HIGH SALT OR LOW VOLUME: A CRITICAL DISSECTION OF A MECHANISTIC CONTROVERSY IN CYSTIC FIBROSIS

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ABSTRACT

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Cystic fibrosis is an autosomal recessive disease caused by a mutation in the sequence coding for a transmembrane chloride channel called the cystic fibrosis transmembrane conductance regular (CFTR). Complications due to chronic lung infections are the primary cause of the decreased quality of life and the leading cause of mortality seen in cystic fibrosis patients. Two main hypotheses for the origin and mechanism of bacterial colonization of cystic fibrosis lungs exist and have been debated for years. The high salt hypothesis equates the colonization of the lungs to an increased concentration of sodium and chloride in the layer of liquid covering the surface of the lungs, known as airway surface liquid (ASL), inhibits the bactericidal activity of airway defensins. The low volume hypothesis suggests that the decreased volume of the ASL, as a result of increased sodium absorption causes the cilia to collapse in the absence of a liquid covering thereby halting mucus clearance. An analysis of available data for sodium and chloride concentrations in liquid lining of lungs and the depth was conducted using an independent samples student's t-test. The evidence evaluated supports that no difference exists between the liquid lining of the normal and cystic fibrosis lungs for salt concentrations (Sodium: $p = 0.316$, Chloride: $p = 0.30$) or depth ($p = 0.16$). The findings of this study suggest an alternative mechanism for initial bacterial colonization of the lungs in cystic fibrosis.

For my Mom and Daddy-o, Cora and Gary Igert, the one who believed in me when I did not, Matthew Marquette, and the little boy who looks at me as though I hang the moon and the stars, Ralph Waldo Emerson Marquette

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KEY TO SYMBOLS AND ABBREVIATIONS

ASL – Airway Surface Liquid

ATP – Adenosine Triphosphate

β-S – Beta-Adrenergic-Sensitive cells

β-I – Beta-Adrenergic-Insensitive cells

CBAVD – Congenital Bilateral Absence of Vas Deferens

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

$CFTR^{-/-}$ – Animal model with no expression of CFTR protein

$CFTR^{\Delta F508/\Delta F508}$ – Animal model homozygous for the ΔF508 mutation

ENaC – Epithelial Sodium (Na^+) Channel

IM – Intramuscular

IV - Intranvenous

$Isc_{(amil)}$ – Short circuit current under amiloride inhibition

ME – Myoepithelial cell

MSD1 – Membrane Spanning Domain 1

MSD2 – Membrane Spanning Domain 2

NBD1 – Nucleotide Binding Domain 1

NBD2 – Nucleotide Binding Domain 2

NHERF – Na^+/H^+ Exchanger Regulator Isoform-1

PDZ1 – PSD-95/Discs-large/ZO-1 Binding Domain 1

PDZ2 – PSD-95/Discs-large/ZO-1 Binding Domain 2

PKA – Protein Kinase A

R Domain – Regulatory Domain

TER – Transepithelial Resistance

YAP65 – Yes-Associated Protein-65

INTRODUCTION

Cystic fibrosis (CF) is the most prevalent genetic disease among Caucasians, occurring once in every 3,400 births (Kosorok et al., 1996). Inheritance occurs in an autosomal-recessive pattern and approximately 2-5% of the Caucasian population are carriers of the mutated gene. In total, around 30,000 people are affected with cystic fibrosis in the United States (Gershman et al., 2006). The underlying etiology of cystic fibrosis is due to over 1,500 different mutations in a 1,480 amino acid membrane-bound glycoprotein of the ATP binding cassette superfamily. A deletion of three base pairs encoding phenylalanine in the 508th amino acid position, known as the cystic fibrosis is due to over 1,500 different mutations in (Kerem et al., 1989). Approximately 70% of cystic fibrosis chromosomes contain this deletion (Morral et al., 1994). These cystic fibrosis transmembrane conductance regulator (CFTR) protein mutations, particularly the ΔF508 mutation, lead to a misfolding of the quaternary protein structure.

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is expressed in multiple tissues; hence cystic fibrosis affects multiple organ systems throughout the body. Systems with interrupted function in CF are the pulmonary, digestive, and reproductive systems, along with sweat glands (Quinton, 1999). Symptomatic presentations of cystic fibrosis involve pulmonary disease, gastrointestinal disease, diabetes, infertility, and failure to thrive. Pulmonary disease and its symptoms appear early on as a productive cough in more than 60% of patients by the age of five, and in all patients by the onset of adult-hood (Penketh et al., 1987). Other pulmonary

symptoms include wheezing, inflammation, airway obstruction due to mucus, and chronic lung infections. The main pathogen in lung exacerbation and infection is the bacterium *P. aeruginosa* (Peterson et al., 1981). Gastrointestinal issues recurrent in cystic fibrosis are loose and fatty stool, malabsorption of nutrients, gallstones, diabetes mellitus, and pancreatic insufficiency (Penketh et al., 1987; Gershman et al., 2006). With the strides in patient care and treatment advances, patients with CF are now living past sexual maturity, leading to a surge of research concerning the reproductive repercussions of cystic fibrosis (Dodge et al., 2007). Due to bilateral absence of the vas deferens, 95% of males with cystic fibrosis are infertile, while otherwise healthy females have a small decrease in reproductive capacity (Gershman et al., 2006).

Normal Physiology of Respiratory System and Function of the Cystic Fibrosis Transmembrane Regulator Protein Channel

The respiratory system, when unaffected by cystic fibrosis, works collectively to create a barrier for pathogens and provide sufficient gas exchange in order to oxygenate tissue. In order to achieve adequate gas exchange, air is breathed in through the nose and mouth, and travels down the trachea, a tube whose structure is maintained through cartilage rings. The trachea then branches into the right and left bronchi, which divide into smaller stems, known as bronchioles and then alveoli. The cystic fibrosis transmembrane conductance regulator (CFTR) protein is present in the membrane of nasal, tracheal, and bronchial epithelial cells and a schematic is shown in Figure 1. However, the primary symptoms of CF lung disease occur in the trachea and upper bronchi. The CFTR protein is bound to the membrane by twelve hydrophobic alpha

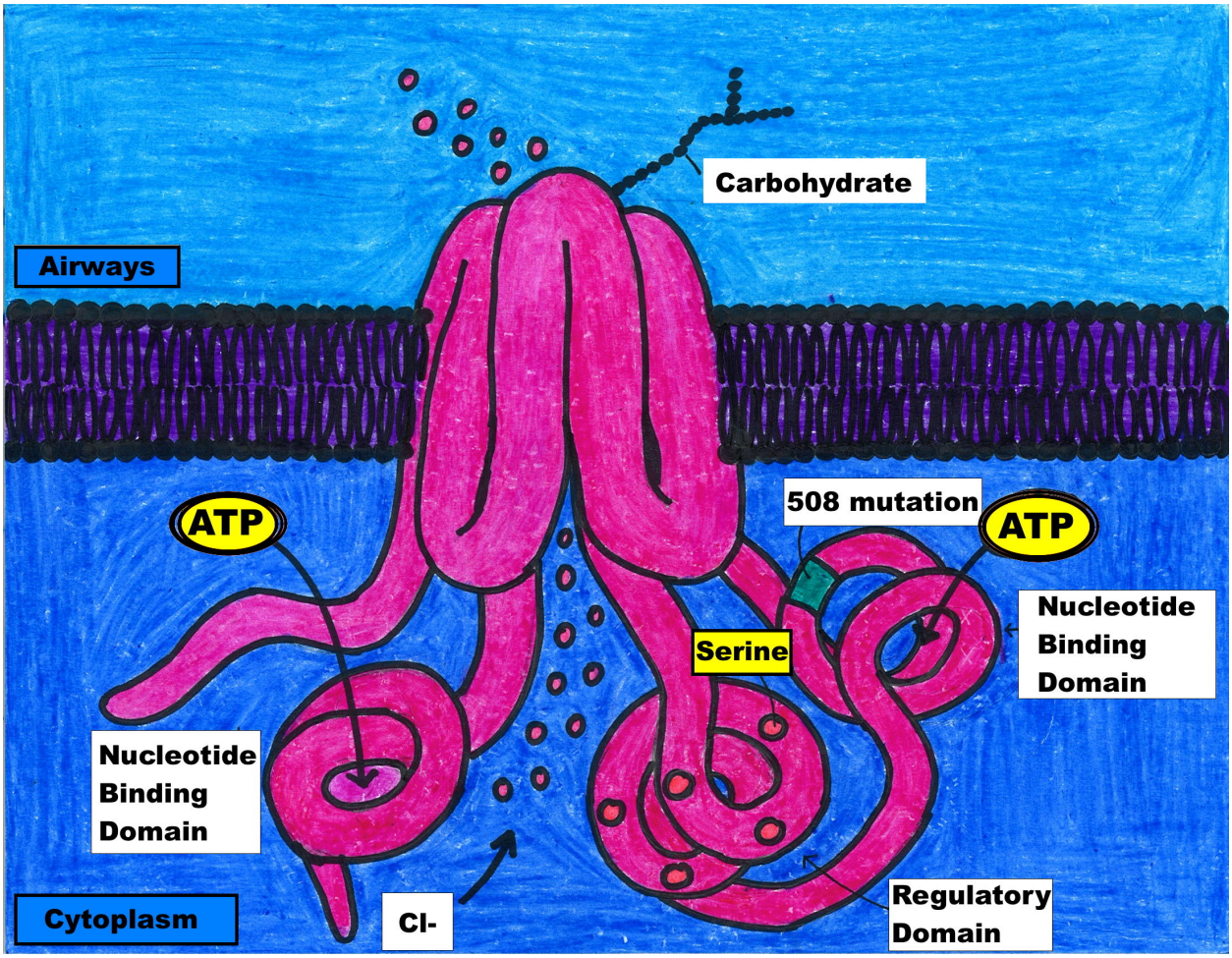

Figure 1: Diagram of Cystic Fibrosis Transmembrane Regulator (CFTR) Chloride Channel. Located on the apical side of airway epithelial cells, the CFTR channel passively transports chloride across the membrane. Shown above are the regulatory and nucleotide binding domains, along with the site of the ΔF508 mutation.

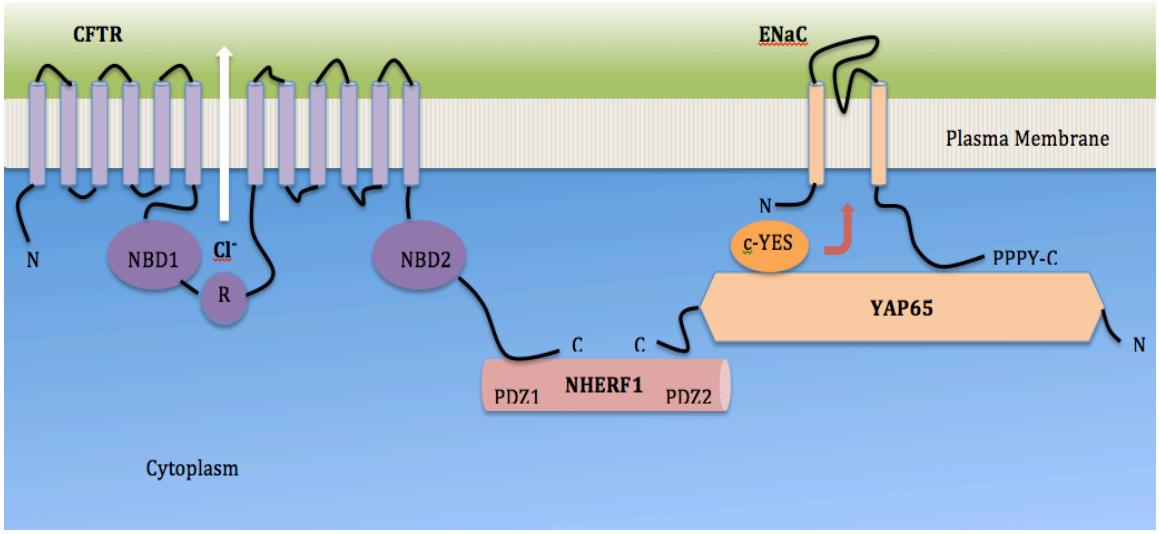

Figure 2: Basic Components of Cystic Fibrosis Transmembrane Regulator (CFTR) Channel and hypothesized interactions with Epithelial Sodium Channel (ENaC). The passive chloride channel, CFTR, consists of 2 membrane spanning domains, MSD1 and MSD2, along with 2 nucleotide-binding domains, NBD1 and NBD2. Amino and carboxyl groups at either end with a regulatory domain, the R domain, in the middle. The PDZ domains of the Na^+/H^+ exchanger regulatory factor isoform-1 (NHERF1) between CFTR and YES-associated protein-65 (YAP65) connecting to ENaC where c-YES, a non-receptor tyrosine kinase, may inhibit activity.

