# Establishing a Methodology to Measure the pH of the Airway Surface Liquid in non-CF and CF Human Airway Epithelial Cells

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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## Abstract

Human lungs maintain proper airway surface liquid homeostasis for mucociliary clearance, hydration, and as the first line of defense against inhaled pathogens. In Cystic Fibrosis, the absence of functional CFTR-mediated $Cl^-$ and $HCO_3^-$ flux causes ENaC hyperactivity at the apical membrane leading to an acidic and dehydrated ASL[1]. Abnormal bicarbonate transport creates an acidic environment, which alters mucus properties, increases bacterial colonization and impairs antimicrobial function[2,3]. I have demonstrated that the ASL pH can be measured reliably in fully differentiated primary human non-CF and CF bronchial epithelial cells. The ASL pH was significantly lower in CF primary bronchial epithelial cells compared to normal bronchial epithelial cells and the use of ouabain and SLC9A3 inhibitor increases the ASL pH in CFB cells. This protocol allows for future research of the altered ASL pH in CF patients, and its possible correction by targeting proton exchangers/channels.

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# List of Abbreviations

**ANOVA:** analysis of variance
**APIP:** Apaf-1-interacting protein
**ASL:** airway surface liquid
**ATP:** adenosine triphosphate
**$Ca^{2+}$**: Calcium ion
**CACC:** calcium activated chloride channel
**Calu-3:** human submucosal gland cancer cell line
**cAMP:** cyclic adenosine monophosphate
**CAP protease:** channel activated protein protease
**CF:** cystic fibrosis
**CFBE:** cystic fibrosis bronchial epithelial cells
**CFTR:** cystic fibrosis transmembrane conductance regulator
**CGS:** Canadian consortium of genetic studies
**$Cl^-$**: Chloride ion
**$CO_2$**: Carbon dioxide
**ddH20:** double distilled water
**DNA:** deoxyribonucleic acid
**EHF:** epithelium specific transcription factor
**ENaC:** epithelial sodium channel
**F508del:** CFTR protein containing a deletion of phenylalanine at position 508
**FBS**: fetal bovine serum
**FVC:** forced vital capacity
**GMS:** genetic modifier study
**GWAS:** genome wide association study
**$HCO_3^-$**: bicarbonate
**HD:** hypothesis driven
**HEPES:** 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, buffering agent
**IFRD1:** interferon-related development regulator 1
**IL-8:** interleukin-8
**kDA:** kilo-Dalton's
**MBL2:** mannose-binding lectin
**MEM:** minimum essential medium
**MI:** meconium ileus
**mRNA:** messenger ribonucleic acid
**NBE:** normal bronchial epithelial cells
**NKCC:** sodium-potassium-chloride co-transporter
**$O_2$**: oxygen
**PBS:** phosphate buffered saline
**PCL:** periciliary liquid
**PFC:** perfluorocarbon
**ROI:** region of interest
**SNP:** single nucleotide polymorphism
**TGFb1:** Transforming growth factor beta 1

**TSS:** Twin and sibling study

# Introduction

## 1.0 Cystic Fibrosis

Cystic Fibrosis (CF) is a life limiting genetic disorder, caused by defective Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein that prevents its functional expression on the apical airway epithelium[4,5]. CFTR conducts the transepithelial flux of chloride and bicarbonate across the cell membrane and its defect affects multiple secretory organs, such as the pancreas[6], liver[7], reproductive tract[8], intestines[9] and lungs[10]. Researchers have identified more than 2000 mutations in the CFTR gene[11]. The different CFTR mutations are grouped into categories based on the way the mutations affect the CFTR protein. The most common CFTR mutation is a deletion of three nucleotides at exon 10, coding for phenylalanine, at position 508 (F508del)[12,10]. Clinical variability is observed among patients with CF, especially regarding the severity of lung disease. 85-90% of CF infants born with CF will suffer from pancreatic insufficiency[13]. Pancreatic fibrosis may often lead to the development of diabetes mellitus (β-cells are unable to secrete sufficient amounts of insulin)[14]. This clinically apparent CF related diabetes occurs in about 34% of patients over the age of 18[15]. CFTR-deficient $HCO_3^-$ secretion in the small intestine can cause improper mucus secretion (viscous accumulation)[16], limited water secretion[5], inflammation and can lead to intestinal obstruction (Meconium Ileus in 15% of newborns and 10-20% of adult CF patients[17]). Many other clinical manifestations arise with CFTR defects, with the leading cause of mortality being progressive lung disease due to chronic lung infections[18].

Two drugs have been developed and successfully approved to target the basic defect in CF, Orkambi and Kalydeco. Orkambi is used to treat patients with the F508del CFTR mutation which affects the intracellular processing and delivery of CFTR to the cell surface resulting in loss of ion secretion[19]. Orkambi is considered a "corrector", which is a pharmacological agents that increase the delivery of CFTR proteins in individuals homozygous for F508del-CFTR, to the cell surface[19,20]. Less common CFTR mutations that impair the ability of CFTR to open (G551D mutation) at the apical surface or chloride and bicarbonate conductance (R117H mutation) are also found in many patients[21]. Pharmacological agents called "potentiators" restore cAMP-dependent chloride channel activity to mutant CFTR at the apical surface[22]. Kalydeco (ivacaftor), increases $Cl^-$ secretion, and the channel opening probability after the signaling pathway is activated[23].

## 1.1 Heterogeneity in CF

The CF gene encodes approximately 1500 amino acid proteins with CFTR activity and of those the Δ508 mutation is found on 50-80% of CF chromosomes (depending on the population being studied)[24]. The F508del mutation is a deletion of phenylalanine at position 508 of CFTR linear sequence[5]. Individuals who are homozygous for F508del often have a severe phenotype such as >95% of patient having insufficient exocrine pancreatic function[25]. The large number of CFTR mutations, and the variability of the complications from patient to patient, creates a wide spectrum of variable phenotype-genotype relationships[26,27,28]. For example, the degree of correlation for CFTR genotype and CF phenotype is highest for pancreatic insufficiency and lowest for pulmonary disease[29,30]. Most individuals with pancreatic insufficiency have two severe CFTR

mutations, whereas individuals with pancreatic sufficient phenotype normally have one or two mild mutations[25]. Different CFTR mutations confer different defects, for example there is a strong clinical association between CFTR mutation R117H and pancreatic sufficient status, therefore it is considered a mild mutation[25]. The lack of association between CFTR genotype and lung disease prognosis creates perplexity[31]. Twin and sibling studies have been used to evaluate the degree of heritability of numerous phenotypes. It was found that there is non-CFTR genetic contribution to lung disease (heritability range of 0.54-1.0)[32]. These studies pave the course of CF research exemplifying the strong non-CFTR genetic modification of the disease[33,31]. Patients with the same CFTR genotype show variability in their clinical phenotypes of CF thus it is crucial to search for genes, other then CFTR, which may modify the CF phenotype.

## 1.2 Modifying Genes in CF

The phenotypic variation in CF has stimulated a search for genes external to CFTR. After evaluating dozens of candidate genes, at least nine were implicated as modifiers of the CF phenotype[34]. One of the first candidates indicated mannose-binding lectin (*MBL2*) because of its role in innate immunity and demonstrated earlier age of infection with *Pseudomonas aeruginosa* with MBL deficient genotype[35]. Transforming growth factor beta 1 (*TGFβ1*) studies found that increased *TGFB1* expression leads to worsened lung function in CF[36]. Interferon-related development regulator 1 (*IFRD1*) and interleukin-8 (*IL-8*) showed gene association with lung function[31,37]. To create a large enough cohort, a consortium was formed containing a Genetic Modifier Study (GMS), the Canadian Consortium of Genetic Studies (CGS) and a CF twin and Sibling Study (TSS). Several Genome Wide Association Studies (GWAS) were undertaken which were able to have

many well-phenotyped CF patients to study modifiers in CF[38]. The numerous analysis resulted in a 800kb SNP region between *EHF (*epithelium specific Ets transcription factor)[39]-*APIP* (Apaf-1-interacting protein)[40] having statistical significance with lung severity[38]. The study concluded that increased expression of *APIP* is associated with decreased lung function due to its inhibition of apoptosis worsens lung disease[38].

A recently published hypothesis-driven genome-wide association study (GWAS-HD) hypothesized that apical epithelial membrane constituents are most likely to modify CF phenotype[41]. The GWAS chose the meconium ileus (MI) status at birth as a CF outcome marker because it is almost entirely genetically based, and occurs in utero before any exposure to environmental bacteria[41]. The study found three apical membrane constituents responsible for ~17% of the variation of MI in North American samples[41]. The three solute carrier genes are, SLC6A14 (a sodium and chloride dependent amino acid transporter), SLC26A9 (a $Cl^-$/ $HCO_3^-$ channel) and SLC9A3 (a $Na^+/H^+$ exchanger localized to the apical membrane of intestinal polarized epithelial cells)[41]. Li *et al.* analyzed a pediatric population, looking at the existence of pleiotropic effects of modifier genes associated with pediatric lung disease severity, the age of onset of P. *aeruginosa* infection and prenatal pancreatic damage. Using a larger CF cohort the results of their study showed that SLC9A3 SNP rs17563161 ($p=1.47x10^{-4}$), was found to be also associated with the severity of pediatric lung disease ($p=1.5x10^{-6}$)[42].

## 2.0 General Airway Physiology

The lung posses a branching structure of increasingly small diameter airways that end in blind sacs called the alveoli, which is the site of gas exchange[43]. The lumen of the respiratory tract is lined with pseudo stratified ciliated airway epithelial cells, mucus secreting goblet cells, basal cells (precursor cells), Clara non-ciliated bronchiolar secretory cells, type I and II alveolar cells of the distal airways for gas exchange, lymphocytes and mucus and serous sub-mucosal glands[43]. The immunological defenses begin with the mucus layer of the airway surface liquid (ASL), located at the interface between surface epithelial cells and air [44]. The first layer of the ASL, the mucus layer, secreted by submucosal glands, is made up of glycosylated macromolecules *(MUC5AC* and *MUC5B*)[45]. The mucus free-zone, otherwise known as the periciliary layer (PCL), is the second layer of the ASL and is a low viscosity liquid layer allowing the cilia to beat rapidly (8-15Hz)[46,47]. In normal airways the PCL height is the length of the outstretched cilia (7μm), whereas the mucus layer height varies (7 to 70μm)[48]. Particulates such as a number of bacteria are deposited onto the PCL and through the mechanical beating of cilia are entrapped[44,40,42]. Studies have identified pH-sensitive antimicrobial enzymes such as lysozyme, leukocytes protease inhibitor, β-defensin (Human β-defensin 1 and 2) and lactoferrin that are secreted into the ASL to suppress bacterial growth[49,50]. Loss of CFTR leads to a rapid depletion of ASL height creating a dehydrated, sticky ASL environment. Water moves out of the ASL mucus layer, dehydrating the CF airway (**Figure 1.**).

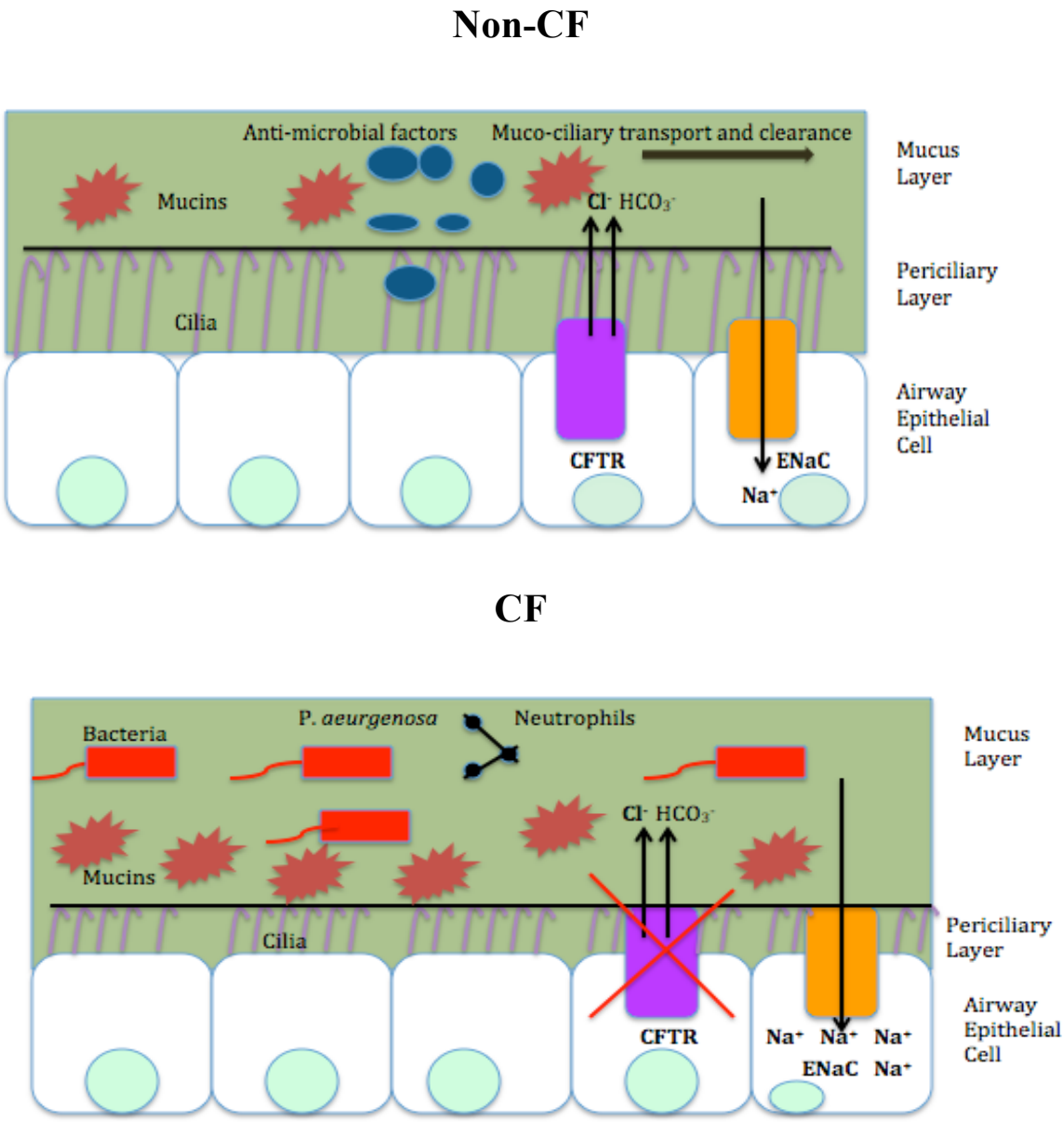


**Figure 1. Comparison of non-CF and CF airway surface liquid**

In non-CF airway (top panel) homeostasis exists between ENaC mediated $Na^+$ absorption and CFTR-mediated $Cl^-$ secretion to maintain PCL height, cilia movement and pathogen clearance. When CFTR is absent or defective (bottom panel) $Na^+$ hypersecretion reduces ASL height and causes the ASL to become dehydrated, stationary and unable to be propelled by cilia or support normal bacteria killing.

