Biomarkers in Lung Diseases: from Pathogenesis to Prediction to New Therapies

Sialic acid-to-urea ratio as a measure of airway surface hydration

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Esther CR Jr, Hill DB, Button B, Shi S, Jania C, Duncan EA, Doerschuk CM, Chen G, Ranganathan S, Stick SM, Boucher RC. Sialic acid-to-urea ratio as a measure of airway surface hydration. *Am J Physiol Lung Cell Mol Physiol* 312: L398–L404, 2017. First published January 6, 2017; doi:10.1152/ajplung.00398.2016.—Although airway mucus dehydration is key to pathophysiology of cystic fibrosis (CF) and other airways diseases, measuring mucus hydration is challenging. We explored a robust method to estimate mucus hydration using sialic acid as a marker for mucin content. Terminal sialic acid residues from mucins were cleaved by acid hydrolysis from airway samples, and concentrations of sialic acid, urea, and other biomarkers were analyzed by mass spectrometry. In mucins purified from human airway epithelial (HAE), sialic acid concentrations after acid hydrolysis correlated with mucin concentrations ($r^2 = 0.92$). Sialic acid-to-urea ratios measured from filters applied to the apical surface of cultured HAE correlated to percent solids and were elevated in samples from CF HAEs relative to controls (2.2 ± 1.1 vs. 0.93 ± 1.8, $P < 0.01$). Sialic acid-to-urea ratios were elevated in bronchoalveolar lavage fluid (BALF) from β-epithelial sodium channel (ENaC) transgenic mice, known to have reduced mucus hydration, and mice sensitized to house dust mite allergen. In a translational application, elevated sialic acid-to-urea ratios were measured in BALF from young children with CF who had airway infection relative to those who did not (5.5 ± 3.7 vs. 1.9 ± 1.4, $P < 0.02$) and could be assessed simultaneously with established biomarkers of inflammation. The sialic acid-to-urea ratio performed similarly to percent solids, the gold standard measure of mucus hydration. The method proved robust and has potential to serve as flexible techniques to assess mucin hydration, particularly in samples like BALF in which established methods such as percent solids cannot be utilized.

mucins; mass spectrometry; dehydration

HEALTHY AIRWAYS finely regulate absorption vs. secretion on airway surfaces to maintain appropriate hydration of the mucus layer, which is critical to effective mucociliary transport (2). These regulatory processes are often disrupted as part of disease pathogenesis, leading to increased mucus concentrations. Cystic fibrosis is perhaps the best known example of this disruption, with loss of the CFTR chloride channel leading to airway surface liquid (ASL) volume depletion and airway mucus dehydration (7). More recently, elevated airway mucus concentrations have also been reported in other diseases including asthma (24) and chronic obstructive pulmonary disease (COPD) (1).

Although measuring mucus concentration is critical to understanding airways disease pathophysiology, assessing airway hydration status in vivo poses considerable challenges. Mucins themselves can be difficult to measure since they are extremely large macromolecules whose heavy glycosylation shields most of the protein backbone from immunological measurement techniques (18). The more exposed NH_2- and COOH-terminal regions can be recognized by antibodies but are prone to proteolytic degradation in the setting of disease that can make immunological detection unreliable (19). Physical methods such as refractometry can be used to assess macromolecular content (18, 22) but require specialized equipment and expertise.

Given the challenges of measuring mucins directly, the practical gold standard for assessing the concentration of airway mucus is to measure its % solid content (4, 18). Percent solids is defined as the dry weight of the solid content, typically dominated by mucins, divided by the total wet weight of the ASL sample. This method has been shown to closely track the intact mucin concentration in sputum as assessed by physical techniques (1). While conceptually simple, accurately assessing dry and wet weights requires collection of neat airway samples without perturbing the hydration state, typically as expectorated sputum. However, this makes % solids difficult to utilize when airway samples volumes are small and/or are diluted during recovery, e.g., with bronchoalveolar lavage (BAL), which is particularly relevant to animal models and children.

