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Original Article

Mucus and mucus flake composition and abundance reflect inflammatory and infection status in cystic fibrosis

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ABSTRACT

Background: Mucus hyperconcentration in cystic fibrosis (CF) lung disease is marked by increases in both mucin and DNA concentration. Additionally, it has been shown that half of the mucins present in bronchial alveolar lavage fluid (BALF) from preschool-aged CF patients are present in as non-swellable mucus flakes. This motivates us to examine the utility of mucus flakes, as well as mucin and DNA concentrations in BALF as markers of infection and inflammation in CF airway disease.

Methods: In this study, we examined the mucin and DNA concentration, as well as mucus flake abundance, composition, and biophysical properties in BALF from three groups; healthy adult controls, and two CF cohorts, one preschool aged and the other school aged. BALFs were characterized via refractometry, PicoGreen, immunofluorescence microscopy, particle tracking microrheology, and fluorescence image tiling.

Results: Mucin and DNA BALF concentrations increased progressively from healthy young adult controls to preschool-aged people and school-aged people with CF. Notably, mucin concentrations were increased in bronchoalveolar lavage fluid (BALF) from preschool-aged patients with CF prior to decreased pulmonary function. Infrequent small mucus flakes were identified in normal subjects. A progressive increase in the abundance of mucus flakes in preschool and school-aged CF patients was observed. Compositionally, MUC5B dominated flakes from normal subjects, whereas an increase in MUC5AC was observed in people with CF, reflected in a reduced flaked MUC5B/MUC5AC mucin ratio.

Conclusion: These findings suggest mucus composition and flake properties are useful markers of inflammatory and infection-based changes in CF airways.

1. Introduction

The airway epithelium is protected from inhaled particles and pathogens by the airway surface liquid (ASL). The ASL is a multiphase gel system comprised of two layers: the mucus layer containing gel-forming polymeric mucins, predominantly MUC5B with lesser amounts of MUC5AC [1,2]; and the periciliary layer (PCL), which contains cilia and the grafted membrane-bound mucins [1].

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In health, ASL facilitates mucociliary clearance (MCC) of inhaled particles. In cystic fibrosis (CF), defective Cl^- secretion by the cystic fibrosis transmembrane conductance regulator (CFTR) channel coupled to persistent Na^+ absorption produces ASL volume depletion, i.e., airway surface dehydration [3–5]. Consequently, the mucus layer becomes hyperconcentrated, with abnormally increased viscous and elastic properties [6], osmotic collapse of the PCL [1,7] and impairment of MCC [8]. Intrapulmonary mucus accumulation predisposes people with CF to infection and pro-inflammatory feedback loops [9], producing the bronchiectasis and respiratory failure that are the primary causes of morbidity and mortality in CF [10–12].

While spirometry is a classical biomarker of adolescent and adult CF lung disease progression and severity, novel techniques, e.g., lung clearance index (LCI) [13] and CT imaging tools [14], are required to detect early disease. Recently, increased mucin concentrations in bronchoalveolar lavage fluid (BALF) from preschool-aged children with CF were observed in conjunction with an increased incidence of insoluble mucus "flakes" [15]. Unlike typical entangled, non-interacting polymeric gels that swell and dissolve into free solution [16], the mucus flakes observed in CF BALF failed to dissolve after prolonged exposure to free solution (PBS) [15]. Flake appearance was correlated with markers of disease severity in CF (e.g., neutrophil numbers), and preceded lung-structural defects seen on CT scans, raising the possibility that flakes may be a sensitive biomarker of early disease. We hypothesized that mucus flakes occur in normal human mucus physiology, and that their abundance, composition and formation mechanism may be altered during infection or inflammatory events.

To test this hypothesis, biochemical and biophysical analyses were utilized to characterize flake number, size, concentration and composition in BALF samples from healthy adults and two CF cohorts: 1) a pre-school age cohort with zero to minimal detectable lung disease; and 2) a school aged cohort that had more advanced CF lung disease. Biochemical measurements tested for prospective biomarker utility included mucin [17] and DNA [15] concentrations and immunofluorescence imaging of mucins and DNA within flakes [15]. Biophysical analyses of mucus flakes were performed using particle tracking microrheology (PTMR), which provided further characterization of flake properties [15,18–20].