## 2.1 ASL

Ion and fluid exchange between epithelial cells and the ASL is critical for the regulation of osmolality and hydration of the ASL[51]. The cAMP-activated Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) regulates chloride secretion$^-$on the apical membrane of ciliated cells[52]. Next to being an anion conductor, CFTR also secretes bicarbonate, which likely plays a role in regulating the ASL pH[53]. Another $Cl^-$ channel is located on the apical membrane of the airway epithelia, the $Ca^{2+}$ activated $Cl^-$ channel, CaCC[52]. CaCC is activated when intracellular $Ca^{2+}$ is elevated to micromolar concentrations and by membrane depolarization at low cellular $Ca^{2+}$ concentrations[46]. When stimulated by a P2Y2 receptor, CaCC influences fluid secretion following luminal nucleotide release. ENaC, selectively transports $Na^+$ across the apical membrane establishing an osmotic gradient that facilitates the movement of water in the same direction[54]. In airways, functional CFTR is believed to be associated with inhibition of ENaC activity by decreasing ENaC opening probability[55]. In CF, CFTR-dependent $Cl^-$ and $HCO_3^-$ inhibition and $Na^+$ upregulation are believed to cause an unfavorable environment leading to depletion of the PCL, mucin overproduction and bacterial upregulation by *Pseudomonas aeruginosa* and other inflammatory mediators[56,57]The $Na^+$-$K^+$ATPase, is one of two contributors for setting up the electrochemical gradients, confined to the basolateral domain of epithelial cells essential to generate forces for ion movement[43]. $Na^+$ is secreted by the $Na^+$-$K^+$ ATPase which provides the driving force for apical $Na^+$ reabsorption, thus $Na^+$-$K^+$ ATPase with ENaC mediates transcellular $Na^+$ reabsorption [43]. Chloride entry across the basolateral membrane is regulated by Na/K/2Cl (NKCC1) co-transporter isoform, the second contributor to the electrochemical gradient,

required for ion and fluid transport.[4] Activation of CFTR results in chloride secretion across the epithelium into the lumen, sodium follows passively and water moves by osmotic forces. In reverse, epithelial fluid absorption is driven by ENaC with passive flux of chloride ions and water across the epithelium[58]. Human airway epithelia controls ASL volume via numerous pathways: intracellularly by ATP via P2Y2-R and adenosine[59], extracellular channel activating protein (CAP) protease[60], and mechanosensitive release of ATP through movement of cilia and phasic sheer stress[1]. Depletion of extracellular ATP leads to a decrease of $Cl^-$ secretion and inhibition of mucus clearance[1]. In healthy individuals The ASL is constantly being hydrated due to the balance of $Na^+$ absorption and $Cl^-$ secretion mediated by the epithelial $Na^+$ channel (ENaC) and the epithelial $Cl^-$ channel, CFTR[48]. In CF, defective $Cl^-$ secretion and upregulation of ENaC activity disrupts the homeostasis of the ASL fluid regulation in the airway[44]. The reduction of ASL volume is dispersed between the two layers: (1) the mucus layer is reduced to a sticky environment; and (2) the periciliary layer water volume is thought to be depleted leading to cilia collapse and a loss of the ability to separate the mucus layer from the apical surface[48,61]. The loss of the PCL (layer of fluid which bathes the cilia) lubricant activity causes adhesion of the mucus to the cell surface[62]. In healthy airways the PCL is as tall as the extended cilia (~7μm) and is much more fluid and viscous[47] (**Figure 2)**. In CF airways, a reduction in PCL height (~4μm), causes cilia to collapse, and consequently, impairs mucocilliary clearance function ultimately leading mucus and bacteria hypersecretion that cause airflow obstruction[63].

## 3.0 ASL pH in non-CF and CF patients

There is increasing evidence that in CF primary bronchial epithelial cells the ASL is acidic compared to healthy bronchial epithelial cells due to loss of CFTR-mediated $HCO_3^-$ secretion[64,53,65,2,](**Figure 2.)**. A potential mechanism has recently emerged with the discovery that the loss of CFTR-mediated bicarbonate secretion and an acidic pH impairs airway host defense mechanisms. Loss of bicarbonate, lowers the ASL pH in CF individuals, inhibits the killing of bacteria in ASL, and alters mucus viscosity[66]. Defective CFTR leads to absence of $HCO_3^-$ secretion that results in the acidification of the ASL, and has been shown to increase lung bacterial infection and reduced bacterial killing[65.67]. Dehydration and acidification of the ASL paired with stationary hypermucin secretion, causes partial occlusions of the submucosal glands[87]. The mucus occlusion and formation creates hypoxic zones that favor growth and colonization of bacteria[63].

The recent finding that CF pigs develop airway disease that mirrors that of CF in humans provided the opportunity to study ASL pathophysiology more closely. One group examined antimicrobial activity in porcine primary epithelial cells and determined a lower ASL pH in CF pigs and a correlation between raising pH with an increase in bacterial killing in CF[65]. The results concluded that a decrease in ASL pH inhibits antimicrobial function in the lung[65]. A separate study examining sputum from patients with CF found the ASL to have lower sputum pH and increased viscosity compared to healthy ASL and through $HCO_3^-$ stimulation in primary airway epithelial cells, an increase in pH showed a reduction of ASL viscosity[66]. Both studies conclude that loss of CFTR reduces ASL pH in cultures of airway epithelia and raising ASL pH has the

potential to repair host defenses. Not all research groups have determined that the ASL of CF epithelial cells are more acidic compared to healthy individuals[69,70]. McShane *et al.* measured ASL pH *in vivo* on nasal epithelial cells using pH-sensitive probes found that there is no significant difference between CF and non-CF airway pH[69]. Jayaraman *et al.* investigated tracheal ASL pH using non-invasive fluorescence indicators and demonstrated that ASL pH did not vary between CF and non-CF mice[70]. The group suggested that the similarity in ASL pH CF values does not contribute to the abnormalities in ASL pH CF pathogenesis[70].

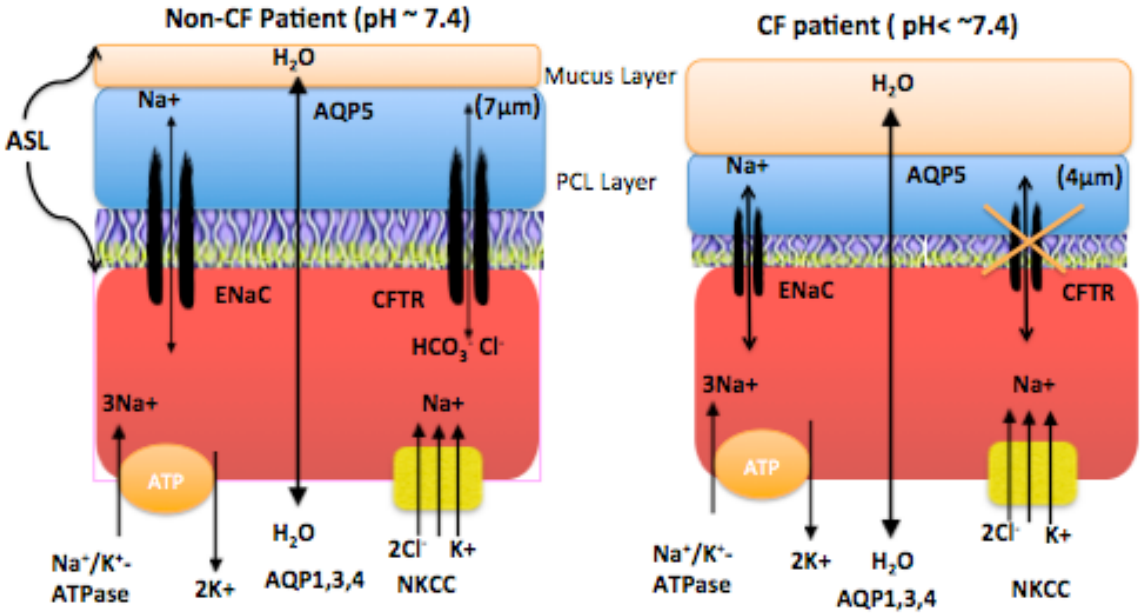


**Figure 2. Major fluid regulating ion transporters in non-CF and CF polarized airways**

Next to chloride conductance, CFTR also conducts bicarbonate. There is increasing evidence that the loss of bicarbonate flux across the apical membrane leads to an acidic ASL pH as compared to non-CF primary cells. The acidic environment alters mucus viscosity and inhibits crucial antimicrobials needed to reduce bacterial killing.

Over the past decade, there have been numerous publications using a variety of techniques to measure ASL pH. Initially, protocols used pH-sensitive microelectrodes in small-volume samples to measure the ASL pH. The establishment of fluorescence indicators such as BCECF and SNARF both conjugated to a dextran allowed for a non-invasive method to measure ASL pH using confocal microscopy. Most recently, Robert Tarran's protocol established a non-invasive technique measuring ASL pH using a pH-sensitive dye, pHrodo Red and pH-insensitive dye, Alexa Green. The pH sensitivity of pHrodo Red is much broader compared to previously used fluorescence indicators such as SNARF and BCECF. Tarran used a microplate reader rather than a confocal microscope for ASL pH measurements because they were able to read numerous wells at once with the same precision as a confocal microscope.

## 4.0 Proton Transporters

Next to CFTR, the airway epithelium expresses $H^+$ channels and transporters that contribute to the regulation of ASL[71]. In absence of functional CFTR, $H^+$ transporters/exchangers may be involved in acid secretion into the ASL. Several proton transporters and channels have been identified in the airway epithelium and apical localization suggests a direct role in ASL pH regulation[71] There is an unclear understanding on how they regulate the pH of the ASL in epithelial cells (**Figure 3.)**.

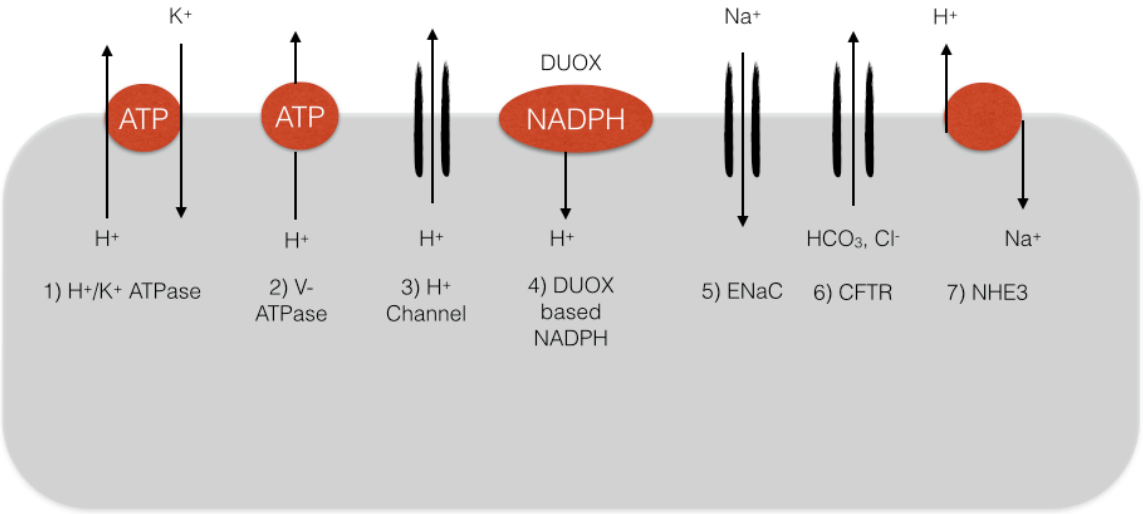


**Figure 3. Overview of the apical membrane of human bronchial epithelial cells**

Proton transporters and channels that have been identified in the airway epithelium and apical localization suggests a direct role in ASL pH regulation. These include $H^+/K^+$ ATPase, V-ATPase, $H^+$ channels, $Na^+/H^+$ exchangers as being responsible for acidic pH in absence of functional CFTR in epithelial cells.

$H^+/K^+$ ATPase, an ATP-dependent transporter was identified on the apical membrane of epithelial cells. Coakley *et al.* detected mRNA expression of the non-gastric isoform of the $H^+/K^+$ ATPase by RT-PCR in cultured bronchial epithelia[2]. Using pH microelectrodes they measured the pH on the apical ASL of polarized primary human bronchial epithelial cells from normal bronchial epithelial cells (NBE) and CF bronchial epithelial cells (CFB) lung explants, after cAMP stimulation and after challenging the cells with a luminal acid load[2]. Under basal conditions the non-gastric $H^+/K^+$ ATPase isoform acidified the ASL, in both the NBE and CFB cultures[2]. Welsh *et al.* also looked at the non-gastric $H^+/K^+$ ATPase isoform in primary porcine bronchial epithelial cells and found that ouabain, an inhibitor of the non-gastric isoform, increased ASL pH in CF and non-CF airway, as well as increasing bacterial killing in porcine bronchial epithelial cells[72]. The Welsh group also predicted that instilling an adenovirus-expressing ATP12A intro the trachea of CF mice lacking ATP12A would decrease ASL pH and create abnormalities in host defense mechanisms. Inhibiting ATP12A mediated $H^+$ secretion provided therapeutic benefits in raising ASL pH[72].

