Culture with apically applied healthy or disease sputum alters the airway surface liquid proteome and ion transport across human bronchial epithelial cells.

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Sputum alters bronchial epithelial cell function.

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# Abstract (250 words)

Airway secretions contain many signalling molecules and peptides/proteins that are not found in airway surface liquid (ASL) generated by normal human bronchial epithelial cells (NHBE) *in vitro.* These play a key role in innate defence and mediate communication between the epithelium, immune cells and the external environment. We investigated how culture of NHBE with apically applied secretions from healthy or disease (Cystic Fibrosis, CF) lungs affected epithelial function with a view to providing better *in vitro* models of the *in vivo* environment. NHBE from 6-8 different donors were cultured at air-liquid interface (ALI), with apically applied sputum from normal healthy donors (NLS) or CF donors (CFS) for 2-4 hours, 48 hours or with sputum reapplied over 48 hours. Proteomic analysis was carried out on the sputa and on NHBE ASL before and after culture with sputa. Transepithelial electrical resistance (TEER), short circuit current (Isc) and changes to ASL height were measured. There were 71 proteins common to both sputa but not ASL. The protease:protease inhibitor balance was increased in CFS compared to NLS and ASL. Culture of NHBE with sputa for 48 hours identified additional factors not present in NLS, CFS or ASL alone. Culture with either NLS or CFS for 48 hours increased CFTR activity, calcium activated chloride channel (CaCC) activity and changed ASL height. These data indicate that culture with healthy or disease sputum changes the proteomic profile of ASL and ion transport properties of NHBE and this may increase physiological relevance when using *in vitro* airway models.

# Introduction

Airway secretions contain signalling molecules and peptides/proteins that play a key role in innate defence and mediate communication between the external environment, inflammatory cells and the epithelium. Reciprocal activity of the cystic fibrosis transmembrane regulator (CFTR) anion channel and the epithelial Na+ channel (ENaC) are important for maintaining the correct hydration of the airway surface liquid (ASL), mucocilliary clearance and innate immune function of the airways. In respiratory diseases such as cystic fibrosis (CF), aberrant CFTR transport results in an altered luminal environment. Changes to mucus structure, ASL volume, pH, protease activity, pathogenic bacteria and their toxins, inflammatory cells and inflammatory mediators all contribute to the development of a thick viscous sputum (1, 11, 24, 31, 39). These changes can then, in turn, further modify CFTR and ENaC activity. Normal human bronchial epithelial cells (NHBE), cultured at air liquid interface *in vitro,* produce a similarly regulated ASL but this lacks contribution from other cell types found *in vivo*. Exposure of NHBE to supernatant from mucopurulent material (SMM) collected from cadaver or post-transplant lungs was shown to increase CFTR activity and airway surface liquid (ASL) height (1, 13). Pro-inflammatory mediators present in SMM were proposed to be responsible for this effect (1, 13). In addition, induced sputum from people with CF (CFS) was shown to evoke an acute protease-mediated effect that activated ENaC, driving dehydration of the airways (39). These findings indicate that factors present in disease sputum can have short-term and long-term effects on ion channel activity *in vitro*.

However, most of the observed effects of exposure to SMM or CFS *in vitro* have been compared to exposure with a buffered salt solution. Normal healthy lungs do not contain mucopurulent material, but sputum can be induced and collected (normal lung sputum, NLS). The effect of NLS on epithelial function is poorly understood but we proposed that culture of NHBE with apically applied NLS could provide an improved physiological control to determine epithelial changes mediated exclusively by the pathophysiological constituents of sputum associated with disease states such as CFS. In addition, exposure to apical factors such as sputum i*n vitro* has often relied on only a single application for the duration of the treatment. *In vivo*, factors are continually secreted into the ASL from the epithelium and inflammatory cells. The mucocilliary escalator has also been calculated to bring 50 µl of new ASL to the tracheao bronchial region every 24 hours (23). Some of these factors may be subject to rapid degradation/inactivation reducing their activity. Accounting for the labile nature of proteins *in vitro* is challenging, nevertheless we wanted to address this by reapplying sputum to airway cultures in order to replenish labile constituents of the sputum in a chronic exposure setting.

