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## Characterizing Mucociliary Clearance in Young Children with Cystic Fibrosis

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### Abstract

**Background:** This research characterized mucociliary clearance (MCC) in young children with cystic fibrosis (CF).

**Methods:** Fourteen children (5–7 years old) with CF underwent: two baseline MCC measurements (Visits 1 and 2); one MCC measurement approximately one year later (Visit 3); and measurements of lung clearance index (LCI), a measure of ventilation inhomogeneity.

**Results:** Median (range) percent MCC through 60 minutes (MCC60) was similar on Visits 1 and 2 with 11.0 (0.9–33.7) and 12.8 (2.7–26.8), respectively ( $p=0.95$ ), and reproducible (Spearman  $Rho=0.69$ ;  $p=0.007$ ). Mucociliary clearance did not change significantly over one year with median percent MCC60 on Visit 3 [12.8 (3.7 – 17.6)] similar to Visit 2 ( $p=0.58$ ). Lower percent MCC60 on Visit 3 was significantly associated with higher LCI scores on Visit 3 ( $N=14$ ; Spearman  $Rho=-0.56$ ;  $p=0.04$ ).

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**Disclosure Statement:** There are no disclosures.

**Informed Consent:** Written, informed consent was obtained from parents, or guardians, of children who participated in the study. The study was approved by the Johns Hopkins Institutional Review Board.

**Conclusions:** Tests of MCC were reproducible and reliable over a two-week period and stable over a one-year period in 5–7 year-old children with CF. Lower MCC values were associated with increased ventilation inhomogeneity. These results suggest that measurements of MCC could be used in short-term clinical trials of interventions designed to modulate MCC and as a new, non-invasive test to evaluate early lung pathology in children with CF.

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## Introduction

Currently, the state of the art, non-invasive method, for evaluating lung pathology in people with CF is pulmonary function testing. However, many children who are <6 years of age cannot perform this test and physicians must rely on radiologic tests, oropharyngeal cultures and cultures of bronchoalveolar lavage to assess lung disease objectively in this population. A non-invasive test that young children can perform is needed to impartially evaluate early lung pathology in CF.

Mucociliary clearance (MCC) measurements are non-invasive, and defective MCC is an integral step in the pathogenesis of CF lung disease. Mutations in the CF transmembrane conductance regulator (CFTR) gene lead to impaired mucus removal, accumulation of bacteria and fungi in the airways and infection (1–4). Chronic infection of the airways is the major cause of morbidity and mortality (5).

Previously, we have measured MCC in children with CF who are 7 years of age and found that MCC declines significantly when quantified over several years (6), and lower MCC values are associated with higher incidence of infection with *Pseudomonas aeruginosa* (PA), increased ventilation inhomogeneity and increased peripheral lung damage (6, 7). However, MCC has not been characterized in children with CF who are 7 years old. Thus, it is unknown if younger children can perform the procedure and if results are reproducible. It is also unknown if MCC values in this population change over time and what factors lead to variability in MCC measurements.

We hypothesized that: (1) MCC measurements are reproducible and reliable in children with CF who are 5–7 years old; (2) MCC values decline significantly over a one-year period; and (3) variability in MCC is associated with PA infection history, mucus composition, ventilation inhomogeneity and/or peripheral lung damage. In two exploratory analyses, we examined variability in MCC in relation to environmental exposures and variants in genes related to pulmonary phenotype in CF, or MCC. Results from these experiments would determine if MCC measurements can be performed in young children with CF and whether they provide a novel test to evaluate early lung pathology.

## Materials and Methods

### Study Overview:

The study consisted of Visits 1 and 2 during a two-week period and Visit 3, approximately one year later. All visits were approved by the Johns Hopkins Institutional Review Board. Written, informed consent was obtained from parents, or guardians, of children who

participated in the study. An historic group of adult subjects who had undergone an MCC procedure previously in our laboratory (8) served as controls for MCC measurements.

**Eligibility Criteria for Children with CF:**

(1) Males and females between the ages of five and seven years; (2) diagnosis of CF by sweat chloride >60 meq/L, or by the presence of two CFTR mutations known to cause CF; and (3) FEV<sub>1</sub> 80 percent predicted. Children who could not perform spirometry reliably, or whose FEV<sub>1</sub> was less than 80 percent predicted, were eligible if they had a minimal history of infection and chronic symptoms, as determined by a physician investigator.

**Eligibility Criteria for Controls:**

Healthy, nonsmoking adults with no history, or evidence, of lung disease.

**Use of Concomitant Therapies:**

Use of concomitant therapies was documented through chart review. Short-acting bronchodilators, dornase alfa and airway clearance with high frequency chest wall oscillation were discontinued for 12 hours and hypertonic saline for 72 hours before all three study visits. Treatment with lumacaftor/ivacaftor were not discontinued. Visits were scheduled at least two weeks following completion of antibiotic treatment for a pulmonary exacerbation.

**Lung Function Measurements:**

Initially, the protocol did not include spirometry. This was amended after the first child completed Visits 1 and 2. For all other children, forced expiratory volume in one second (FEV<sub>1</sub>) was measured on Visits 1 and 3, after MCC, using a MicroLab Mk8 spirometer (MD Spiro; Lewiston, ME), according to accepted guidelines (9). FEV<sub>1</sub> was reported as percent of predicted values, calculated using Global Lung Function Initiative 2012 equations (10).

**High Resolution Computed Tomography (HRCT) Lung Scan:**

Peripheral lung damage was assessed from HRCT scans performed on Visit 3 and scored as described by Brody et al. (11). A total combined score for severity of bronchiectasis, mucous plugging, peribronchial thickening, air trapping and parenchymal opacities in both lungs was reported. HRCT scans were not performed on Visit 1 to avoid additional radiation exposure.

**Multiple Breath Washout Test:**

Multiple breath washout (MBW) tests measure lung ventilation inhomogeneity (12). Lung Clearance Index (LCI), measured by MBW, is considered to be a more sensitive indicator of early lung disease than FEV<sub>1</sub> (13–17). Ventilation inhomogeneity was evaluated after MCC on Visits 1 and 3, in terms of absolute LCI<sub>2.5%</sub>, according to consensus guidelines (12). MBW tests were performed using the EXHALYZER® D (ECO Medics AG; Bubikonstrasse, Switzerland).

**Pseudomonas aeruginosa (PA) Infection and Hospitalization History:**

Age of initial PA+ culture, number of PA+ cultures/year and percent of respiratory cultures per year that were PA+ prior to Visit 2 and between Visits 2 and 3 were documented through chart review. Number of hospitalizations for a pulmonary exacerbation and/or treatment with antibiotics between Visits 2 and 3 were documented through questionnaires and review of medical records.

**Environmental Exposures:**

Caregivers completed questionnaires documenting their child's exposure to pets, pests, secondhand tobacco smoke, gas combustion in the home and respiratory syncytial virus (RSV) infection prior to Visits 2 and 3.

**Genes of Interest:**

The following genes were of interest. Each could have a deleterious clinical effect on CF lung disease: *TGFβ1* (18), *MBL2* (19), *MUC4/MUC20* (20, 21), *SLC9A3* (20), *AGTR2/SLC6A14* (20), *EHF/APIP* (22), *DNAH14*, *DNAAF3*, *DNAH6* (23), *DCNT4* (24), *MUC5AC* (25) and *MUC5B* (26).

