Methods and Data Supplement:

Human Nasal Epithelial (HNE) cell sampling and culturing:

Subjects were asked to blow their nose before the sampling. A standard otoscope was used to visualize the inferior turbinate and a cytology brush was used to collect cells from along the lower aspect of the turbinate in both nostrils. The cytology brushes were placed in a 15 mL centrifuge tube containing sterile, ice-cold PBS, maintained on ice, and processed within 1 hour of sampling. In a sterile tissue culture hood, cells were dislodged from brushes by gently rubbing the brushes together, pelleted, and resuspended in Accutase for 10 min at 4 °C with rocking. Accutase was aspirated following centrifugation, and cells resuspended in "Georgetown" media in preparation for expansion.

Human nasal epithelial cells (HNEs) were expanded through co-culture with irradiated, 3T3 J2 feeder cells (irrJ2's), prepared ahead of nasal sampling. One day before sampling, two 100 ml petri dishes per subject were coated with 1x10^6 irrJ2's suspended in 10 ml/plate complete growth media and incubated in a 37 °C tissue culture incubator. After the fresh HNE were prepared, the complete growth media was aspirated from the petri dishes and replaced with 10 ml of the HNE preparation. Plates were returned to the 37 °C incubator and fed daily with Georgetown media. After the HNE cells reached 98% confluency, a second expansion phase was performed. HNE were dissociated from the original 2 plates with 0.05% trypsin, distributed to four 100 ml petri dishes precoated with irrJ2's, and maintained at 37 °C with daily feeding until 98% confluent.

Upon reaching 98% confluency, HNE were trypsinized (0.05%) from the plates and resuspended in BEGM, then seeded onto collagen-coated 0.33-cm^2 Costar Transwell filters at a density of 2 x $10^5/cm^2$; apical media was removed 24 hours after seeding and the filters grown at an air-liquid interface thereafter. Filters were fed 3x weekly with BEGM + Ultroser G differentiation media and used in assays between 0.5 and 2 months of seeding.

Media Composition:

Georgetown Media: Complete Growth Media(1x): 500 mL DMEM (H) (Gibco #11965-092) + 50 mL fetal bovine serum (Gibco #16140-071) + 5.5 mL 100x glutamine (Gibco #25030-081) + 5.5 mL pen/strep (Gibco #15140-122). Sterile. Store at 4°C.; F-12 Nutrient Mix (1x): Gibco #11765-054. Sterile. Store at 4°C. Hydrocortisone/EGF Mix (1000x): Dissolve hydrocortisone (Sigma H0888) in 100% ethanol at 0.5mg/ml. Mix 1 mL of this with 19 mL DMEM containing 2.5 ug EGF (Invitrogen PHG0311 or PHG0311L). Store sterile 1.1 mL aliquots at -20°C (hydrocortisone= 25 ug/mL; EGF=0.125 ug/mL). Insulin (5mg/mL): Dissolve 100 mg insulin (Sigma I5500 or I2643) in 20 mL distilled water containing 200 uL glacial acetic acid. Filter sterilize and store 1.1 mL aliquots at -20°C. Fungizone/Amphotericin B (250 ug/mL): Fisher #BP264550. Store sterile 1.1 mL aliquots at -20°C. Gentamicin (10 mg/ml): Gibco #15710-064. Sterile. Store at room temperature. Cholera toxin (11.7 uM): Dissolve 1 mg vial of cholera toxin (Sigma C8052) in 1 mL distilled water and filter sterilize. Stable at 4°C for one year. Y-27632: Axxora #ALX-270-333-M025. Dissolve 25 mg with sterile water to a concentration of 5 mM; make 1 mL aliquots to store at -20°C. *Volumes to make 500 ml*: Complete DMEM 373 mL, F12 nutrient mix 125 mL, Hydrocortisone/EGF mix 0.5 mL, Insulin 0.5 mL, Fungizone/Amphotericin B 0.5 mL, Gentamicin 0.5 mL, Cholera toxin 4.3 uL, Y-27632 (add last) 0.5 mL.