An ATP-dependent transporter located in the apical bronchial epithelia is V-ATPase, which contributes to apical proton secretion[100]. Inglis *et al.* discovered through the use of primary porcine bronchial epithelial cells that bafilomycin, an inhibitor for V-ATPase, reduced both the magnitude and the rate of acidification in NBE and CFBE cell cultures[73]. CFTR and V-ATPase are co-localized and undergoing cAMP regulated trafficking indicating a functional association between the two[73]. A rat intestinal model discovered that the activity of V-ATPase pumps at the apical membrane were dependent

on the function of CFTR. Inhibition of either V-ATPase or CFTR resulted in a decrease in intracellular pH[74].

Voltage-gated, highly selective $H^+$ channels have been localized in the apical membrane, which has been demonstrated using apical patch clamp recordings in human primary tracheal cells. Fisher *et al.* showed that $H^+$ secretory channels in human airway cells are activated during airway inflammation in CF, which in turn, acidifies the ASL and compromises epithelial integrity[71]. The data suggested that an electrochemical $H^+$ gradient across the epithelium exists that drives luminal $H^+$ secretion[71]. $ZnCl_2$ was able to block 50-70% of $H^+$ secretion and the remaining fraction was partially inhibited with bafilomycin A1 (11%) or ouabain (15%)[71].

SLC9A3 (rs17563161) is one of the modifier genes that have been found to be statistically associated to the severity of lung disease in CF[75,76]. SLC9A3 is a $Na^+/H^+$ antiporter, thus its role as a proton exchanger provides reason to investigate it as a potential regulator of ASL pH. The results of an earlier mice experiment further this association between CF and SLC9A3[22]. Double heterozygous mice were mated, creating CFTR-null mice lacking one or both copies of the SLC9A3 gene[22]. These mice exhibited increased fluidity of their intestinal contents, which prevented intestinal obstructions and increased survival[22]. This data established a major role of SLC9A3 in regulating the fluidity of the intestinal contents and showed that reduced SLC9A3-mediated absorption reverses some of the intestinal pathologies of CF[22]. Another $Na^+/H^+$ anti-porter, SLC9A1, was detected by RT-PCR in all bronchial regions of the human lung[18]. pH stat recordings of acid secretion found that inhibiting SLC9A1 suppressed acid secretion by 34% in CF

bronchial cells[18]. Inhibition of SLC9A1 is expected to inhibit $H^+$ channels in the apical membrane and reduce $H^+$ channel-mediated acid secretion

# Rationale

Cystic Fibrosis is the most common fatal genetic disorder in Canada with an incidence of 1 in 3600 live births. Respiratory infection caused by defective CFTR ion transport and abnormalities in fluid secretion underlie many of the clinical manifestations of CF[77]. F508del is the most common CFTR mutation and 48.8% of the patients have this on both alleles[78]. There is considerable variation in the phenotype of patients, who are homozygous for F508del mutation in CFTR protein[79]. Individuals with F508del have a spectrum of associated clinical phenotypes including pancreatic insufficiency, meconium ileus and the severity of lung disease. The severity of lung disease is partially contributed by the degree of mucociliary transport, bacterial colonization, ASL viscosity and ASL pH. The exact reason to why ASL pH is acidic is not fully understood. ASL could be acidic for several reasons including impaired CFTR-mediated bicarbonate secretion and changes in non-CFTR $H^+$ transporters and exchangers. By optimizing a novel methodology I can reliably measure ASL pH and potentially establish important determinants regulating ASL pH. To achieve this, I have created an optimized protocol using a microplate reader and fluorophores to measure ASL pH in non-CF and CF patients to better understand $H^+$ and $HCO_3^-$ transport. The microplate reader allows for non-invasive, efficient measurements of ASL pH in patient-specific samples that will help us better understand the variability in ASL pH which is crucial for individualized CF therapy. Individualized therapy is necessary for CF research because individuals who share the same mutant CFTR protein may respond to treatment differently. It is prudent to develop a protocol to measure the ASL pH, so that this research can be applied to a more detailed characterization of the non-CF and CF airways, and also to investigate

different channels and transporters that may contribute to ASL pH regulation. The plate reader method allows us to add $H^+$ inhibitors/antagonists apically to the human primary airway cells to observe changes in ASL pH. In current research, the Welsh group has targeted ATP12A (an $H^+/K^+$ ATPase localized on the apical membrane) and inhibited it with ouabain thus increasing and normalizing ASL pH[72]. A recent GWAS study identified SLC9A3, a $Na^+/H^+$ exchanger, as a gene that may potentially modify the effects of CF lung disease[80,41]. Further understanding of $H^+$ secretory mechanism and its role in regulating ASL pH may lead to this likely candidate, SLC9A3, as a key regulator of ASL pH. The ASL pH of CF individuals can then be correlated with the presence of an SLC9A3 risk allele, to discover if an association between the presence of the allele and an acidic ASL pH exists. An accurate, reliable measurement of ASL pH will allow for a detailed understanding of the role of ASL pH in CF pathology.

## Hypothesis

SLC9A3 and CFTR are important regulators of airway surface liquid pH and that changes in CFTR expression and SLC9A3 function may alter ASL pH.

## Specific Aims:

The specific aims of this graduate project are:

1) Establish a reliable methodology for ASL pH measurement in human bronchial epithelial cells using a pH-sensitive fluorescence dye
2) Determine if their are differences in ASL pH between human bronchial epithelial cells from non-CF and CF patients
3) Evaluate the concept of targeting proton secretory mechanisms to alter the acidic ASL pH in CF patients

# Methods

## 1.0 Primary Culture

Primary human bronchial epithelial cells were derived from lung explants of CF and non-CF patients and were obtained thanks to a collaboration between the Toronto General Hospital Lung Transplant Centre (Dr. Shaf Keshavjee) and the cell facility at the University of Iowa (Dr. Michael Welsh). The cells were plated on a porous membrane (Costar Transwell Clear) with a 0.4μm pore size. This encourages formation of a fully differentiated monolayer of cells, and also allows for independent access of both the apical and basolateral chambers (**Figure 4)**. The transwells were coated with human placental collagen IV, which promotes cell attachment and spreading, allowing the cells to be visualized during culture. The cells were grown and differentiated in an air-liquid interface.

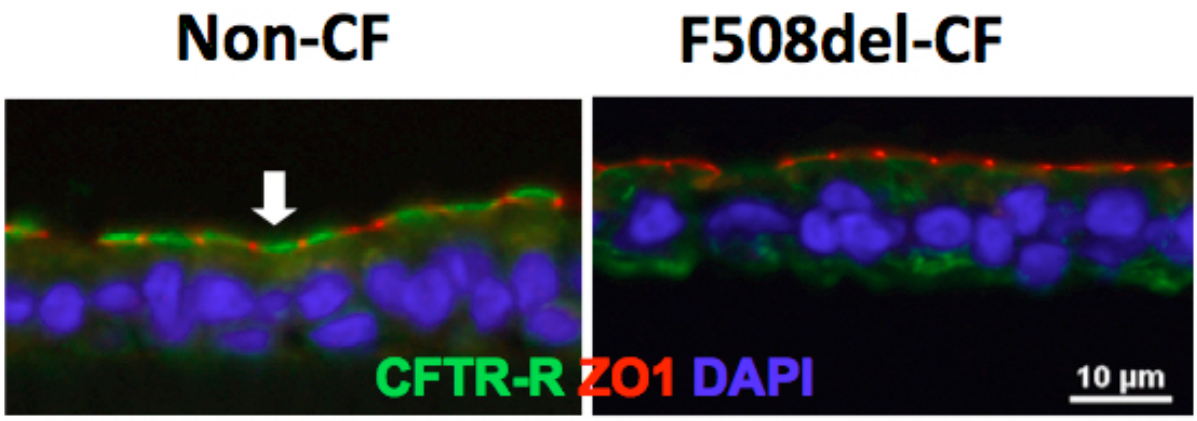


**Figure 4. Cyrosection of human primary bronchial epithelial cultures from non-CF and F508del patients.**

The epithelial cells were stained with ZO1 to show tight junctions (red) as well as with CFTR-antibodies, which shows CFTR localization at the apical membrane in non-CF cells, but not in the F508del cells (immunofluorescence images provided by Dr. Kai Du).

## 2.0 Transepithelial Electrophysiological Analysis

Human primary airway epithelial cells were mounted in an Easy Mount Ussing Chamber to allow access to the apical and basolateral membrane. The cells were bathed in Krebs bicarbonate buffer maintained at $37^0$C and bubbled with 95% $O_2$ and 5% $CO_2$. Transepithelial resistance (Rte) was recorded by measuring the response of the transepithelial potential difference to 1mA current pulses every 60 seconds.

# 3.0 Imaging ASL Height

The primary bronchial epithelial cells height was visualized using a well-established method[81]. The primary bronchial epithelial cells were labeled with calcein-AM to stain the lives cells in the epithelial layer. The ASL is labeled with Texas Red conjugated to a 10kDa dextran, which dissolves in the ASL and the 10kDa dextran is relatively impermeable to the epithelial membrane. To standardize the ASL height measurements, the cultures were prewashed three times with 800µL PBS to remove the previously formed ASL and mucus. 5µM of PBS-calcein-AM solution was added apically to the transwells containing primary bronchial epithelial cells to stain the epithelial cells. The primary cells were incubated for 60 minutes at $37^0$C and 5% $CO_2$. The PBS and calcein mixture were then aspirated and the cells were transferred into culturing media (**Figure 9**). Cultures were placed on a coverslip mounted in a chamber, which is heated to $37^0$C and 5% $CO_2$. A total volume of 40µL consisting of PBS, aprotinin (1.5 units/ml) (a sodium transport inhibitor added to replace the natural sodium inhibitors washed away with PBS) and Alexa Red conjugated to a 10kDa dextran are added to the mucosal surface. Finally, perfluorocarbon (PFC) was added to prevent evaporation after incubation of the primary bronchial cells. 50µL of PFC was added to ensure that the entire apical side is covered. The Nikon A1R confocal microscope was used to image cells using the Nikon acquisition software. The images were analyzed using the software program, volocity. To measure the ASL height, an average of 5 height measurements at different areas along the ASL were calculated to accurately depict the ASL height.

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# 4.0 ASL pH Measurements

ASL pH was measured with a fluorescence-sensitive microplate reader, Spectramax i3x microplate at $37^0$C and 5% $CO_2$. The human primary bronchial epithelial cells grown in an air-liquid interface were washed three times from the apical side with PBS supplemented with $Mg^{2+}$ (0.5mM) and $Ca^{2+}$(0.9mM), that allows the cells to adhere and grow, and were incubated at $37^0$C and 5% $CO_2$ for 5 minutes before ASL pH measurements. At the beginning of the experiment the fluorescence intensity of each transwell containing primary bronchial epithelial cells was measured without any fluorescent dyes added. This background reading was subtracted from the experimental measurements to account for any background noise. A 25µL apical bolus containing pH-sensitive dye, pHrodo dextran (0.5mg/mL, 585nm), pH-insensitive dye Alexa 647 dextran (0.5mg/mL, 668nm) and 23.5µL Ringers solution was added to the primary bronchial cells. The Ringers solution was adjusted to a pH of 7.4. Channel or transporter antagonists were added to the apical bolus. Once the ASL has been pipetted apically onto the NBE and CFBE cells they were immediately incubated at $37^0$C and 5% $CO_2$ for 5 minutes to equilibrate the ASL. PFC was added to the cells post incubation to prevent evaporation. The ASL pH of the 12-well plate containing the primary bronchial cells was then measured in the microplate reader. The first measurement in the microplate reader is considered time point 0h. The cells were incubated for 5 minutes up to 24 hours, and the length of the incubation period when removed to be measured in the microplate reader is considered the time point.

## 4.1 Standard Curve Measurements

Following the ASL pH measurements, the same primary human bronchial epithelial cells were washed with PBS three times to remove the previous bolus. 4 Ringers solutions with a large amount of HEPES buffer (100mM) was set to a specific pH (pH of 6, 6.75, 7.5, and 8) were added apically with the pHrodo red and Alexa green dye to create a final volume of 25µL. The cells were then incubated at $37^0$C and 5% $CO_2$ for five minutes. PFC was added to the cells and then placed into the microplate reader to obtain a fluorescence intensity reading. The fluorescence intensity was calculated into a standard curve that will translate to an ASL pH.

# 5.0 Solutions and Chemicals

To measure ASL pH and to image the ASL the fluorescence-sensitive dyes (pHrodo red dextran, alexa green dextran, calcein-AM and Texas Green) were purchased from Thermofisher scientific. The Modified Ringers solution to create the standard curve consisted of 86mM NaCl, 5mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 100mM HEPES buffer. ). In some experiments, to eliminate the effect of $HCO_3^-$ transport on ASL pH, basolateral cell culture media was replaced with HEPES-buffered solution containing 135mM NaCl, 2.4mM $K_2HPO_4$, 0.6mM $KH_2PO_4$, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 25mM HEPES, and 10mM glucose set to pH 7.4. The proton inhibitors, ouabain ($H^+/K^+$-ATPase inhibitor,10µM) was purchased from Sigma-aldrich and the SLC9A3 inhibitor were provided by Sanofi-Aventis (Frankfurt, Germany). The ionophore, monensin (1mM) was purchased from Sigma-aldrich (Oakville, Ontario).

# 6.0 Statistics

The microplate reader generates 5 fluorescence intensity readings for both dyes separately over a period of 2 minutes. These 5 time points were averaged, and a ratio between the pH-sensitive and insensitive dye was calculated. The ratio was then inserted into a linear equation computed by the standard curve that results in an ASL pH (**Equation 1.)**. Data are presented as means ± standard deviation or ± 95% confidence intervals. Experiments on primary cells were analyzed in a paired fashion using a two-way ANOVA comparison test. Data were analyzed using GraphPad Prism (v.6).