**Genetic Testing:**

Blood was collected on Visit 1. DNA was sequenced by Broad Genomics (Cambridge, MA) using bead-based capture. Genetic variants were called using the Genomic Analysis Tool Kit (GATK) pipeline (Broad Genomics). All samples passed sample quality thresholds for contamination, chimera rate and coverage, and only variants that passed Variant Quality Score Recalibration conducted by Broad Genomics were assessed. Variants in genes of interest were annotated with cDNA and protein change and frequency. Null variants (i.e. nonsense, frameshift, canonical +/- 1, or 2, splice sites, initiation codon, single exon, or multi-exon deletion) and candidate variants (i.e. reported in the literature [27 and 28], or pathogenic in ClinVar [29]) were evaluated.

**Mucus Composition:**

Sputum samples were collected on Visit 1, after MCC, using a standardized sputum induction procedure (30). MUC5B and MUC5AC quantities were determined by dot-blot ELISA, using rabbit-anti-MUC5B (H-300, Santa Cruz Biotechnology, 1:5,000) and rabbit anti-MUC5AC (MAN5AC, provided by Dr. David Thornton, University of Manchester, 1:2,000). Irrelevant rabbit antisera used to verify observed signals were specific for each primary antibody, when used at the dilutions above. Standard curves for MUC5B were calibrated against mucin purified from human saliva (provided by Dr. Thornton). Standard curves for MUC5AC were generated using a sputum sample from a previous study (6). Because MUC5AC standards were not tested against purified protein, MUC5AC data are presented in arbitrary units rather than mass concentrations.

**Quantification of MCC:**

MCC was quantified immediately following inhalation of an aerosol containing the radioisotope <sup>99m</sup>technetium sulfur-colloid (radioaerosol) and for 90 minutes, thereafter,

using a gamma camera (6, 31, 8). On all three visits, MCC endpoints were calculated for the right lung as average percent clearance through 60 minutes (**MCC60**) and 90 minutes (**MCC90**), as described previously (6, 31, 8). Between 60 and 90 minutes, children performed 30 programmed-coughs (9).

#### **Radioaerosol Deposition Pattern:**

To determine if MCC was influenced by the initial radioaerosol deposition pattern, we compared deposition in outer (O) and inner (I) regions of the lung for each visit as described previously (6, 31). Deposition patterns were compared in terms of Outer:Inner (O:I) ratio, as recommended by a consensus of imaging experts (32).

#### **Sample Size:**

Based on demographics of patients who attended the Johns Hopkins CF Clinic at the time of study design, we estimated there would be approximately 35 eligible children on the projected study start date and approximately 20 of those children would be willing to participate in the study. The sample size of 20 was fixed by this estimate.

#### **Statistical Analysis:**

The following primary endpoints were selected *a priori* according to a pre-study protocol: percent MCC60 and MCC90, FEV<sub>1</sub> percent predicted, absolute LCI<sub>2.5%</sub>, HRCT score, O:I ratio, PA infection history and MUC5B and MUC5AC concentrations.

Due to the small sample size, all MCC, FEV<sub>1</sub>, LCI<sub>2.5%</sub>, HRCT, O:I ratio, PA, MUC5B and MUC5AC data are presented as median (minimum-maximum) and non-parametric tests were used to test for statistical differences in comparisons and associations.

Data from Visits 1 and 2 were used to determine MCC reproducibility and reliability over the two-week period. Data from Visit 2 were used for comparisons with data obtained on Visit 3 and for comparisons with other endpoints obtained during the two-week period. We chose not to average MCC data on Visits 1 and 2 for comparisons because an average could lead to an artificially more stable estimate of the baseline value. We chose not to use MCC data from Visit 1 for comparisons for the following reason. Consistency in performing the breathing maneuvers during radioaerosol administration is key to reproducible aerosol deposition in the lungs, which leads to more reproducible MCC results. Children were first introduced to the breathing maneuvers on Visit 1. On Visit 2, children remembered the breathing maneuvers learned on Visit 1 and became more proficient with additional practice. We assumed the additional proficiency gained on Visit 2 would lead to the most reproducible results for comparisons.

*Ad hoc* comparisons were performed between percent MCC60 and O:I from historic controls (8) and children with CF in the current study. Comparisons between percent MCC90 for the two groups were not performed because control subjects coughed 60 times between 60 and 90 minutes and children with CF coughed 30 times.

Reliability of MCC measurements was quantified in terms of intraclass correlation coefficients (ICCs), with ICCs >0.70 indicating good reliability (33).

In two exploratory analyses, we examined variability in MCC60 and MCC90 in relation to different environmental exposures and variants in genes related to pulmonary phenotype in CF, or MCC. Associations between each environmental exposure (independent variable) and percent MCC60, or MCC90, (dependent variable) were assessed using mixed effects models adjusted for sex and age and nested by individual to account for values obtained on Visits 2 and 3.

Analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina), Microsoft Excel 2016, or Stata IC 14 (College Station, Texas). All tests were two-sided and significance was set at  $p < 0.05$ .

## Results

### Timeline:

Visits 1 and 2 occurred between April 2014 and September 2018. Visit 3 occurred between July 2015 and July 2019.

### Children with CF:

At the start of the project, the number of eligible children who attended the CF Clinic at Johns Hopkins was less than projected. To increase recruitment capabilities, we added two additional sites: the CF Centers at Walter Reed National Military Medical Center (Bethesda, MD) and Children's Hospital Colorado (Aurora, CO). Nevertheless, we were only able to enroll 17 children from all three sites and three of the children discontinued their participation before completing any study visits. Therefore, a total of 14 children ( $n=5$  males) completed all three study visits. Nine children were referred by the CF Clinic at Johns Hopkins. Three children were referred by Walter Reed National Military Medical Center and two children were referred by Children's Hospital Colorado.

Thirteen children were between 5–6 years old and one child was 7 years old at the time of Visits 1 and 2. Median age at Visit 1 was 5.7 (5.2–7.2) years. Two children were full genetic siblings. Twelve children were Caucasians. Seven of 14 children were homozygous for F508del CFTR mutations. Five children had one F508del CFTR mutation, including one residual function mutation (R117H). Two children had no F508del CFTR mutations, but other minimal function mutations. Those genotypes were I507del/621 + 1G>T and exon 2,3 del/R553X. Thirteen children were pancreatic insufficient.

### Control Subjects:

There were 10 control subjects for the MCC60 measurements ( $n=6$  males). Median age was 24 (18–37) years. Median FEV<sub>1</sub> was 103 percent predicted (82–115).

### Use of Concomitant Therapies in Children with CF:

The use of concomitant therapies is shown in Table 1. The effect of treatment with lumacaftor/ivacaftor varied between children. In the two children treated with lumacaftor/ivacaftor at the time of Visit 2, percent MCC60 on Visit 2 was faster in one child and slower in the other, with 26.8% and 5.0%, respectively. In the five children treated with

lumacaftor/ivacaftor after Visit 2, MCC60 at Visit 3 was slower compared to Visit 2 in two children and faster in three children.

### **Reproducibility, Reliability and Stability of MCC Measurements in Children with CF:**

Percent MCC60 and MCC90 on Visits 1 and 2 are shown in Figures 1A and 1B. Median number of days between the two visits was seven (4–14). Median percent MCC60 and MCC90 were similar on Visits 1 and 2 (Table 2).