Based on previous studies (1, 13, 27, 39), we therefore tested the hypotheses that culture of NHBE *in vitro* with apically applied NLS would not change the function of NHBE and that any responses to normal or disease sputum would be dependent on duration of application in culture. We investigated the proteomic constituents of NLS, CFS, and the NHBE ASL before and after culture with apically applied sputa, short circuit current (Isc) and ASL height. We used an 2-4 hour ‘acute’ (39) and a 48 hour ‘chronic’ culture with apically applied sputa (1) using primary bronchial airway epithelial cells from 6-8 different donors without respiratory disease. We studied the effects of reapplication of apical sputa over 48 hours in order to better replicate conditions *in vivo.* The aim was to understand how secretions from the normal luminal environment modify airway epithelial function in order to provide better *in vitro* models of airway function.

# Materials and methods

## Primary Normal Human Bronchial Epithelial Cell Culture

Normal human bronchial epithelial cells (NHBE), male and female, were obtained from endobronchial brushings or extracted from explanted non-diseased lungs as previously described and in accordance with ethical approvals obtained by The University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #03-1396) (10). Donor demographics are shown in Supplementary Table 1a, [https://doi.org/](https://doi.org/10.6084/m9.figshare.14791926)[10.6084/m9.figshare.14791827](http://10.6084/m9.figshare.14791827). Cells were cultured on permeable supports and maintained at air-liquid interface (ALI) in a modified bronchial epithelial growth medium as described (10). Cells were studied 28-35 days after seeding on 12-mm T-clear inserts (Corning-Costar, Corning, NY)

## Sputum preparation

Airway sputum samples were obtained as previously described (3, 28, 29) and in accordance with ethical approvals obtained by The University of North Carolina at Chapel Hill Biomedical Institutional Review Board (protocol #15-2431). Donor demographics are provided in Supplementary Table 1b, <https://doi.org/10.6084/m9.figshare.14791827.v1>. An ultrasonic nebuliser was filled with 30 ml of 5% hypertonic saline (NaCl). After a 12-min inhalation period subjects underwent a cleansing procedure: gargle and rinse the mouth with water, scrape and clear the back of the throat (to avoid the inclusion of non-airway fluid samples) and blow nose. The subjects were asked to deliver a chesty cough and expectorated the secretions into a sterile specimen jar. Samples were placed on ice and stored at -80oC.

Un-refined sputum samples were thawed on ice and centrifuged at 4000 x g for 20 minutes to remove cells, bacteria and macromolecules and the supernatant used for all downstream experimentation. NLS or CFS was pooled from 10 donors and 20 μl was applied to the apical surface of ALI cultures. Cells were cultured with apically applied CFS or NLS for 2-4 hours (acute), or 48 hours (chronic) or to emulate the replacement of labile constituents in the luminal lung environment, 4 μl of NLS or CFS was reapplied apically twice a day for 48 hours and 4 hours before taking the first measurement (reapplied).

## Mass spectrometry based proteomic analysis

NHBE ASL for was acquired for mass spectrometry by incubation of NHBE cultures at 37oC with 100 µl apical applied PBS for 10 minutes and collecting the resulting apical fluid. Samples were prepared for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis using filter-aided sample preparation (FASP) (41). Peptides for peptidomic analysis were collected using Amicon Ultra 4 10 kDa filters prior to the proteomic sample preparation. Samples were reduced by 10 mM dithiothreitol (Sigma-Aldrich) and alkylated in 50 mM iodoacetamide (Sigma-Aldrich), followed by digestion overnight using trypsin (20 ng/μl) in 50 mM ammonium bicarbonate at 37°C. Peptide eluates were vacuum freeze-dried and dissolved in 25 μl of 1% acetonitrile and 0.1% trifluoroacetic acid. Solubilised peptide material (5μl) was loaded into a trap column for proteomic analysis in a Q Exactive mass spectrometer coupled to an UltiMate 3000 nano HPLC system, and data acquisition was performed as described (17).

