metabolic pattern of purine nucleotides on these airway surfaces.

Regardless of the underlying mechanism(s), the strong correlation with neutrophil counts indicates that airway levels of ATP and AMP are potential biomarkers of neutrophilic inflammation. Our data suggest that measurement of ATP in EBC may hold promise as a simple and non-invasive method to assess the degree of neutrophilic airway inflammation in children. Indeed, the approximately 3-fold reduction in EBC ATP levels after treatment of CF pulmonary exacerbation parallels the reduction of neutrophil cell counts and IL-8 levels in sputum after similar treatment (40).

However, our results also highlighted many of the known limitations to EBC analysis. The measured ATP concentrations were low and near the threshold for detection, suggesting that small technical artifacts or variations could significantly impact our results. Furthermore, the small volumes of EBC collected precluded measurement of both ATP and dilution markers. Consequently, systematic differences in sample dilutions among groups could not be assessed and could contribute to the observed differences. Therefore, further improvements in
methodology are needed to more thoroughly evaluate the clinical potential of EBC purine measurements.

While ATP and AMP emerged as potential biomarkers, we did not observe similar findings for adenosine. While the levels of adenosine in bronchoalveolar lavage were similar to previously published values (27), we did not observe the elevated levels of airway adenosine (27, 28) or correlation to disease severity previously described for asthma (29). This difference may reflect fundamental differences in airway pathophysiology between asthma and CF. CF is characterized by a pathogen and neutrophil dominated airway inflammation that differs significantly from the less cellular, eosinophil rich, and relatively sterile airway inflammation observed in asthma. These findings suggest that factors responsible for increased adenosine levels in asthma are not common in the neutrophilic bronchitis that characterizes CF.

In addition to exploring the potential of airway purines as biomarkers of inflammation, our studies also provide new insights into purinergic signaling pathways in airways disease. While we were unable to accurately determine the dilution in our BALF samples, both the ATP and adenosine levels in BALF were consistent with previous reports (25, 27), and previous studies suggest that airway secretions in BALF were likely diluted 10-100 fold (35). Given this level of dilution, in situ ATP levels would be above the EC_{50} value of for the P2Y_{2} ATP receptor in airway epithelia (0.24 \mu M) (44) and approach the levels needed to activate P2Y_{2} receptors in inflammatory cells (13, 45). These considerations suggest that ATP mediated purinergic signaling may be adaptively increased in bacterially infected airways, leading to higher mucociliary clearance and greater activation of inflammatory cells. Similarly, the average
adenosine levels in both healthy and CF airways are predicted to be above the EC50’s of the A1 (EC50 0.31 µM) and A2A (EC50 0.73 µM) adenosine receptors found on inflammatory cells (46, 47), though somewhat lower than the EC50 of the dominant adenosine receptor (A2B) expressed in airway epithelia (32). Because adenosine levels did not correlate with markers of disease, we would not anticipate that adenosine mediated signaling would increase with neutrophilic inflammation, and the increased ATP to adenosine ratio might favor a more pro-inflammatory environment (20). However, recently published evidence suggests that AMP may serve as an adenosine receptor agonist, either directly (48) or after conversion to adenosine at the epithelial surface (49). Thus, increased adenosine receptor signaling, mediated through AMP, could modulate increased inflammation.

In conclusion, we demonstrate that purines recovered in airway secretions are biomarkers of neutrophilic airway inflammation and are elevated in CF airways disease. Our results suggest that measurement of purines in EBC may be useful as a non-invasive method to assess airway inflammation, although further refinements are necessary.

Acknowledgements.