**Equation 1. Linear Equation: Slope-intercept form**

$$y=mx+b,\ m=\text{slope},\ b=\text{y-intercept},\ x=\text{pH}$$

# Results

## 1.0 Imaging ASL

I imaged the ASL to demonstrate good quality of the primary bronchial epithelial cells and also to demonstrate that the ASL is a distinct layer assessable to pH measurements (**Figure 5**.). I verified through imaging and electrophysiological results that the human primary bronchial epithelial cells are tight (no leakages) with very good transepithelial resistances (Rte NBE=597.3$\mu cm^2$ and Rte CFBE= 488.4$\mu cm^2$) and express polarity. (**Figure 6(A) and 6(B))**.

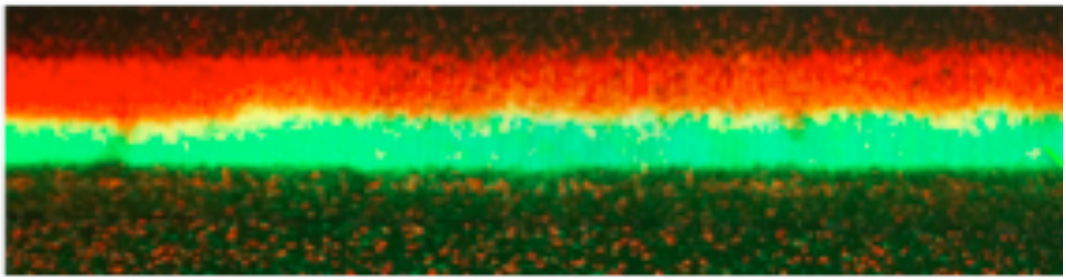
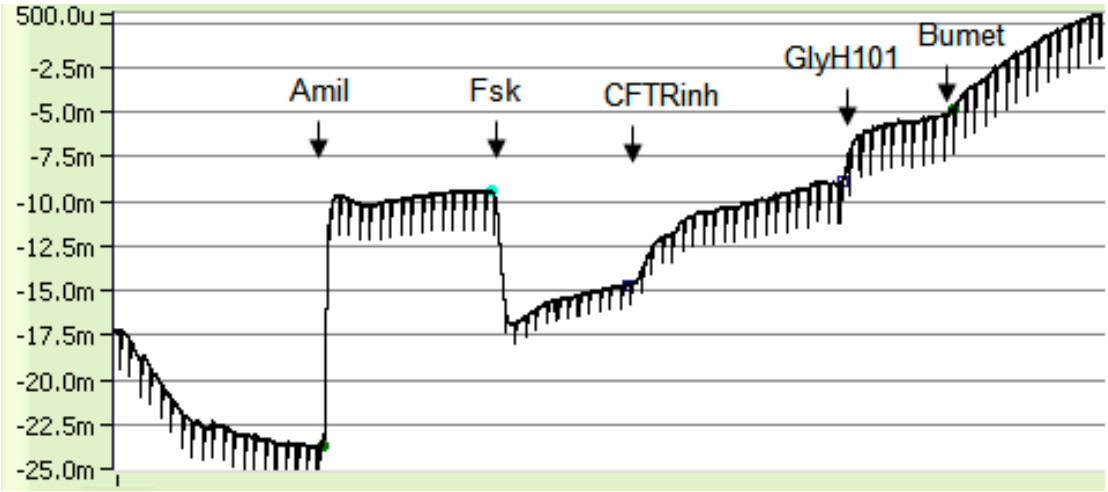


**Figure 5. XZ confocal image of airway surface liquid**

The ASL (red) and cell membrane (green) are stained with fluorescence dye. The ASL is stained with the addition of Ringers solution in a 25µL bolus. The ASL can be assessed distinctively from the epithelial membrane because the Texas red dye is conjugated to a 10kDa dextran that is impermeable to the membrane.

**(A)**




**(B)**

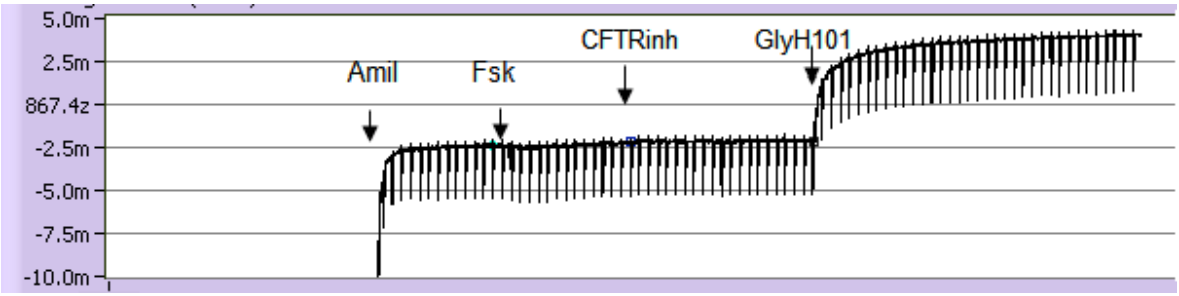



**Figure 6 (A) and (B). Ussing recordings of NBE (A) and CFBE (B) cell cultures**

The high transepithelial resistance of (A) and (B) ensures polarity and tight junctions in both NBE and CFBE cells (Amil- amiloride, Fsk-forskolin)

# 2.0 Establishing a Technique to Measure ASL pH

## 2.1 Optimization of a previously established protocol

### 2.1.1 Microplate Reader

Initially, an older model that was more accessible at the start of the experiments, SpectraMax Gemini EM Thermofisher plate reader was used and yielded a low resolution. After comparing the resolution to a newer model microplate reader, Spectramax i3x, it yielded a higher resolution, and I decided to change plate readers to more accurately measure ASL pH.

### 2.1.2 Alterations in 12-well plate

Tarran's protocol advised that the 12-well plate lid should be left on immediately after removing the cultures from the incubator to prevent degassing. Initially, when using the Gemini EM the lid remained on during the readings. The fluorescence intensity signal was weaker due to the optical reflection caused by the lid. The Spectramax i3x does not allow for the lid to be on because the port of entry is too small to fit a lid. Studies using similar methodologies confirmed that the time required to remove the cultures from the incubator to 2 minute microplate readings did not lead to a change in ASL pH due to $CO_2$ with the lid off[68]. I noticed that the bolus inside the well tends to evaporate faster with the lid on, leading to a concentration change that leads to a signal change. Subsequent ASL pH measurements were measured with the lid off. These changes I made resulted in a reduction of variability in ASL pH measurements between different non-CF donor cells as well as lead to a better comparatibility to results published by other groups (**Figure 7**.) The mean ASL pH of the initial protocol at t=0h was 7.97 ±0.79 (n=6) and the mean ASL

pH of the optimized protocol was 7.27±0.39(n=6). The measurements shown in the graph are taken at t=0h. The optimized protocol reduced the standard deviation. The initial protocols results are more alkaline with a wider pH range, 9.43-7.21, as compared to the optimized protocols pH range, which is within the physiological range, 7.92-6.82.

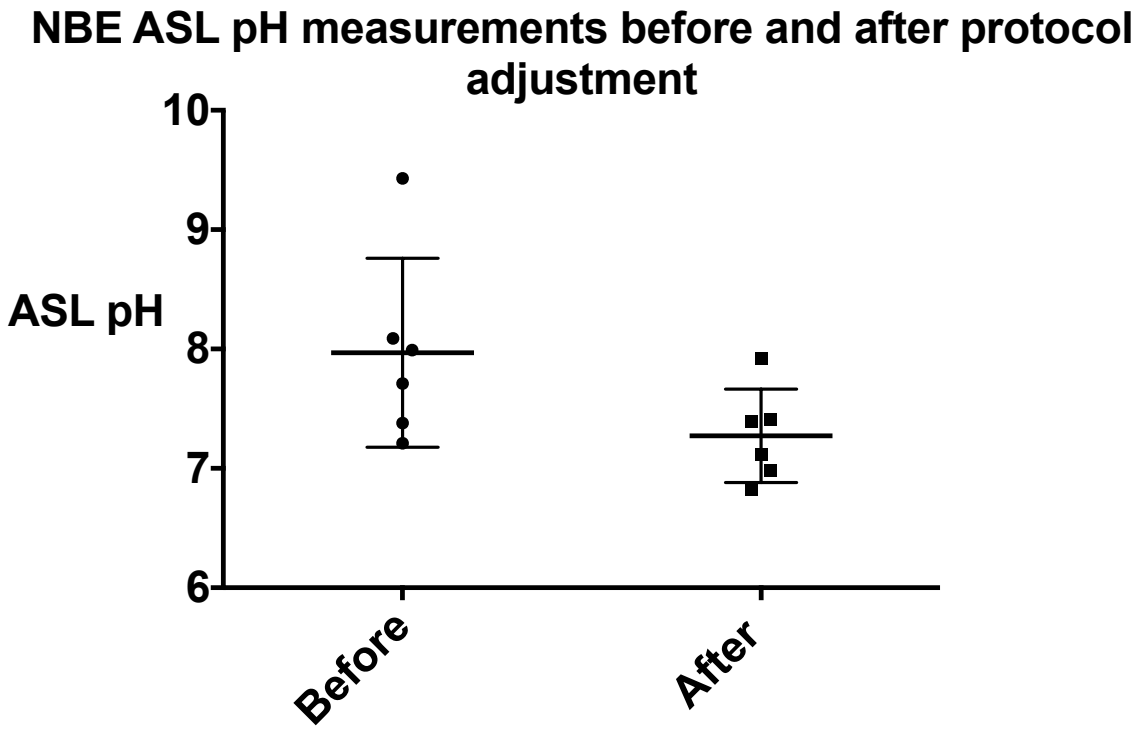



**Figure 7. Comparison of the ASL pH in normal bronchial epithelial cells at 0 hours after apical addition of the fluid bolus using the initial and the optimized protocol**

The initial protocol used the Spectramax Gemini EM, with the lid on at an interval of 0h, and 5 minutes. The optimized protocol uses the Spectramax i3x, with the lid off at time intervals over 2 hours and once more at 24 hours. Bars indicate means±SD.

## 2.2 Effects of varying concentrations of HEPES buffer solution

In order to establish optimal parameters for studying ASL pH in normal human primary bronchial epithelial cells it was important to determine the contents that make-up the apical bolus. Tarran's protocol used Ringers solution with 25mM HEPES in solution[87]. HEPES is used in solution because it maintains physiological pH, however, I am interested in measuring changes of ASL pH of NBE and CFBE cells to various channel agonists and antagonists. Therefore, I elucidate the effect of different buffer concentrations using two different concentrations of HEPES, 25mM and 0mM. At the 0h, and 24h time point the ASL pH of the NBE cells with 25mM HEPES in the Ringers solution was more alkaline compared to NBE cells with 0mM HEPES ($p<0.004$, $n=3$). At the time point 0h, the mean ASL pH using 25mM HEPES containing Ringers solution was 7.80±0.12, and the 0mM HEPES containing Ringers solution had a mean pH of 7.31±0.36. At the 24h time point the mean ASL pH with 25mM HEPES containing Ringers solution was 7.1±0.38 and the 0mM HEPES containing Ringers solution mean pH was 6.44±0.53 (**Figure 8**). However, at 2h both solutions, containing HEPES and without HEPES, are not significantly different. At 2h the mean ASL pH with 25mM HEPES containing Ringers solution was 6.96±0.49, and the 0mM HEPES containing Ringers solution had a mean pH of 6.77±0.42. Therefore, going forward using either a 1hr or 2h time point for my ASL pH measurements, the use or not use of HEPES addition did not alter the ASL pH measurements. I have decided to use 0mM HEPEs-containing solution in subsequent experiments to eliminate any buffering effect caused by HEPES before the 1hour time point

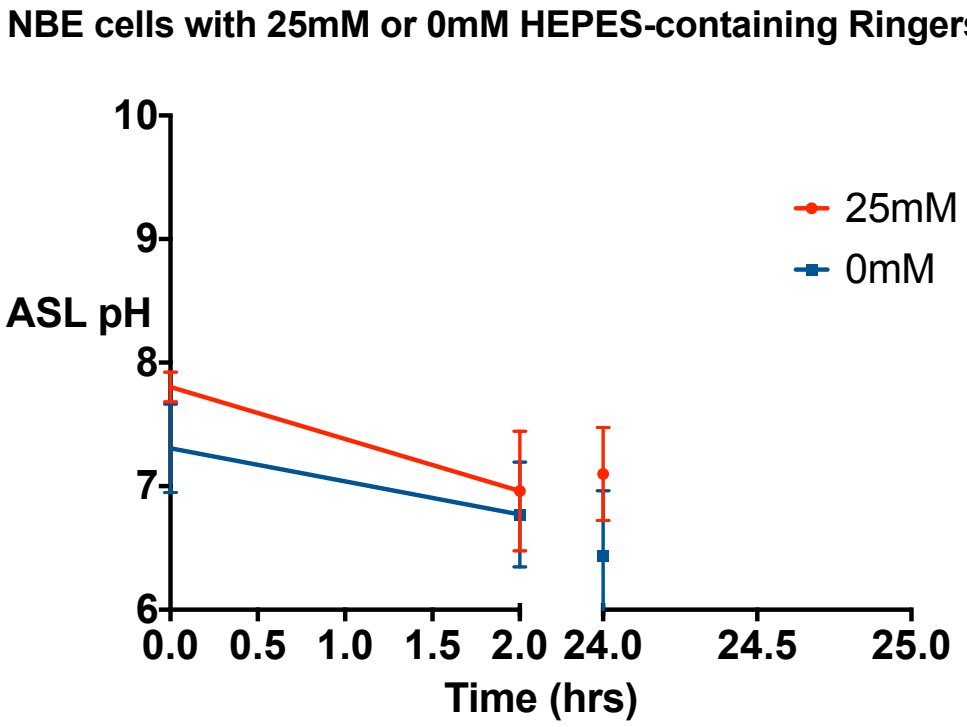



**Figure 8. ASL pH of NBE cells with 25mM and 0mM HEPES containing Ringers solution over 24 hours**

NBE cells were exposed to an apical bolus containing two fluorophores and either 25mM or 0mM HEPES containing Ringers solution for 24 hours. At t=0h there was a statistical difference between the HEPES containing solution, and without HEPES solution ($p<0.004$). At t=2h there was no significant difference. Points represent mean ± SD, n=3.