## Proteomic data analysis

The raw data was processed and searched against the Uniprot protein database (Homo sapiens, April 2019) using the Proteome Discoverer 1.4 (Thermo Scientific) software. Parameters used in the Sequest search engine: 10 ppm mass accuracy for parent ions and 0.02 Da accuracy for fragment ions, 2 missed cleavages allowed. Carbamidomethyl modification for cysteines was set to fixed and methionine oxidation to variable. Scaffold 4.7.5 (Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The heat map and Euclidean hierarchical clustering was generated from Log10 mean ion current data obtained from samples and normalised to a common protein found at similar levels in all samples (TXN) using Morpheus at software.broadinstitute.org.

## Electrophysiological measurements

Transepithelial ion transport was measured using the Ussing technique as previously described (43). Cultures were mounted onto sliders and inserted into EasyMount Ussing Chamber System. Apical and basolateral chambers were filled with 5 ml of buffer (117 mM NaCl, 2.5 mM CaCl2, 4.7 mM KCl, 1.2 mM MgSO4, 25 mM NaHCO3, 1.2 mM KH2PO4, 11 mM D-glucose, 5 mM HEPES (pH 7.4)). The solution was maintained at 37°C and bubbled with 21% O2+5% CO2 premixed gas throughout the course of the experiment. The epithelium was voltage clamped at 0 mV and short-circuit current (Isc) and transepithelial resistance (TEER) were measured. Cultures were equilibrated for a minimum of 20 min prior to the addition of amiloride, 100 μM (apical) to inhibit ENaC; forskolin, 10 μM (bi-lateral) to elevate cAMP and activate CFTR; CFTRinh172, 10 μM (apical) to inhibit CFTR; UTP, 100 μM (apical) to activate Ca2+-activated Cl− channels (CaCC) and ouabain, 100 μM.

## Airway surface liquid height measurements

PBS, CFS or NLS (20 μl) containing 0.5 mg/ml of 10 kDa dextran-tetramethylrhodamine (Life Technologies, USA) was added to the apical surface of NHBE. Cells were labelled with CellTrace™ Calcein Green, AM (Thermofisher, USA) (5 µg/ml in media). ASL height stabilized by 120 minutes. 100 nM vasoactive intestinal peptide (VIP; Life Technologies, USA) was added basolaterally to induce CFTR mediated secretion. Perfluorocarbon (3 M Fluorinert FC-770) was added apically to prevent ASL evaporation. Images were obtained immediately before and 60 minutes after basolateral VIP addition in XZ-scanning mode by using a Leica SP8 confocal microscope with a ×63/1.3 numerical aperture (NA) glycerol immersion lens. 10 ASL images per culture were acquired by using an automatic stage with the “Mark-and-Find” function as described (7).

## Statistics

. Raw data from cells from individual donors before and after treatment (as shown in supplementary data) was analysed using a non-parametric paired Wilcoxon test. Normalised summary data shown in the manuscript was analysed using a non-parametric Kruskal–Wallis test with a post hoc Dunn’s test. Data are shown as individual points (scatterplot) with mean ± standard deviation. Significant differences are indicated with \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. Data analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