helical domains. Monomer channels form a multi-protein assembly, likely utilizing two PDZ domains consisting of 80-90 amino acids each (Short et al., 1998; Ponting, 1997). PDZ1, a domain on the Na^+/H^+ exchanger regulatory factor isoform-1 (NHERF1) as shown in Figure 2, binds to a small portion of the C-terminus of CFTR through beta sheet augmentation, anchoring the transmembrane channel into place (Ponting, 1997; Guggino and Stanton, 2006). The regulatory domain (R domain) of CFTR contains several serine residues that receive protein kinase A (PKA) and are phosphorylated resulting in channel activation (Riordan et al., 1989; Cheng et al., 1991). As a member of the ATP-binding cassette superfamily, CFTR has two nucleotide-binding domains (NBD1 and NBD2) and two membrane-spanning domains (MSD1 and MSD2) with six α-helices each, as seen in Figure 1 and 2 (Rowe et al., 2005). The CFTR channel has been shown to transport chloride from the airway lumen to the interstitium through the opening of a pore formed by the membrane spanning domains (Bear et al., 1992; Rowe et al., 2005). The NBD1 and NBD2 located on the CFTR channel both bind ATP while the second domain, NBD2, is hypothesized to hydrolyze ATP to generate energy for the activation of the channel (Berger et al., 2005).

Alongside the CFTR channel on the apical membrane of airway epithelial cells and the serous cells of the submucusal gland is an epithelial sodium channel (ENaC). Consisting of α, β, and γ subunits, ENaC is believed to be inhibited by the activation of the CFTR channel, thus slowing the movement of sodium into the interstitium (Canessa et al., 1993; Lingueglia et al., 1993; Canessa et al., 1994; Stutts et al., 1997). The inhibition of ENaC is thought to be controlled by a complex relationship between the

PDZ binding domains of the Na^+/H^+ exchanger regulatory factor isoform-1 (NHERF1), which bind both the C-termini of CFTR and the YES-associated protein-65 (YAP65) to PDZ1 and PDZ2, respectively (Guggino and Stanton, 2006). The non-receptor tyrosine kinase, c-YES, interacts with YAP65. The c-YES kinase is a member of the c-src family, which are known strong inhibitors of ENaC and may inhibit ENaC channel activity through said interaction with YAP65 and the PDZ domains (Guggino and Stanton, 2006).

The apical surface of the airways is protected by two layers; a periciliary liquid layer or as it will be called in this paper, the airway surface liquid (ASL), and a mucus layer of mostly mucin glycoproteins produced by mucosal cells. These two layers work as an innate immune system that is the first line of defense against bacterial pathogens (Morgenroth et al., 1985; Hollingsworth et al., 2004). The airway surface liquid is thought to be approximately 10 μm in depth (Widdicombe, 2002). Cilia of the airway epithelial cells are about 7 μm, an facilitate mucociliary clearance towards the mouth as a component of the innate immune system (Wanner et al., 1996; Widdicombe, 1997). Due to the low viscosity of ASL, the mucus layer, floating on top, is pushed by the cilia at a rate of 3 mm/min for bacterial clearance (Widdicombe, 2002).

Reproductive System Complications Present in Cystic Fibrosis

As noted previously, several organ systems are affected in cystic fibrosis, including the reproductive, digestive systems, and sweat glands. In men with CF, there is over a 95% chance of infertility, mostly due to congenital bilateral absence of the vas deferens

(CBAVD). The absence of the vas deferens results in a lack of sperm in the seminal fluid, a condition known as azoopsermia (Lissens et al., 1996). In mild cases of cystic fibrosis, infertility has led to the detection of a CFTR mutation, with no other symptoms present (Anguiano et al., 1992).

In women with CF, the reproductive system is minimally affected for those who maintain a normal weight, body fat percentage, and bone mass (Edenborough et al., 2001). The CFTR protein is highly expressed in the cervix, less so in the endometrium and fallopian tubes, and not at all in the ovaries (Shawker et al., 1983; Tizzano et al., 1994; Tizzano et al., 1995). The columnar epithelia in the cervix of women with CF are filled with mucin and their normal column-like shape is often distorted (Tizzano et al., 1995). Furthermore, there is no increase in water content, as is typical near ovulation, but instead the mucus maintains a steady concentration of water (Shawker et al., 1983). Endocervical polyps, mucus plugs, and obstruction of the cervical canal have been reported in cystic fibrosis patients (Oppenheimer et al., 1970; Kopito et al., 1973). Aside from mucus plugs in the cervix, there is no evidence of significantly reduced fertility in women with CF that are otherwise healthy.

Effects of Cystic Fibrosis on the Digestive System

The digestive system also is plagued by problems due to mucus build-up in patients with CF. Complications in the pancreas include the blockage of pancreatic ducts by mucus plugs, preventing the pancreatic enzymes amylase, lipase, and protease from entering the duodenum (Reis and Damaceno, 1998). One of the most common

symptoms of digestive disease in CF is pancreatic exocrine insufficiency (Park and Grand, 1981). A decrease in sodium bicarbonate secretion, which lowers pH, negatively affects the efficacy of pancreatic enzymes and precipitation of bile salts, in the duodenum thereby preventing the proper breakdown and absorption of food (Reis and Damaceno, 1998; Koscik et al., 2004; Panagopoulou et al., 2013; Haack et al., 2013). Over the course of a lifetime, damage to the pancreas can decrease insulin secretion and result in CF-related diabetes (Brennan et al., 2004).

Abnormal Fluid Secretions in Cystic Fibrosis

CFTR plays a major role in fluid secretion. Hence, proper function of the sweat glands is also affected by CF. Children with cystic fibrosis have characteristically abnormal salty skin during perspiration (Andersen, 1944). The secretory coil of sweat glands consists of three types of cells; myoepithelial (ME), beta-adrenergic-insenstive (β-I), and beta-adrenergic-sensitive (β-S). However, only the β-S cells in the secretory tissue of the sweat glands are affected in CF. Skin chloride concentrations greater than 60 mEq/l are indicative of CF in children; due to the lack of reabsorption of chloride ions by sweat duct cells prior to secretion onto the skin. In sweating, primary fluid begins as an isotonic solution, and under normal physiologic conditions would be secreted as a hypotonic solution. In CF, the lack of chloride absorption results in a final fluid that remains isotonic (Di Santns as a and Powell, 1962; Lobeck and McSherry, 1967; Shwachman and Mahmoodian, 1967; Emrich et al., 1968).

Decreased mucus clearance allows for a build up within the airways of mucus with

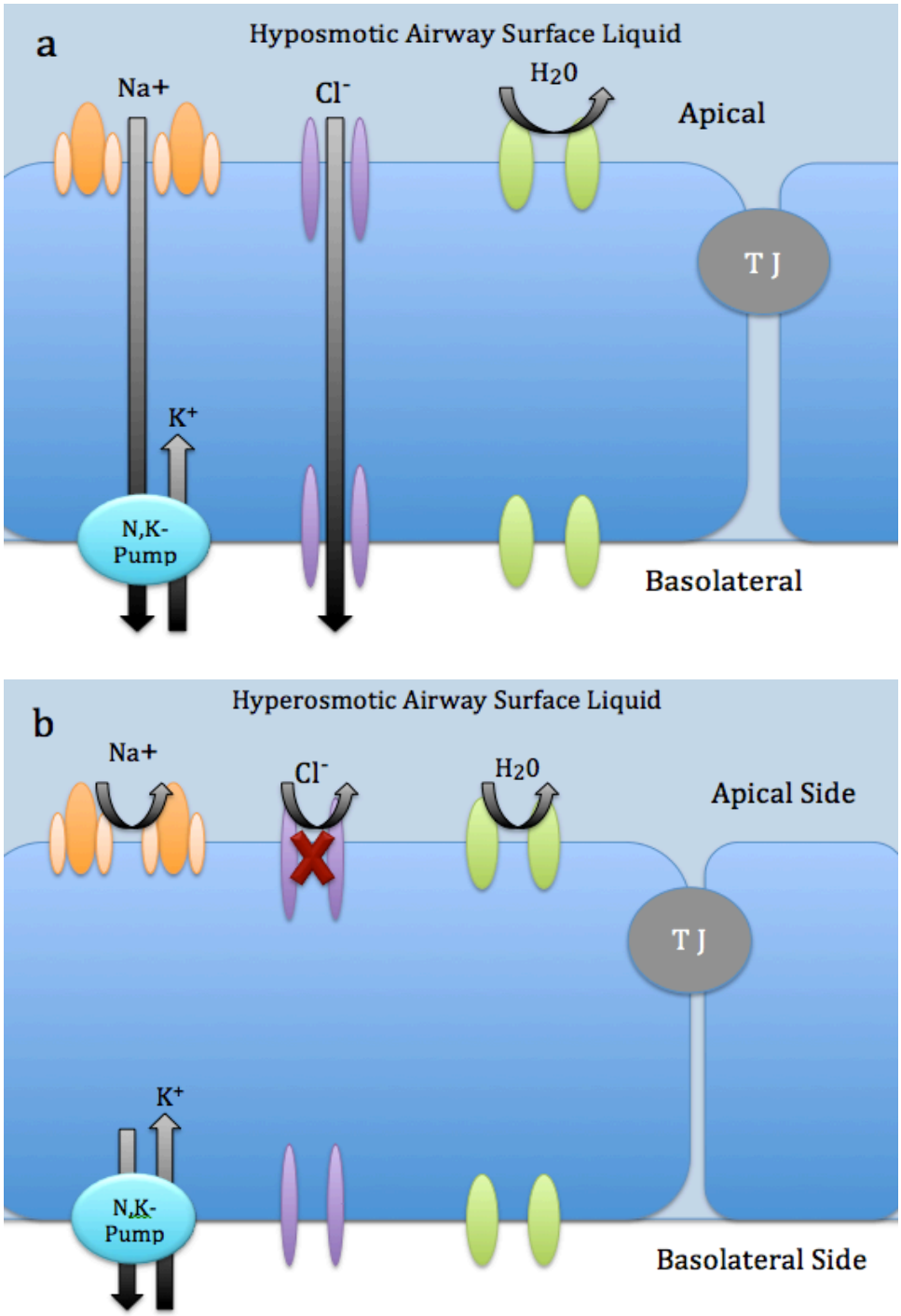

Figure 3: Model of the High Salt Hypothesis. In Figure 3a, a normally functioning lung epithelial cell while 3b is a lung epithelial cell with defective CFTR channels following the pulmonary disease progression model of the high salt hypothesis.