Differentiation Media: bronchial epithelial growth medium (BEGM) supplemented with Ultroser G (BEGM/USG; Pall Corporation, Crescent Chemical Company). Contained 5 μg/ml insulin, 10 μg /ml transferrin, 0.07 μg /ml hydrocortisone, 0.6 μg/ml epinephrine, 0.8% vol/vol bovine hypothalamus extract, 0.5 mg/mL BSA, 0.5 μM ethanolamine, 15 ng/ml retinoic acid, 0.5 ng/ml human epidermal growth factor, 10 nM triiodothyronine, 0.5 μM phosphoethanolamine, and 0.5% vol/vol USG in Dulbecco's MEM (DMEM) + F12 nutrient mix.

Methods for measuring apical Tc-DTPA absorption in HNEs (cell ABS):

We measured the absorption rate of 99m Technetium labeled Diethylenetriamine pentaacetate (Tc-DTPA) from the apical surface of the HNEs (Cell ABS). This *in vitro* measurement parallels the measurement of Indium 111-DTPA absorption in the *in vivo* ABS measurement. Tc-DTPA was produced by adding 5 mCi Technetium 99m pertechnetate to a Draximage DTPA kit vial and adding saline until the total volume is 5ml. Strip chromatography was then performed to ensure proper binding. A volume of 10 μL of the Tc-DTPA solution was then diluted with 990 μL of Ringer's. A 10 μL volume of this diluted solution was added to the apical side of the HNEs (0.1 μCi of Tc-DTPA, DTPA mass=0.4 μg). Retained activity in the ASL, cell layer, and filter surface was measured by briefly withdrawing the filter and placing it above a rate counter and measuring emitted radioactive counts for 30 s. Measurements were made at=0, 2, 4, 6, 8, 12, and 24 hours. The normalized percentage of decay-corrected radioactive counts absorbed over 24 hours is reported as Cell ABS. Each reported measurement represents the average of 6 filters.

FRAP methods:

The ASL layer was labeled with 10 μL of 70 kDa FITC-labeled dextran (20 mg/mL, Sigma-Aldrich, St. Louis, MO). The following morning, 50 μL of perfluorocarbon (FC-770, ACROS organics, ThermoFisher, Waltham, MA) was applied to the apical surface to prevent evaporative losses during evaluation. The cultures were then placed on a modified stage of a Nikon TiE inverted microscope equipped with a Nikon confocal A1 scanner and the ASL was visualized with a 40× water immersion objective (Nikon Apo LWD 1.15 NA). A baseline image was obtained and then a small region (6 × 18 μm) in the middle of the ASL was photobleached for 400 milliseconds. Following photobleaching, serial images of the region were acquired. The data was fit to an exponential *rise to max* function to determine the time constant (τ) for fluorescence recovery associated with dye diffusion. FRAP is expressed as the ratio of the time constant of recovery in the ASL relative to saline (τ_{ASL}/τ_{saline}). Higher FRAP values are associated with longer diffusion times and slower diffusion rates.

Ussing Chamber measurements:

HNE monolayers cultured on filter supports (0.33-cm^2, Costar Transwell) were mounted in Ussing chambers and continuously short circuited with a VCC MC6 automatic voltage clamp (Physiologic Instruments, San Diego, CA) to measure their transepithelial short-circuit current (I_{sc}). Transepithelial resistance (TER), an indicator of monolayer integrity, was measured by applying a 2 mV bipolar pulse every 90 s at the start and end of the experiments and calculated using Ohm's law. A TER ≥ 200 Ωcm^2 was considered indicative of acceptable monolayer integrity. Ringer's solution composed of 115 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5 mM glucose was used to fill the apical and basolateral chambers, which were continuously gassed with a mixture of 95% O_2 and 5% CO_2 to maintain solution pH at 7.4. All experiments were performed at 37° C. Following chamber flooding and voltage clamping, the I_{sc} was allowed to

stabilize until a steady state was reached (~10-30 min). Next, amiloride (10 μM, apical) was added to block the flow of Na^+ through ENaC; the resulting change in Isc is reported as the Na^+ current. After 5 min, forskolin (10 μM, basolateral) was added to activate CFTR via its phosphorylation by protein kinase A; the Cl^- current is measured as the stimulated increase in Isc at its plateau, typically 3 min after addition of forskolin.