As an alternative approach, we hypothesized that small molecule biomarkers that correlate with the solid (mucin) and the fluid phases of ASL could be used to develop a surrogate measure of mucus concentration. Mucins contain a large number of terminal sialic acid residues that can be released through acid hydrolysis (5, 8), and sialic acid concentrations measured

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after hydrolysis could in theory serve as a marker of the solid, mucin phase of ASL. The fluid phase could be assessed through analysis of urea, which is found in a relatively narrow range in most mammals and diffuses freely throughout fluid compartments (9). Therefore, we hypothesized that the sialic acid-to-urea ratio (SA/Urea) could function as a marker of mucus concentration similar to percent solid measures. An advantage of the SA/Urea ratio is that it could be applied to sample types such as BALF in which dilution of airway secretions during lavage makes percent solid measurements impossible. Furthermore, since both sialic acid and urea can be measured via mass spectrometry (11, 32), we hypothesized that using sensitive mass spectroscopic methods would permit application in small volume samples as well as simultaneous measurement of other metabolomic biomarkers.

To test these hypotheses, we developed mass spectrometric methods to measure sialic acid and urea, then applied these methods to model systems known to exhibit altered mucus concentrations including cystic fibrosis (CF) human airway epithelia and mouse models of disease (26). We then determined whether these methods could be used to address factors in early CF lung disease associated with changes in mucin hydration.

METHODS

Human airway epithelia (HAE) were grown at air-liquid interface, and mucus was obtained from the apical surface as previously described (16, 21). Briefly, excess surgical tissue was procured by the University of North Carolina (UNC) Tissue Core Facility. Normal human bronchial epithelial cells were cultured on 0.4-mm pore-sized Millicell membranes (Millipore, Billerica, MA) coated with collagen and maintained in air-liquid interface medium (UNC Tissue Core) as described in Ref. 20. Over a period of 6 wk, confluent cultures developed cilia and transported mucus. Apical mucus samples were obtained by gently placing preweighed nitrocellulose filters on the apical surface of the HAE and allowing them to equilibrate over 12 h.

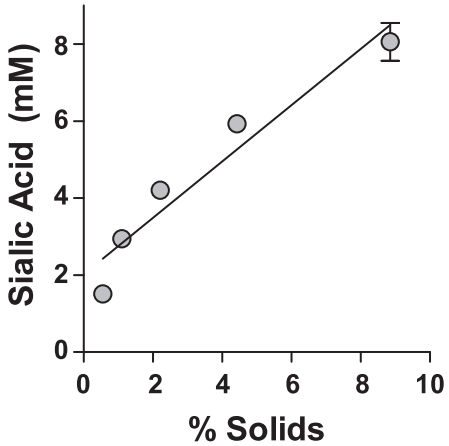

Fig. 1. Relationship between mucin concentration and acid hydrolyzed sialic acid concentrations. Human airway secretions were isolated from human airway epithelial (HAE) allowed to accumulate mucins to high concentration, measured for percent solid content, then serially diluted over the range 0.45 to 9% solids. Each dilution was treated with formic acid and heat as described, and the sialic acid concentrations were measured by mass spectrometry (n = 3 per dilution). Sialic acid concentrations were highly correlated to percent solids. There was a significant linear relationship between percent solids and sialic acid ($r^2 = 0.92$, $P < 0.001$), although the curve of best fit was a second-order polynomial ($r^2 = 0.98$).

Urea was added to the basolateral medium at a concentration of 10 μM at least 24 h before the filters. Filters were removed, weighed, and dried to completeness in a 100°C oven and reweighed to obtain percent solids measures defined as (dry weight of filter − filter weight)/(wet weight of filter − filter weight).