# Results

## Sputum/ASL proteomes

We firstly analysed the proteomes of the NLS and CFS (pooled samples n=10). We then analysed NHBE ASL (pooled n=8) before and after culture with sputum (pooled n=4 each). A total of 268 proteins were identified in NLS, 262 in CFS and 1016 in NHBE ASL (Figure 1a and b). The variation in the number of proteins identified is likely due to differences in protein concentration associated with the different methods of sample acquisition. The signal intensity for MUC5B in the NLS analysis was ~3 times lower than for a NHBE ASL samples (Supplementary Table 2, <https://doi.org/10.6084/m9.figshare.14791827>). Thus, there was an increase in sensitivity for acquiring proteins in ASL over that of induced sputum. 136 proteins were common to all samples whilst 22 proteins were exclusively found in NLS, 19 in CFS and 805 in NHBE ASL (Figure 1b). The proteins exclusively found in CFS were tightly correlated with immunity pathways including regulated exocytosis, neutrophil degranulation, immune response, cell activation and transport (alignments made via analysis through the STRING database) (p<0.001). No such correlations were identified with proteins exclusively found in NLS. Both NLS and NHBE ASL contained a lower abundance of identified proteases compared to CFS. In particular, neutrophil elastase, cathepsins, myeloperoxidase and myeloblastin (Figure 1c). NLS contained more serpin family protein members than CFS and NHBE ASL, although key protease inhibitors such as alpha-1-antitrypsin, anti-thrombin and leukocyte elastase inhibitor were elevated in CFS (Figure 1c). Innate immune factors such as Neutrophil defensin 3, BPIFB2 and Lipocalin-1 were detected in both NLS and CFS. SPLUNC1, reported inhibitor of ENaC and a potential therapeutic peptide for CF was detected in NHBE ASL (Supplementary Table 2, <https://doi.org/10.6084/m9.figshare.14791827>).

Analysis of the ASL from NHBE cultured with NLS or CFS identified 7 new proteins resulting from chronic exposure to either sputa (Figure 2a and b), which included brain-specific protease 4 (PRSS22) a serine protease found in airway cells, lung and oesophagus) and the tumour necrosis factor alpha (TNF-α) induced protein 2 (TNFAIP2) which mediates many of the effects of TNF-α. There were 37 proteins exclusively identified in NLS exposed ASL and 23 in CFS exposed ASL, indicating that these proteins were released from NHBE in response to sputum exposureEvidence indicates that PRSS22 is constitutively expressed in airway epithelium, is resistant to protease inhibitors such as a1-anti-typsin and that urokinase-type plasminogen activator is a preferred substrate, suggesting that PRSS22 could have a role in lung injury and repair (35, 42). TNFAIP2 is a factor in the early response to inflammatory stress. The other 37 proteins were aligned to biological processes in the STRING database and were found to be tightly correlated with cellular transport and localisation processes, including protein localisation and vesicle mediated transport (p<0.001) (Figure 2a and c). This is a well-documented response to inflammatory signalling in eukaryotic cells, in which transcriptional induction of genes that have functions associated with increasing the ER volume and capacity for protein folding are upregulated (16, 32, 33). A number of factors in sputum (including proteases) could elicit the release of these proteins via direct or receptor mediated mechanisms.  More work is required to understand how/why sputum induces release of these proteins from airway cells *in vitro.*

## Effect of NLS and CFS on epithelial electrophysiology

Mean donor resistance ranged from 276 Ω.cm2 and 1169 Ω.cm2 but with little variation within samples from the same donor (Table 1, Supplementary Table 1a, <https://doi.org/10.6084/m9.figshare.14791827>). TEER changed in untreated NHBE at both the acute and chronic time points (Supplementary Figure S1 a-h, <https://doi.org/10.6084/m9.figshare.16628725.v1>). Taking these changes into account, NLS had no further effect on TEER. The only consistent response we observed was a reduction in TEER (~20%) after acute and reapplied culture with CFS compared to untreated NHBE (p<0.05; n=6, respectively) (Figure 3a). Incubation of CFS for 30 minutes with cOmplete™ protease inhibitor cocktail prevented the decrease in TEER (p<0.01, n=4) (Figure 3b). These data indicated that proteases present in CFS acutely reduce epithelial TEER.