Percent MCC60 on Visit 1 was correlated with percent MCC60 on Visit 2 and 10/14 data points fell on, or near, the line of identity, indicating good reproducibility (Figure 2A). Percent MCC90 on Visit 1 was also correlated with percent MCC90 on Visit 2 (Spearman  $Rho=0.73$ ;  $p=0.003$ ; data not shown). ICCs for the two MCC60 and MCC90 measurements were 0.72 and 0.77, respectively, indicating good reliability

There was no significant difference between percent median MCC60 and MCC90 on Visits 2 and 3, showing stability over the one-year period (Table 2). However, percent MCC60 on Visit 2 was not correlated with MCC60 on Visit 3 and intrasubject variability in MCC varied considerably over the longer time period (Figure 2B). Percent MCC90 on Visit 2 also was not correlated with MCC90 on Visit 3 (Spearman  $Rho=0.14$ ;  $p=0.63$ ; data not shown). ICCs for the two MCC60 and MCC90 measurements were 0.25 and 0.17, respectively, indicating poor reliability.

### **Outer:Inner (O:I) Ratios:**

O:I ratios on Visits 1 and 2 were similar (Table 2) and significantly correlated (Figure 3A), indicating good reproducibility. ICC for these O:I measurements was 0.72, indicating good reliability. O:I ratios on Visits 2 and 3 were also similar (Table 2). However, O:I ratios on Visits 2 and 3 were not correlated (Figure 3B), indicating poor reproducibility and the ICC for these O:I measurements was 0.35, indicating poor reliability. There were no significant associations between O:I ratio and MCC60 on any of the three visits. (Spearman  $Rho=0.26$ ;  $p=0.38$ ;  $-0.38$ ;  $p=0.18$  and  $-0.18$ ;  $p=0.53$  on Visits 1, 2 and 3, respectively). Similar results were obtained for O:I ratio and MCC90 (Spearman  $Rho=0.24$ ;  $p=0.40$ ;  $-0.40$ ;  $p=0.16$ ;  $-0.17$ ;  $p=0.57$  on Visits 1, 2 and 3, respectively).

Absolute change in O:I ratio on Visits 2 and 3 was significantly related to absolute change in MCC90 on Visits 2 and 3. Greater changes in O:I ratio were significantly correlated with greater changes in MCC90 (Spearman  $Rho=0.56$ ;  $p=0.04$ ). Absolute change in O:I ratio on Visits 2 and 3 was similarly associated with absolute change in MCC60. However, that relationship was not statistically significant (Spearman  $Rho=0.51$ ;  $p=0.06$ ).

### **Ad Hoc Analysis in Controls Subjects:**

Median percent MCC60 for control subjects was 12.4 (0.7–19.7), which was similar to median percent MCC60 reported for children with CF on Visit 2 (Table 2) (Mann-Whitney U test;  $p=0.73$ ). Median O:I for control subjects was 0.52 (0.24–0.83), which was similar to median O:I reported for children with CF on Visit 2 (Table 2) (Mann-Whitney U test;  $p=0.33$ ).

**Relationship between MCC and Age of Initial PA+ Culture:**

Seven children were PA<sup>-</sup> prior to Visit 2. Age of initial PA+ culture in the other seven children was 21 (3–69) months. There were no statistically significant associations between age of initial PA+ culture and percent MCC60, or MCC90, on Visit 2 (Spearman Rho= -0.20; p=0.50 and -0.17; p=0.57, respectively) (n=14).

**Relationship between MCC and PA+ Cultures/Year:**

Number of bacterial cultures performed/year prior to Visit 2 was 3.6 (1.1–8.0). Number of PA+ cultures/year prior to Visit 2 was 0.06 (0–1.08). Percent MCC60 and MCC90 in children with zero PA cultures/year prior to Visit 2 were not significantly different from MCC60 and MCC90 in children with positive PA cultures prior to Visit 2 (Mann-Whitney U test; p=0.57 and p=0.52, respectively). Percent MCC60 and MCC90 on Visit 2 were not significantly associated with number of PA+ cultures/year prior to Visit 2 (Spearman Rho= -0.33; p=0.24 and -0.36; p=0.21, respectively), or with percent of respiratory cultures that were PA+/year, prior to Visit 2 (Spearman Rho= -0.24; p=0.40 and -0.28; p=0.34, respectively) (n=14).

Ten children were PA<sup>-</sup> between Visits 2 and 3. Median number of bacterial cultures performed between Visits 2 and 3 was 5.0 (1.0–8.0). Median number of PA+ cultures/year between Visits 2 and 3 was zero (0–1.2). Percent MCC60 and MCC90 on Visit 3 were not significantly associated with the number of PA+ cultures/year between Visits 2 and 3 (Spearman Rho= 0.35; p=0.22 and 0.28; p=0.33, respectively), or with percent of respiratory cultures that were PA+/year between Visits 2 and 3 (Spearman Rho= 0.35; p=0.22 and 0.28; p=0.32, respectively).

**Relationship between MCC, MUC5B, MUC5AC and MUC5AC/MUC5B:**

Three children could not produce a sputum sample. Concentrations of MUC5B, MUC5AC and MUC5AC/MUC5B in the sputa were, therefore, determined from the remaining 11 children. Concentrations of MUC5B and MUC5AC were 77.6 (3.1–245.4) µg/mL and 65.6 (4.6–3984.0) AU/mL, respectively. Ratios of MUC5AC/MUC5B were 1.0 (0.4–16.23). There were no significant associations between MUC5B, MUC5AC, or MUC5AC/MUC5B and number of PA+ cultures/year prior to Visit 2 (Spearman Rho= 0.49; p=0.12, 0.03; p=0.92 and -0.03; p=0.93, respectively). Percent MCC60 was not significantly correlated with MUC5B, MUC5AC, or MUC5AC/MUC5B (Spearman Rho= -0.40; p=0.22, -0.01; p=0.98 and -0.03; p=0.94, respectively). Percent MCC90 was not significantly correlated with MUC5B, MUC5AC, or MUC5AC/MUC5B (Spearman Rho= -0.46; p=0.16, -0.01; p=0.98 and 0.05; p=0.89, respectively).

**Relationship between MCC and LCI<sub>2.5%</sub>:**

LCI<sub>2.5%</sub> was measured in 13 children on Visit 1 (Year 1) and 14 children on Visit 3 (Year 2). Median LCI<sub>2.5%</sub> increased from 7.7 to 8.2 between Visits 1 and 3 (n=13), but was not statistically different on the two visits (Table 2).

Percent MCC60 on Visit 2 was not significantly associated with LCI<sub>2.5%</sub> in Year 1 (Figure 4A), whereas lower percent MCC60 on Visit 3 was associated with higher LCI<sub>2.5%</sub> in Year



2 (Figure 4B). Lower percent MCC90 on Visit 2 was associated with higher LCI<sub>2.5%</sub> in Year 1 (Figure 5A), whereas, percent MCC90 on Visit 3 was non-significantly associated with LCI<sub>2.5%</sub> in Year 2 (Figure 5B).

Correlation tests between the directional change in percent MCC60 and percent MCC90 and the directional change in LCI<sub>2.5%</sub> between Years 1 and 2 showed no significant associations (Spearman Rho= 0.06; p=0.86; and 0.03; p=0.92, respectively).