BAL fluid (BALF) samples from mice were collected as previously described (23) from mice overexpressing the *Scnn1b* gene, which codes for the β-subunit of ENaC, a sodium channel expressed, in airway epithelial cells (βENaC-Tg mice) (27). These mice were backcrossed to the C57BL/6N background, and the colony was maintained by breeding transgenic to wild-type (WT) mice. Offspring were genotyped, and WT littermates served as controls. BALF was obtained by lavage with PBS at 7.5 mo of age. BALF was also collected via these methods from 2- to 3-mo-old C57/B6J mice sensitized to house dust mite antigen (HDM) (Greer, Lenoir, NC) as previously described (33). Briefly, mice were sensitized by intranasal inhalation of 1 μg HDM daily × 2 days, then exposed to 10 μg HDM by intranasal inhalation daily × 7 days. BALF was collected 12–24 h after the last HDM exposure. All animals were housed in individually ventilated microisolator cages in a facility maintained at the UNC at Chapel Hill on a 12-h day/night cycle. They were fed a regular chow diet and given water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee of the UNC at Chapel Hill and performed according to the principles outlined by the Animal Welfare and the National Institutes of Health (NIH) guidelines for the care and use of animals in biomedical research.

BALF samples from human subjects were collected as part of a previous studies (10, 14) using 1–2 aliquots of 1 ml/kg of sterile saline as the lavage fluid. The sample set included samples collected at the UNC (n = 10) and Australian Respiratory Early Surveillance Team for CF (AREST CF) (n = 4). All studies were approved by the UNC IRB (IRB nos. 07-0787, 12-1538).

Acid hydrolysis to release sialic acid was performed by adding formic acid to a final concentration of 1% and incubating the samples at 80° for 1 h. Before incubation, an internal standard solution of isotopically labeled purines, urea, and amino acids was added to the samples as previously described (12, 13). The internal standard was modified to include [^{13}C]sialic acid at a final concentration of 100 μM. Samples from HAE were centrifuged at 11,000 *g* for 5 min, and the supernatant was utilized for mass spectrometry as described below. BALF samples were centrifuged through a 10-kDa size-selection filter (EMD Millipore, Billerica, MA) before mass spectrometry.

Mass spectrometry was performed via a previously described method (10) using a Quantum-Ultra triple quadrupole mass spectrometer (Thermo-Finnigan, San Jose, CA) in multiple reaction monitoring mode with chromatographic conditions similar to those previously described [UPLC T3 HSS C18 column, methanol/formic acid gradients (11, 12)]. The previous method was modified to include monitoring transitions specific for sialic acid (*m/z* 308→87 in negative mode) and [^{13}C]sialic acid (*m/z* 311→90 in negative mode). All samples were filtered through a 10-kDa size selection filter (EMD Millipore) before analysis. Biomarker signals were defined as ratios to the relevant internal standard using the stable isotope dilution method (6) to control for losses during processing and matrix effects on mass spectrometric detection. Concentrations were calculated via comparisons to standard solutions run in parallel.

RESULTS

To determine whether sialic acid could serve as a surrogate marker for mucins, we made serial dilutions of ASL isolated from cultured HAE cells in which the mucin content had been determined (21). Previously described methods for acid hydrolysis of terminal mucin sialic acid residues (8, 29) were modified to use formic acid, which is more compatible with our mass spectrometric analysis technique. After treatment with 1% formic acid at 80°C for 1 h, we observed a strong corre-

lation between sialic acid and percent solid measurements in these human airway samples (Fig. 1). The correlation was generally linear ($r^2 = 0.92$), though due to modest deviations at high percent solids the curve of best fit was a second-order polynomial.

We next assessed whether this approach could be utilized to directly measure mucus concentrations in a cell culture model of HAE grown at air-liquid interface. As a marker of the fluid phase of ASL, we chose to measure urea, since it freely diffuses through fluid compartments and is commonly utilized as a marker of airway fluid dilution (28). However, since we observed that HAE do not typically produce significant concentrations of urea (data not shown), we added urea to the basolateral medium to simulate in vivo conditions. Urea was allowed to equilibrate throughout the fluid phase for at least 24 h before experiments.