2. Methods

Full descriptions of the methods are given in the online supplement. Bronchoscopy and bronchoalveolar lavage (BAL) in preschool and school-age children with cystic fibrosis were performed per clinical routines as previously described [21,22]. Healthy adult control BALF was obtained from nonsmoking volunteers [23]. Mucin concentrations of BALF samples were measured via gel permeation chromatography in series with multi-angle laser light scattering and differential refractometry as previously described [7]. DNA concentrations were assessed via a Quant-iT PicoGreen Assay (Invitrogen). Immunofluorescence microscopy (IFM) staining of mucus flakes for MUC5AC, MUC5B, and DNA and scanning electron microscopy was performed as described in [15]. IFM staining, imaging, and analysis were performed blinded to infection status. PTMR was performed as detailed in the Supplemental Information. Mucus flake volume fraction was performed as previously described in [15] while blinded to other clinical details.

3. Results

3.1. Subject demographics

To study mucus flakes, and mucin and DNA concentrations as markers of CF airway disease-related derangements, three cohorts were studied: 1) healthy volunteers (n=11, mean age = 29.8 years); 2) a previously published set of preschool CF samples (n=46, mean age = 3.3 years) obtained through the AREST CF study [15]; and 3) a school-aged CF cohort (n=37, mean age = 10.5 years). Demographic information, treatment history, spirometric data, and air-

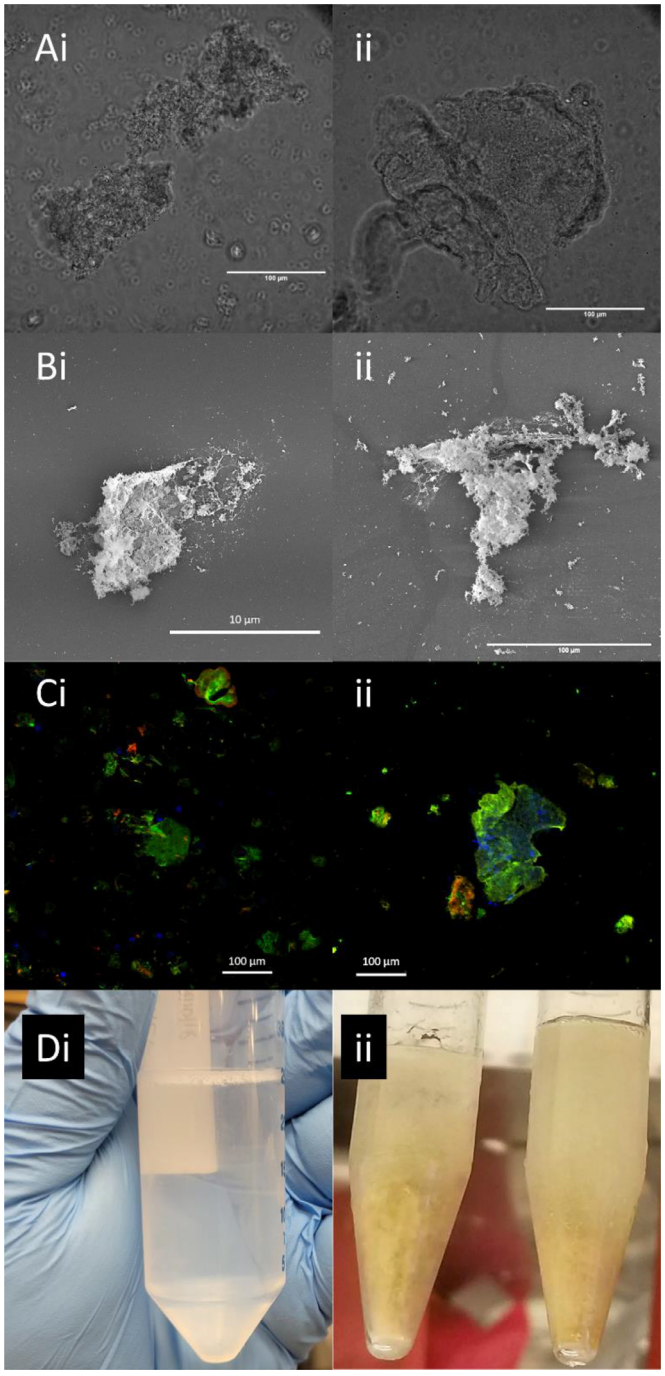

Fig. 1. Image-based characterization of mucus flakes from healthy control BALF (i) compared to those in school age CF BALF (ii). (A) Under 40x magnification with brightfield illumination, mucus flakes appeared as gel-like structures in a distinct phase from the surrounding fluid. (B) Scanning electron microscopy of mucus flakes revealed their underlying polymeric network structure, including both short- and long-scale network elements. (C) MUC5AC (red) and MUC5B (green) staining of healthy BALF revealed that mucus flakes consisted of insoluble gels of mostly mucins and minimal cell debris, including intranuclear DNA (DAPI, blue. (D) Gross visual appearance of BALF illustrating the difference in turbidity between control (in 50 mL conical) and CF (in 15 mL conicals) BALF. All scale bars 100 µm except Ci (10 µm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