Sweat Chloride:

A Macroduct® system was used to collect sweat from the forearms of subjects after pilocarpine iontopheresis. The collection apparatus remained in place for 30 minutes. The procedure was performed on both arms. Samples were drawn from the Macroduct tubing for the measurement of Chloride concentration (mMol/L).

Multiple-breath washout (MBW):

MBW was performed with a photoacoustic spectroscopy system (Innocor, Innovision Medical, Denmark) and a re-breathing technique. The test gas mixture contained 0.2% sulfurhexafluoride (SF6) and air. After establishing a regular breathing pattern, the rebreathing bag was filled with the test gas. The subject breathed from this bag and wash-in is continued until an equilibrium was reached for a stable SF6 concentration. A valve then opened, and the patient breathed room air for the washout, which finished when the tracer gas (SF6) concentration fell below 2.5% (1/40) of the starting level. Functional residual capacity (FRC) was calculated from the cumulative volume of expired SF6 divided by the difference between end-tidal gas concentration at the start and end of the washout. Lung clearance index (LCI) was calculated as the cumulative expired volume, divided by the FRC. Three measurements were attempted and the average of 1-3 successful measurements was reported.

MCC/ABS nuclear scans:

Participants first lay recumbent while 4-minute anterior and posterior (256 x 256 pixel) background images were collected. A 90s Cobalt-57 transmission scan was then performed. These posterior images were used to locate the lung perimeter for use in subsequent analysis. Subjects then rose and were seated for radiopharmaceutical delivery. A DeVilbiss 646 nebulizer containing 1.5 mCi (55.5 MBq) of Indium 111-DTPA (In-DTPA) and 8 mCi (296 MBq) of Technetium 99m-sulfur colloid (Tc-SC) in 3 ml of normal saline was used. The nebulizer was driven by a DeVilbiss 8650D compressor attached through a Spira dosimeter which triggered the nebulizer for 0.7s after the participant had inhaled 100ml of air. Peak compressor flow during nebulization was 10-13 L/min. These methods were intended to preferentially deliver aerosol to the airways. Radiopharmaceutical delivery was performed for 2 minutes (ages 12 or 13) or 4 minutes (older participants). After delivery participants returned to the camera and lay recumbent for 80 minutes while a series of sequential images was collected. Imaging was conducted in three energy level windows: 140 keV (Tc99m), 210 keV (mid-window), and 247 keV (In111). Medium energy collimators were used. After 10 minutes of imaging, while continuing to lie in the camera, all participants inhaled nebulized isotonic saline using a special tubing arrangement. Aerosol was delivered for 10 minutes. Imaging continued throughout. On a separate study day (order randomized) the CF group inhaled 7% hypertonic saline (Pulmosal) during this period instead of isotonic saline.

Posterior right lung images were analyzed. The left lung was not used to avoid interference from the stomach. A whole lung region of interest (ROI) was drawn from the lung outline on the transmission scan and placed onto the clearance images and used to determine whole lung measurements (red outline on **Figure S2**). A rectangle was drawn that exactly surrounded this lung outline (blue dashed line box on Figure S2). Its size was then decreased to one-half of its original height and width. This box was placed at mid-height of the lung and designated as the central lung ROI (yellow box on Figure S2). The portion of the lung inside the whole lung region but outside of the central lung region was designated as the peripheral lung ROI. Central lung zones contain large and small airways and alveoli. Peripheral lung zones contain small airways and alveoli. No adjustment is made for peripheral lung clearance into the central lung zone.