Filters were placed on the apical surface of HAE, allowed to absorb mucus, then removed and assessed for percent solids (wet-to-dry ratios). The dried filters were then acid hydrolyzed to release mucin sialic acid and analyzed by mass spectrometry. To ensure that the acid hydrolysis did not alter urea concentrations, we obtained aliquots from three filters before treatment as well as 1 h and 2 h after incubation with 1% formic acid at 80°C. In these experiments, sialic acid concentrations increased >40-fold after 1 h of treatment (Fig. 2*A*), but urea concentrations were not changed. Extending the 80°C incubation time to 2 h did not significantly increase sialic acid or alter urea concentrations.

We then measured SA-to-urea ratios (SA/Urea) in a larger number of filters and compared results with percent solid measures obtained by standard wet-to-dry methods. We observed an overall positive correlation between SA/Urea and

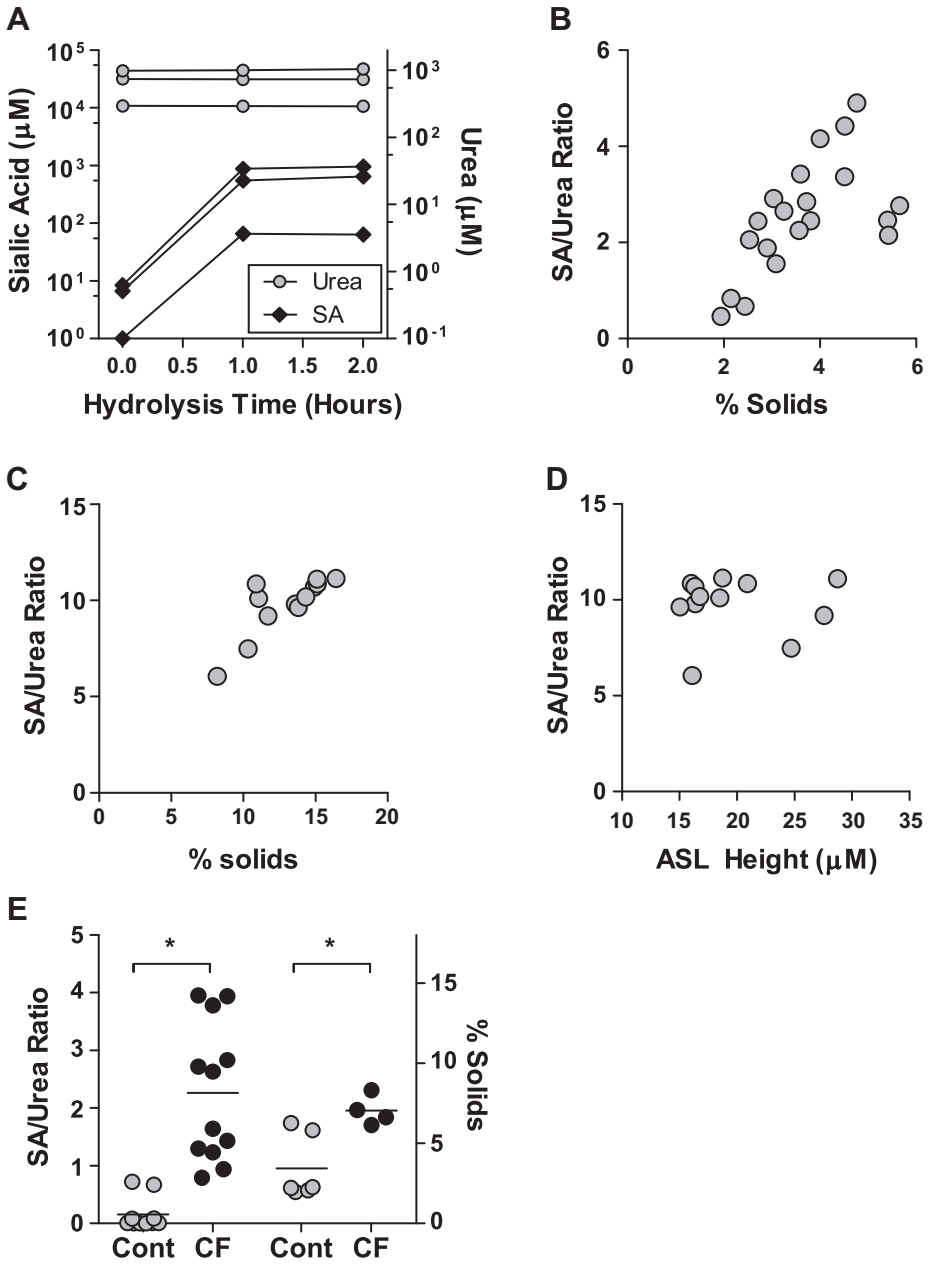

Fig. 2. Sialic acid (SA)-to-urea ratios (SA/Urea) in human airway epithelia. *A*: treatment of filters ($n = 3$) applied to the apical surface of HAE with 1% formic acid at 80° for 1 or 2 h resulted in a significant increase in SA concentrations (solid diamonds) but did not impact urea concentrations (shaded circles). *B*: SA/Urea was correlated with % solids ($r = 0.61$, $P < 0.01$) except at very high % solid concentrations (>5%). *C*: SA/Urea was correlated with % solids in HAE allowed to accumulate mucus to higher % solid concentrations. *D*: airway surface liquid (ASL) height was not correlated with SA/Urea ($r < 0.1$, $P > 0.5$) or to % solids ($r < 0.1$, $P > 0.5$, not shown), reflecting the fact that the thickness of the mucin layer is not necessarily proportional to its concentration. *E*: SA/Urea ratio was elevated in filters obtained from HAE from patients with cystic fibrosis (CF) relative to non-CF controls (Cont). *$P < 0.05$. Results were comparable to % solids measures.