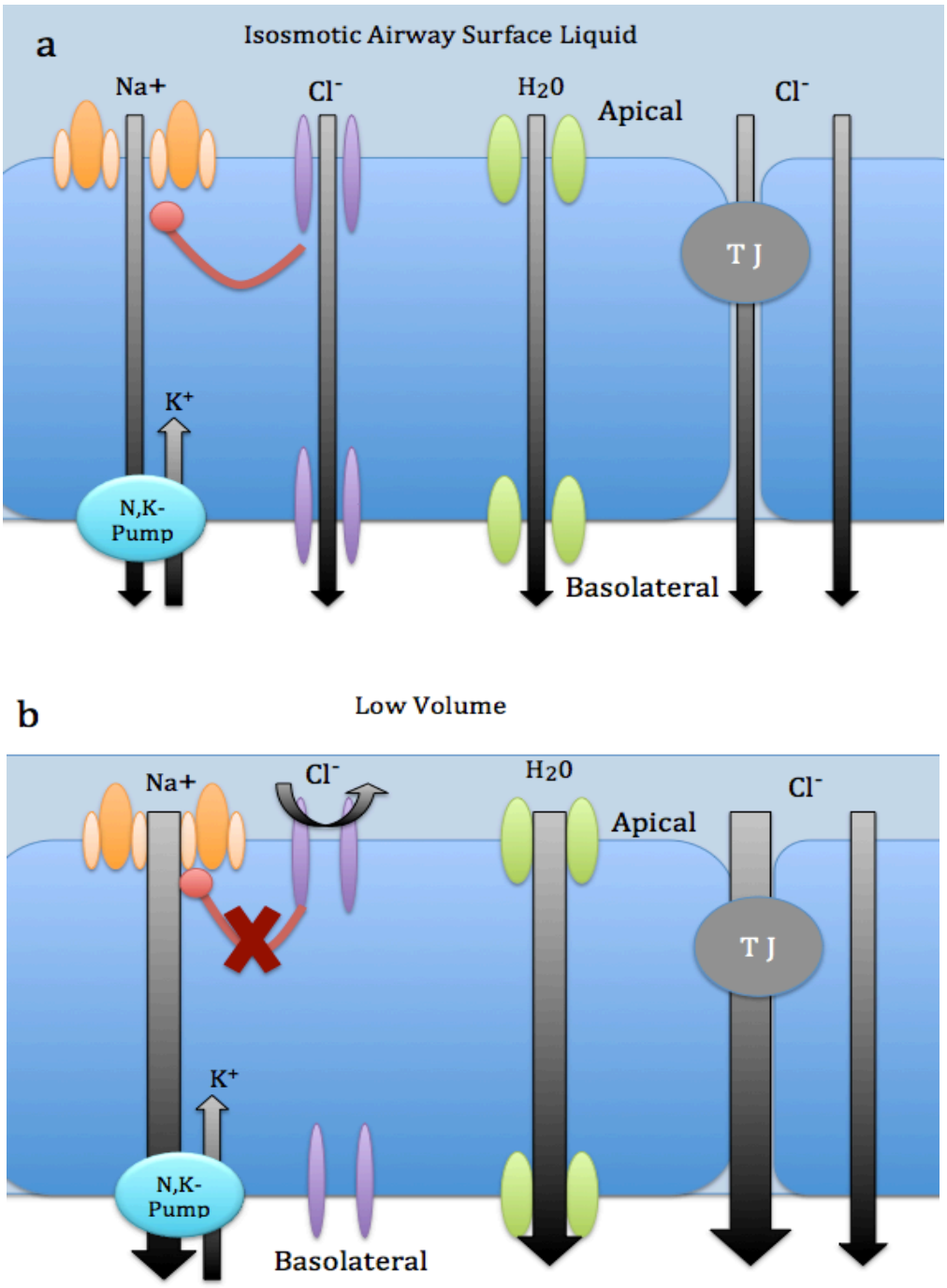

Figure 4: Model of the Low Volume Hypothesis. In Figure 4a, a normally functioning lung epithelial cell while 4b is a lung epithelial cell with defective CFTR channels following the pulmonary disease progression model of the low volume hypothesis.

increased viscoelasticity. The mucus layer is the first line of defense against pathogens but abnormalities caused by a mutation of the CFTR protein results in chronic pulmonary infections and bronchiectasis. The main pathogen seen in these infections are bacteria (Peterson et al., 1981; Wat, 2003). At birth the lungs of CF patients are mostly normal and not infected. From 1 to 2 years old, until 10, children are commonly infected with *Staphylococcus aureus* and *Haemophilus influenza*. After 10 years of age, leading cause of mortality in cystic fibrosis patients (Emerson et al., 2002). Currently, the main pathogen is *Pseudomonas aeruginosa* (Armstrong et al., 1995; Plummer and Wildman, 2013).

The Cystic Fibrosis Controversy: High Salt or Low Volume Airway Surface Liquid

Respiratory complications as a result of chronic lung infections are the there are two hypotheses concerning the origin of the abnormal bacterial colonization seen in CF airways; the "high salt" hypothesis and the "low volume" hypothesis. In 1996, Smith et al. showed that ASL collected from primary lung epithelial cultures of non-CF patients exhibited bactericidal effects on *P. aeruginosa* and other strains of bacteria (Smith et al., 1996). However, when bacteria were introduced to ASL collected from cultures generated from CF patients, bacteria thrived. Smith et al. suggested that normal ASL may contain a small defensin-like molecule of less than 10 kDa with broad-spectrum bactericidal activity, whose activity was absent in the ASL of CF patients. Ion concentrations were measured in the ASL of both non-CF and CF cell cultures, showing that CF ASL had an increased concentration of chloride (Cl^-) ions, and that this most likely was inhibiting the ability of the salt-sensitive bactericidal molecule (Smith et al.,

1996). This study by the Welsh and Smith group from the University of Iowa, in conjunction with similar previous studies laid the foundation for the high salt hypothesis: Namely, elevated Cl^- concentrations, as a result of defective Cl^- transport by CFTR, inhibits the broad-spectrum bactericidal activity of a small defensin-like molecule, allowing for bacterial colonization within the mucus of the airways (Gilljam et al., 1989; Joris et al., 1993). The high salt hypothesis is modeled in Figure 3.

However, in 1997, Knowles et al. argued, that it is unlikely that airway Cl^- concentrations are higher than plasma levels, as this is not compatible with the normal physiologic processes involved (Knowles et al., 1997). In their study, ion composition of the airway surface liquid was compared between normal participants and non-infected CF patients in nasal passages and lower airways. No significant differences in Na^+ or Cl^- concentrations were found between CF and normal participants. The concentrations of Na^+ observed in the nasal passages were lower than that of plasma (~109mM), whereas Cl^- concentrations were comparable to plasma levels (~125 mM). Looking at ion composition in the lower airways, Na^+ and Cl^- were found to be lower than plasma levels and hypo-osmotic in comparison to the results of Smith et al.. As a result, the low volume hypothesis, supported by Boucherrcomparison to the results of Smith r airwaysat Chapel Hill, gained credence. The low volume hypothesis states that Cl^- concentrations are similar to plasma levels in CF and non-CF airway surface liquid, but that increased Na^+ absorption leads to an intracellular flux of Cl^- (via a non-CFTR

pathway) and a net movement of water to maintain isotonicity, thereby collapsing the cilia and halting mucus clearance modeled in Figure 4.

For years, these two groups have supported their respective hypotheses with experimental data. With no clear resolutions concerning the mechanism underlying CF, a critical examination of the data are needed to determine which, if either or both, the high salt or low volume hypothesis is correct.

METHODS

The techniques used by both sides of the controversial airway surface liquid composition debate have been categorized into relative sections and reported side by side to distinguish similarities and differences in protocol. In addition the methods by which data was compared and evaluated in this study are explained.

Cell Culture Techniques

The most important aspects in studies of cell culture are origin of the cells cultured, the transepithelial resistance, use of antibiotics in media, and technique of measurement. These cell culture study characteristics are displayed in Table 1. As shown, most studies cultured human bronchial epithelial cells while Zabner et al. cultured human nasal, tracheal, and bronchial epithelial cells in the same apparatus (Zabner et al., 1998). However, this difference in cell origin is not likely to be causative of any differences across findings as all express the CFTR protein and ENaC.

How technique differences might influence findings: The transepithelial resistances (TER) described in Table 1 differ quite widely across studies and within studies. TER is measured in $\Omega x cm^2$ and is the electrical resistance of the tight junctions between cells of the monolayer that was measured. For example, Song et al. reports a TER range of 400 -1000 $\Omega x cm^2$ in 2009, which results in a large difference in the passive flow of fluid and ions through tight junctions between cells (Matsui et al., 1998; Stewart et al., 2012). Significant differences in data could potentially arise from TER measurements that are

Table 1: Cell Culture Study Characteristics. The transepithelial resistance (TER), cell type and origin, methods of measurement, presence (+) or absence (-) or antibiotics in the media, and whether the research group supports the high salt or low volume hypothesis are shown. Independent research groups are denoted as such.

Study	Origin and Cell Type	Antibiotics	TER	Measurement Method	Group
Zabner et al, 1998	Human Nasal, Tracheal and Bronchial Epithelia	+	$\geq 800\ \Omega xcm^2$	Radio-labeled water and sodium or chloride in Ussing Chambers	Welsh and Smith
Matsui et al, 1998	Human Bronchial Epithelial	-	$300 – 600\ \Omega xcm^2$	Microcapillary Collection	Boucher
Jayaraman et al, 2001	Bovine Tracheal Epithelial	-	$> 300\ \Omega xcm^2$	Fluorescent Indicator	Independent
Song et al, 2009	Human Nasal Epithelial	+	$400 – 1{,}000\ \Omega xcm^2$	Rhodamine B-Dextran	Independent
Thiagarajah et al, 2010	Human Bronchial Epithelial	+	$400 – 1{,}000\ \Omega xcm^2$	Rhodamine B-Dextran	Independent
Harvey et al, 2010	Human Bronchial Epithelial	+	Not Reported	Rhodamine B-Dextran	Boucher

abnormally high because the flow of ions may be artificially low in monolayers with abnormally high TER measurements. Several variations of measurements in cell culture studies are seen in Table 1, however Zabner et al. and Matsui et al. are the only two whose variation may result in incompatible findings. Both studies report on Na^+ and Cl^- ASL concentrations in cell culture yet in 1998, Zabner et al. used radioactive isotopes of sodium or chloride in Ussing chambers, a well-established method of measuring the short circuit current of monolayer to determine the net ion transport across epithelia. In contrast, Matsui et al. collected representative samples in microcapillary tubes for measurement. The use of a microcapillary tube could result in the puncture of the cell walls and lead to skewed data that are higher than typically reported. Yet Matsui found lower ion concentrations than peers so this limits concern. Song et al., Thiagarajah et al., and Harvey et al. used similar measurement methods to each other, cell types, and the known TERs in their ASL depth experiments (Song et al., 2009; Thiagarajah et al., 2010; Harvey et al., 2010). For further details on methods used, see Appendix 1. Thus, no significant differences in findings are likely to be attributed to methodological variations.

Cystic Fibrosis Murine Model

There is a variety of murine models for cystic fibrosis. Zeiher et al.., of the Welsh and Smith group, developed of the first CF murine model in 1995. These mice had the be attributed to methodological va targeting and a cross between the C57/BL6 and 129 murine strains (Zeiher et al., 1995; McCray et al., 1999).

In a 1999 study, McCray et al. generated these ΔF508 mice in the same manner as Zeiher. For their study, mice were kept in normal conditions and the control models were ΔF508 mutation carriers (McCray et al., 1999). ASL depth and salt concentrations were measured using radioisotopes of water, sodium, and chloride. However, several studies use wild type and other CF murine models instead of the ΔF508 mice.

How animal differences might influence findings: The mouse strains used in the studies examined are from Jayaraman et al., Tarran et al., Song et al., and Morgan et al.. Wild type C57/BL6 and CD1 strains were used for normal murine models in comparison to CF mice. However, Jayaraman et al., Song et al., and Morgan et al. only investigate ASL characteristics of normal mice in 2001, 2003, and 2013 respectively. A description of the murine model used in Jayaraman et al., 2001 is not present in the methods except for a range of body weights (Jayaraman et al., 2001). In contrast, Tarran et al. makes use of $Cftr^{tm1Unc}$ CF mice of a mixed strain to form a truncated Cftr mRNA protein (Tarran et al., 2001; Jin et al., 2006). Thus the mice studied in these four publications are not identical and in particular the Tarran et al. group is an outlier because a CFTR knockout mouse was used in in the 2001 paper in comparison to the ΔF508 mice that retained low-level expression of CFTR mRNA.

On the other hand, any empirical differences in findings across studies could be attributed to either/or model differences and measurement method variations, however, while the values obtained between studies may differ, there is typically no difference found in the pulmonary system between CF and normal mice within each study

(Colledge et al., 1995; O'Neal et al., 1993; Zeiher et al., 1995). Given these authors are supporting the position of "no difference", it greatly limits concern.