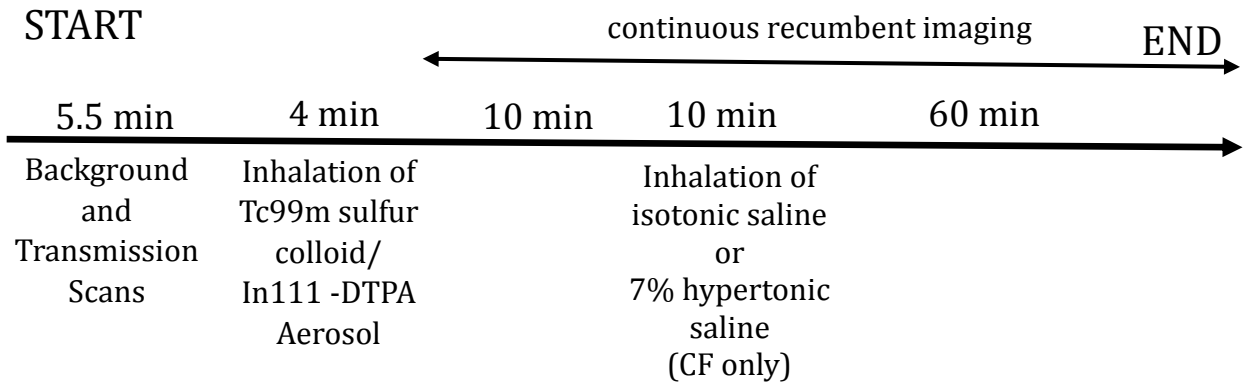


Figure S1: Timeline for imaging procedures. All subjects inhaled isotonic saline from a nebulizer while recumbent from t=10-20 min of the imaging period. CF subjects performed an additional imaging day where they inhaled 7% hypertonic saline during this period.

Measurements are adjusted for decay, background, and the spillover of Indium-111 into the Technetium-99m window. The latter correction was performed using the counts measured in the 210 KeV mid-window, which is midway between the two energy levels for In111 (173 and 247 KeV). Camera specific phantom studies were performed to determine the relative contributions of Indium 111 sources to the 140 KeV Tc99m window based on measures with known doses assessed in the 210 KeV window and the 140 KeV window.

Radioactive counts were normalized by starting counts in the ROI. MCC is the percentage of starting Tc-SC radioactive counts cleared at the end of the 80-minute study period, based on the average of the last 4 time points (t=74, 76, 78, and 80 min). Total In-DTPA clearance is the percentage of starting In-DTPA radioactive counts cleared at the end of the 80-minute study period, based on the average of the last 4 time points (t=74, 76, 78, and 80 min). ABS is total In-DTPA clearance minus MCC.

<u>Adjusted MCC rate</u>:

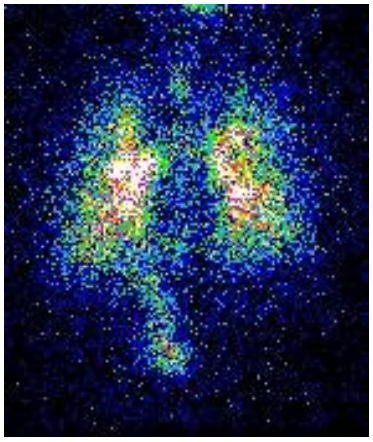
Previous studies have demonstrated that differences in the distribution of the deposited radioisotope aerosol in the lung can have a confounding effect on measurements of mucociliary

clearance (Locke et al, ERJ, 2016). Aerosol deposited in the larger, well-ciliated central airways is cleared more quickly that aerosol deposited in small airways or alveoli. To allow for comparisons of subjects with different distribution patterns we calculated an adjusted measure of MCC based on the amount of aerosol deposited in the central lung ROI (Cen%):

$MCC_{adjusted} = MCC_{measured} - S\ (Cen\% - Cen\%_{ave})$

where S is the slope of the relationship between $MCC_{measured}$ and Cen% in a multivariable model of MCC including central deposition % and group (HC, CR, or CF) as shown in table S2, S=1.33% cleared/% central deposition. Cen% is the subject's central deposition percentage and $Cen\%_{ave}$ is average of all three groups on the isotonic saline day (52%).

Use of $MCC_{adjusted}$ allows for comparisons between different subjects or different study days even if there is some variation in deposition pattern. It is utilized here to calculate MCC therapeutic response which involves two separate MCC measurements.



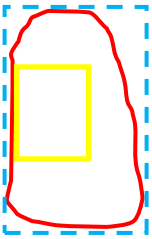


Figure S2: Regions of interest used for image analysis. Red is the whole lung region. Yellow is the central lung region. The peripheral lung region is the portion inside the whole lung but outside of central lung region. Technetium window shown.