percent solids ($r = 0.61$, $P < 0.01$, Fig. 2*B*). In a small subset of samples with high percent solids (>5%), SA/Urea were lower than predicted, and excluding these high outliers strengthened the correlation considerably ($r = 0.90$, $P < 0.001$). To determine whether the presence of outliers reflected a limitation of the method at high percent solid values, we repeated this experiment with HAE allowed to accumulate mucus over a longer period of time. SA/Urea remained strongly correlated with percent solids even at the higher values ($r = 0.80$, $P < 0.01$, Fig. 2*C*). Notably, neither SA/Urea nor percent solids were correlated with total ASL height (Fig. 2*D* and not shown), as ASL height does not adequately capture mucin concentration when cultures with variable mucin secretion rates are studied over prolonged intervals with the mucus layer present.

We next explored whether this method could detect disease related changes in mucus concentrations by measuring SA/Urea from filters applied to cultured HAE from patients with CF compared with those obtained from non-CF patients as controls. Consistent with the known airway dehydration phenotype of cultured CF HAE, SA/Urea in filters from CF cells were elevated relative to those from controls. The differences in SA/Urea were comparable to percent solid measures obtained in parallel (Fig. 2*E*).

To assess whether the SA/Urea approach could detect mucus concentrations in vivo, we assessed respiratory samples obtained from βENaC-Tg mice that overexpress the β subunit of the ENaC sodium channel and have airway surface dehydration and increased airway mucus concentrations (27). In BALF from these animals, we observed significantly higher sialic acid concentrations after acid hydrolysis (Fig. 3*A*) without significant changes in BALF urea (Fig. 3*B*) in βENaC-Tg mice relative to WT controls. As a result, the SA/Urea ratio was elevated in the βENaC-Tg mice, consistent with previously published measures of airway mucus hydration in this model (26).