Ex Vivo Samples From Bovine Tissue, Porcine Models, and Human Subjects

In lieu of cell culture and mice studies that have been demonstrated to be less effective as means of studying cystic fibrosis lung disease (CF mice don't have airway disease), researchers moved to examine intact tissue from larger animals and humans. The study of intact tissue from an animal model expressing airway disease, or from humans, is more likely to yield relevant data enhanced by the presence of submucosal glands and goblet cells, as well as ideal ciliary length and density (Song et al., 2009; Wanner et al., 1996; Widdicombe et al., 1997; Matsui et al., 1998).

In 1997, Widdicombe collected bovine tracheas within 5 minutes of death from normal animal specimens. From these collected bovine tracheas, tissue samples were dissected away with submucosal glands intact (Widdicombe, 1997). The depth of the airway surface liquid was then measured from cryoprobe frozen tissue samples using microscopy (Widdicombe, 1997).

Before the development of the CF pig model, tissue samples were collected from normal pigs at slaughterhouses with 12 hours of death. Song et al. utilized this method in their 2009 study of changes in ASL depth in porcine tracheal samples. Instead of a CF model to compare normal values to, a CFTR inhibitor, MalH-2, also known as di-

sulfonate glycine hydrazide was used for porcine tissue samples to simulate a lack of CFTR channels (Song et al., 2009).

In 2008, Rogers et al. generated the CFTR-null porcine model, which lacked CFTR protein expression completely. $CFTR^{-/-}$ pigs exhibit all the manifestations of cystic fibrosis such as pancreatic insufficiency, inflammation, airway obstruction, infections, and increased mucus (Meyerholz et al., 2010; Stoltz et al., 2010). Since the generation of both CFTR-null and $CFTR^{\Delta F508/\ \Delta F508}$ porcine models in 2010, studies have been conducted using *ex vivo* measurements of ASL (Ostedgaard et al., 2010). Chen et al. euthanized newborn $CFTR^{-/-}$ and normal pigs within 8-15 hours after birth to obtain approximated 1200 ASL depth measurements/pig (Chen et al., 2010).

Several researchers have also gathered *ex vivo* measurements of human tissue. For example, in 2001 Jayaraman et al. excised intact pieces of only normal human bronchial tissue from airways. The ASL depth of these samples was measured using fluorescent indicators (Jayaraman et al., 2001). An attempt at mimicking the lack of CFTR expression in *ex vivo* measurements of human tracheal samples was made by Song et al. (2009). In order to achieve a lack of CFTR channel activity, a CFTR inhibitor, also known as $CFTR_{inh\text{-}172}$ was used to collect data on transient changes in ASL depth when stimulated by various secretagogues (Song et al., 2009).

How tissue differences might influence findings: The use of *ex vivo* tissue samples from

various organisms including human subjects can result in a wide range of measurements due to anatomical differences in airway size and time of harvest. Due to the lack of a CF model in the organisms used for tissue studies until recently, inhibitors were also used. CFTR inhibitors likely do not translate in to CF model-like conditions, unless it is a CFTR-null model that lacks expression of the protein. The ΔF508 porcine model however, is not a null model. These anatomical and CFTR expression variations across studies must be taken into consideration.

Human In Vivo ASL Measurements

To measure the sodium and chloride concentrations in the ASL of normal and cystic fibrosis patients, four filter paper studies report on these values. Gilljam et al. in 1989, Joris et al. in 1993, Knowles et al. in 1997, and Hull et al. in 1998 utilized various forms of filter paper ASL sampling to measure sodium and chloride concentrations in the airway. In 1989, Gilljam et al. sampled from bacterially-infected cystic fibrosis subjects and as a normal/control group, subjects with severe chronic bronchitis through a bronchoscopy (Gilljam et al., 1989). The patient population of Joris et al., 1993 consisted of normal subjects, subjects who had been intubated for 6 hours – 3 days, subjects with acute infection in the airway but did not have CF, asthma patients, and finally, CF patients (Joris et al., 1993). Similarly to the 1989 Gilljam et al. study, a bronchoscope was used to collect the ASL sample on filter paper from the distal trachea or proximal bronchus. The most recent studies of *in vivo* ASL salt concentrations are by Knowles et al. in and Hull et al. in 1997 and 1998, respectively. Supposedly, non-

infected CF patients were measured through bronchoscopy using filter papers, as well as in normal subjects for comparison (Knowles et al., 1997; Hull et al., 1998).

How technique differences might influence findings: Variations in the patient cohorts sampled in the *in vivo* ASL measurement studies can lead to artificially high or skewed data. Several studies, Gilljam et al. and Joris et al. in 1989 and 1993 respectively, use less than healthy subjects for the control groups. Chronic bronchitis and patients undergoing other medical procedures may not reflect ASL conditions in a healthy non-CF individual (Gilljam et al., 1989; Joris et al., 1993). Additionally, the Joris et al. 1993 paper had a sample size of 3 CF patients compared to 17 control non-CF subjects. Those 3 CF patients also varied widely in age, were undergoing medical treatment, and 2 had likely had inflammation from previous or current lung infections (Joris et al., 1993).

As mainly two separate groups, the Boucher group and the Welsh group, support the two conflicting hypotheses results are categorically organized by “Low Volume” for the Boucher group or “High Salt” for the Welsh group with an “Independent” category for groups not affiliated with either hypothesis.

RESULTS

Sodium Concentration in ASL of Normal and Cystic Fibrosis Models

Various models of cystic fibrosis have been used to examine the Na^+ concentration in the airway surface liquid lining the lungs given that CFTR may inhibit ENaC channels of lung epithelial cells. Over the years, six papers have reported comparisons of Na^+ in ASL between normal and cystic fibrosis cells; Joris, Hull, and Jayaraman from independent groups, Knowles and Matsui from the Boucher group, and Zabner from the Smith and Welsh group. The 1998 Hull paper as well as the two Boucher group papers fall into the "no difference" category supportive of the low volume hypothesis in Figure 5; given they found similar concentrations of Na^+ in normal conditions compared to cystic fibrosis. While the 1993 Joris paper and Zabner paper fell into the "high salt" category as they found an increase in ASL Na^+ concentrations in cystic fibrosis. Jayaraman found a slight decrease in sodium ion concentration in cystic fibrosis, however given the margin of error was not provided it is likely that the difference seen is insignificant and thus this will be considered more supportive of the "no difference" result.

For each of the six studies examined in Figure 5 for Na^+ concentration in ASL, the difference between cystic fibrosis and normal models was calculated and replotted in Figure 6 as a percentage. The largest difference in normal and CF models was found in Zabner et al., 1998 with a 100% difference, or twice as much sodium, in the CF model.

Joris et al., 1993 exhibited an approximately 50% increase in sodium in the CF model compared to normal. The other four papers from Hull in 1998, Jayaraman in 2001, Knowles in 1997, and Matsui in 1998 had less than a 20% difference with the difference lying in an increased amount of Na^+ in the normal models.

Chloride Concentration in ASL of Normal and Cystic Fibrosis Models

As CFTR is a chloride channel, chloride concentration also has been heavily investigated in the ASL of normal and cystic fibrosis models over the years. Nine studies have measured the tracheal and bronchial Cl^- concentration of normal and cystic fibrosis models using various methods and yielding varying results, as seen in Figure 7. Ion concentrations are in mM and organized according to research group. There is some variation in trends for the independent category with Joris et al., 1993 indicating an increase in ASL Cl^- concentration in cystic fibrosis and possibly in Gilljam et al., 1989 as well. However, their values have a larger standard error measure and no conclusions can be drawn from these data as the error bars largely overlap. Investigators associated with Boucher group show results that support their proposed low volume hypothesis. They found ion concentration to be unchanged in normal and cystic fibrosis models and thus that CF lung disease is caused by decreased ASL volume. Investigators associated with the Welsh group found an increase in Cl^- ion concentration in cystic fibrosis compared to normal in the 1998 (Zabner et al. 1998). Yet in a 1999 study the Welsh group (McCray et al. 1999) found no significant difference between the two conditions.

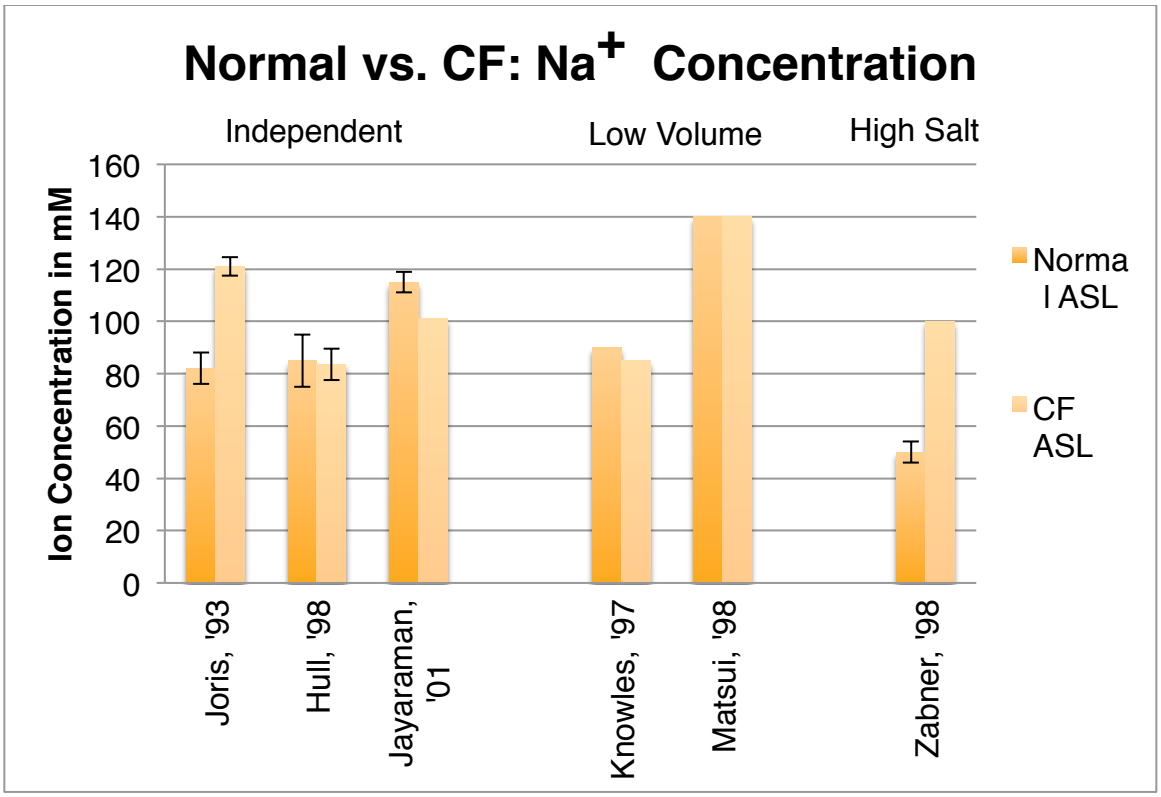

Figure 5: Normal vs. CF: Na^+ Concentration. Comparison of the concentration of Na^+ ions in normal ASL and CF ASL from a multitude of model systems, grouped according to research group affiliations. ("Independent" for all non-affiliated research groups, "Low Volume" for the Boucher Research Group at the University of North Carolina at Chapel Hill, and "High Salt" for Welsh and Smith of the University of Iowa)