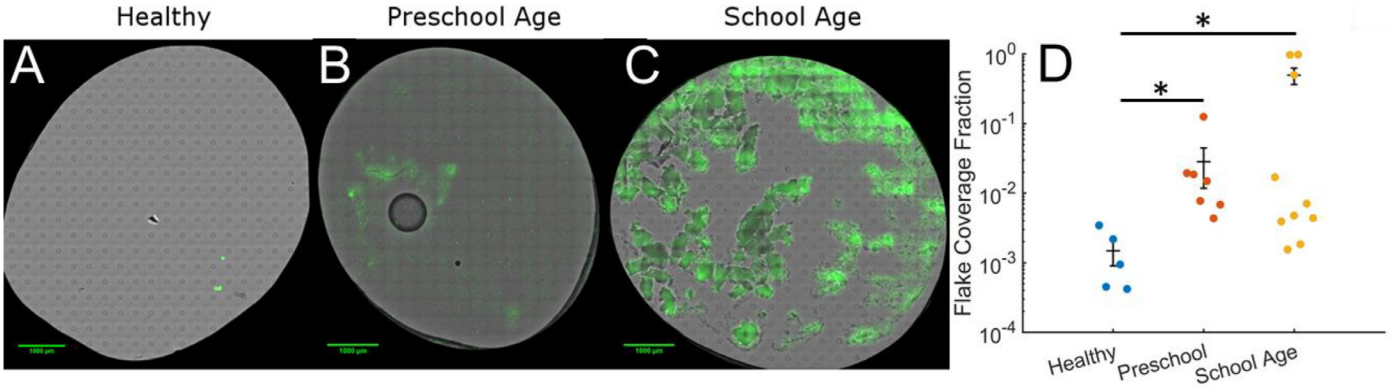

Fig. 2. Mucus flake sample fraction and composition. (A-C) Fluorescent beads (green) used in PTMR experiments concentrated in mucus flakes to make them appear brighter than background when imaging entire 5 μL sample droplets via tiling. All scale bars 1 mm. (D) Total sample area occupied by flakes was increased in both pre-school CF samples (n=7) and school age CF samples (n=10) as compared to healthy adults (n=5). Note log-scale on ordinate; *, $p<0.05$). Error bars represent mean ± SEM. A total of 3 technical replicates per subject was performed for each subject. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

way infection status for each cohort are presented in Supplemental Table 1.

3.2. Mucus flakes in health and CF

In addition to those previously reported in preschool CF BALF, mucus flakes were present in both healthy adult (Fig. 1, subpanels i) and school aged CF BALF (Fig. 1, subpanels ii). Mucus flakes viewed via brightfield microscopy appeared as gel-like masses in the surrounding BALF (Fig. 1A). SEM of mucus flakes from both cohorts revealed a polymer network structure consistent with the organization of gel-forming mucins and globular proteins (Fig. 1B), though it was difficult to identify flakes on the 100 µm-scale in healthy control samples via SEM (Fig. 1Bi). Flake substructure was nearly identical in appearance to previously reported flakes, which were shown to be susceptible to dissolution via chemical reduction [15]. IFM employing mucin antibodies showed that flakes contained the gel-forming mucin MUC5B, occasionally admixed with small amounts of MUC5AC. Small amounts of DNA were also occasionally detected which were mostly within flake-entrapped cells (Fig. 1Ci) in health, while extracellular DNA was also present in CF flakes (Fig. 1Cii). Mucus flakes viewed in all modalities from healthy controls varied modestly in size but rarely exceeded a few hundred microns in diameter in healthy subjects, whereas CF flakes demonstrated significant variability in size, from a few microns to several millimeters. Fig. 1D illustrates that the hyperabundance of flakes in CF produced dramatic increases in turbidity compared to healthy BALF due to the presence of both micro- and macroscopic flakes. Such turbidity is characteristic of CF and is not observed in BALF from infants with non-CF lung disease [15].

3.3. Flake abundance, DNA and mucin concentration in Health and CF

Tiled fluorescence imaging of BALF samples was used to quantitate the prevalence of mucus flakes in healthy and CF BALF (Fig. 2A-C). In BALF from healthy adults, the fractional area of BALF images occupied by mucus flakes was small (mean=0.15% Fig. 2D), which was consistent with the low total mucin concentration in control BALF (Fig. 3A) and the low relative fraction (7%) of mucins contained in flakes vs. the total in BALF solution (Fig. 3B).