Supplementary Data:

	Group I **CF ivacaftor (n=4)**	**Group TL** **CF ivacaftor + tezacaftor or lumacaftor (n=7)**	**Group N** **CF no corrector or potentiator (n=14)**	**All Groups** **p**	**I vs N** **p**	**TL vs N** **p**	**I vs TL** **p**
Age (years)	42 (27-61)	21 (17-39)	25 (18-31)	0.19	0.12	0.45	0.10
Female/male	1/3	2/5	9/5	0.45	0.26	0.36	0.29
FEV1 % pred	54 (48-83)	63 (47-73)	89 (67-98)	0.16	0.21	0.12	0.44
FVC % pred	86 (74-98)	89 (63-104)	102 (80-108)	0.51	0.43	0.46	0.48
FEF2575 % pred	25 (21-71)	26 (17-60)	59 (26-66)	0.39	0.39	0.30	0.43
LCI	13 (9-14)	9 (9-13)	9* (8-11)	0.31	0.25	0.30	0.31
Sweat Chloride (mMol/L)	25 (16-71)	95 (91-105)	107 (95-111)	0.06	**0.04**	0.13	0.22
MCC (WL)	46 (19-64)	27 (26-40)	40 (28-50)	0.39	0.41	0.32	0.26
MCC (PL)	46 (22-61)	27 (7-43)	37 (21-42)	0.33	0.25	0.27	0.24
MCC adjusted	34 (13-60)	26 (19-46)	38 (29-46)	0.63	0.37	0.50	0.68
ABS (WL)	5 (3-26)	24 (8-25)	23 (11-26)	0.56	0.43	0.47	0.37
ABS (PL)	9 (7-28)	27 (11-34)	18 (15-28)	0.54	0.44	0.29	0.40
% central aerosol deposition	55 (51-61)	51 (45-57)	51 (47-53)	0.44	0.36	0.44	0.24

Table S1: Comparing *in vivo* measurements in the CF group based on use of CFTR modulators. 4 CF subjects were using ivacaftor, 3 lumacaftor/ivacaftor, 4 tezacaftor/ivacaftor. 14 did not use modulators. 1 had unknown status. Data is Median (interquartile range). P values comparing all groups by Kruskal-Wallis (non-parametric). Group comparisons by Dunn's test with Holm correction (non-parametric, multiple comparisons). *-n=11.

Model Number	Model of	Central Deposition %		HC vs		Model		
		β	p	β	p	R^2	p	β 0
1	Whole Lung MCC	1.33	<0.001	CR -3.10 CF -7.02	0.55 0.19	0.23	0.01	-27
2	Peri Lung MCC	0.97	0.02	CR -3.02 CF -9.43	0.56 0.10	0.15	0.10	-10

Table S2: Multivariable linear regression model of mucociliary clearance (MCC) including central deposition percentage to indicate distribution of the aerosol and comparing healthy controls (HC), to Cystic Fibrosis (CF) and carrier (CR) groups.

	CF (n=26) IS	CR (n=16)	HC (n=12)	CF (n=26) HS	p	p(CFvHC)	p(CFvCR)	p(CRvHC)	p CF (HSvIS)
MCC (WL) ADJ	36 (23-46)	35 (30-48)	39 (34-52)	51 (40-62)	0.38	0.26	0.49	0.25	0.0002

Table S3: Adjusted mucociliary clearance (MCC) measurement. Since deposition of the radioisotope aerosol can affect measurements of MCC we calculated an adjusted MCC based on the slope of the relationship between MCC and central deposition %.

The formula used was MCC adjusted = $MCC_{measured} - S (Cen\% - Cen\%_{ave})$ where S is the slope of the relationship between MCC measured and Cen% in the multivariable model shown in Table S2, S=1.33% cleared/% central deposition. Cen% is the subject's central deposition percentage and $Cen\%_{ave}$ is average of all three groups on the isotonic saline day (52%).