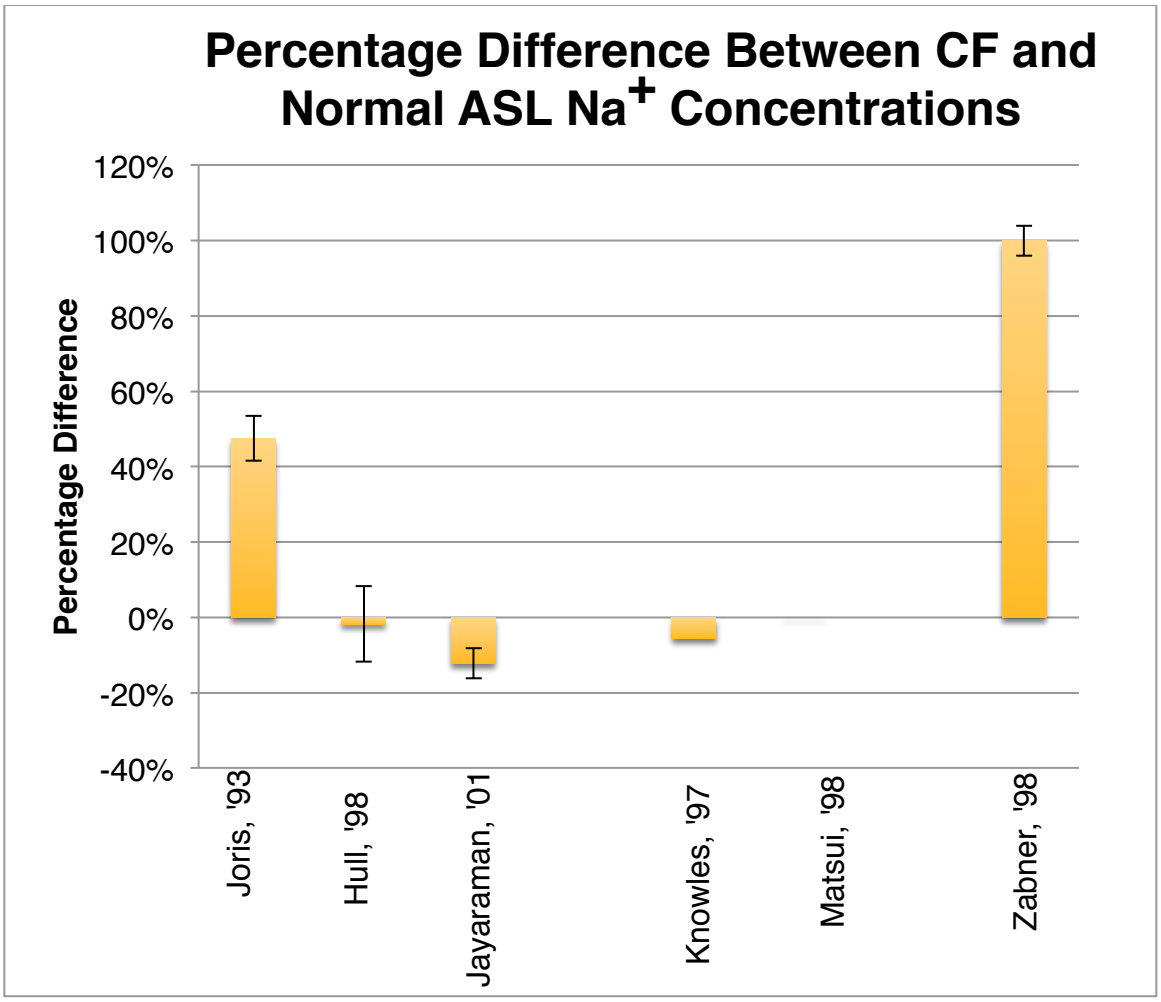

Figure 6: Percentage Difference Between CF and Normal ASL Na^+ Concentrations. Difference between each studyyn CF and Normal$^+$ concentration and CF ASL Na^+ concentration as a percentage. Figure 1 data was represented in this manner to focus on normal Na+ concentrations compared to CF Na^+ concentrations in ASL rather than actual values, which may differ across models. Hull, Jayaraman, Knowles, and Matsui found no significant difference between normal and CF.

To focus on the relationship between the concentration of chloride in the ASL of cystic fibrosis and normal models instead of actual values due to different models and measurement techniques, the difference between normal and CF was calculated and shown in Figure 8 as a percentage. A significant difference is seen in Joris et al., 1993 with an over 50% difference between cystic fibrosis and normal Cl^- concentration measurements in ASL, while Zabner et al., 1998 also showed a large difference of over 125%, meaning that the Cl^- concentration was over twice as much as normal levels. Again, Gilljam et al., 1989 may be significantly different with Cl^- concentration approximately twice as high as normal in CF models but error estimations are so large that a conclusion cannot be made regarding its significance. Several studies show little to no difference in cystic fibrosis ASL Cl^- concentrations; Hull et al., 1998; Jayaraman et al., 2001; Knowles et al., 1997; Matsui et al., 1998; Tarran et al., 2001; and McCray et al., 1999.

In Vivo Sodium and Chloride Concentrations in ASL of Normal and Cystic Fibrosis Human Subjects

As mentioned previously, several model systems were used for the data in Figures 5-9, which can account for the large variations in concentration values. For example, the murine model has been shown to lack the pulmonary symptoms of cystic fibrosis unless repeatedly exposed to a large number of colony forming units of bacteria (Colledge et al., 1995; O'Neal et al., 1993; Zeiher et al., 1995; Davidson et al., 1995). In cell culture studies, variations in measurements depend on the donors for trans-epithelial resistance and

capability to differentiate into goblet cells or be ciliated (Stewart et al., 2012). Therefore, to examine physiological ion concentrations relevant to the treatment of cystic fibrosis in humans, results for human *in vivo* measurements of Na^+ and Cl^- of normal and cystic fibrosis patients were compiled in Figures 9− 12.

The Na^+ concentrations in the ASL of normal human subjects when measured *in vivo* were within the range of 82 to 103 mM, with the highest margin of error being an subjects when measured 9. The mean concentration of normal human Na^+ ASL concentrations was 90 mM.

In comparison, Figure 10 shows human *in vivo* ASL Na^+ concentrations of subjects with cystic fibrosis. Measurements *in vivo* are within a range of 82 ts with , with the highest margin of error being fibrosis. Measuremen$^+$ concentration of 96 mM. Thus, the ranges of values and the mean for human ASL Na^+ concentration measured *in vivo* were slightly higher in cystic fibrosis patients. Standard error measures are shown but are not utilized in statistical tests to analyze significance, as data are across several models.

When the ASL Cl^- concentration was measured in normal human subjects, the range of concentrations was between 78 and 108 mM, with ± 18 mM being the largest margin of error from the five studies shown in Figure 11. The mean Cl^- concentration value for ASL of normal subjects was 89.4 mM. In cystic fibrosis patients, *in vivo* ASL Cl^-

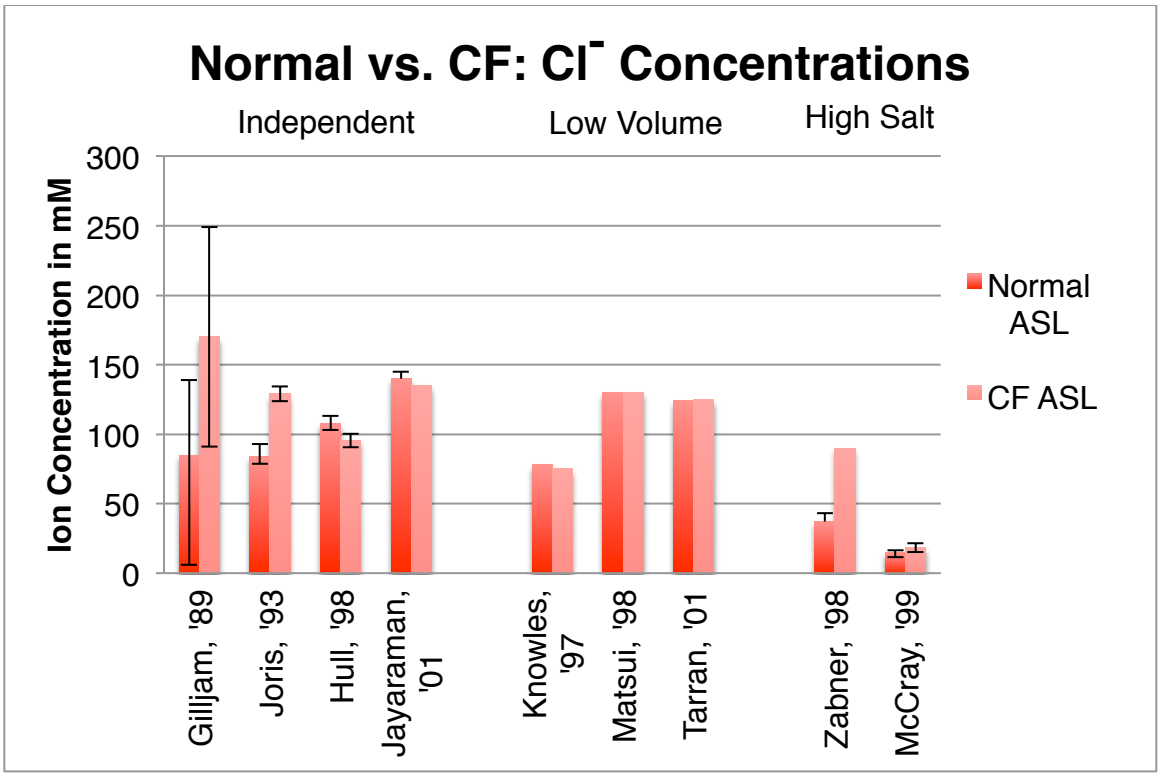

Figure 7: Normal vs. CF: Cl^- concentrations. Comparison of the concentration of Cl^- ions in normal ASL and CF ASL, from a multitude of model systems, grouped according to research group affiliations. ("Low Volume" refers to research affiliated with the Boucher group at UNC, "High Salt" for work from the Welsh group at Iowa, while the term "Independent" refers to research groups not affiliated with UNC or Iowa)

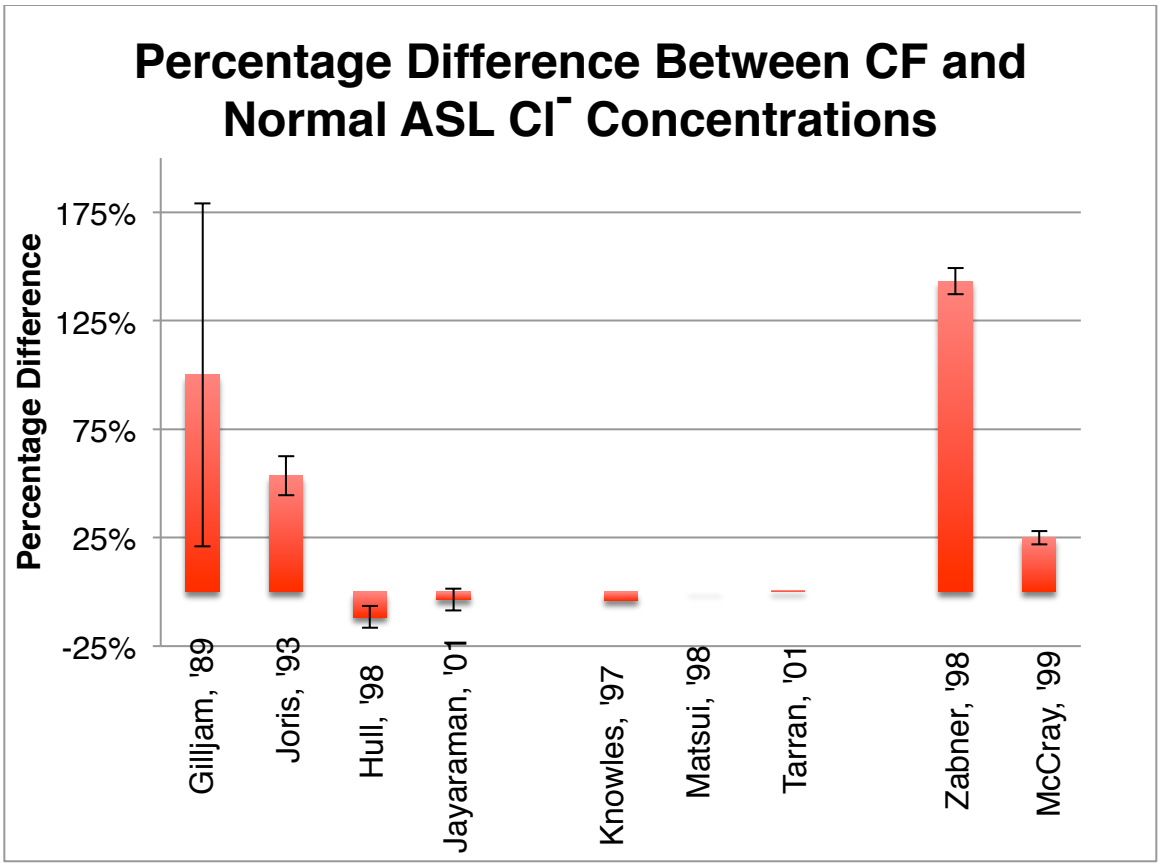

Figure 8: Percentage Difference Between CF and Normal ASL Cl^- Concentrations. Difference between each studyn Cnormal ASL Cl^- concentration and CF ASL Cl^- concentration as a percentage to eliminate discrepancies between models and focus on normal Cl^- concentrations compared to CF Cl^- concentrations in ASL. Hull, Jayaraman, Knowles, Matsui, and Tarran all demonstrate an insignificant difference between normal and CF Cl^- concentration in ASL.