In CF BALF, flake coverage fraction was greater than in healthy subjects and progressively increased in pre-school (mean=2.8%) and school-aged CF (mean=49%) subjects compared to controls (Fig. 2D). Flake coverage paralleled age-dependent increases in to-

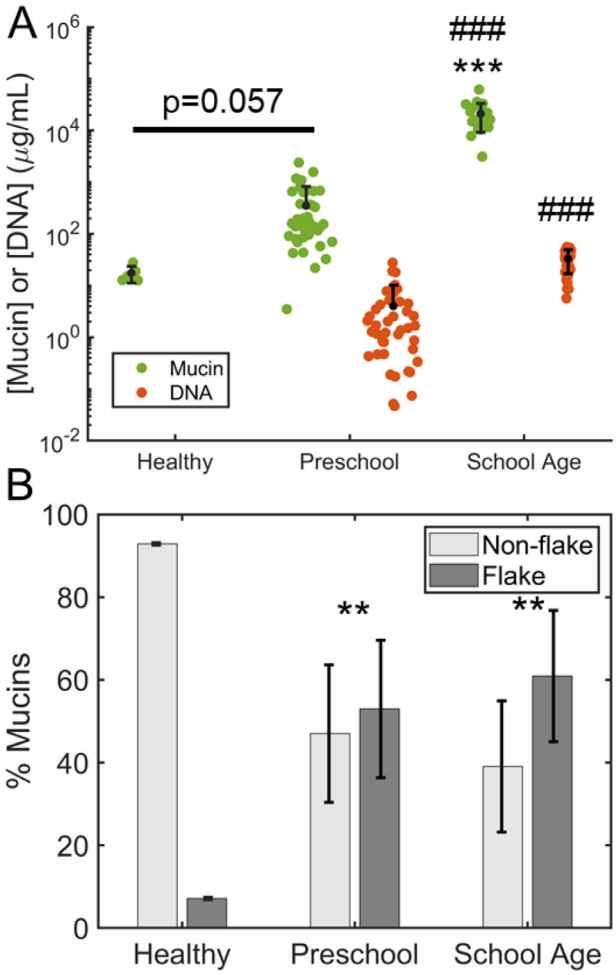

Fig. 3. (A) Total mucin concentration is increased compared to healthy controls in school age CF (***, $p<0.005$) samples. School age CF samples have increased mucin content compared to pre-school samples as well (###, $p<0.005$). DNA content was below detection limit in healthy BALF, but school age CF samples had increased DNA concentration vs. pre-school CF samples (###, $p<0.005$). (B) While 7% of mucins are found in flakes in health, mucin proportions in flakes increased (**, $p<0.01$, z-test for proportions corrected for multiple comparisons via Bonferroni method) to 53% and 59% in pre-school and school age CF samples, respectively..

tal mucin concentrations in the CF samples (Fig. 3A, note logarithmic scale). CF BALF DNA concentrations were increased compared to controls (which had undetectably low DNA concentrations) and increased with age in CF (Fig. 3A). In BALF from both CF cohort groups, the percentage of total mucins found in flakes increased to >50% (Fig. 3B).

3.4. Mucus flake composition in health and CF

IFM imaging of mucus flakes was used to analyze the mucin composition of mucus flakes. In health and disease, MUC5B was the predominant mucin in mucus flakes (Supplemental Figure S1A-C). MUC5AC signals were increased relative to MUC5B signals in the CF cohorts, which resulted in decreased MUC5B/MUC5AC ratios compared to flakes from healthy subjects (Supplemental Figure S1D). Ratios of MUC5B/MUC5AC were similar between the CF cohorts. These images also confirmed the scant presence of DNA in normal flakes and its increased presence in CF flakes.

3.5. Mucus flake rheology

We utilized PTMR to assess the rheological characteristics of flakes suspended in BALF solution from each cohort (Fig. 4A). Beads diffusing in the dilute BALF solution and mucus flakes were filtered into distinct clusters using Gaussian Mixture Modeling (GMM) [15]. In healthy subject BALF, 81% of particles exhibited a complex viscosity (η^*) consistent with water ($\eta^* \approx 0.001$ Pa•s), and 19% were clustered separately and referred to as the "mucus flake signal". The fraction of particles reporting η^* reflective of mucus flake signal was increased to 64% in preschool CF BALF and 83% in school-age CF BALF ensembles (Fig. 4B). Values of mean, variance, and bead number in each ensemble and cluster for each cohort are reported in Supplemental Table S2.