All groups inhaled isotonic saline (IS) during the MCC/ABS scan. CF subjects performed an additional study day where they inhaled 7% hypertonic saline (HS) during the scan.

Data is Median (interquartile range). P values comparing all groups by Kruskal-Wallis (non-parametric). Group comparisons by Dunn's test with Holm adjustment (non-parametric, multiple comparisons). HS vs IS comparison for CF group by Wilcoxon matched-pairs signed-ranks test (non-parametric, paired).

CF=cystic fibrosis, CR=single CFTR disease-causing mutation (parents of CF participants), HC=healthy controls, HS=hypertonic saline (7%), IS=isotonic saline (0.9%). WL=whole lung, PL=peripheral lung, ADJ= adjusted values based on % of central lung aerosol distribution. Cen %=percentage of whole lung radioactive counts found in the central lung zone.

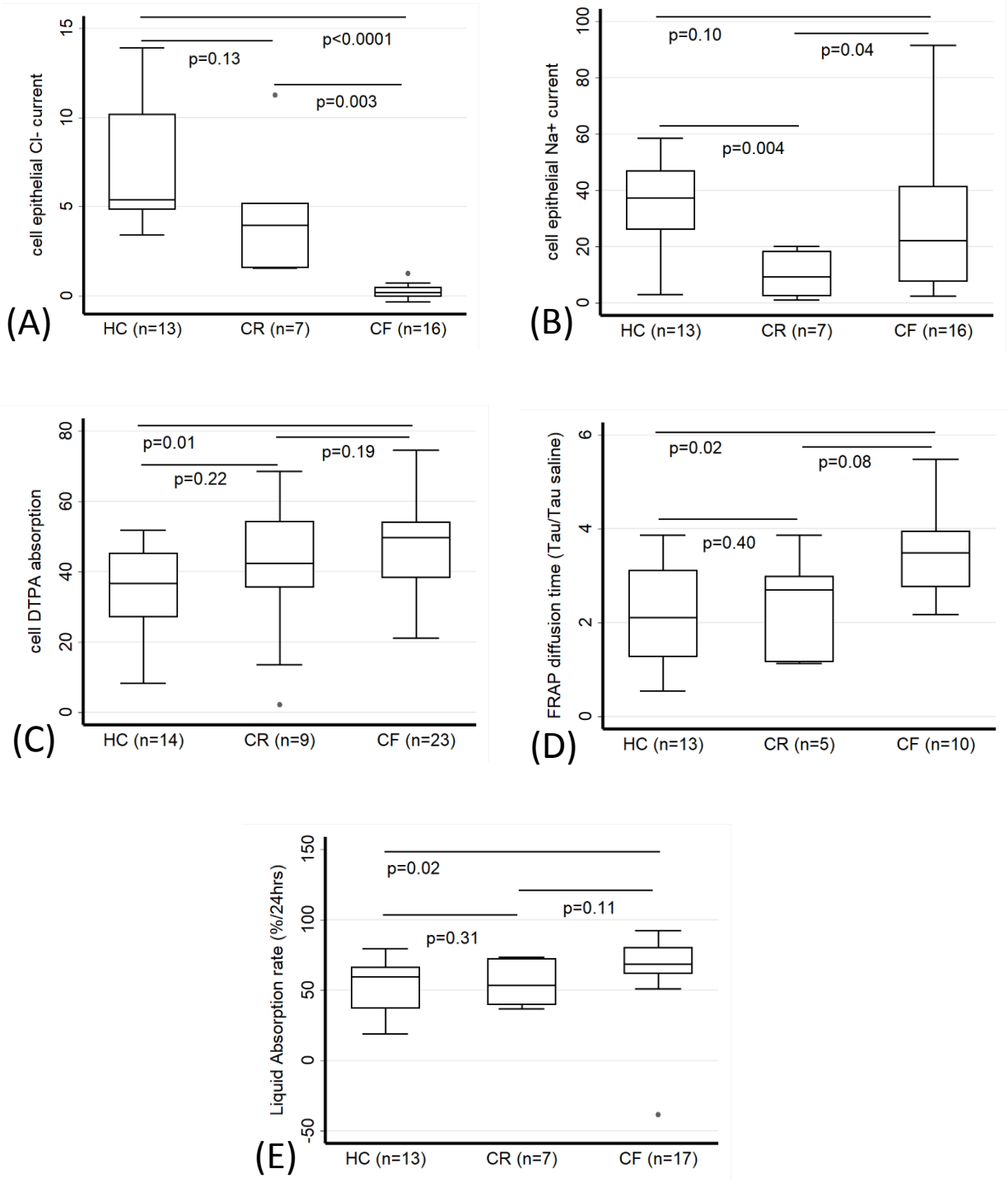


Figure S3: Graphical comparisons of *in vitro* measurements from HNE cultures presented in table 2. (A) Epithelial Cl- current from Ussing chamber. (B) Na+ current, (C) Cell ABS = Tc-DTPA absorption rate from apical surface of cells (% cleared/24 hrs.) (D) Fluorescence recovery after photobleaching (FRAP). (E) Airway surface liquid absorption rate (%/24hrs) as measured using an optical method Ref. 6) Group comparisons by Dunn's test with Holm adjustment (non-parametric, multiple comparisons).

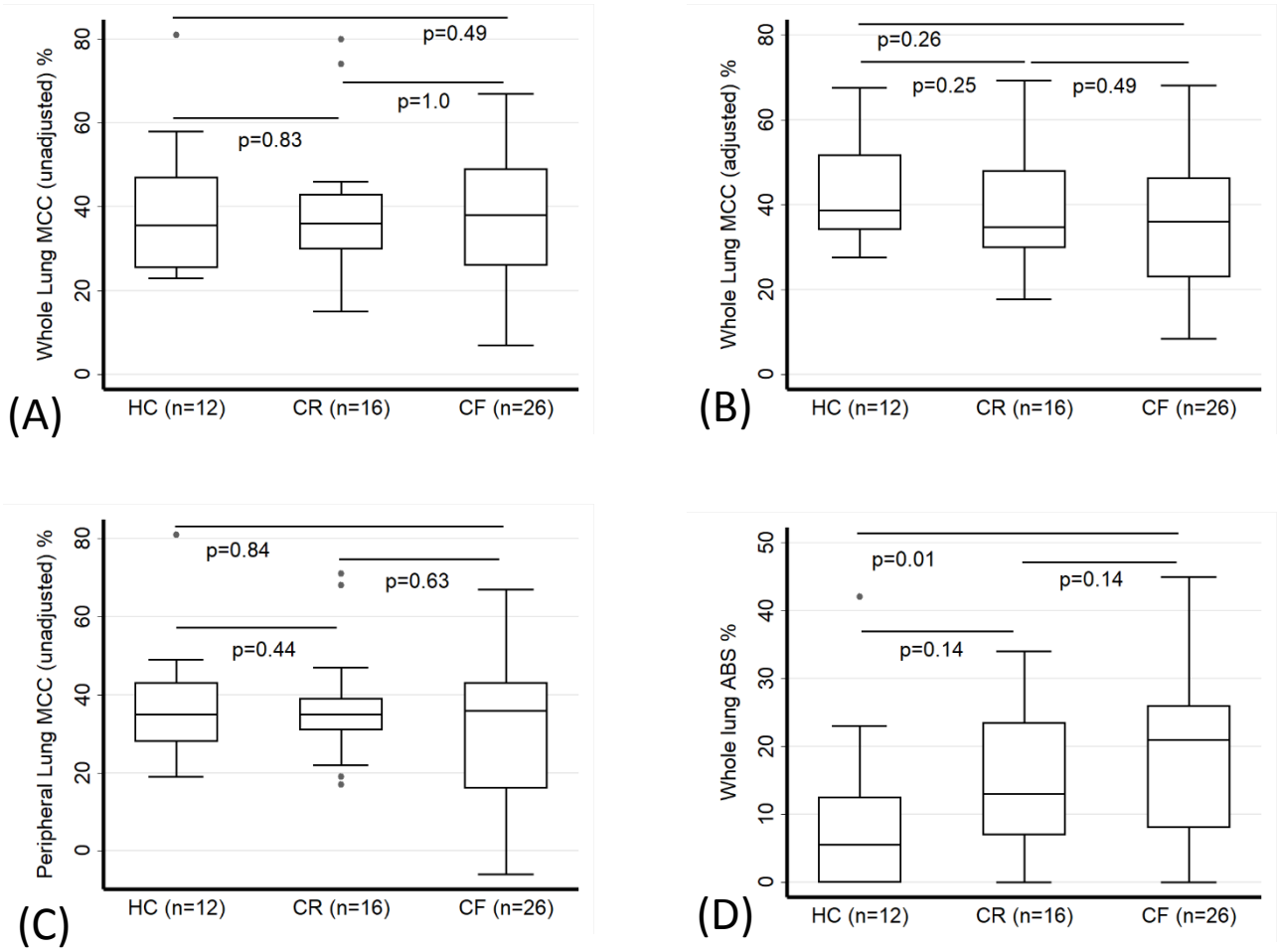
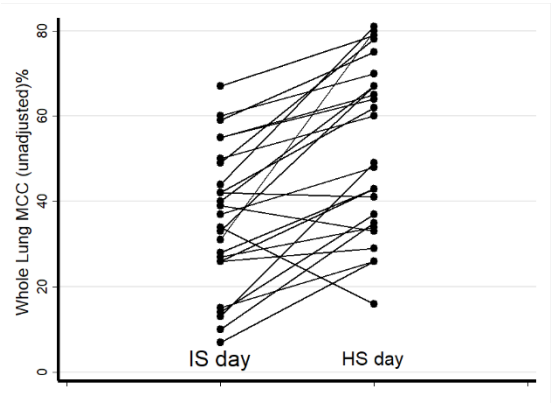
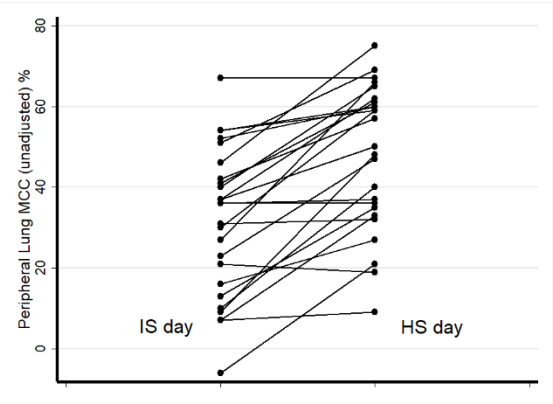