There was significant variation between basal short circuit current (Isc)recorded for each donor and there were donor dependent differences in the magnitude of the response to pharmacological drugs when different sputa treatments were applied. Nevertheless, specific sputum exposures resulted in consistent responses in all donors as exemplified bydonor DD0028N (Figure 3c).

## Effect of NLS

Acute culture with apically applied NLS increased UTP-sensitive Isc (p<0.05, n = 7) but had no consistent effect on any other parameter tested (Figure 4d). Chronic culture with NLS however, resulted in an increase in both forskolin-stimulated, CFTRinh172-sensitive (p<0.05, n =8) and UTP-stimulated Isc (p<0.01, n =8) (Figures 4b, c and d). Reapplying NLS increased UTP-stimulated Isc (p<0.05, n =6) (Figure 4e). However, we observed that the changes in Isc were generally lower than those elicited by chronic application of NLS. Thus, reapplying NLS tempered the effects of treatment on CFTR and CaCC activity. The responses to culture with/without NLS by individual donors are shown in Supplementary Figure S2 a-i, <https://doi.org/10.6084/m9.figshare.14792010>.

## Effect of CFS

Consistent with previous findings, acute culture with apically applied CFS increased the amiloride-sensitive Isc in each donor (p<0.05; n=7) (Figure 4a). It also elicited a modest decrease in forskolin-stimulated Isc and CFTRinh172-sensitive Isc compared to the untreated samples (p<0.05; n=7, respectively) (Figures 4b & c). Thus, acute exposure to CFS increased ENaC and decreased CFTR activity.

Chronic culture with apically applied CFS increased forskolin-stimulated, CFTRinh172-sensitive and UTP-stimulated Isc (p<0.05, n = 8, respectively) (Figures 4b, c and d). These data indicate that chronic exposure to CFS increased both CFTR and CaCC activity. When CFS was reapplied over a period of 48 hours the predominant effect determined was that Forskolin-stimulated Isc remained elevated (p< 0.05, n=6) (Figure 4b). But again, we observed that the changes in Isc were generally lower than those elicited by chronic culture with CFS. Thus, reapplying CFS tempered the chronic effects CFTR and CaCC activity. The responses to culture with/without CFS by individual donors are shown in Supplementary Figure S3 a-i, <https://doi.org/10.6084/m9.figshare.14791926>.

## Effect of NLS and CFS on ASL height

Vasoactive intestinal peptide (VIP) acts as an agonist of basolateral VPAC1 receptors which subsequently increases intracellular cAMP concentration to increase CFTR activity (similar to the mechanisms of forskolin) (8). As VIP requires basolateral application only, it allowed us to better control apical fluid volume and the measurement of ASL height.  In our model, the concentration of VIP used produced similar changes in Isc to that of forskolin (Figures 5a-d).  Treatment with VIP increased ASL height in NHBE acutely cultured with NLS but not acutely cultured with CFS (p<0.05; n=4) (Figure 5e and f). VIP also increased ASL height after chronic culture with NLS or CFS (p<0.05; n=4) (Figure 5e and f) consistent with increased Cl- driven fluid secretion. VIP did not increase ASL height after acute culture with CFS which correlated well with reduced Cl- secretion and increased Na+ driven fluid absorption. Culture with reapplied NLS and CFS were unsuitable for ASL height imaging as 4 µl additions were insufficient in volume to produce homogenous dispersal of labelled dextran across the NHBE culture.

# Discussion

Our data support and expand on findings that the complex luminal environment of the lung modifies airway epithelial function. Our demonstration that normal lung sputum changes NHBE function raises questions as to whether the current *in vitro* culture methods provide an appropriate model of *in vivo* ASL. In this study we compared the proteomic composition of induced sputum from healthy individuals (NLS) to that from people with CF (CFS) and tested their effects on NHBE isolated from 6-8 different donors. There was significant variability in the response to culture with apically applied sputa associated with different donors and surprisingly, neither the TEER nor basal Isc was predictive of the outcome. Nevertheless, some treatments generated similar response in all donors and, importantly, we were able to determine consistent acute and chronic effects of culture with both NLS and CFS on epithelial function lending power to our study.