#### **Relationship between MCC and FEV<sub>1</sub>:**

FEV<sub>1</sub> was obtained in 12 of 14 children on Visit 1. Spirometry was not included in the protocol at the time of Visit 1 for one child and one child could not perform spirometry reliably. FEV<sub>1</sub> was below 80 percent predicted in two of the 12 children, with 75.1 and 76.9 percent, respectively. All four children remained in the study because they had a minimal history of infection and no chronic symptoms.

FEV<sub>1</sub> values for the 12 children were similar on Visits 1 and 3 (Table 2). Percent MCC60 and MCC90 on Visit 2 were not significantly associated with FEV<sub>1</sub> values measured on Visit 1 (Spearman Rho= -0.09; p=0.78 and -0.06; p=0.85, respectively). Percent MCC60 and MCC90 on Visit 3 were not significantly associated with FEV<sub>1</sub> values measured on Visit 3 (Spearman Rho= -0.22; p=0.45 and -0.32; p=0.27, respectively).

#### **Relationship between MCC and HRCT Scores:**

HRCT scans were performed in 14 children on Visit 3. Median HRCT score for both lungs combined was 5.0 (0–58.0). There was no evidence of mucus plugging (n = 10), or bronchiectasis (n = 6), in either lung. Percent MCC60 and MCC90 on Visit 3 were not significantly associated with HRCT scores on Visit 3 (Spearman Rho= -0.10; p=0.75 and -0.16; p=0.59, respectively).

#### **Relationship between MCC and Genetic Variants:**

Null variants and candidate variants were identified in the following genes: *MBL2*, *DNAH14*, *MUC5B*, *MUC5AC* and *APIP*. Seven children carried at least one candidate variant. Four children carried one null variant. The genetic variant and the number of individuals with each is shown in Tables 3A and 3B. Two variants were identified in three individuals (#3, #4, and #13). Three variants were identified in one individual (#9). Median MCC60 and MCC90 on Visit 2 were not significantly different in children with no candidate variants [14.7 (2.7–26.8) and 18.9 (3.5–29.1), respectively], compared to children with at least one variant [10.3 (5.0–25.1) and 13.4 (6.9–29.8), respectively] (Mann-Whitney U test; p=0.28 and p=0.37, respectively). The sample size was too small to test for differences between MCC endpoints in children with one null variant.

#### **Relationship between MCC and Environmental Exposures:**

Table 4 shows the percent of children whose caregivers reported exposures to pets, pests, secondhand smoke, gas combustion in the home and RSV infection prior to Visits 2 and/or 3. MCC was not significantly associated with any of these exposures (Table 5).

## Discussion

To our knowledge, this is the first study to show that mucus clearance from the lungs by ciliary mechanisms (MCC60) and ciliary/cough mechanisms combined (MCC90) can be measured and is reproducible and reliable over a two-week period in 5–7 year-old children with CF. In addition, median MCC60 for these children, as well as the range of values, appear to be normal, since they were similar to values observed in a group of healthy, adult controls. Healthy, aged-matched children would have been the preferred control group. However, ethical considerations do not allow healthy children to be exposed to ionizing radiation for research purposes.

We hypothesized that MCC values would decline over the one-year study period. However, this was not the case. Median MCC values for Visits 2 and 3 were not statistically different, indicating MCC measurements were stable during this time period. Interestingly, PA infection rate in the majority of children was low over the one-year period and there was only one hospitalization for a pulmonary exacerbation. This lack of infection with PA and hospitalization might account for the lack of a significant decline in MCC between Visits 2 and 3.

MCC measurements on Visits 2 and 3 were less reproducible and reliable than on Visits 1 and 2. These results suggest that the change in MCC over time varies and multiple observations will be most reproducible when made over a short time interval. Reduced reproducibility and reliability in MCC on Visits 2 and 3 could have been related to changes in the distribution of the radioaerosol used to measure MCC within the lung, possibly due to alterations in ventilation homogeneity. It is well-known that changes in the deposition pattern of the radioaerosol, measured in this study in terms of O:I ratio, can influence MCC measurements (32) and absolute change in MCC90 between the two visits was significantly associated with absolute change in O:I ratio.

LCI<sub>2.5%</sub> did not change significantly over the one-year study period. However, MCC90 measurements on Visit 2 (Year 1) and MCC60 measurements on Visit 3 (Year 2) were significantly, negatively correlated with LCI<sub>2.5%</sub> values obtained at the same time points. These results suggest that variability in MCC is related to differences in ventilation homogeneity in this age group and lower MCC values are associated with increased ventilation inhomogeneity. It is unknown if variability in MCC and changes in ventilation homogeneity are independent markers of lung disease, or if altered ventilation homogeneity impacts MCC tests directly via changes in deposition. It is also unclear why MCC60 on Visit 2 and MCC90 on Visit 3 were not correlated with LCI<sub>2.5%</sub> values. This could be due to the small number of study subjects.

Inter-subject variability in MCC measurements in these children does not appear to be due to differences in therapy, since the majority of children in the study were treated with similar drugs during the study protocol. A small number of children were treated with lumacaftor/ivacaftor during the study. However, the effect of this drug combination on MCC is unclear, since some children demonstrated faster clearance with treatment and others demonstrated slower clearance after starting the drug.

Inter-subject variability in MCC measurements was not associated with age of initial PA+ culture. This finding is similar to what we found in older children with CF (6). However, unlike the previous study, there was no significant association between MCC and PA infection rate. Also, unlike another study (7), there was no difference in MCC between children who were PA<sup>-</sup> vs. PA<sup>+</sup>, prior to the MCC test. It is unknown why variability in MCC in these young children was not associated with PA infection rate. However, only four of 14 children in the present study were PA+ between Visits 2 and 3, with median PA+ cultures/year at zero. In the previous study (6), PA+ cultures/year between visits was much higher, averaging 0.90, and the time between visits was much longer with a median of 4.6 (3.1–10.0) years. This suggests that changes in MCC may be related to the length of time the infection is present. With such a low infection rate and short study period, it is not surprising that there was no association between MCC measurements and infection rate in the present study. Similar to studies in older children with CF (6, 7), FEV<sub>1</sub> in these younger children did not change significantly during the one-year study period and MCC measurements were not associated with FEV<sub>1</sub> values.

In contrast to previous studies (6,7), there was no association between percent MCC and HRCT scores in these young children with CF. It is not clear why this was the case. However, the lack of an association could be due to the large percentage of children with scores of zero for both mucus plugging and bronchiectasis, two components of HRCT scoring that directly pertain to MCC. Additionally, the effects of changes in MCC on the parenchymal damage observed in CT scanning may be cumulative, such that abnormalities appear over time (6, 7).

Other investigators have compared MUC5B and MUC5AC concentrations in healthy adults and adults with CF. Henke *et al.* showed that MUC5AC and MUC5B are increased in adult patients with CF during a pulmonary exacerbation (34) and Henderson *et al.* showed that concentrations of these two mucins are higher in young adults with CF who were infected with PA, compared to normal, uninfected adults (35). However, neither of these studies explored possible relationships between MCC and MUC5B and MUC5AC concentrations in these adults with CF.