## 2.3 Time Point Analysis

Previous studies demonstrated that human bronchial epithelial cells absorb excess ASL volume within 12 hours, after which ASL height is maintained at 7μm in non-CF airway cells and 4μm in CF airway cells[19]. Further, Coakley *et al.* showed that over 48 hours there is an acidification that naturally occurs in both NBE and CFBE cells[79]. Using the previously established 0mM HEPES containing Ringers solution ASL pH was measured over 48 hours to assess the ASL pH change over time. NBE cells were measured at varying time points over a period of 48 hours (0h, 2h, 24h, 48h). Over 48hours the ASL pH becomes more acidic (NBE n=5, $p<0.0005$)(**Figure 9.**). The mean ASL pH at 0h is 7.57±0.69, at 2h is 6.92±0.69 at 24h is 6.69±0.38, and at 48h is 6.42±0.33. Previous studies have used 1h, 2h, and 6h[87]. Subsequent studies were measured at time points in the first 2 hours and once more at 24 and/or 48 hours. I choose a few time points in the first two hours because both Tarran and Welsh's group used either a 1h or 2h time point. Both groups stated that after the 1hr time point the NBE and CFB cells would have enough time to equilibrate to $CO_2$ and temperature in the incubator.

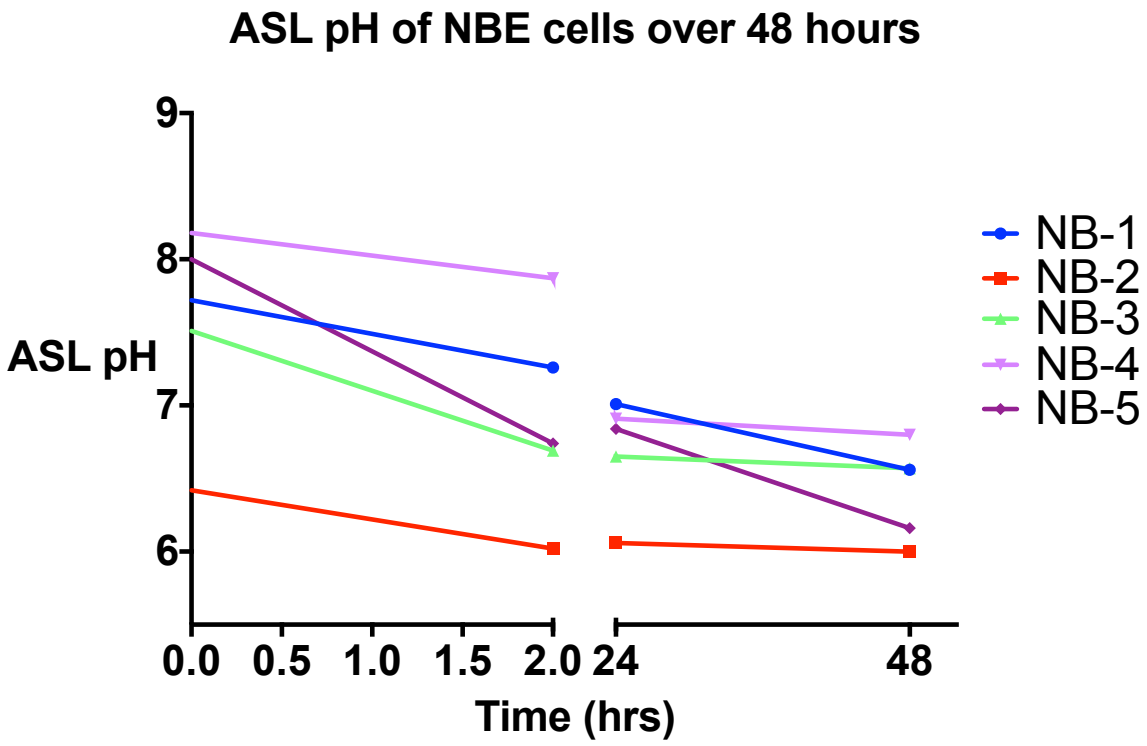



**Figure 9. Acidification of NBE cells ASL pH over 48 hours**

Normal primary bronchial epithelial cells (NBE) measured from 5 patients (1-5) over a period of 48 hours. ASL pH was measured at 0h, 2h, 24h, and 48 hours.

## 2.4 Reproducibility of ASL pH measurements

I receive primary cultures containing ~5-6 transwells of human primary bronchial epithelial cells from each patient's lung explant. For the repeatability studies I used two transwells containing human primary bronchial epithelial cells from each patient and measured the ASL pH subsequently for 3 times at time points 0h, 5min, 15min and 1h (**Figure 10.)**. Using a 2-sided ANOVA statistics with adjustments using Tukey's multiple comparison test there was no significance between the two transwells from the same primary bronchial cell donors. Nevertheless, between the three different cell donors (3 individual patients) there were significant differences ($p<0.0001$) between the measured ASL pH. These results confirm that reproducibility of my technique and established protocol measuring ASL pH.

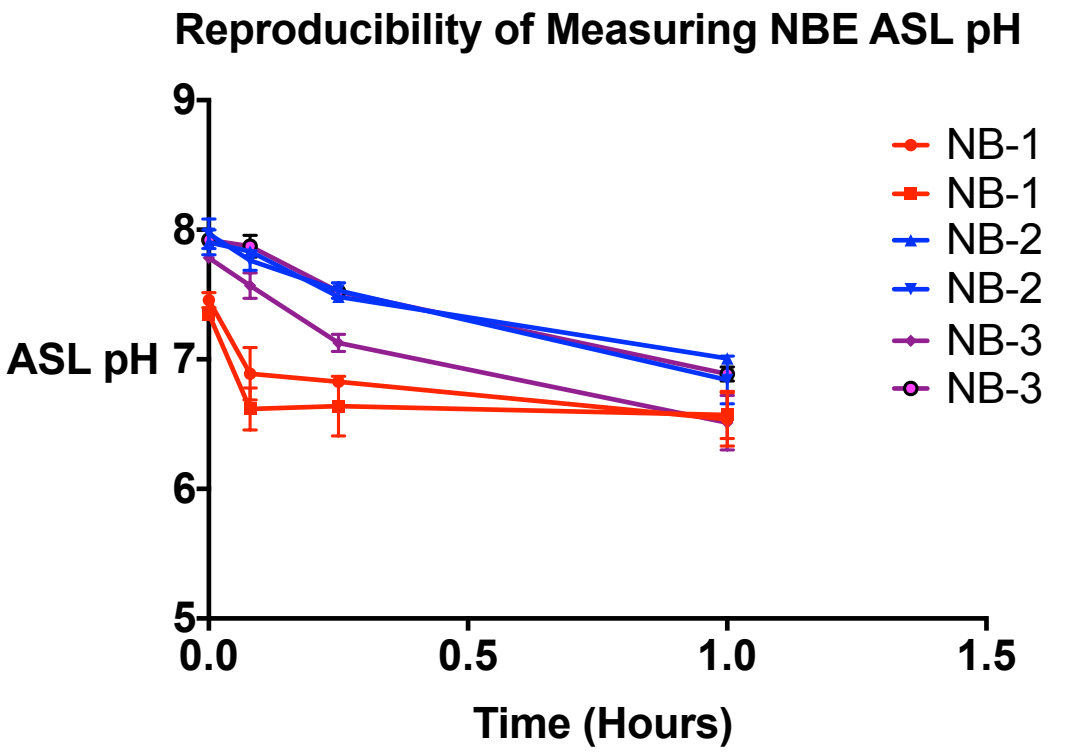



**Figure 10. Reproducibility in the technique for measuring ASL pH**

Two transwells of normal human bronchial epithelial cells from three different lung transplant donors, were measured three times at time points 0h, 5min, 15min and 1h. There were no significant differences between the measurements of the 2 transwells of the same donor, but significant differences between transwells of different donors ($p<0.0001$). Points represent means ±SD, n=3.

To investigate variability as well as repeatability in normal human primary bronchial epithelial cells I used two transwells from the same donor and measured the ASL pH over a period of 48 hours (**Figure 11)**. There was no difference between the two transwell's from each donor($p<0.05$), but there was variation amongst all 5 donors up to 1h ($p<0.001$). The mean pH at 0h was 7.05 ±0.38, at 1h was 6.68±0.15 and acidified over 24 hours to 6.52±0.065 (n=5), which is a trend previously noted. The variation was least at the 1hour time point with a standard deviation of ±0.14 as compared to 0h of ±0.38

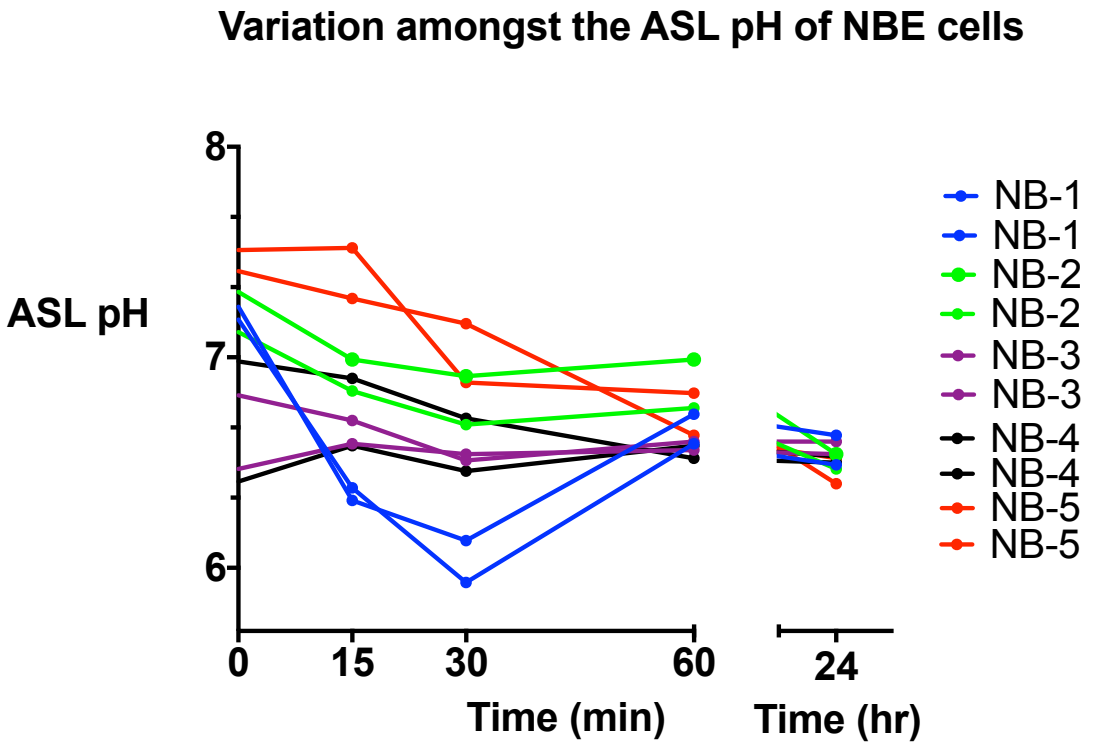



**Figure 11. Variability of ASL pH measurements amongst normal primary bronchial epithelial cell cultures**

Two transwells of normal primary bronchial epithelial cells from lung transplant donors were measured over 48 hours. There was no statistical significant difference between the measurements of the two transwells (n=5). Amongst different cell cultures there was significant differences in ASL pH up to 1h($p<0.001$, Two-Way ANOVA). There is variability amongst each patients ASL pH ranging from ~6.5-7.5.

## 2.5 Effect of $CO_2$-equilibration in the microplate reader

The accessibility to a $CO_2$ tank that can be connected to the microplate reader in order to ensure continuous $CO_2$ flow varied throughout my experiments. All prior experiments were $CO_2$ equilibrated in an incubator at $37^0$C and with 5% $CO_2$ for a minimum of 5 minutes before the 2 minute measurement in the microplate reader. I measured if differences exist in the ASL pH measurements with and without $CO_2$ using the same 2 transwells containing primary bronchial epithelial cells from the same donor (**Figure 12**). There were significant differences at time point 0h ($p<0.001$, $n=3$) in the ASL pH when measured with $CO_2$ and in the absence of continuous $CO_2$ equilibration. However, in all the time points after the 0h time point there was no significant difference in ASL pH measurements. The mean ASL pH in the absence of continuous $CO_2$ at 0h is 7.64±0.08, and with $CO_2$ was 7.25±0.09 ($p<0.001$, $n=3$). At 1h the mean ASL pH in the absence of continuous $CO_2$ is 7.33±0.08 and with $CO_2$ was 7.18±0.01 ($n=3$), and were not statistically significantly different from one another. This can be explained with the fact that at the 0h time point I manipulate the apical membrane by washing it with PBS and adding an apical bolus. In the presence of $CO_2$ there is less acidification of the NBE cell cultures over the 1hour period than in the absence of $CO_2$. Ideally, all experiments measuring the ASL pH of bronchial epithelial cells should be performed using a $CO_2$ equilibrated microplate reader. However, in cases in which this was not available, I can be re-assured of the ASL pH measurements, as at the later time point the presence of $CO_2$ did not further affect the ASL pH results.