Similarly, in individual subjects, the mean percentage of particles reporting η^* in the mucus flake range progressively increased from healthy controls to pre-school and then to school-aged people with CF (Fig. 4Ci). The increase in mucus prevalence drove an increase in average weighted η^* across cohort groups (Fig. 4Cii).

3.6. Infection Effects on CF Flakes and BALF composition

Airway infection has been implicated in worsening mucus accumulation in CF airways and the associated biochemical and biophysical changes of increased mucus burden [2,9]. Mucus flakes in BALF from the school-age CF cohort positive for bacterial culture (n=23) were roughly 100-fold larger and more abundant than bacterial culture negative (n=5) samples (Supplemental Figure S2). DNA concentrations in BALF were also significantly increased in infected samples (Supplemental Figure S3). A similar trend towards increased mucin concentration with infection was also observed (p=0.1).

IFM was used to compare the macromolecular composition of mucus flakes in CF BALF with negative vs. positive bacterial cultures. Average intensities in mucin and DNA channels were significantly increased in culture positive vs. culture negative flakes (Fig. 5A, B) by roughly five- and 35-fold, respectively. Culture positive CF flakes also exhibited increased roughness, a measure of flake network granularity previously shown to be distinct in CF [15], compared to culture negative samples (Supplemental Figure S4). Infected CF BALF flakes also exhibited increased mucus fraction, weighted average η^*, and mucus signal η^* (Fig. 5C-E). GMM-PTMR parameters and DNA concentration also correlated with increased levels of neutrophilic inflammation (Figure S5). Taken together, these immunofluorescence and biophysical results indicate that bacterial infection may have increased the number, size, and viscoelasticity of CF mucus flakes.

4. Discussion

Hyperconcentrated mucus is a common feature of muco-obstructive airway diseases [12]. Accordingly, mucus-based biomarkers, including mucin concentration [8,17,24], osmotic pressure [1,8,25], and mucus rheology [19,25], have been employed to investigate disease severity and therapeutic efficacy in these diseases. In addition to these traditional biomarkers, recent reports that half of the mucins present in BALF from preschool aged patients with CF are contained in mucus flakes motivates further investigation into the prevalence of mucus flakes in patients with CF. The advent of highly effective modulator therapies (HEMTs) has brought a new era of CF therapeutics, with patients exhibiting dramatically increased lung function [26] and decreased sputum production [27]. As the availability of HEMTs has expanded, new mucus-based biomarkers that are sensitive to the exacerbation-related events in HEMT-treated CF airway disease are needed to complement CT scans [28] and LCI [13] technologies. Recently, it was reported that BALF mucin and DNA concentrations from preschool-aged people with CF were elevated prior to the onset of structural lung damage or changes to pulmonary function [15]. We pursued these findings by studying BALF of healthy adults and a school-aged cohort with CF to determine if these measurements are affected by infection and inflammation found in CF airway disease.

Our results describe a reference level for flake expression in normal physiology. In healthy adults, mucus flakes were scarce (~0.1% BALF coverage area) and contained a small fraction (< 10%) of the total mucins in BALF (Fig. 1 i subpanels, 2A, 3A). Immunostaining of normal flakes confirmed that they were largely composed of MUC5B admixed with small amounts of MUC5AC and no detectable extracellular DNA (Fig. 2). We speculate that the mucus flakes recovered from healthy individuals arose from a mixture of submucosal gland (SMG) secretions, which are dominated by MUC5B, and superficial airway epithelia, which in health are dominated by MUC5B but are also a source of MUC5AC, particularly in the upper airways [29]. Bronchoscopy with lavage likely stimulated SMG secretion, though we do not rule out the possibility of flake formation from tonic SMG mucus secretion. Flakes could also be formed from dysfunctional mucin secretion [30], crosslinking due to environmental oxygen [31], or mucin adhesed onto inhaled particulates.

Our report of insoluble flakes in healthy BALF has implications for normal lung mucus clearance. It is currently unclear whether airway mucus forms a single, continuous layer ("blanket") [32] over the airway surface, a multi-phasic/discontinuous layer [33], or a "lumpy blanket" intermediate of the two. Newer studies suggest that the mucus layer may have two distinct transport modalities: 1) a faster, swellable phase; and 2) a slower phase composed of non-swelling gel components, e.g., flakes and/or strands [34,35]. The swellable phase theoretically could concentrate as it moves cephalad to avoid occluding airways as mucus moves into central airways with far less surface area than distal airways [36]. Discontinuous mucus flakes could ascend the airway tree without jamming and hyperconcentrating as they move from distal to central airways [37]. The relative roles of these two phases in normal mucus clearance remains to be determined.