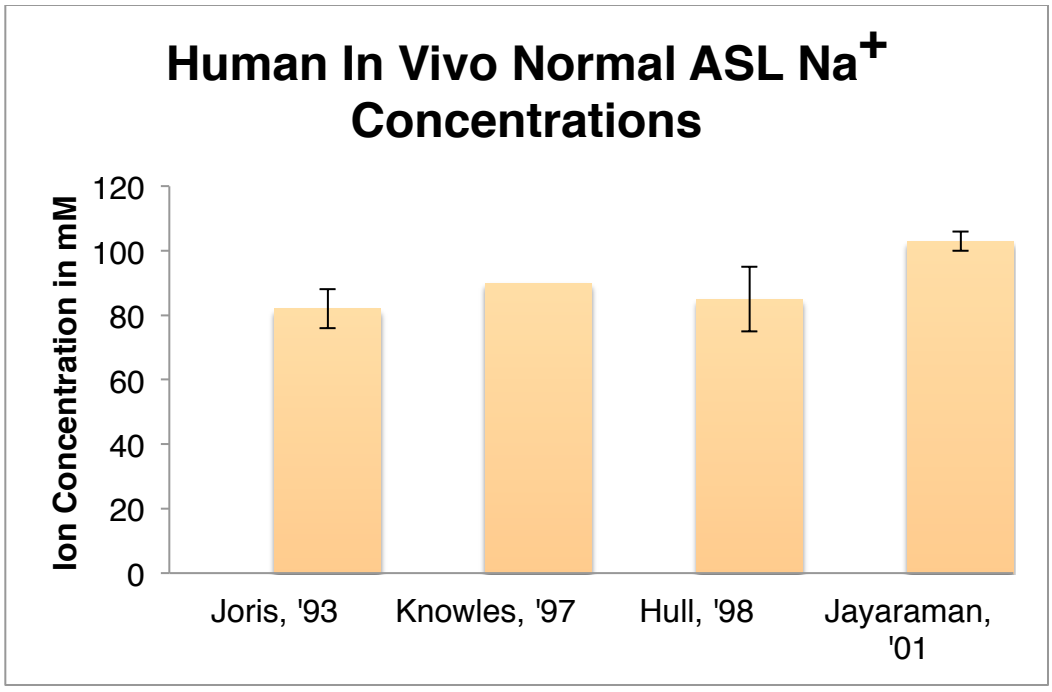

Figure 9: Human *In Vivo* Normal ASL Na^+ Concentrations. Four *in vivo* measurements of the concentration of Na^+ ions in human ASL demonstrating a general agreement on a range of 82 ce between nor$^+$ in normal subjects from Joris, Knowles, Hull, and Jayaraman. After conducting a Grubbs' test for outliers, a p-value of 0.291 for the furthest lying data point, 103 mM by Jayaraman et al was calculated.

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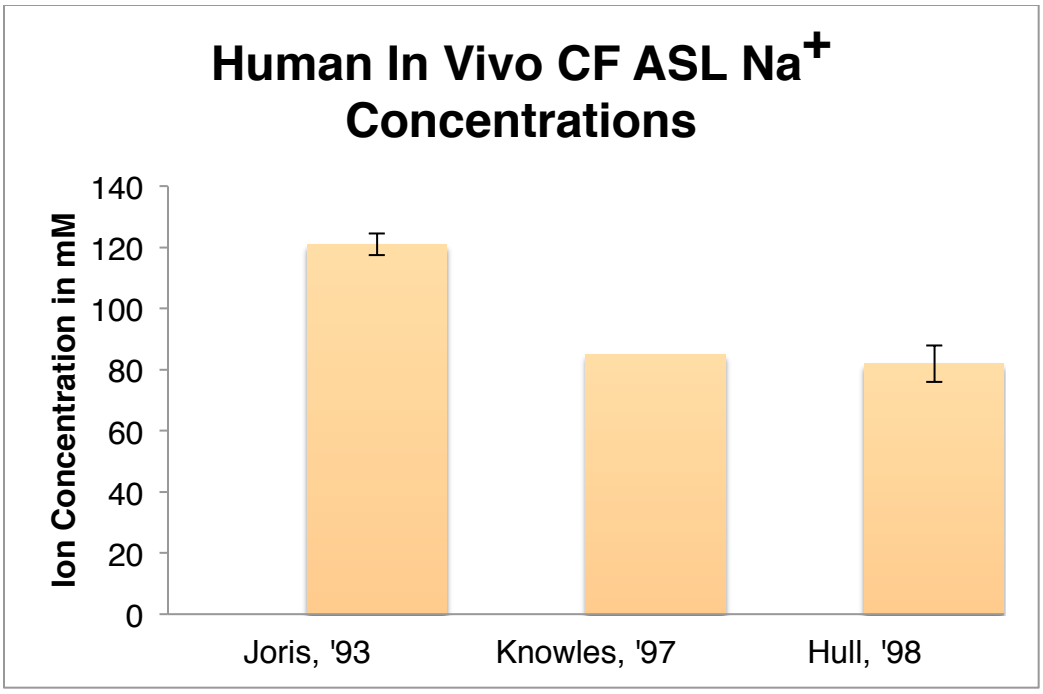

Figure 10: Human *In Vivo* CF ASL Na^+ Concentrations. Three *in vivo* measurements of the concentration of Na^+ ions in human ASL of CF subjects showing agreement between the values of Knowles' 1997 study and Hull's 1998 study. An independent samples *t*-test determined a p-value of 0.316 between human *in vivo* ASL Na^+ concentrations in normal and CF subjects.

measurements, shown in Figure 12, were within the range of 75 − 170 mM with the largest margin of error at ± 22.6 mM from the Gilljam, 1989 paper. The mean Cl^- concentration value calculated from the four studies shown was 116.25 mM. Thus, the Cl^- concentration in ASL of cystic fibrosis patients had a greater range and mean value than the normal subjects.

In Figure 13, the mean Na^+ and Cl^- ASL concentrations in CF from several models and human subjects are shown according to ion and group. Plasma concentrations of sodium and chloride are included to the far right in Figure 13 (Wolford et al., 1986). The mean values for CF ASL Na^+ concentration were 94 mM for researchers in the Independent category, 100 mM for those in the High Salt category, and 115 mM for the Low Volume category. CF ASL Cl^- mean values were 132.4 mM, 100 mM, and 110 mM for the independent, high salt, and low volume categories respectively. The provided plasma concentrations are 140 mM for Na^+ and 100 mM for Cl^- (Wolford et al., 1986).

Depth of ASL in Normal and Cystic Fibrosis Models

The low volume hypothesis, proposed by Boucher of the University of North Carolina at Chapel Hill, insists that a decrease in ASL height or fluid depth prevents proper muco-ciliary movement of bacteria out of the airways and causes cilia to collapse which allows for bacterial colonization of these standstill islands. Thus, ASL depth has been measured extensively in various models such as human lung epithelial cell culture, bovine tissue, and porcine tissue. In Figure 14, measurements of normal

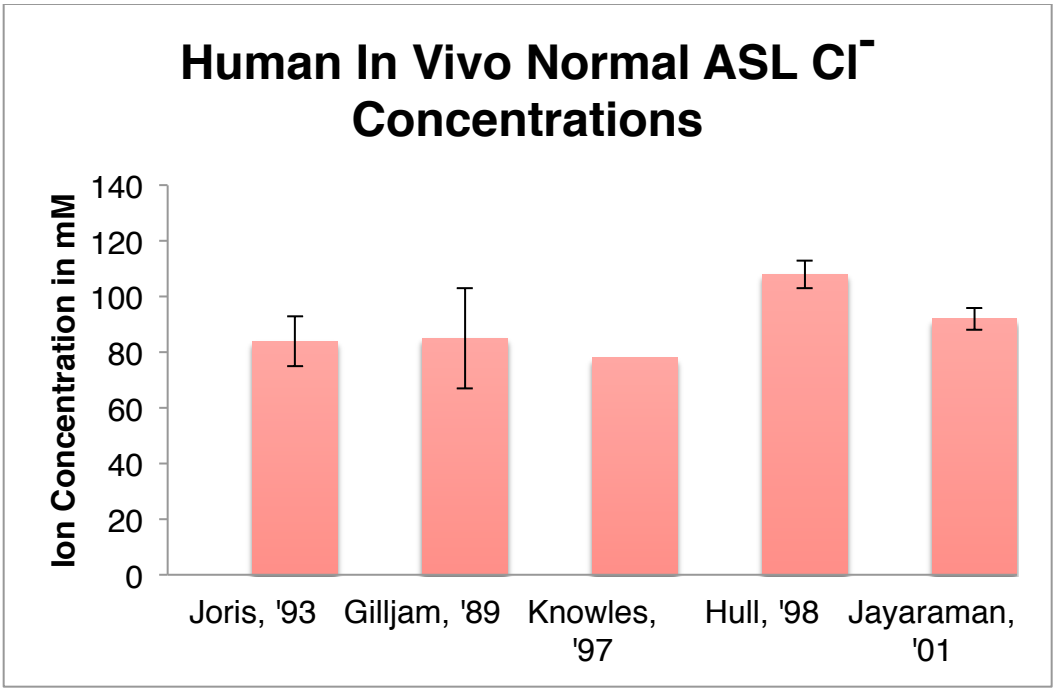

Figure 11: Human *In Vivo* Normal ASL Cl^- Concentrations. Five *in vivo* measurements of the concentration of Cl^- ions in human ASL showing a general agreement on a range of 85 – 100 in normal subjects from Joris, Gilljam, Knowles, and Jayaraman. The furthest data point from the mean, 108 mM, had an insignificant p-value of 0.181.

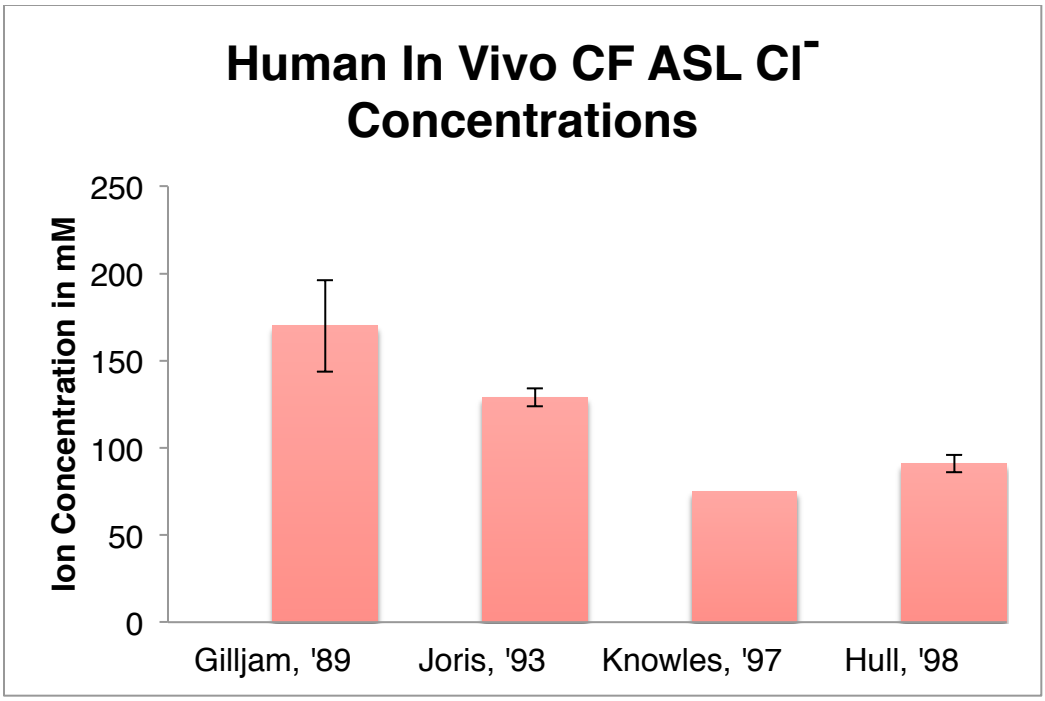

Figure 12: Human *In Vivo* CF ASL Cl^- Concentrations. Four *in vivo* measurements of Cl^- ions in human ASL of CF subjects demonstrating an agreed range between Gilljam, Joris, Knowles, and Hull with a p-value of 0.30. An independent samples *t*-test was done between human *in vivo* ASL Cl^- concentrations in normal and CF subjects with a p-value of 0.105.