We also explored the utility of the SA/Urea in an allergic model of mice sensitized and exposed to HDM. Similar to the βENaC-Tg mice, HDM-treated mice had higher levels of sialic acid than control animals (Fig. 3*D*), consistent with the known increase in airway mucin production after HDM exposure (30). BALF urea concentrations were similar in HDM-treated and control groups (Fig. 3*E*), resulting in an increased SA/Urea (Fig. 3*F*). These data suggest that mucus concentration were increased in HDM-treated mice, similar to findings previously reported in asthma (15, 25).

Having demonstrated the utility of the SA/Urea method within in vitro and in vivo model systems, we next determined whether this method could provide insight into mucus concentrations in human disease. CF airways disease is associated with accumulation of excessively concentrated mucus, but the factors that influence the onset and progression of changes in mucus hydration in early disease are not well established. We applied the SA/Urea method on BALF from young (≤6-yr-old) children with CF available from a previous study (14) grouped by the presence or absence of pathogens on BALF culture (Table 1). In these samples, both sialic acid (Fig. 4*A*) and urea (Fig. 4*B*) concentrations were elevated in samples with respiratory infection (>50,000 pathogens/ml). Furthermore, the SA/Urea was also elevated in culture-positive samples, suggesting that infection is associated with mucus dehydration (Fig. 4*C*). Using our mass spectrometric method, we were able