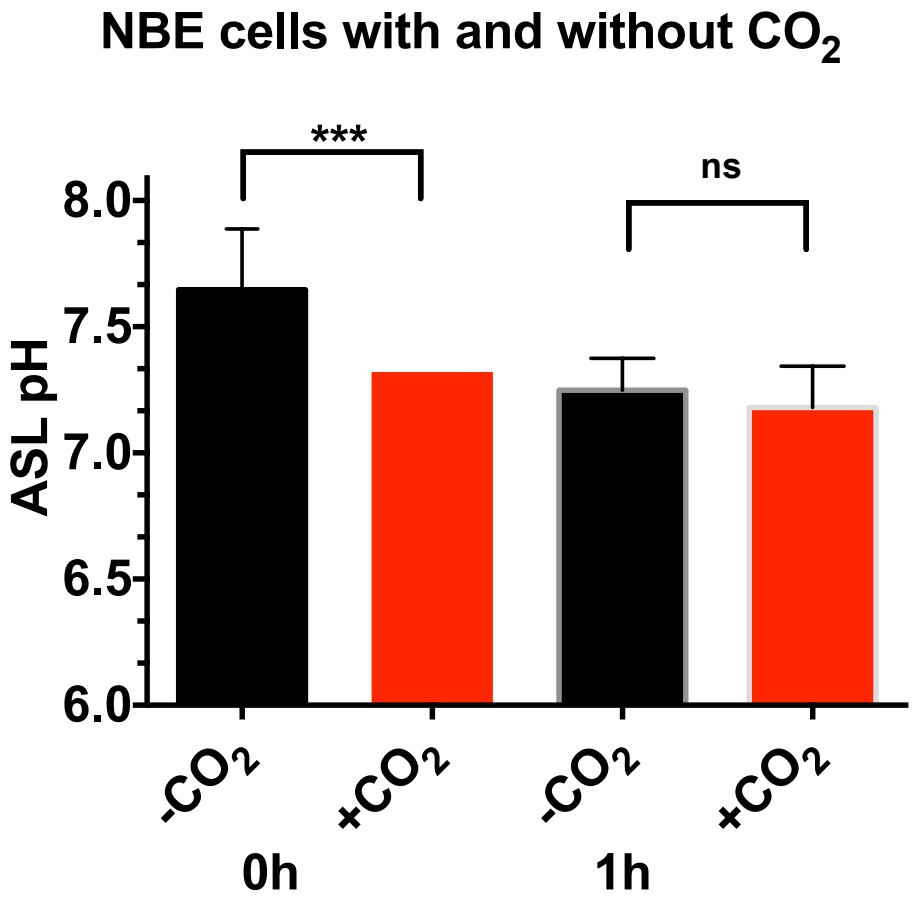



**Figure 12. ASL pH measurement of NBE cells with and without $CO_2$-regulated microplate reader**

Using two transwells containing human primary bronchial epithelial cells from the same donor in two conditions, $CO_2$ present in the microplate reader and $CO_2$ absent, I measured the ASL pH of normal primary bronchial epithelial cells. There are only statistical significant differences between the two conditions at 0h. Points represent means±SD.

I wanted to measure the stability of the measurements using $CO_2$-equilibratation during the microplate reader measurements as the $CO_2$ tank became available for my use (**Figure 13)**. In the presence of $CO_2$ there was a greatest range in ASL pH at time point 0h (pH range from 7.54-6.98) between the donor NB-1, and donors NB-2, and NB-3 ($p<0.0001$). By the 1h time point the ASL pH range was smaller (pH range from 7.39-7.04) and there were no significant differences amongst all three donors. The mean ASL pH at 0h was 7.35±0.21, and at 1h it was 7.21±15 (n=3).

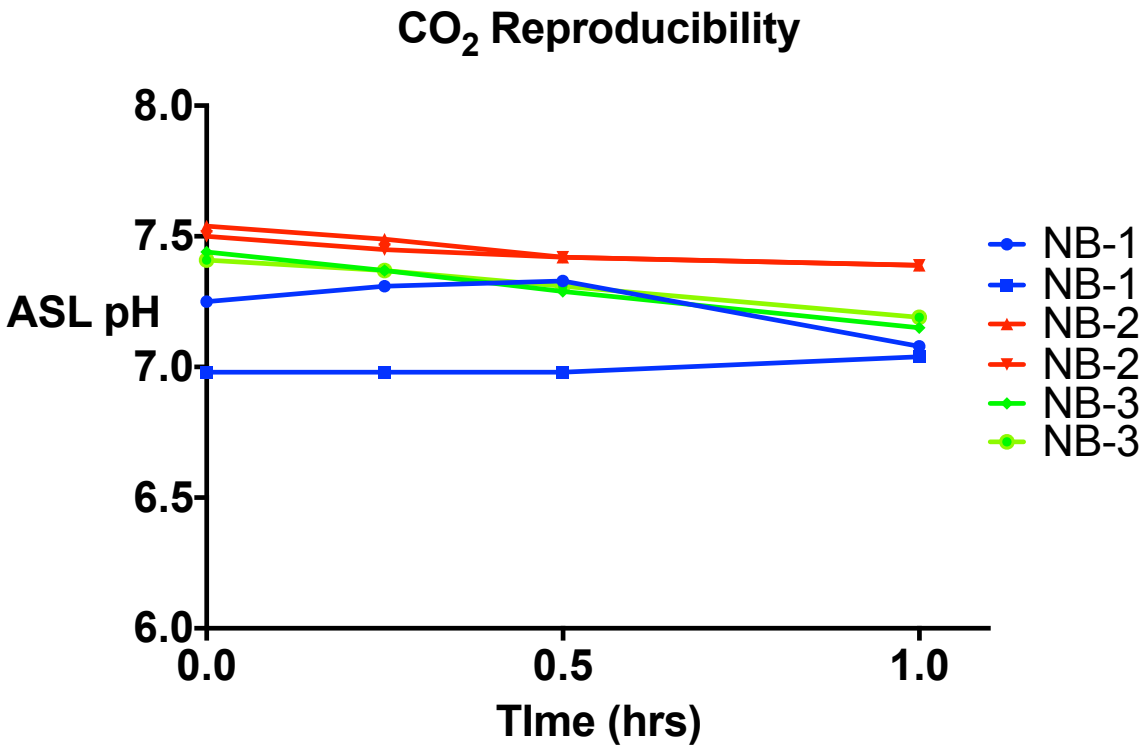



**Figure 13. Reproducibility of ASL pH measurements amongst two transwells from the same patient in a $CO_2$-regulated microplate reader**

Two transwells containing human primary bronchial cells from three patients were placed in a $CO_2$-equilibrated microplate reader. At time point 0h there were statistical significant differences in ASL pH between patient 1 and patients 2 and 3 at time point 0h ($p<0.0001$, n=3, 2-sided ANOVA with Tukey's multiple comparison test). At 1h there were no significant differences amongst all transwells from all three patients.

## 2.6 Effect of basolateral bicarbonate on the ASL pH

In all previously mentioned experiments, the human primary bronchial epithelial cells were measured in the presence of media containing 1.2mg/mL sodium bicarbonate. Coakley *et al.* performed ASL pH experiments without basolateral bicarbonate (in a bicarbonate free HEPES buffered solution) and demonstrated that basolateral bicarbonate contributed to ASL alkalization in normal primary bronchial epithelial cells. To measure the effect of HEPES-buffered solution in place of media containing bicarbonate on the ASL pH the transwells were placed in a basolateral pH 7.4 HEPES buffered solution (**Figure 14.**). The mean pH at time point 0h with media was 7.73±0.29 (n=3) whereas the mean pH at 0h with HEPES buffered solution was lower 7.42±0.37 (n=3, $p<0.0001$). In all three experiments there was significance difference between the media and the HEPES buffered solution up to the 1h period ($p<0.0001$). At the 1h time point, there was no significant difference between the ASL pH with either basolateral solutions. Moving forward I suggest to use HEPES buffered solution rather then media because basolateral transporters would not be moving bicarbonate to the apical membrane thus allowing us to detect small changes in ASL pH.

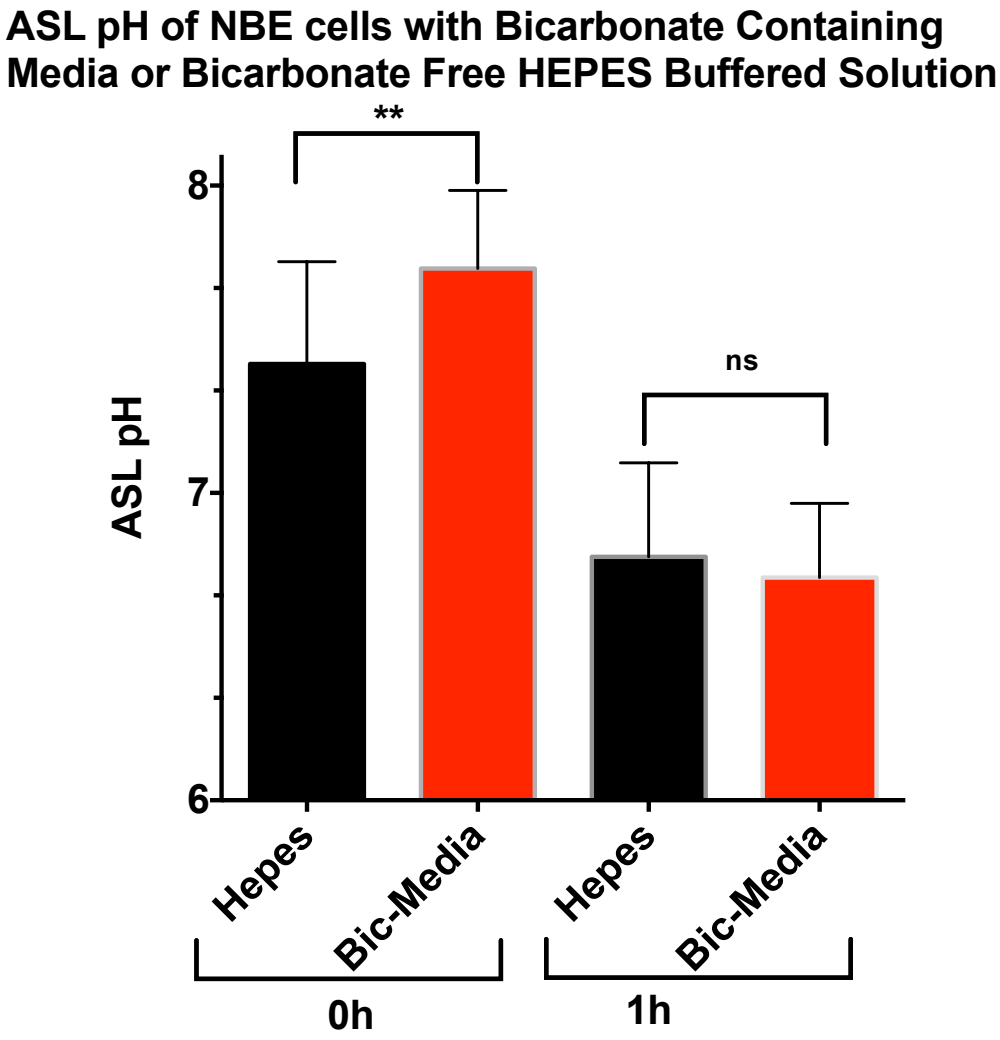



**Figure 14. ASL pH measurements of NBE cells with basolateral media or pH 7.4 HEPES-buffered solution**

The ASL pH of NBE cells with media and HEPES-buffered solution over a period of 1 hour. There is a statistical significant difference between HEPES-buffered solution and bicarbonate containing media at 0h ($p<0.0001$) but no differences at the 1h time point. Points represent mean ±SD.

I repeated the same experiment for 1 hour in the presence and absence of $CO_2$ using the $CO_2$-equilibrated microplate reader. At time point 0h the ASL pH was statistically significant different between the HEPES- buffered solution and media in the absence and presence of $CO_2$ ($p<0.0001$, $p<0.005$, respectively, $n=3$). I attribute it to the apical manipulation of washing the cells with PBS and adding the bolus at this time point. At the 1h time point the cells have had a chance to $CO_2$ and temperature-equilibrate and there is no significant difference between the HEPES buffered solution and the media in the presence and absence of $CO_2$ (**Figure 15a and 15b)**. Future experiments will have HEPES-buffered basolateral solution to eliminate bicarbonate transport to the apical membrane which affects the ASL pH, and will be measured in the presence of $CO_2$ when available.