To our knowledge, this is the first study to examine the relationship between MCC and these two mucins in young children with CF. Our results indicate that, during a period of stability (i.e. low infection rate with PA), MCC is not significantly related to MUC5B, or MUC5AC, concentrations, or MUC5B/MUC5AC ratio, measured in induced sputum in 5–7 year-old children with CF. Because of the small sample size, additional studies investigating the amounts and integrity of MUC5AC and MUC5B in larger cohorts are warranted.

In the two exploratory analyses, we found that MCC on Visit 2 was not significantly different in children with no candidate gene variants, compared to children with at least one variant. We also found no significant associations between MCC and reported environmental exposures.

In conclusion, this was the first study to characterize MCC in children with CF who were 5–7 years old. Findings from this study showed that measurements of MCC were

reproducible and reliable over a two-week period and stable over a one-year period. In addition, variability in MCC between children was associated with differences in ventilation homogeneity, such that children with lower MCC values had increased ventilation inhomogeneity. These results suggest that measurements of MCC could be used in short-term clinical trials of interventions designed to modulate MCC and as a new, non-invasive test to evaluate early lung pathology in children with CF.

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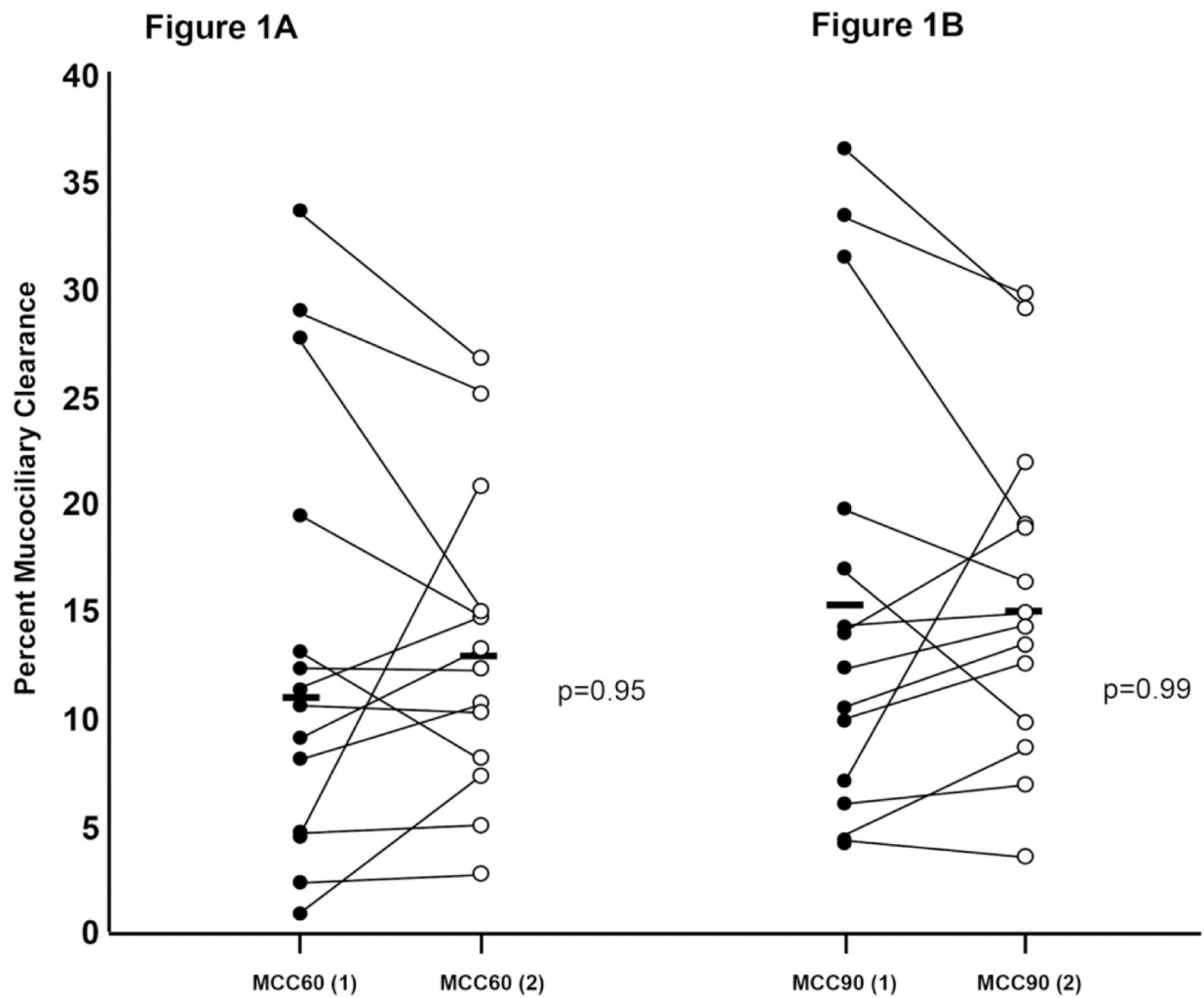
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**Impact Statement:**

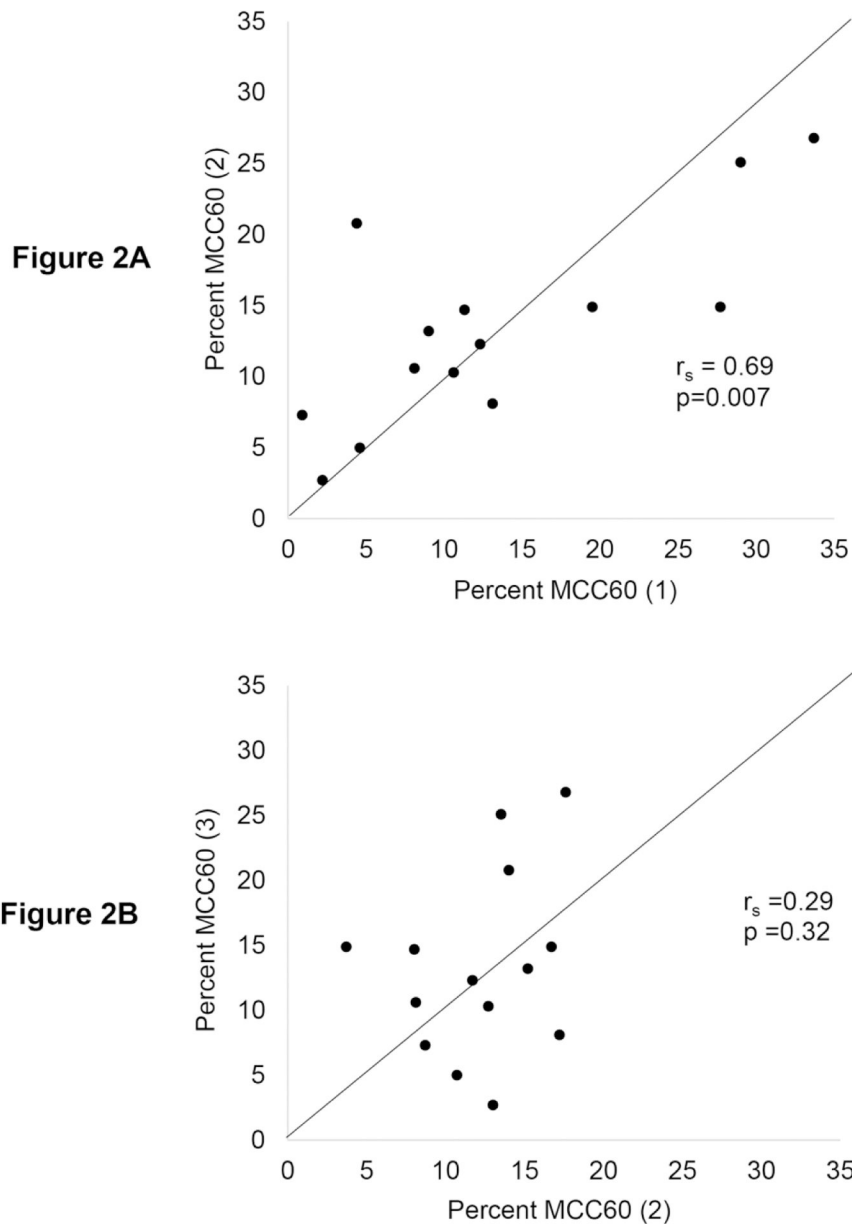
- This is the first study to characterize mucociliary clearance (MCC) in children with cystic fibrosis (CF) who were 5–7 years old.
- Measurements of mucociliary clearance were reproducible and reliable over a two-week period and stable over a one-year period.
- Variability in MCC between children was associated with differences in ventilation homogeneity, such that children with lower MCC values had increased ventilation inhomogeneity.
- These results suggest that measurements of MCC could be used in short-term clinical trials of interventions designed to modulate MCC and as a new, non-invasive test to evaluate early lung pathology in children with CF.