In CF, flakes were larger and biochemically distinct from mucus flakes in health. It is unclear whether CF flakes reflect a continuum from "normal" flakes or emerge as a unique consequence of CF pathogenesis. They may have emerged by growth of a single large mucus flake on airway surfaces or reflect growth of aggregates of multiple flakes (Fig. 2A-D), as noted in rats [33]. Additionally, increased mucin concentrations present in BALF from people with CF would increase the likelihood that mucus flakes would form and merge on the assumption that free mucin entanglement

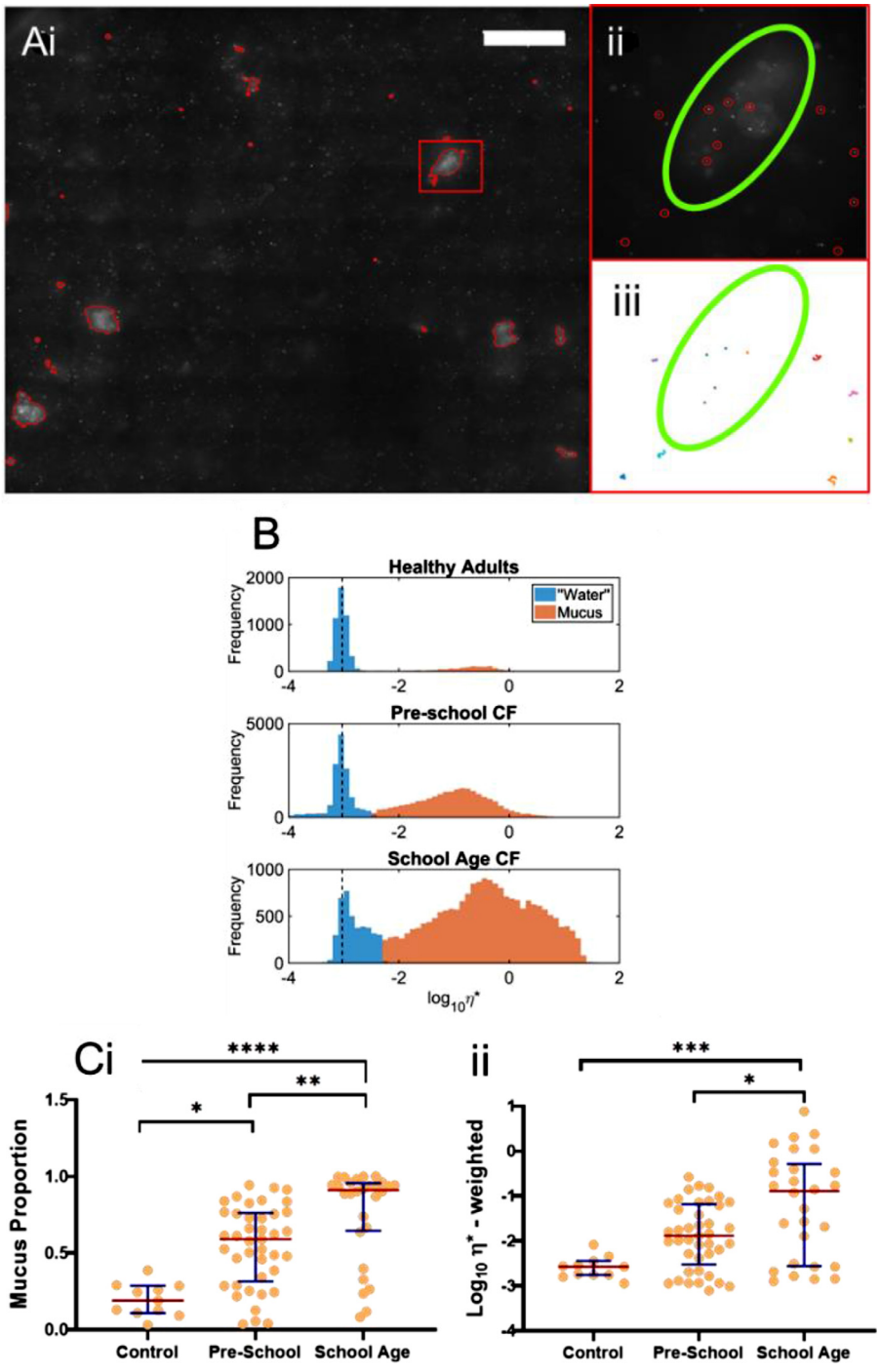

Fig. 4. Imaging and rheologically characterizing mucus flakes. (Ai) Tiled and stitched 10×10 image of mucus flakes (outlined in red) in a pre-school CF BALF sample (scale bar 250 nm). (Aii) Specific flakes (red box in Ai, green outline in Aii and Aiii) were imaged and beads in and near the flake were automatically identified and tracked. (B) Smaller bead trajectories reflected increased viscoelasticity present in mucus flakes. Histograms of watery and mucus clusters of η^* in healthy, pre-school, and school age CF BALF revealed increasingly pronounced mucus peaks in CF samples. Age-dependent increases in the proportion of beads in mucus (Ci) and weighted η^* (Cii) were found from health to pre-school CF to school age CF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is a means by which flakes form and grow. The increased abundance of flakes present in CF airways may reflect the initial growth of mucins adhesed to airway surfaces, i.e., a product of failed clearance, but they also may contribute to failed clearance in a positive feedback cycle.