Figure S4: Graphical comparisons of *in vivo* imaging measurements presented in table 3. (A) Mucociliary clearance rate (MCC) for the whole lung region, (B) MCC for the whole lung region adjusted for aerosol distribution, (C) MCC for the peripheral lung region (unadjusted), (D) ABS – absorptive component of Indium 111-DTPA clearance. Group comparisons by Dunn's test with Holm adjustment (non-parametric, multiple comparisons).

(A)



(B)



(C)

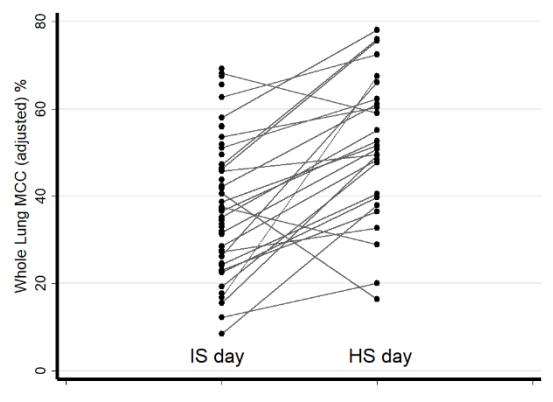


Figure S5: Mucociliary clearance (MCC) in CF group comparing inhalation of isotonic saline (IS day) to hypertonic saline (HS day). (a) Whole lung MCC with no adjustment for aerosol distribution ($p=0.0001$), (b) peripheral lung MCC with no adjustment ($p<0.0001$), (c) whole lung clearance adjusted for aerosol distribution ($p=0.0002$). Comparisons by Wilcoxon matched-pairs signed-ranks test (non-parametric, paired).