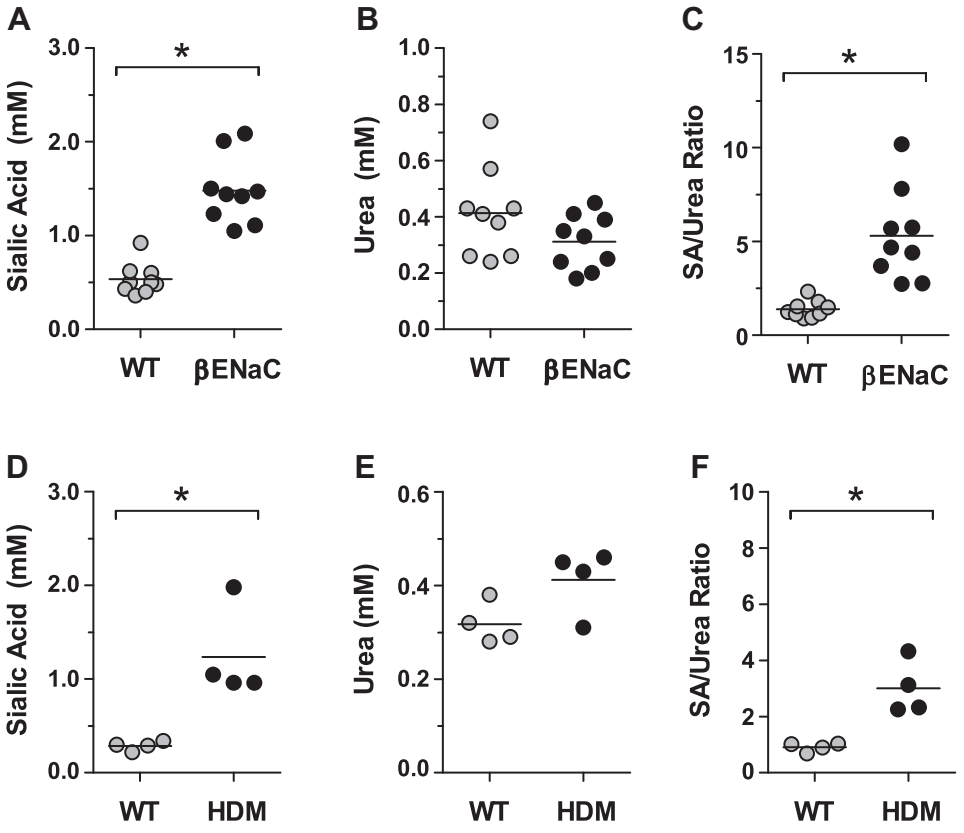

Fig. 3. Sialic acid, urea, and SA/Urea ratios in mouse models of airways disease. *A–C*: sialic acid and urea were measured in BALF obtained from mice overexpressing the β-subunit of the epithelial sodium channel (ENaC) sodium channel (βENaC) as well as wild-type (WT) controls. Sialic acid concentrations were elevated in the βENaC-overexpressing mouse relative to WT (*A*), though urea concentrations were not significantly different (*B*). *C*: SA/Urea ratios were elevated in the βENaC-overexpressing mouse, consistent with its known airway dehydration phenotype. *D–F*: similar findings were observed in BALF obtained from mice sensitized then exposed to house dust mite (HDM) allergen, with increased sialic acid (*D*), no change in urea (*E*), and increased SA/Urea ratios (*F*) relative to controls. *$P < 0.05$.

Table 1. *Demographics of human CF subjects*

	Infected	Not Infected	*P* Value
Age, yr	3.6 ± 1.5	4.1 ± 2.3	0.663
Sex, % male	67%	38%	0.592
% Neutrophils in BALF	72.5% ± 21.5%	24.5% ± 20.3%	0.001

Values are means ± SD.

to simultaneously measure other established biomarkers and observe patterns consistent with previous observations (10, 34): elevated AMP, hypoxanthine, and phenylalanine in samples from patients with infection (Fig. 4*D*).

DISCUSSION

In this study, we demonstrate the utility of a relatively simple method to estimate mucin concentrations within respiratory samples using the ratio of acid to hydrolyzed sialic acid as a marker of secreted mucins and urea as a marker of the fluid phase of ASL. The SA/Urea ratio performs similarly to percent solid measures in cell culture models and is elevated as predicted in an animal model of airway dehydration. Using this method, we observed evidence suggesting that infection in early CF lung disease exacerbates mucus dehydration, likely by triggering increased mucin, but not liquid secretion. While this is consistent with known CF pathophysiology, it has been difficult to measure in early disease using previous methods.

The SA/Urea offers a straightforward method to estimate mucus hydration when other methods are not feasible or practical. Although we chose to utilize mass spectrometry for detection, more conventional colorimetric assays are commercially available for both sialic acid and urea, which broadens the potential application of this approach. One advantage of using mass spectrometry is the ability to measure other inflammatory biomarkers simultaneously with mucus concentration, including compounds such as purines that modulate both inflammation and hydration (3). Indeed, we were able to observe changes in inflammatory biomarkers with infection in young children with CF that parallel findings in our previous studies (10, 14, 34).

One potential limitation to this approach is that sialic acid concentration in airway samples could be altered by changes in the sialic acid content of individual mucin molecules. Previous studies have reported that total mucin sialic acid content is elevated in CF (8, 35), though other studies have suggested that this difference is primarily related to increase in total mucins (29). Another disadvantage reflects the limitations of using urea as a dilution marker in BALF. Urea likely diffuses into lavage fluid during lavage, limiting its accuracy as a dilution marker, though no superior marker has been established (17). Notably, we have observed increased urea concentrations in BALF from subjects with more significant disease (10), which may reflect accumulation of secretions in airways with reduced mucociliary clearance. Despite these limitations, we were able to observe the biologically plausible differences in SA/Urea in BALF both mouse models and human subjects, suggesting that these issues do not obviate the utility of this approach.