A disease-specific mechanism for flake formation related to CF mucus hyperconcentration and inflammation is consistent with observed biochemical differences between CF and healthy flakes. The relative decrease in MUC5B/MUC5AC ratios present in CF BALF (Supplemental Figure S1D) is consistent with other muco-inflammatory diseases [17,38] and CF cell culture models [9,39,40]. This observation may also indicate that the ratio of MUC5B/MUC5AC in BALF is useful as a biomarker of mucus pathology in CF. Given that MUC5AC is selectively secreted by airway surface epithelia, the relative increase in MUC5AC suggests that surface-secreted mucins contribute more to flake formation in

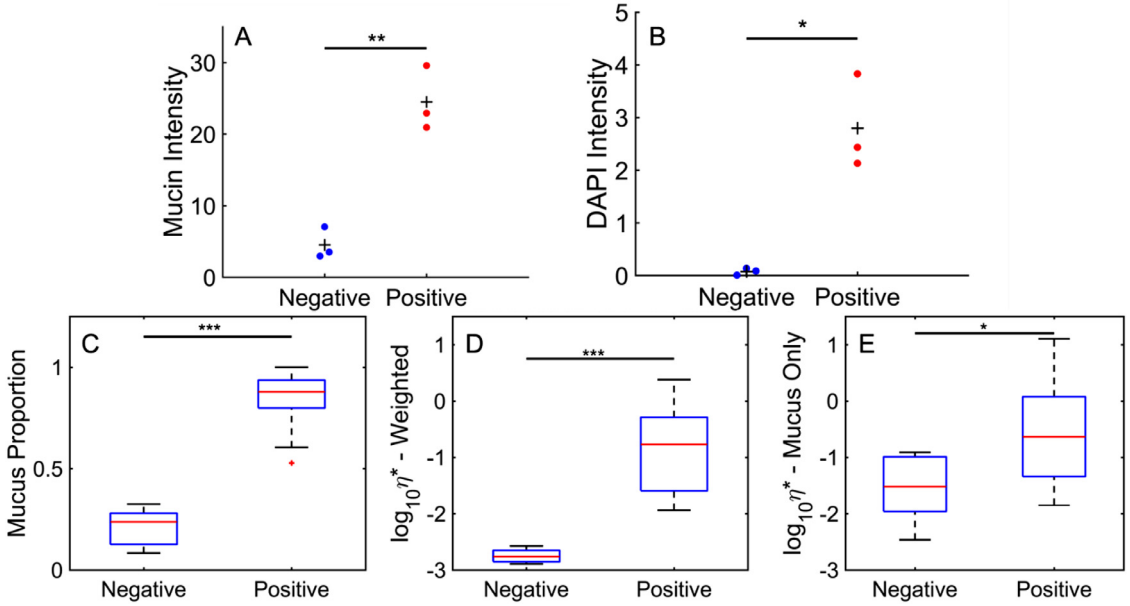

Fig. 5. Biochemical and biophysical differences in mucus flakes in school aged patients with CF based on culture status. Culture positive (n=3) flakes were confirmed to have higher (A) mucin and (B) DNA stain intensity than culture negative (n=3). (C) The fraction of beads in mucus flakes, (D) weighted η^*, and (E) η^* of the individual patient mucus component means were increased in (n=23) culture positive samples vs. n=5 culture negative samples. *, $p<0.05$, *, $p<0.01$, ***, $p<0.005$ positive compared to negative.

CF than mucus generated from submucosal glands. The combination of increased mucin and DNA concentrations in CF BALF (Fig. 3A) may reflect inflammatory responses triggered initially by microaspiration and viral infections and ultimately by an increasing bacterial burden. IL-1α/β stimulation, common to all these pathogens, increases airway mucus concentration, disproportionately increases MUC5AC secretion, and triggers neutrophil accumulation [9,41].