**Figure 1:**

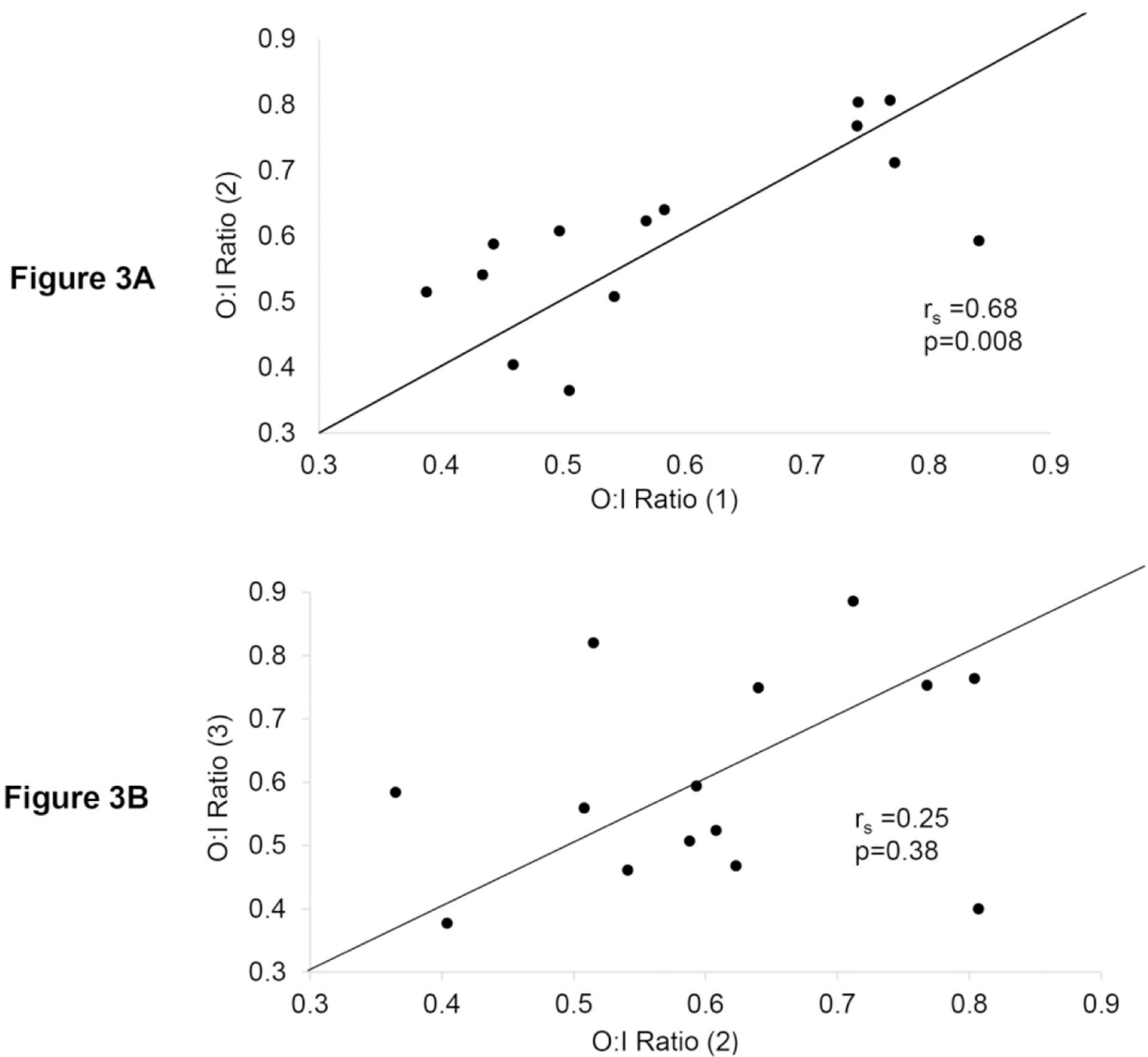
Comparison of percent MCC60 (A) and MCC90 (B) for each of the 14 children on Visits 1 and 2. Visit 1 is represented as (1) and Visit 2 is represented as (2). Horizontal black lines represent median values. MCC60 and MCC90 were similar on the two visits ( $p=0.95$  and  $p=0.99$ , respectively). Abbreviations: MCC60, average percent mucociliary clearance through 60 minutes; MCC90, average percent mucociliary clearance through 90 minutes. P values were determined by Wilcoxon signed rank tests.





**Figure 2:**

(A) Line of identity for average percent MCC60 measurements (N=14 pairs) on Visits 1 and 2. Visit 1 is represented as (1) and Visit 2 is represented as (2). Percent MCC60 on the two visits was significantly correlated ( $p=0.007$ ). A similar result was obtained for MCC90 ( $r_s = 0.73$ ;  $p=0.003$ ; data not shown). (B) Line of identity for average percent MCC60 measurements (N=14 pairs) on Visits 2 and 3. Visit 2 is represented as (2) and Visit 3 is represented as (3). Percent MCC60 on the two visits was not significantly correlated ( $p=0.32$ ). A similar result was obtained for MCC90 ( $r_s = 0.14$ ;  $p=0.63$ ; data not shown).  $R_s$  values were determined by Spearman correlation test. Abbreviation: MCC60, average percent mucociliary clearance through 60 minutes; MCC90, average percent mucociliary clearance through 90 minutes.



**Figure 3:**

(A) Line of identity for O:I ratio (N=14 pairs) on Visits 1 and 2. Visit 1 is represented as (1) and Visit 2 is represented as (2). O:I ratio on the two visits was significantly correlated ( $p=0.008$ ). (B) Line of identity for O:I ratio (N=14 pairs) on Visits 2 and 3. Visit 2 is represented as (2) and Visit 3 is represented as (3). O:I ratio on the two visits was not significantly correlated ( $p=0.38$ ).  $R_s$  value was determined by Spearman correlation test. Abbreviation: O:I ratio, percent of radioactivity deposited in outer region of right lung divided by percent of radioactivity deposited in inner region of right lung.