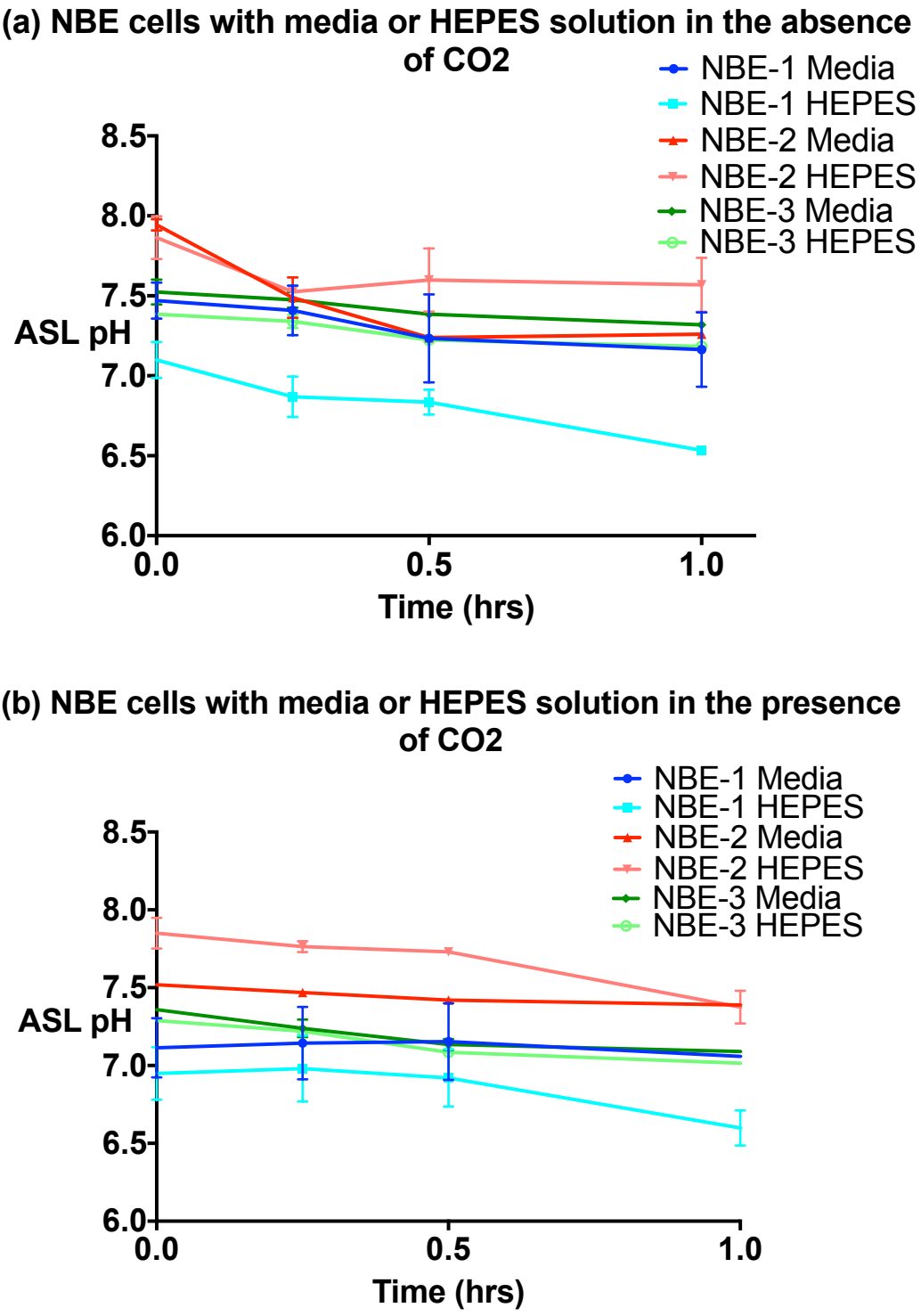



**Figure 15. ASL pH of NBE cells in the absence (a) or presence (b) of $CO_2$**

The ASL pH was measured in normal primary bronchial epithelial cells in the absence or presence of $CO_2$ with two different basolateral solutions, media and HEPES-buffered solution. A similar trend is observed in the absence and presence of $CO_2$. At 0h, there is a significant difference between the ASL pH using HEPES buffered solution and media in absence ($p<0.0001$) and presence ($p<0.005$) of $CO_2$. After this time point there is no statistical difference between the ASL pH using HEPES-buffered solution and media in both $CO_2$ and without $CO_2$ conditions. Points represent mean ±SD.

In a previous publication, Coakley *et al.* demonstrated that acidification of media occurs over time, reflecting bicarbonate depletion and lactate accumulation leading to an acidic ASL pH. The study showed that it is possible to re-alkalinize the ASL pH with bicarbonate replenished basolateral solution reflecting transcellular and paracellular bicarbonate transport to the apical membrane. Using my protocol I tested whether I can equally pick up a change in ASL pH following replacement of the basolateral media and thus replenishing the basolateral bicarbonate concentration (**Figure 16.**). In fact, replacement of basolateral media at a time point of 24 hours resulted in an increase of the ASL pH to 7.67±0.19, compared to an ASL pH from 6.74±0.17 in cells in which media was not replaced ($p<0.0004$) and confirms transepithelial ASL pH regulation via basolateral influx of bicarbonate. This is an agreement with Coakley *et al.* and confirms the sensitivity of my methodology to measure changes in ASL pH.

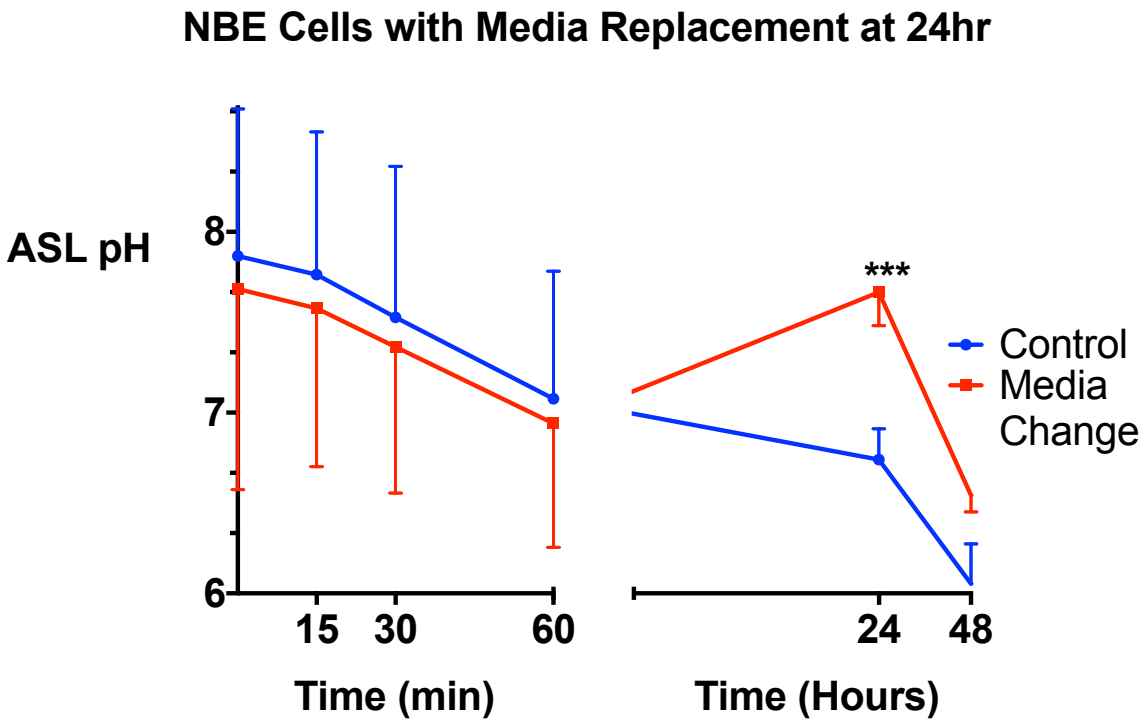



**Figure 16. ASL pH measurement is responsive to replacement of basolateral media change**

The basolateral bicarbonate concentration was replenished by changing to fresh media at 24 hours. There is a statistical significance increase in ASL pH with the media change at 24h ($p<0.0004$). Points represent mean ±SD.

## 2.7 Addition of an $H^+$- ionophore

I have established that at time point1 hour, which is a time point commonly used in other publications to compare ASL pH[87,99], there is no difference in ASL pH in the presence or absence of $CO_2$, as well as with HEPES buffered solution or media containing bicarbonate. I wanted to see if my optimized technique reliably measures different ASL pH by adding a $H^+$-ionophore apically causing a change in pH. Monensin, a monovalent selective ionophore, catalyzes the transport of $Na^+$ into the cell across the membrane in exchange for $H^+$ and thus acidifies the ASL pH. I added monensin apically to facilitate proton transport at time 0hrs. The mean ASL pH (n=3) of NBE cells treated with monensin was 6.87±0.34, which is statistically different compared to the NBE cells without monensin with an ASL pH of 6.96±0.47 (**Figure 17)**. After 30min the mean pH of the NBE cells treated with monensin was 6.35±0.30 and the ASL pH of the NBE cells without monensin was 6.82±0.30. Prior publications presented data showing extracellular pH rapidly responded to monensin, decreasing the extracellular pH for at least 30 minutes. My results indicate that the addition of monensin reduces ASL pH, and thus my technique is sensitive enough to identify changes in ASL pH following targeted acidification.

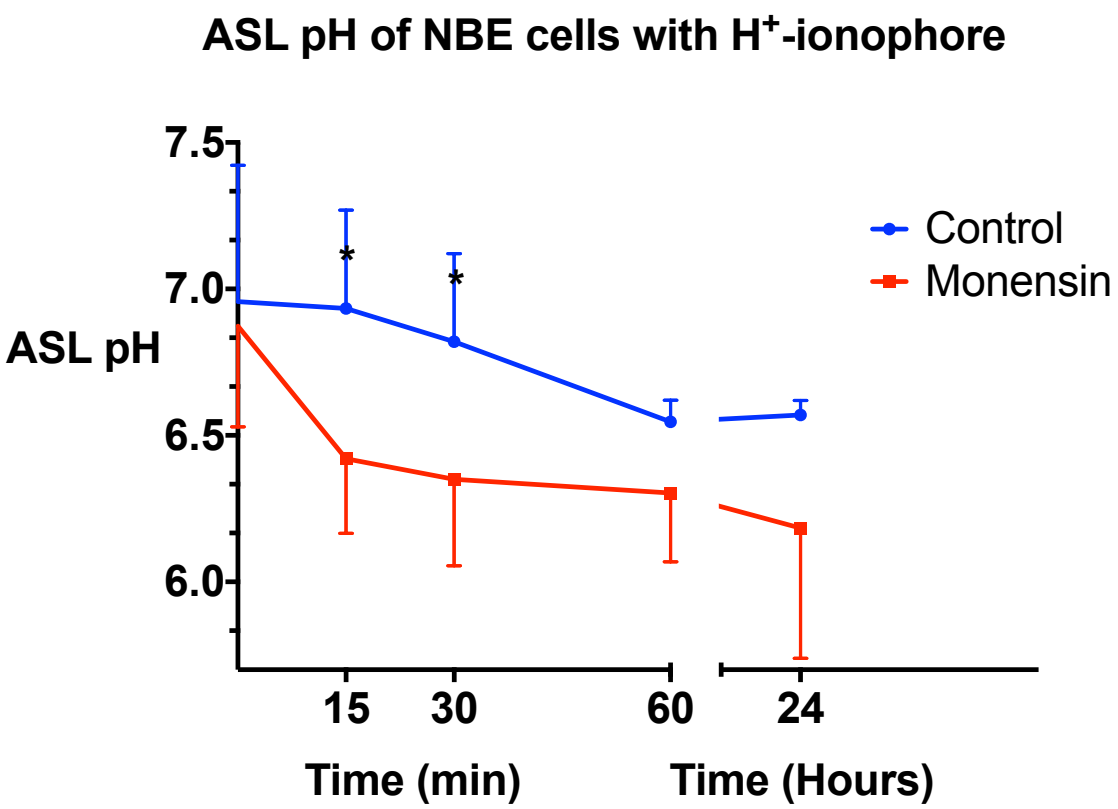



**Figure 17. ASL pH of NBE cells with Monensin added apically**

With the addition of $H^+$-ionophore, monensin there was a statistically significant reduction in ASL pH at 15 and 30min time points ($p<0.01$, $n=3$). Points represent mean ±SD.

# 3.0 Differences in non-CF and CF ASL pH

Earlier studies have indicated that human airway epithelia exhibit CFTR-dependent bicarbonate secretion and in its absence such as seen in airway epithelial cells from CF, the ASL pH is reduced. I measured the ASL pH of non-CF patients (n=7) and CF-patients (n=6) at a 1hour time point and observed that the mean ASL pH in bronchial epithelial cells from CF patients is statically significantly lower 6.57±0.37, when compared to bronchial epithelial cells from non-CF patients 7.14±0.19 ($p<0.05$) **(Figure 18.)**.

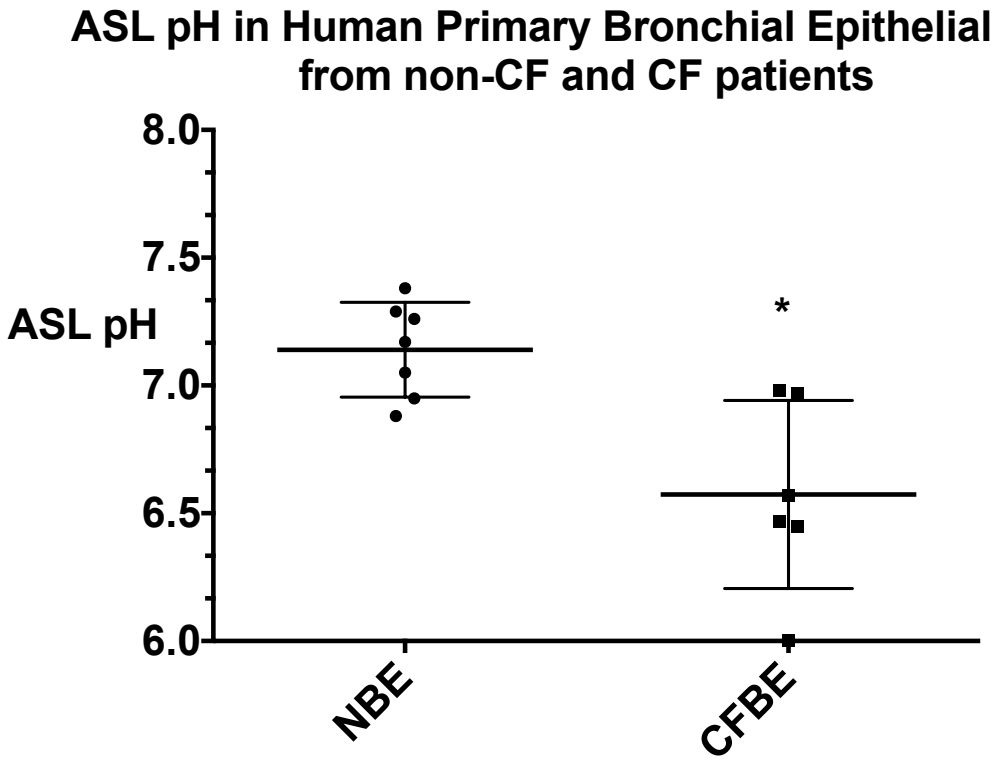



**Figure 18. ASL pH is more acidic in CF than non-CF**

ASL pH was measured in NBE and CFBE cells in HEPES buffered solution. n=7 for non-CF and n=6 CF primary bronchial epithelial cells. Measurements were taken at 1h where NBE and CFBE are statistically significantly different ($p<0.001$). Points represent means ±SD.