Disease-state and age-associated differences were also observed in biophysical analyses of CF vs. normal flakes. An increased prevalence of mucus flakes with elevated viscoelastic properties was observed in each CF cohort (Fig. 4B, orange bins). Only mucus flakes in school-aged people with CF exhibited viscosities consistent with pathological mucus hyperconcentration ($\eta^* > 1$ Pa•s), particularly during infection (Fig. 5E) [6,18,42]. Importantly, our data indicate that there are more mucus flakes in CF, and the viscoelastic properties of mucus flakes reflected more concentrated mucus in older patients with CF.

Our findings suggest that airway infection and associated inflammation resulted in fundamental changes in the composition and behavior of CF flakes (Fig. 5A, B). DNA concentration (and PTMR values) were correlated with neutrophil counts, suggesting neutrophil accumulations produced the increased DNA concentrations (Supplemental Figure S5). We speculate that extracellular DNA, which is both stiff and "sticky" [16,43], could act as a nidus for flake growth/aggregation in the ASL, particularly in bacterially infected lungs. Although the relative roles of increasing DNA vs. mucin concentrations on flake formation and growth are yet unknown, increased DNA concentration may contribute to increased mucus flake size (Figure S2A) and η^* (Fig. 5E) in culture positive people with CF. Notably, the increased DNA concentration in flakes and whole CF BALF (Supplemental Figure S2) could reflect the presence of NETs [41], which may contribute to the crosslinking, aggregation, and stiffening of mucus flakes due to their oxidative properties and DNA content [31]. If changes in flake properties and formation are, indeed, related to increased DNA concentrations, our observations may partially explain why DNase therapy is more effective in older than younger people with CF [15,44,45]. However, we cannot rule out other factors that may contribute to flake formation in bacterially infected CF airways, e.g., debris from infection/inflammation, fibrin, and/or actin.

In summary, mucus flakes are a feature of normal airway physiology. However, mucus flakes in patients with CF differ in size, number, and composition from those in healthy subjects. These findings suggest that CF pathophysiologically impacts the formation, size, viscoelasticity, and clearance of mucus flakes. We postulate that increased CF flake numbers may be related to ASL dehydration, inflammation, altered/increased concentrations of mucins MUC5AC and MUC5B, and/or increased DNA concentrations. The associations with infection and inflammation suggest the presence of positive feedback loops that accelerate mucus flake formation and disease progression. These findings indicate that mucin and DNA concentration, mucus flakes, and the MUC5B/MUC5AC ratio, may serve as biomarkers of CF disease status and may have utility in evaluating modulator therapies. Our data also indicate that mucolytic therapies designed to dissolve mucus flakes found in CF and other muco-obstructive lung diseases are rational.

Declaration of Competing Interest

The authors have no competing or conflicting interests to disclose that are relevant to this work.

CRediT authorship contribution statement

Matthew R. Markovetz: Conceptualization, Methodology, Software, Formal analysis, Investigation, Validation, Data curation, Writing – original draft, Visualization, Funding acquisition. **Ian C. Garbarine:** Investigation, Validation, Writing – original draft. **Cameron B. Morrison:** Investigation, Validation, Visualization, Writing – review & editing. **William J. Kissner:** Investigation. **Ian Seim:** Investigation, Software. **M. Gregory Forest:** Resources, Writing – review & editing, Funding acquisition. **Micah J. Papanikolas:** Investigation. **Ronit Freeman:** Supervision, Resources, Funding acquisition. **Agathe Ceppe:** Formal analysis, Visualization. **Andrew Ghio:** Methodology, Investigation, Resources. **Neil E.**

Alexis: Methodology, Investigation, Resources. **Stephen M. Stick:** Resources, Project administration, Funding acquisition. **Camille Ehre:** Methodology, Supervision, Resources. **Richard C. Boucher:** Supervision, Resources, Writing – original draft, Project administration, Funding acquisition. **Charles R. Esther:** Conceptualization, Investigation, Resources, Writing – original draft, Project administration, Funding acquisition. **Marianne S. Muhlebach:** Conceptualization, Investigation, Resources, Writing – original draft, Funding acquisition. **David B. Hill:** Conceptualization, Resources, Writing – original draft, Supervision, Funding acquisition.

Author contributions

MRM, NEA, SMS, CE, RCB, CRE, MSM, and DBH designed the experiments. MRM, ICG, CBM, WJK, IS, and MJP performed the experiments. MRM, CBM, IS, MGF, RCB, CRE, MSM, and DBH analyzed the data. AG, SMS, RF, CE, CRE, MSM, and DBH provided reagents. MRM, RCB, CRE, MSM, and DBH wrote the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2022.04.008.

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