Figure 4A

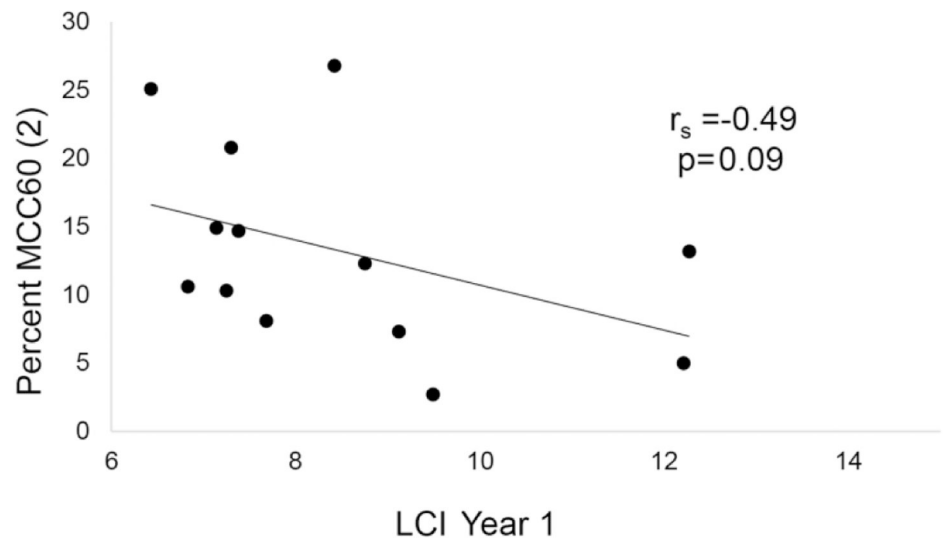
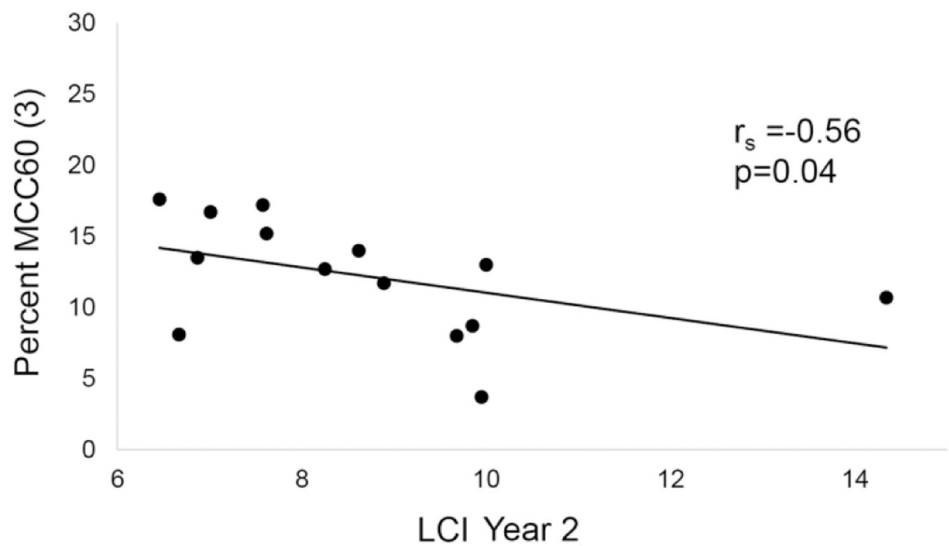


Figure 4B

**Figure 4:**

(A) Non-significant correlation between percent MCC60 on Visit 2 (2) and  $LCI_{2.5\%}$  in Year 1 (N=13) ( $p=0.09$ ). (B) Significant correlation between percent MCC60 on Visit 3 (3) and  $LCI_{2.5\%}$  in Year 2 (N=14) ( $p=0.04$ ). Linear trendlines, or best-fit straight lines, for the data, are included.  $R_s$  values were determined by Spearman correlation test. Abbreviation: MCC60, average percent mucociliary clearance through 60 minutes.

Figure 5A

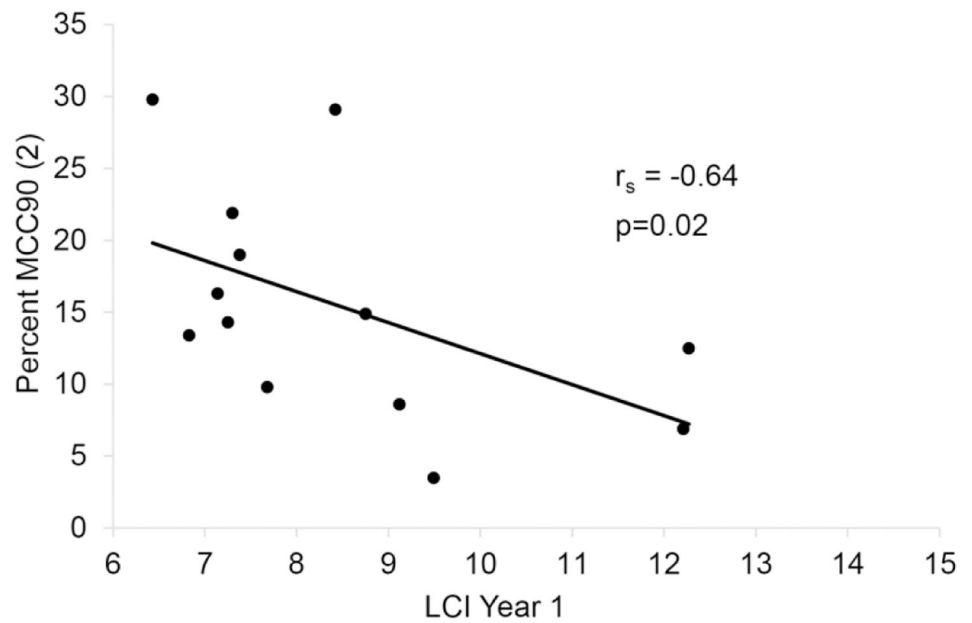
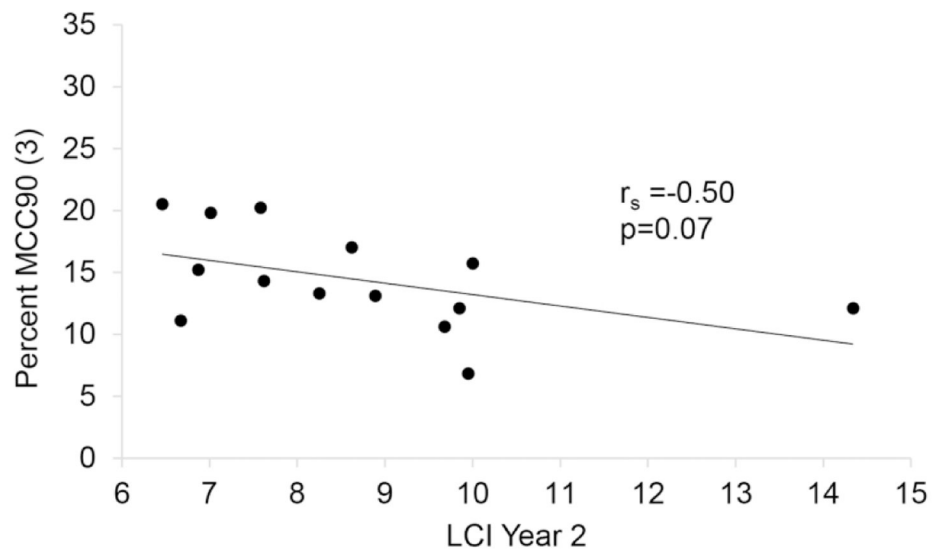