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References


Figure Legends

Figure 1. Airway purines are elevated in CF. A. Sputum was obtained by spontaneous expectoration from adults with CF (n=7) and after induction in healthy controls (n=14), and purines measured in isolated supernatants. Both ATP and AMP were significantly elevated in CF compared to control, while ADP and adenosine (Ado) levels were similar between groups. B. Supernatant of mucopurulent material (SMM) was obtained from the explanted lungs of adults with end stage cystic fibrosis at time of lung transplantation (n=13). The pattern of purine levels in SMM was similar to that observed in CF sputum.
Figure 2. **Purines in BALF from children are elevated in CF and correlate with neutrophilic airway inflammation.** A. BALF was obtained from children with CF (n=25) and disease controls (n=12) with other respiratory diseases. Measurement of purines in the supernatant of this BALF revealed elevated levels of ATP and AMP in the CF group. B. Statistically significant correlations were observed between neutrophil counts and levels of both ATP and AMP in BALF.
Figure 3. Purine ratios correlate with neutrophilic airway inflammation and lung function.

Ratios of ATP to adenosine (Ado) and AMP to adenosine were calculated as markers of purine levels corrected for variable dilution of airway secretions in BALF. A. Both ATP/Ado and AMP/Ado ratios were strongly correlated with percentage of neutrophils as a dilution independent marker of inflammation. B. Purine ratios were compared to spirometry data available from a subset of subjects (n=28, 20 CF and 8 disease controls). Both ATP/Ado and AMP/Ado ratios were negatively correlated with percent predicted FEV₁.
Figure 3

A

![Graph A](image)

- ATP/Ado: $r = 0.67$, $P < 0.0001$
- AMP/Ado: $r = 0.76$, $P < 0.0001$

B

![Graph B](image)

- ATP/Ado: $r = -0.43$, $P = 0.0240$
- AMP/Ado: $r = -0.41$, $P = 0.0335$
Figure 4. ATP in EBC is elevated in CF and decreases with treatment of a CF exacerbation.

ATP levels in EBC were measured by luminometry. A. Pilot EBC collections were obtained from four healthy controls (1-4) and four CF subjects (5-8). A luminescent signal was detected in all samples that was stable to incubation at 37 °C for 30 minutes (compared to samples kept at 0° on ice), and disappeared after incubation with the 2 units/ml of the ATP degrading enzyme apyrase (Apy). B. ATP levels were measured in EBC from CF (n=14) and healthy controls (n=14). ATP was elevated in the CF group. C. ATP levels in EBC were measured at the start and end of a course of antibiotics to treat a pulmonary exacerbation in children with CF (n=14). ATP levels were lower at the end of treatment.
Figure 4

A

![Bar chart showing ALU levels across different subject numbers and temperatures.]

B

![Scatter plot comparing ATP levels in control and CF conditions.]

C

![Line graph showing ATP levels from start to end.]

P = 0.0441

P = 0.0175
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<tr>
<td>Sputum</td>
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<td>26.9 ± 5.4</td>
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<td>6M/8F</td>
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<tr>
<td>BALF</td>
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<tr>
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<tr>
<td>FEV1 (% predicted)*</td>
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<tr>
<td>Age (yrs)</td>
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<tr>
<td>Gender</td>
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<tr>
<td>FEV1 (% predicted)*</td>
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* Lung function results were not available for 3 CF subjects in the sputum study, 4 CF and 4 disease controls in the BALF study, and 1 subject in the EBC study.
Table 2

Purine composition of airway secretions

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<th>AMP</th>
<th>Ado</th>
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<td>36.2 ± 4.3</td>
<td>51.2 ± 4.9</td>
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<td>CF</td>
<td>Sputum 8.0 ± 3.5</td>
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<td>84.8 ± 7.0</td>
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<td>SMM 14.1 ± 3.1</td>
<td>9.1 ± 1.9</td>
<td>69.5 ± 5.5</td>
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</tr>
<tr>
<td></td>
<td>BALF 2.3 ± 0.2</td>
<td>2.7 ± 0.8</td>
<td>82.9 ± 4.0</td>
<td>12.0 ± 3.9</td>
</tr>
</tbody>
</table>

Relative percentage of each purine is listed by subject and sample type. The pattern of airway purines is similar in various airway samples, but differs in healthy versus CF subjects.