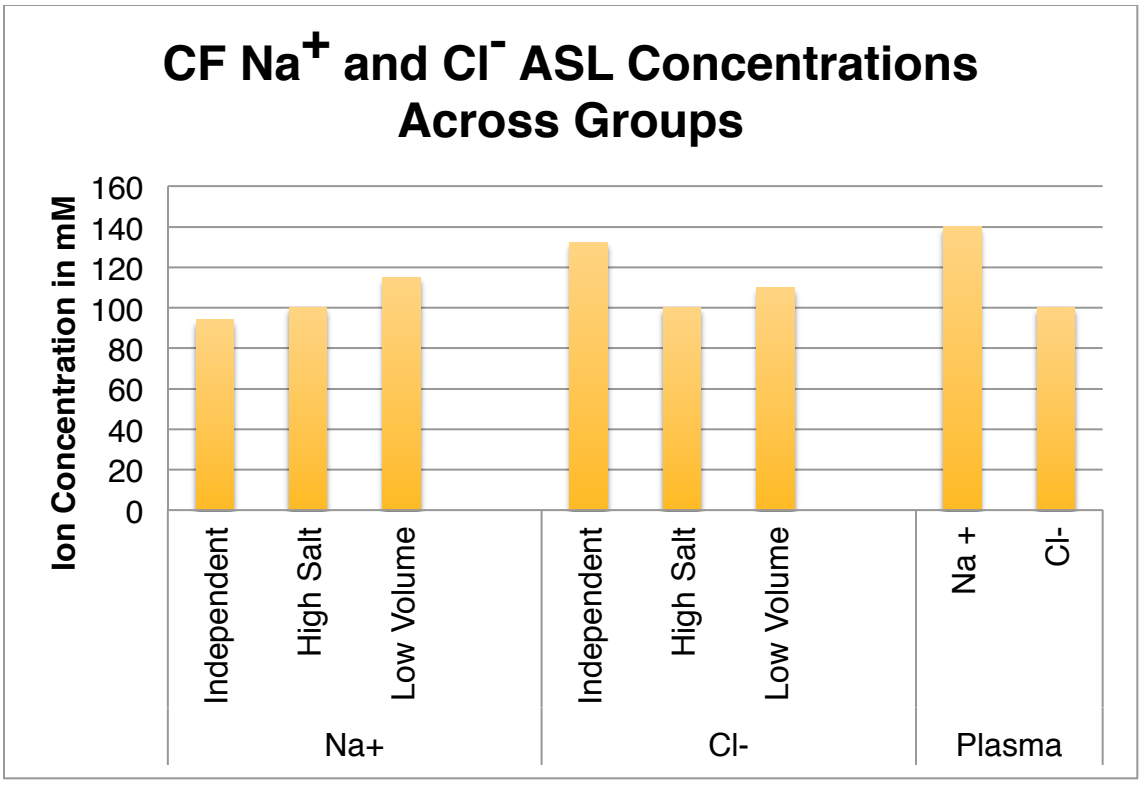

Figure 13: CF Na^+ and Cl^- Concentrations Across Research Groups. Comparison of the averaged ASL concentrations of Na^+ and Cl^- ion in mM according to group with plasma concentrations of each ion on the right for reference. ("Low Volume" refers to research affiliated with the Boucher group at UNC, "High Salt" for work from the Welsh group at Iowa, while the term "Independent" refers to research groups not affiliated with UNC or Iowa)

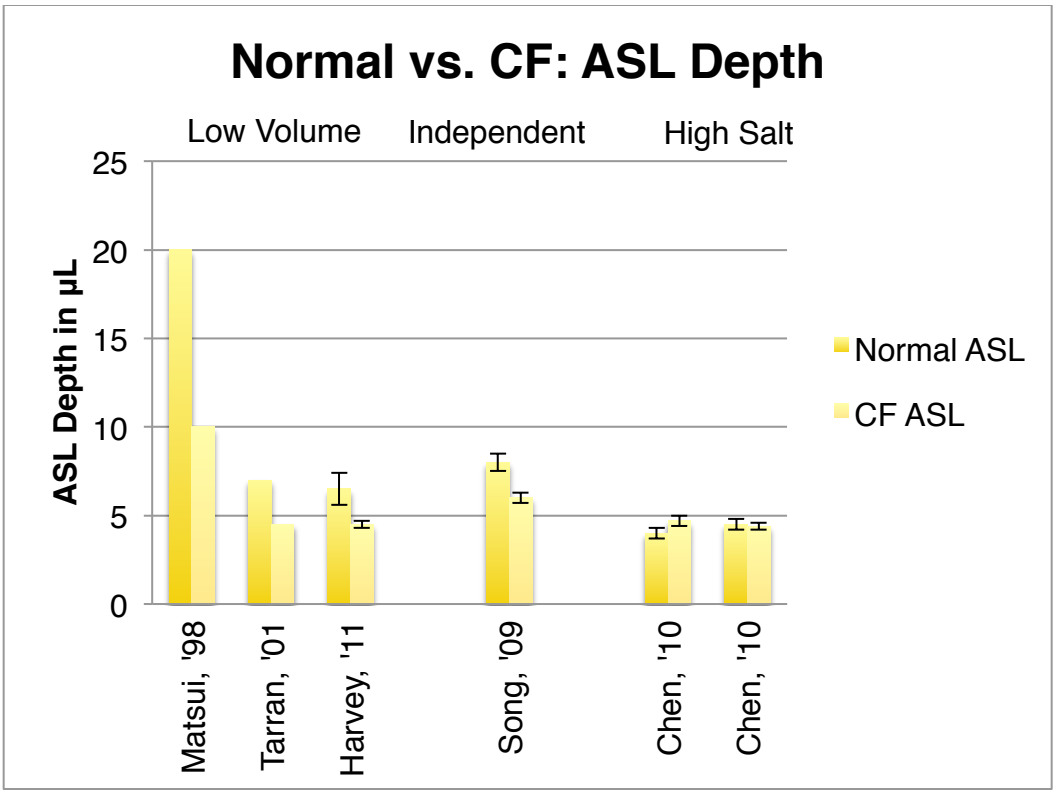

Figure 14: Normal vs. CF: ASL Depth. Comparison of the depth of normal ASL and CF ASL in dependent" refers to research groups not affiliate Matsui et al., 1998 at 20 the depth of normal ASL and CF. A Grubbs's test for outliers with a p-value of 0.01. The CF outlier of 10 r of 10rom Matsui et al., 1998 with a p-value of 0.013. An independent samples *t*-test resulted in a p-value of 0.16. ("Low Volume" refers to research affiliated with the Boucher group at UNC, "High Salt" for work from the Welsh group at Iowa, while the term "Independent" refers to research groups not affiliated with UNC or Iowa)

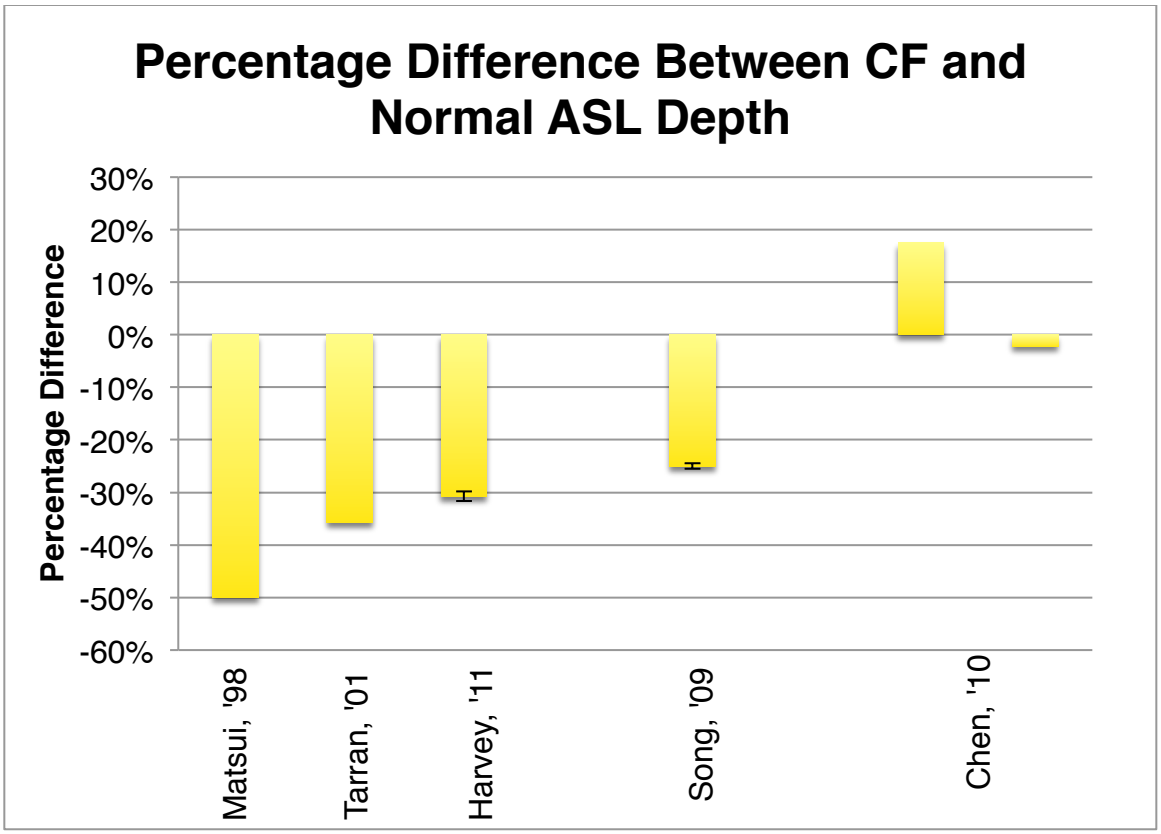

Figure 15: Percentage Difference Between CF and Normal ASL Depth. Difference between normal ASL depth and CF ASL depth as a percentage to eliminate discrepancies between models and focus on comparing ASL depth changes from normal to CF ASL.