We used proteome analysis to characterise the sputa and to identify changes in the NHBE ASL in response to chronic culture with sputa. The data we obtained indicated that NLS, CFS and ASL had distinct proteomic fingerprints. Consistent with the findings of others, CFS contained more immunity related proteins and inflammation related proteases compared to NLS (4, 25, 26). NLS contained a broader range of protease inhibitors supporting the notion that the protease/protease inhibitor balance is increased in CF disease (1, 14, 24, 39). Interestingly, whilst NHBE ASL showed commonality with both NLS and CFS the protease/protease inhibitor balance was more akin to that of NLS.

Consistent with the proteomic data, culture of NHBE with CFS for 2-4 hours reduced TEER in a protease dependent manner. Proteases have been shown to significantly increase transepithelial diffusion, decrease tight junction protein formation and reduce epithelial apical junctional complex reformation during wound healing *in vitro* (11, 38, 44). CFS but not NLS also increased ENaC activity, reduced CFTR activity and ASL height as previously reported (39). This finding was interesting in the face of a reduction of TEER which might be expected to increase fluid flux into the ASL, but the acute response to CFS mimicked CF disease where ASL height is reduced *in vivo* (12, 37). Protease activity was also likely to underpin the increase in ENaC and decrease in CFTR activity. For example, neutrophil elastase (which we found to be ~10-fold more abundant in CFS than NLS) has been shown to rapidly increase ENaC activity in human nasal epithelial cells and decrease CFTR channel function within 6-hours in NHBE and mice *in vivo* (20, 30). Cathepsins, serine proteases and matrix metalloproteinases, which we show are elevated in CFS (corroborating with other work: (5, 11, 24, 36)), are known to cleave and activate ENaC (6, 15, 40). In addition, the inhibitor of ENaC, SPLUNC1 although present in NHBE ASL was not detected in CFS or CFS treated cultures where it is reportedly degraded (39).

We showed that chronic culture with apically applied sputa evoked changes to the proteome of the NHBE ASL that were distinct from NLS and CFS and signposted an epithelial response to the apical application of the sputum that further changed the luminal environment. Disproving our hypothesis, we also found that chronic culture with either NLS or CFS consistently increased anion transport via CFTR and CaCC activity in all donor NHBE and the increased capacity for Cl- efflux correlated with increased fluid secretion into the ASL. Chronic exposure to supernatant from mucopurulent material (SMM) was shown to increase CFTR and CaCC activity (1, 13). While we cannot exclude indirect effects that modify the driving force for Cl- movementour findings indicate that chronic culture with sputum from healthy donors or CF patients with FEV1s ≥ 0.92 (i.e., cohorts still responsive to treatments and not requiring lung transplant) had a similar effect to that of SMM on electrophysiological readouts. This novel finding raises the possibility that factors present in both sputa and/or induced/released by epithelial cells in response to apical culture with sputa were responsible for the changes we observed.