# 4.0 Low ASL pH in CF is influenced by ATP12A and SLC9A3

As reported by Welsh *et al.*, ATP12A inhibition with apical ouabain raises ASL pH in primary cultures of non-CF and CF porcine and human airway epithelia cells[72]. Apical ouabain inhibits the effect of ATP12A activity. I applied ouabain (non-gastric $H^+/K^+$-ATPase inhibitor) to the apical surface of the CF primary bronchial epithelial cellsand measured the ASL pH. For these experiments a HEPES-buffered solution was used. The CF primary bronchial epithelial cells were measured at 1h in the presence of $CO_2$ (**Figure 19.)**. The ASL pH increased with ouabain to a mean ASL pH of 6.76±0.51 from a mean ASL pH of 6.31±0.27 ($p<0.05$, n=3). These results are in accordance with Welsh *et al.* data showing a similar result with ouabain, the ASL pH raised to 6.79 from 6.40 in CF primary bronchial epithelial cells. I also applied an SLC9A3 inhibitor and demonstrated that the mean ASL pH increased with SLC9A3 inhibitor to 6.88±0.12, as compared to the ASL pH without SLC9A3 inhibitor, 6.31±0.27 ($p<0.01$, n=3). These results suggest that inhibiting ATP12A and SLC9A3 increase the ASL pH in CF human primary bronchial epithelial cells.

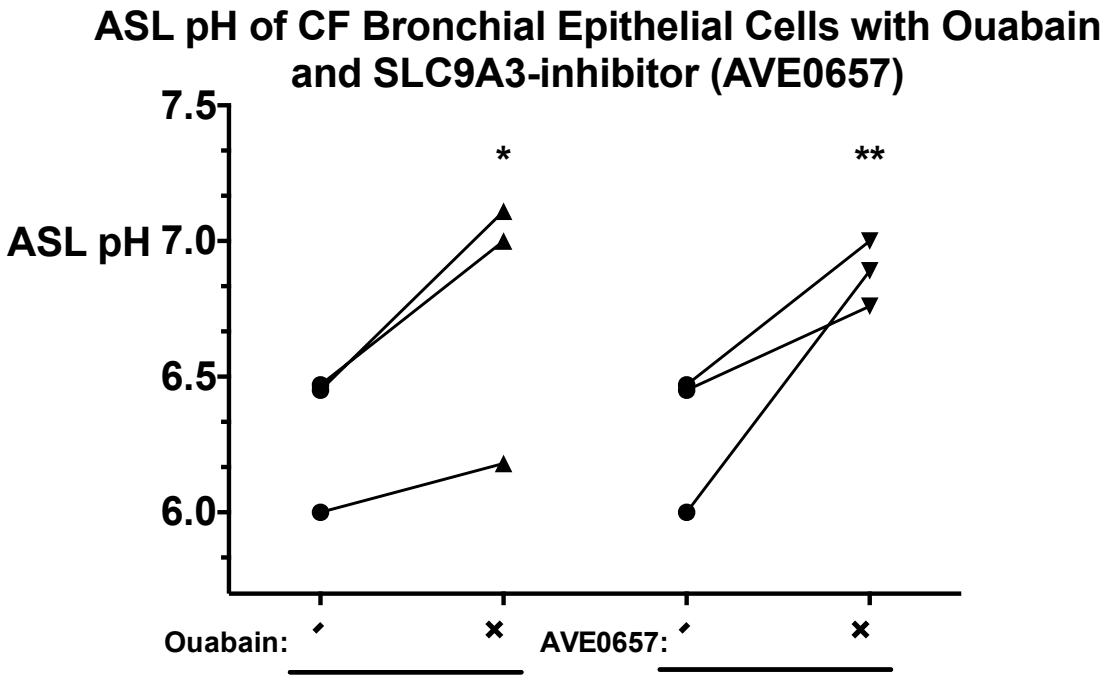



**Figure 19. Inhibiting ATP12A and SLC9A3 increases ASL pH in CFB cells**

CF cultured human airway epithelial cells were treated with 10uM apical ouabain and 10uM SLC9A3 inhibitor. Measurements occurred at 1h in the presence of $CO_2$ and HEPES-buffered basolateral solution. The ASL pH measurements with the SLC9A3 inhibitor and ouabain were significantly different from CFB epithelal cells ($p<0.01$, $n=3$) Each data point is from epithelia from a different human.

# Discussion

## 1.0 Methodology

My results indicate that I have established an accurate and reliable methodology for measuring ASL pH in human primary bronchial epithelial cells using a microplate reader and fluorescence dyes. I choose to adopt Tarran's methodology using pHrodo Red conjugated to a dextran because the previously used fluorescent dyes SNARF-dextran and BCECF have a short pH range ~6-7.5, whereas pHrodo Red has a larger pH range, 4-10. Alternate methodologies for measuring ASL pH accurately exist, such as using a glass pH electrode but in comparison to the plate reader, the pH electrode is invasive and has been criticized because the volumes being sampled can be greater then the expected fluid volume of the thin ASL film. The sampled ASL also may be contaminated changing the ASL pH readings. I adapted Tarran's methodology because my initial results had much variability in ASL pH measurements. The revisions I have made to Tarran's protocol include, measuring the importance of $CO_2$-equilibration on ASL pH, removing the lid of the 12-well plate in the microplate reader, and artificially induced basolateral and apical manipulations to detect ASL pH changes. The changes made exemplified the sensitivity and precision of my methodology.

## 2.0 Environmental $CO_2$ Alters ASL pH

I demonstrated that in the presence of a $CO_2$-equilibrated microplate reader there were differences in ASL pH when compared to the absence of $CO_2$ equilibration before the 1hour time point. After the 1hour time point there were no differences in ASL pH

between the presence and absence of a $CO_2$-equilibrated microplate reader. I attribute the differences in ASL pH before the 1hour time point to the initial manipulation, washing the primary bronchial epithelial cells with PBS and addition of the bolus that may affect the cells equilibration. Another possible explanation for the differences in ASL pH in the first hour is the length of time the human primary bronchial epithelial cells are kept in the 5% $CO_2$ incubator. The primary epithelial cells are kept in the incubator for 5 min before the first read, and 30 min before the 1hour read. The increased length of time may allow for greater equilibration thus reducing the variability in ASL pH and may explain why there is no statistical significant difference in ASL pH with or without $CO_2$ after the first hour. In recent literature, both Welsh *et al.* and Tarran *et al.* published ASL pH measurements after a 1hour time point[68,72]. Tarran measured the ASL pH at the 2 hour time point and stated that offgassing of $CO_2$ did not occur during the time required to remove the cultures from the incubator to the ASL pH measurements in the plate reader in the absence of $CO_2$[68]. Thus, my results are comparable to Tarran *et al.* because after the 1hour time point there was no statically significant difference in ASL pH measurements with or without $CO_2$-equilibration.

## 3.0 Basolateral Bicarbonate Alters ASL pH

Human primary bronchial epithelial cells require media containing bicarbonate to buffer the media pH in the range of 7.2-7.4 to maintain physiological pH and reduce metabolic stress. In some experiments I wanted to eliminate bicarbonate transport to regulate the induced ASL pH changes, that may alkalize the ASL pH, by replacing the media with a HEPES buffered solution. I was able to measure a change in ASL pH at 0h between

media containing bicarbonate and HEPES-buffered solution without bicarbonate. A simple explanation for the more acidic ASL pH before the 1hour time point using the HEPES-buffered solution is in the absence of bicarbonate in the media there is a reduction in bicarbonate secretion. In response to replenishing the basolateral bicarbonate with fresh media there was an increase in ASL pH presumably caused by CFTR-dependent $HCO_3^-$ secretion, which is comparable with Coakley *et al.* observations[2]. $HCO_3^-$ enters the cell across the basolateral membrane either through the sodium-bicarbonate co-transporter NBCe1b or the anion exchanger 2 (AE2)[82]. In the absence of bicarbonate in the basolateral solution, the ASL pH is more acidic due to decreased bicarbonate transport when compared to using bicarbonate-rich media. After the 1hour time point, where there was no difference between the two basolateral solutions. One possible explanation for this finding is that the epithelial cells self-regulate ASL pH changes, thus the addition of bicarbonate in the media would reach a homeostasis with only minimal apical bicarbonate secretion. Bicarbonate also depletes in the media over time, thus at the 1hour time point there are no significant difference between media-containing bicarbonate and HEPES-buffered solution.

## 4.0 ASL pH in non-CF and CF airway cells

Consistent with previous publications I demonstrated that ASL in CF primary bronchial epithelial cells is more acidic then normal primary bronchial epithelial cells. My results reported that the ASL pH of CF patients to be between 6.3-7.1, which is supported by the Welsh group who identified an ASL pH range between 6.5-7.1[72]. My results are not only in agreement with previously reported data in primary epithelial cells, but also in

submucosal glands, breath condensate and porcine bronchial and tracheal epithelial cells studies[53,83,65]. The ASL pH of normal primary bronchial epithelial cells was measured in a range between 6.5 and 7.5. I attribute these differences in CF patients to the loss of CFTR-dependent $HCO_3^-$ secretion, causing a reduced ASL pH.

To ensure that the ASL pH measurements were accurate, repeatability measurements from the same donor demonstrated no significant differences in ASL pH. However, there was variability amongst different donors ASL pH measurements. This variability is in agreement with those made by Welsh *et al.* observed in porcine epithelial cells, that bicarbonate increases proportionally to the number of CFTR channels localized at the apical surface[84]. Individual samples overexpressing CFTR channels have increased bicarbonate flux, and a more alkaline ASL pH[84]. Understanding the roles of acid secretion of major transporters, channels and exchangers in the apical membrane and modifier genes, such as SLC9A3, may account for the variability in ASL pH and potentially recover the acidic ASL pH defect in CF.

# 5.0 Potential Role of $H^+$ Secretory Mechanisms in Regulating ASL pH

CFTR is not solely responsible for ASL pH regulation; the apical airway epithelium expresses $H^+$ channels and transporters that contribute to the ASL pH. My preliminary results targeting proton transporters and exchangers have lead to rescuing the acidic ASL pH. I wanted to duplicate the Welsh group's finding using the pharmacological inhibitor ouabain. Ouabain apically removes $K^+$ preventing $H^+$ secretion and inhibits ATP12A

located on the apical membrane, increasing the ASL pH in CF airways[72]. Consistent with the Welsh groups findings, I demonstrated that ouabain increased ASL pH.

According to UniGene database SLC9A3 is widely expressed in a variety of tissues, yet there is little published whether SLC9A3 is located on the apical membrane of epithelial cells. Assessment of SLC9A3's expression in airway epithelial cells is challenged due to the existence of multiple pseudogenes. A similar $Na^+/ H^+$ antiporter, SLC9A1, was detected by RT-PCR in all bronchial regions of the human lung; therefore I propose that SLC9A3 may also be expressed in the lung. Given the significant association between SLC9A3 disease gene and severity of CF lung disease I nevertheless wanted to assay whether pharmacological modifications of SLC9A3 affects ASL pH.[38] The data revealed an increase in ASL pH in CF primary bronchial epithelial cells with the use of a pharmacological inhibitor, which corroborates with my hypothesis identifying SLC9A3 as a major player on the apical membrane. This data is the first example of an SLC9A3 inhibitor used on the apical airway epithelia to increase ASL pH in CF human primary airway epithelial cells. SLC9A3, ATP12A and other proton transporters and exchangers should be investigated as future drug targets rescuing the acidic ASL pH in CF patients.

# 6.0 Experimental Limitations

The human primary bronchial epithelial cells used are a powerful model, yet a limited resource. The limited supply lead to a small sample size analyzed using the methods presented here. It will take a larger sample size to elucidate differences in ASL pH that may result from SNP expression.

Using the pHrodo Red I would like to compare the ASL pH on a confocal microscope, to assess the validity of my technique. Garland *et al.* who introduced the use of pHrodo Red fluorescent indicator, matched their experiments with confocal microscope experiments. I did not have access to an upright confocal microscope that was large enough to fit into the transwells containing primary bronchial epithelial cells with the appropriate filters.

# 7.0 Summary

I have established a protocol based on previous publications that allows for the quantification of ASL pH in lung epithelial cells. Using this protocol, I have demonstrated that there is a difference in ASL pH between non-CF and CF individuals. I have looked into two proton transporters/exchangers of particular interests, since $H^+/K^+$ ATPase has been shown to increase ASL pH and bacterial killing in CF airways, and SLC9A3 has been associated with increased severity of CF disease in the lungs. I was able to increase the ASL pH by inhibiting both $H^+/K^+$ ATPase and SLC9A3, thus counteracting the negative effect of loss of CFTR-mediated bicarbonate secretion on ASL pH. These studies are some of the first to interrogate the functional role of SLC9A3 in cystic fibrosis and pave the way for further studies into the role of SLC9A3's association in ASL pH regulation and lung disease.

# 8.0 Future Directions

Future experiments will be directed at investigating if there is correlation between extracellular ASL pH and intracellular pH. Investigations of intracellular pH would

strengthen the investigation of how proton transporters and channels regulate extracellular ASL pH and if there is a compensation that occurs intracellulary for the extracellular acidity. I would also like to determine the consequences of the SLC9A3 SNP on ASL pH. Investigations into the functional expression of SLC9A3 would be strengthened by RT-PCR experiments to determine the expression of SLC9A3 in the airways. This would strengthen the understanding of exchangers on the airway, and the implications for CF and airway disease. Quantitative RT-PCR could provide information about changes in expression as a result of SLC9A3 risk SNP expression.

SLC9A3 could potentially interact with ASL-dependent bacterial killing, such as ATP12A. Microscope-based experiments would investigate the impacts of SLC9A3 on mucocilliary clearance, bacterial expression, and fluid regulation. This knowledge would not only be valuable for general lung physiology, CF pathophysiology research and treatment, but it would help to further support an important role of SLC9A3 in ASL homeostasis.

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