In summary, we report a relatively simple measure to assess mucus hydration that is applicable to a variety of respiratory

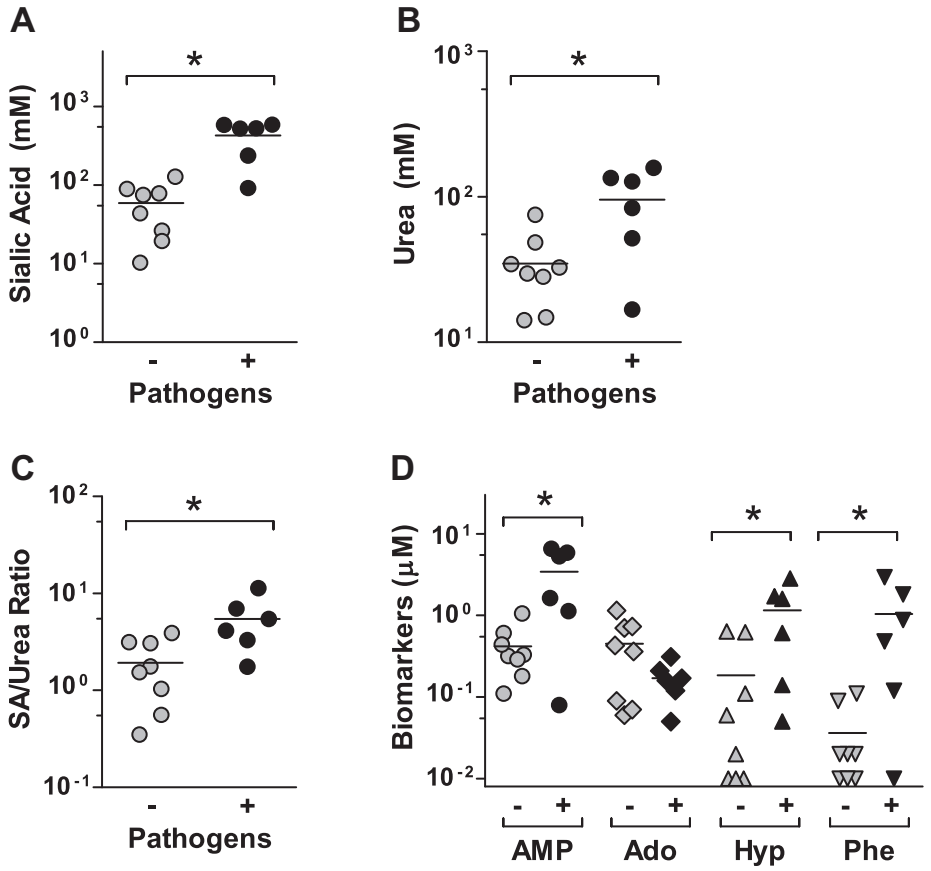

Fig. 4. SA/Urea ratios in CF airways disease. Sialic acid, urea (*B*), and other biomarkers were measured in BALF obtained from young (≤6 yr old) children with CF. Sialic acid (*A*), urea (*B*), and the SA/Urea ratio (*C*) were elevated in BALF from which respiratory pathogens were recovered on culture compared with culture negative samples. *D*: other metabolomic biomarkers measured simultaneously with sialic acid and urea conformed to previously observed patterns in CF, with elevated concentrations of AMP, hypoxanthine (Hyp), and phenylalanine (Phe) in culture positive (+) vs. culture (−) samples. Adenosine (Ado) was nonsignificantly decreased in culture (+) samples ($P = 0.26$). *$P < 0.05$.

sample types. While this method does not replace more sophisticated mucin measures or gravimetric approaches, it does permit estimates of mucus concentration when more established methods such as % solid measures are not practical.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.R.E., D.B.H., B.B., S.S., C.M.J., E.A.D., C.M.D., G.C., and S.M.S. performed experiments; C.R.E., S.S., G.C., S.M.S., and R.C.B. analyzed data; C.R.E., C.M.D., and S.R. interpreted results of experiments; C.R.E. prepared figures; C.R.E. drafted manuscript; C.R.E., D.B.H., B.B., S.S., C.M.D., G.C., S.R., and R.C.B. edited and revised manuscript; D.B.H., B.B., S.S., C.M.J., E.A.D., C.M.D., G.C., S.R., S.M.S., and R.C.B. approved final version of manuscript.

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