Of the candidate proteins common to both CFS and NLS and in addition to those already described, olfactomedin-4 was present. Its role in the airway is not well understood, but it is reported to inhibit Cathepsin C mediated protease activity (25) (such as activation of immune cell-associated serine proteases (2, 18)) and play a role in epithelial differentiation (21) both of which are known to affect epithelial ion transport (6, 15, 20, 30, 45). Pro-inflammatory factors that were identified or that increased in response to exposure to sputa included TNFα -induced protein 2 (TNFAIP2) and IL-6. There were also a number of proteins associated with macromolecule localisation, protein localisation and vesicle-mediated transport. Others have proposed inflammatory mediated increases in CFTR expression via transcriptional and post-transcriptional mechanisms (1, 13, 22, 27). The CaCC, TMEM16A is also reported to be upregulated during inflammation and by associated endoplasmic reticulum Ca2+ store expansion (22, 27). Proteomic studies do not effectively identify pro-inflammatory cytokines or provide information on metabolites (9, 25, 26) and so the specific mechanisms upregulating Cl- efflux via CFTR and CaCC need further investigation. Furthermore, the net change of proteases present in the extracellular environment may affect protease-activated receptors, which are linked to a plethora of epithelial functions, including ion transport (19, 34). Nevertheless, we speculate that long term culture with sputa *per se* to NHBE induces a pro-inflammatory/stress response that modifies CFTR and CaCC function. Finally, reapplying NLS or CSF resulted in a midway response in CFTR and CaCC activity. This implies a balance between factors involved in the acute vs chronic effects and questions the dominance of such factors *in vivo*.

In conclusion, our evidence indicates that culture of epithelial cells from different donors with healthy human sputum or sputum from people with CF disease changes the proteomic signature of the ASL. We show that there was a consistent response to chronic culture with apically applied sputa that is independent of a disease specific effect. Our data support that acute acting components in sputum (that may be replenished *in vivo* but not *in vitro)* can modify outcomes. As a corollary to these novel findings, because the response to NLS was different to that reported for PBS (1, 13), we propose that culture of airway epithelial cells with NLS provides a more physiologically relevant control for the luminal environment and might provide a new method for functional studies investigating interventions/therapeutics for respiratory disease.

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## Author Contribution

MW carried out all the experimental work, data analysis and drafted manuscript. BR and MK helped with MS and proteomic analysis and reviewed manuscript, RT provided cell and sputa resources and supervised. DB obtained funding, conceived study, supervised, helped with data analysis and drafted manuscript.

**List of abbreviations**

ANOVA, analysis of variance

ASL, airway surface liquid

ALI, air-liquid-interface

BPIFB2,  bactericidal permeability increasing fold-containing family B member 2

cAMP, cyclic adenosine monophosphate

CF, cystic fibrosis

CFS, cystic fibrosis sputum

CFTR, cystic fibrosis transmembrane regulator

CaCC, calcium activated chloride channel

ENaC, epithelial sodium channel

ER, endoplasmic reticulum

FASP,  filter-aided sample preparation

LC-MS/MS, liquid chromatography tandem mass spectrometry

NHBE, normal human bronchiolar epithelial cell

NLS, nornal lung sputum

PBS, phosphate buffered saline

SMM, supernatant from mucopurulent material

SPLUNC1,  short palate, lung, and nasal epithelial clone 1(or BPIFA1)

TEER, transepithelial electrical resistance

TNF, tumor necrosis factor alpha

TNFAIP2, tumour necrosis factor induced protein 2

IL-6, interleukin 6

UTP, uridine triphosphate 

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# Table and Figure legends

**Table 1. Transepithelial electrical resistance (TEER) of NHBE from different donors.**

Donor number and passage number (p1) are shown with corresponding TEER (Ω.cm2) as mean ± SD, for the number of individual cultures as shown (n). Donor demographics are given in Supplementary Table 1a <https://doi.org/10.6084/m9.figshare.14791827.v1>

**Figure 1. Distribution and relative abundance of proteins detected in samples of CFS, NLS and ASL from NHBE before and after exposure to sputa.** (a) Heat map with Euclidean distance hierarchical clustering showing distribution and relative abundance of proteins identified in NLS, CFS (pooled sputum samples) (n=10) and ASL before culture with sputum (ASL) (n=8) and after culture with apically applied NLS (NLS48) or CFS (CFS48) for 48 hours (both n=4). (b) Venn diagram showing number of unique proteins found in: NLS, CFS or NHBE ASL. (c) The identity and abundance of proteases and protease inhibitors related to CF lung disease pathology in NLS and ASL before exposure to sputum. Total ion current (TIC) is relative to the abundance of proteins within a 5 µl sample, for each row intensity of shading (red for proteases; green for protease inhibitors) highlight the highest value.