Figure 5B

**Figure 5:**

(A) Significant correlation between percent MCC90 on Visit 2 (2) and LCI<sub>2.5%</sub> in Year 1 (N=13) ( $p=0.02$ ). (B) Non-significant correlation between percent MCC90 on Visit 3 (3) and LCI<sub>2.5%</sub> in Year 2 (N=14) ( $p=0.07$ ). Linear trendlines, or best-fit straight lines, for the data, are included.  $R_s$  values were determined by Spearman correlation test. Abbreviation: MCC90, average percent mucociliary clearance through 90 minutes.

**Table 1.**

Use of concomitant therapies during study protocol.

Therapy	N (%)
Dornase alfa	12 (86)
Hypertonic saline	9 (64)
Airway clearance with high frequency chest wall oscillation	12 (86)
Albuterol	13 (93)
Lumacaftor/ivacaftor at time of Visit 2	2 (14)
Lumacaftor/ivacaftor at time of Visit 3	5 (36)
Any oral antibiotic	10 (71)
1–2 courses of an oral antibiotic	8 (57)
3 or more courses of an oral antibiotic	2 (14)
Inhaled tobramycin (single 28-day course)	2 (14)
Intravenous antibiotic course during hospitalization	1 (7)

Abbreviation: N, number of children; %, percent of 14 children

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**Table 2.**

Median (min-max) assessments in children with CF on three Visits.

Assessment	Visit 1	Visit 2	P value	Visit 3	P value
Percent MCC60 (n =14)	11.0 (0.9–33.7)	12.8 (2.7–26.8)	0.95 <sup>*</sup>	12.8 (3.7–17.6)	0.58 <sup>**</sup>
Percent MCC90 (n =14)	13.2 (4.2–36.5)	14.6 (3.5–29.8)	0.99 <sup>*</sup>	13.8 (6.8–20.5)	0.68 <sup>**</sup>
O:I Ratio (n =14)	0.56 (0.39–0.77)	0.60 (0.36–0.81)	0.42 <sup>*</sup>	0.57 (0.38–0.89)	0.93 <sup>**</sup>
Percent Predicted FEV <sub>1</sub> (n=12)	89.4 (75.1–123.6)	----	----	92.5 (59.5–113.4)	0.94 <sup>+</sup>
LCI <sub>2.5</sub> % (n=13)	7.7 (6.4–12.3)	----	----	8.2 (6.5–14.3)	0.28 <sup>+</sup>

All P values obtained from Wilcoxon signed rank tests.

<sup>\*</sup> P value for MCC Visit 1 vs. MCC Visit 2.<sup>\*\*</sup> P value for MCC Visit 2 vs. MCC Visit 3.<sup>+</sup> P value for MCC Visit 1 vs. MCC Visit 3.

Abbreviations: CF, Cystic Fibrosis, MCC60, average percent mucociliary clearance through 60 minutes; MCC90, average percent mucociliary clearance through 90 minutes; O:I ratio, percent of radioactivity deposited in outer region of right lung divided by percent of radioactivity deposited in inner region of right lung; FEV<sub>1</sub>, forced expiratory volume in one second; LCI, lung clearance index.

**Table 3A.**

Null variants in genes of interest.

Gene	Chr	Position (hg38)	nucleotide change	Protein change	Consequence	gnomAD freq. (v2.1.1)	# inds.	ID
<i>DNAH14</i>	1	225147216	CAATT>C	Ile1637ArgfsTer30	Frameshift	2.29E-03	1	11
<i>DNAH14</i>	1	225360679	A>G	.	Splice site	7.56E-05	1	9
<i>MUC5AC</i>	11	1190841	CA>C	Thr4233GlnfsTer57	Frameshift	0.000	1	4
<i>APIP</i>	11	34883367	C>CA	Trp200LeufsTer13	Frameshift	8.01E-06	1	3

**Table 3B.**

Candidate variants in genes of interest, determined through variants reported in literature, or pathogenic in ClinVar\*.

Gene	rsID	chr	position (hg38)	Change	Reference	Consequence	gnomAD freq. (v2.1.1)	# inds.	ID
<i>MBL2</i>	rs1800450	10	52771475	C>T (Gly54Asp)	VCV000014350.1 <sup>‡</sup>	missense	0.138	4	3, 5, 8, 9
<i>MUC5B</i>	rs35705950	11	1219991	G>T	23321605 <sup>‡</sup>	upstream	0.077	2	1, 13
<i>MUC5B</i>	rs885454	11	1222161	G>A	15709052 <sup>‡</sup>	upstream	0.105	1	4
<i>MUC5B</i>	rs7115457	11	1222830	G>A	15709052 <sup>‡</sup>	upstream	0.130	2	9, 13

\* ClinVar is a freely accessible, public archive of reports of the relationships among human variations and phenotypes, with supporting evidence (<https://www.ncbi.nlm.nih.gov/clinvar/>).

<sup>‡</sup>: ClinVar accession number.

<sup>‡</sup>: PubMed ID.

Abbreviations: Chr, chromosome; freq., frequency; inds., individuals; ID, identification #.



**Table 4.**

Percent of children with CF (n =14) with environmental exposures during study protocol.

<b>Environmental Exposure</b>	Pets (% any yes)	64%
	Pests (% any yes)	29%
	Secondhand Smoke (% any yes)	14%
	Gas Combustion in the Home (% any yes)	71%
	History of RSV (% any yes)	21%

Abbreviations: CF, Cystic Fibrosis; RSV, respiratory syncytial virus.

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**Table 5.**

Effect of environmental exposures on mucociliary clearance.

Exposure	MCC60		MCC90	
	Coefficient* (95% CI)	P value	Coefficient* (95% CI)	P value
Pets (no=0; yes=1)	1.4 (-3.0, 5.8)	0.54	1.3 (-3.3, 5.9)	0.57
Pests (no=0; yes=1) (cockroaches, mice, rats, mold)	1.2 (-4.0, 6.7)	0.65	2.0 (-3.5, 7.5)	0.48
Secondhand Smoke (no=0; yes=1)	6.0 (-1.2, 13.2)	0.10	6.8 (-1.1, 14.6)	0.09
Gas Combustion in the Home (no=0; yes=1)	3.1 (-1.6, 7.8)	0.19	4.1 (-0.5, 8.7)	0.08
History of RSV (no=0; yes=1)	3.0 (-2.6, 8.7)	0.29	3.6 (-2.0, 9.3)	0.21

\* Coefficients represent the change in percent MCC with a given exposure. Positive numbers represent an increase in MCC and negative numbers represent a decrease. For each regression, there were 14 individuals with 2 measurements each.

Abbreviations: MCC60, average percent mucociliary clearance through 60 minutes; MCC90, average percent mucociliary clearance through 90 minutes; RSV, respiratory syncytial virus.