**Figure 2.The number, identity and relative abundance of key proteins detected in ASL from NHBE chronically cultured with apically applied CFS or NLS.** (a) Venn diagram showing unique proteins identified in NLS, CFS (pooled, n=10)ASL before (NHBE ASL) (n=8) or after 48 hour culture with NLS (48hr NLS) (n=4) or CFS (48hr CFS) (n=4). (b) The 7 proteins exclusive to both chronically exposed NLS and CFS are shown. (c) The 60 proteins found exclusively in chronically cultured NHBE were aligned to biological processes through the STRING database, the top 5 matches are shown alongside their false discovery rate (p<0.001).

**Figure 3**. **Culture with apically applied CFS but not NLS decreased NHBE TEER but culture with either sputa changed short circuit current (Isc)**.(a) Percent change in TEER of NHBE before and after culture with NLS or CFS for 4 hours (acute) or 48 hours (chronic) or reapplication of sputa over 48 hours (Re-ap) or CFS incubated with protease inhibitor (acute CFS+PI), normalised to change in untreated cultures over the same time period. Presented as individual points, mean±SD, for n=6 different donors. \*Statistically different from untreated control, p<0.05. (b) Percent change in TEER of NHBE before and after culture with CFS for 4 hours with (+) and without (-) cOmplete™ protease inhibitor cocktail \* statistically different, p<0.05. (c) Exemplar Isc traces from NHBE from one donor that were untreated (dotted line) or after acute (4 hours: solid lines) or chronic (48 hours: dashed lines) exposure to NLS or CFS, showing addition of specific activators and inhibitors of ion transport (marked by black arrows); amiloride (100 μM), forskolin (10 μM), CFTR inhibitor 172 (CFTRinh, 10 μM) UTP (100 μM) and ouabain (100 μM). All drugs were added apically with exception of forskolin which was also added basolaterally.

**Figure 4. Acute or chronic culture of NHBE with sputa induced different effects on Isc in multiple donors**. Isc across NHBE before (untreated) and after culture with NLS or CFS for 4 hours (acute) or 48 hours (chronic) or reapplication of sputa over 48 hours (Re-ap) for n=6-8 individual donors. The dotted line at zero represented no change, positive variation indicates increased Isc and negative variation indicates decreased Isc in response to treatment. (a) amiloride-sensitive Isc, (b) forskolin-stimulated Isc, (c) CFTRinh172–sensitive Isc, (d) UTP-stimulated Isc. Mean values are shown as horizontal bars ± SD. Significantly different from untreated control \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

**Figure 5. ASL height is modified by culture with CFS and NLS.** (a-c) Example Isc traces from NHBE with: (a) forskolin addition prior to VIP addition (b) VIP addition prior to FSK (c) and overlay. Addition of specific activators and inhibitors of ion transport (as detailed below) are shown at points indicated by arrows. (d) Summary of bioelectric data for NHBE showing ΔIsc in response to CFTRinh172 (CFTRinh, 10 μM) (n=3). All drugs were added apically with the exception of forskolin which was also added basolaterally. (e) Representative XZ images from one donor of ASL labelled with dextran (red layer as indicated to right of image) overlying NHBE labelled with CellTrace™ Calcein Green, AM (green) following acute culture with CFS, NLS or PBS (Untreated). (f) Change in ASL height (µm) 1 hour after basolateral addition of vasoactive intestinal peptide (VIP) after CFS and NLS culture for 4 hours (acute) or 48 hours (chronic). The dotted line represents no change after VIP treatment. Positive deflections from line represent increased ASL height after VIP treatment. Data are presented as individual points representing donor average values with mean values shown as horizontal bars ± SD (n=2-4 from 4-7 different donors, untreated not shown). \* Significantly different from untreated control p<0.05.