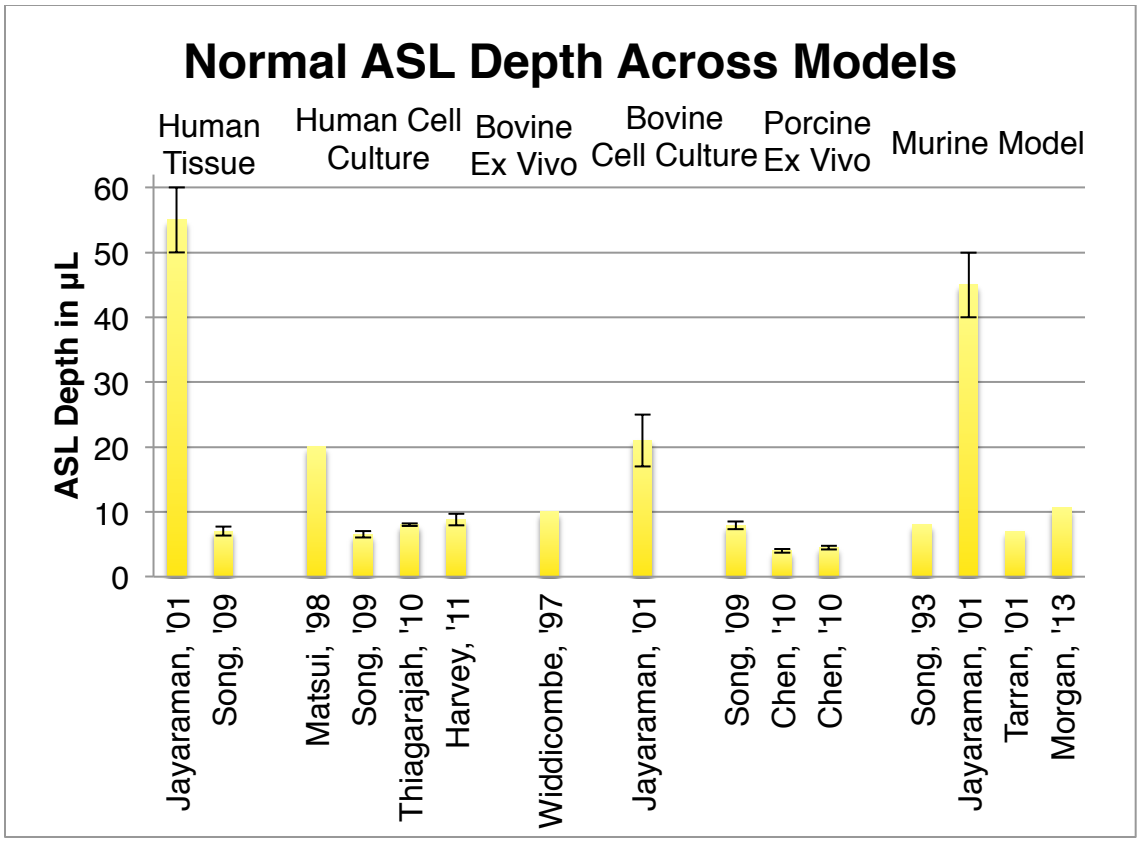

Figure 16: Normal ASL Depth Across Models. Normal ASL depth in µL for *in vivo* and *ex vivo* human tissue (labeled “Human Tissue”), human lung epithelial cell cultures (labeled “Human Cell Culture”), ex vivo bovine tissue, bovine lung epithelial cell cultures (labeled “Bovine Cell Culture”), ex vivo porcine tissue (labeled “Porcine Ex Vivo”), and samples from murine models to show differences in ASL height across models and sampling procedures.

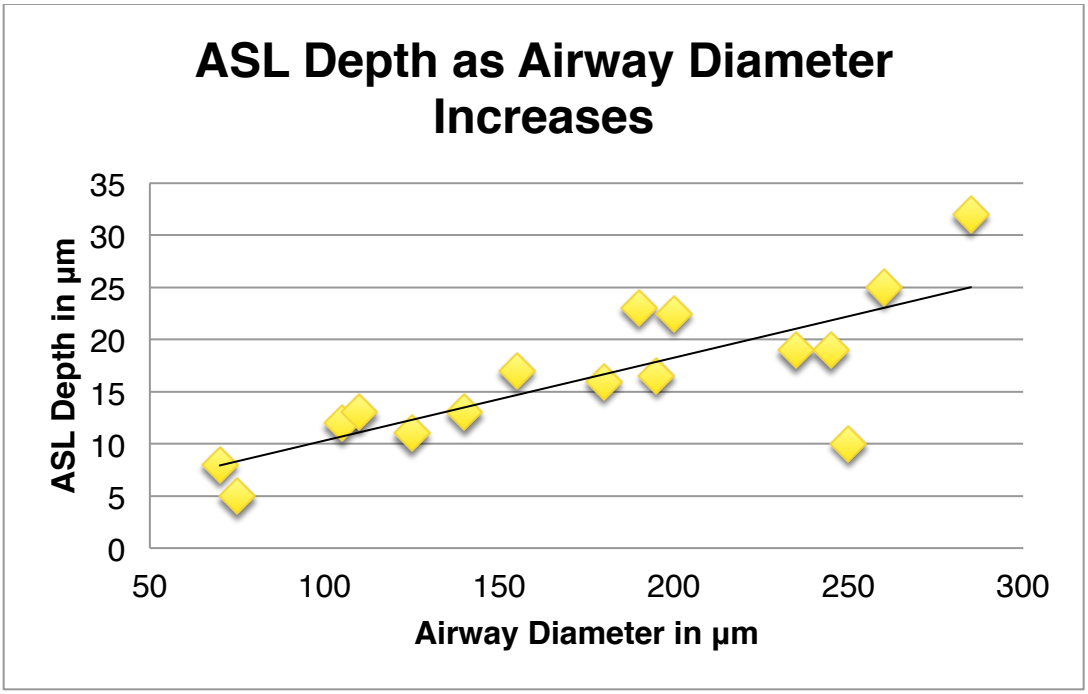

Figure 17: ASL Depth as Airway Diameter Increases. Measurements of ASL depth in µm over increasing airway diameters to show that as the airway decreases in diameter towards the more distal regions, the depth of ASL decreases to limit air flow resistance throughout the lower airways. (Adapted from Song et al, 2003)

ASL depth are compared to measurements of cystic fibrosis ASL depth. Researchers use volume units as an indirect measurement of depth. In the low volume category, which is supported by the Boucher group, the 1998 Matsui et al. study showed a large (10 μL) difference in ASL depth between cells harvested from normal subjects and those of CF individuals. However, Tarran et al. in 2001 and Harvey et al. in 2011 reported smaller overall differences in ASL depth even though Harvey et al. used human cell cultures as well. Investigators not affiliated with Welsh or Boucher (independent category) include a 2009 study by Song et al., which had similar findings to those of Tarran and Harvey. Papers from the Welsh group (high salt category) found lower ASL depths than the other studies, especially for the normal model in a 2010 paper by Chen et al (2010). To remove focus of differing depth values due to model system and place it on the relationship between the depth of ASL and the presentation of pulmonary symptoms in CF, the difference between CF and normal ASL depth is shown in Figure 15 as a percentage. Matsui reported a normal ASL depth that was 50% greater than that for the airways of CF individuals. Tarran and Harvey both reported over a 30% greater depth in normal ASL than in CF ASL. While Song et al. found a 25% difference. In Chen et al., 2010, two data sets found slight variations in depth but not significant differences.

The ASL depth would understandably differ across different animal models due to smaller or larger airways in murine, porcine, bovine, human, or cell culture studies. Therefore, in Figure 16, the amount of difference a model and method of measurement can make on depth values is shown. Reported values vary from 4 μL to 55 μL.

Airway diameter appears to have an effect on the depth of ASL as seen in Figure 17. Song et al., 2009 measured ASL depth at various airway diameters to demonstrate that as the diameter of the airway decreases when moving from proximal to distal airways, the depth of ASL decreases in a linear fashion to minimize the airflow resistance in the smaller airways (Song et al., 2009)

DISCUSSION

Effective treatment of patients with cystic fibrosis depends heavily on understanding the disease mechanism in affected organ systems. However, currently, the field of cystic fibrosis research is in a debate over the mechanism of pulmonary disease, the leading cause of mortality in cystic fibrosis. Dr. Michael Welsh's research group at the University of Iowa has put forward the "high salt" hypothesis; where the lack of CFTR chloride channels increases Cl^- (salt) in the ASL, which inactivates antimicrobial defenses (Figure 18). In contrast, Dr. Richard Boucher's group from the University of North Carolina supports the "low volume" hypothesis; where defects in CFTR lead to abnormal activity of Na channels (ENaC) removing Na^+ ions (salt) and water from the ASL (draining it of its volume; Figure 19).

These two competing hypotheses invoke very different mechanisms permissive for bacterial colonization of the lungs and all the resulting respiratory complications associated with these infections. To definitively test these opposing models, all literature was carefully re-evaluated and the focus of this study's meta-analysis of published data was whether, (i) the salt concentration of ASL and/or (ii) the height of ASL, differ between normal and CF models.

Applications of Cell Culture in Cystic Fibrosis Research

The use of cell culture in research has many benefits and is a valuable source of knowledge in the field of cystic fibrosis. Cell cultures are easily maintained and

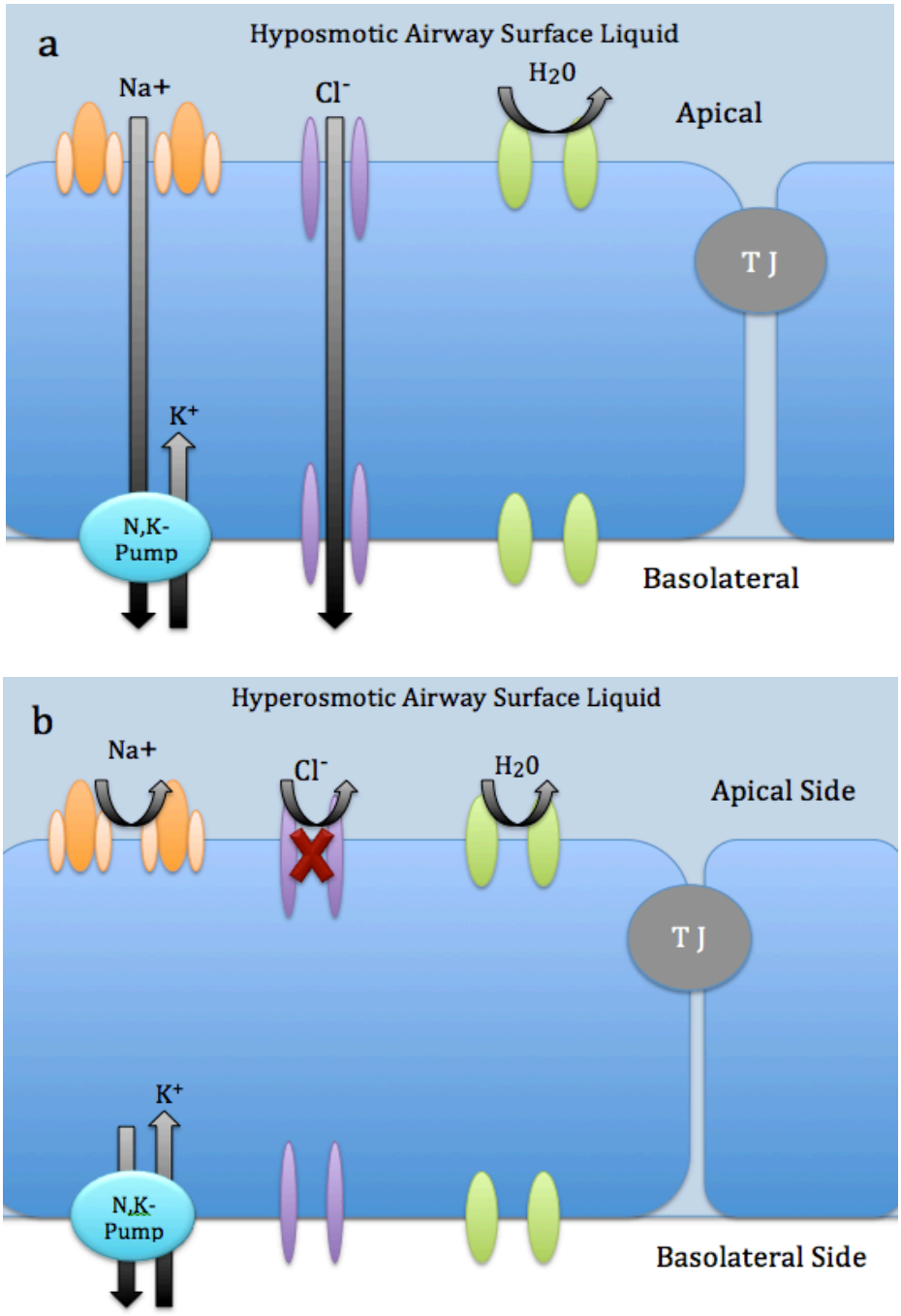

Figure 18: Model of the High Salt Hypothesis. In Figure 18a, a normally functioning lung epithelial cell while 18b is a lung epithelial cell with defective CFTR channels following the pulmonary disease progression model of the high salt hypothesis.

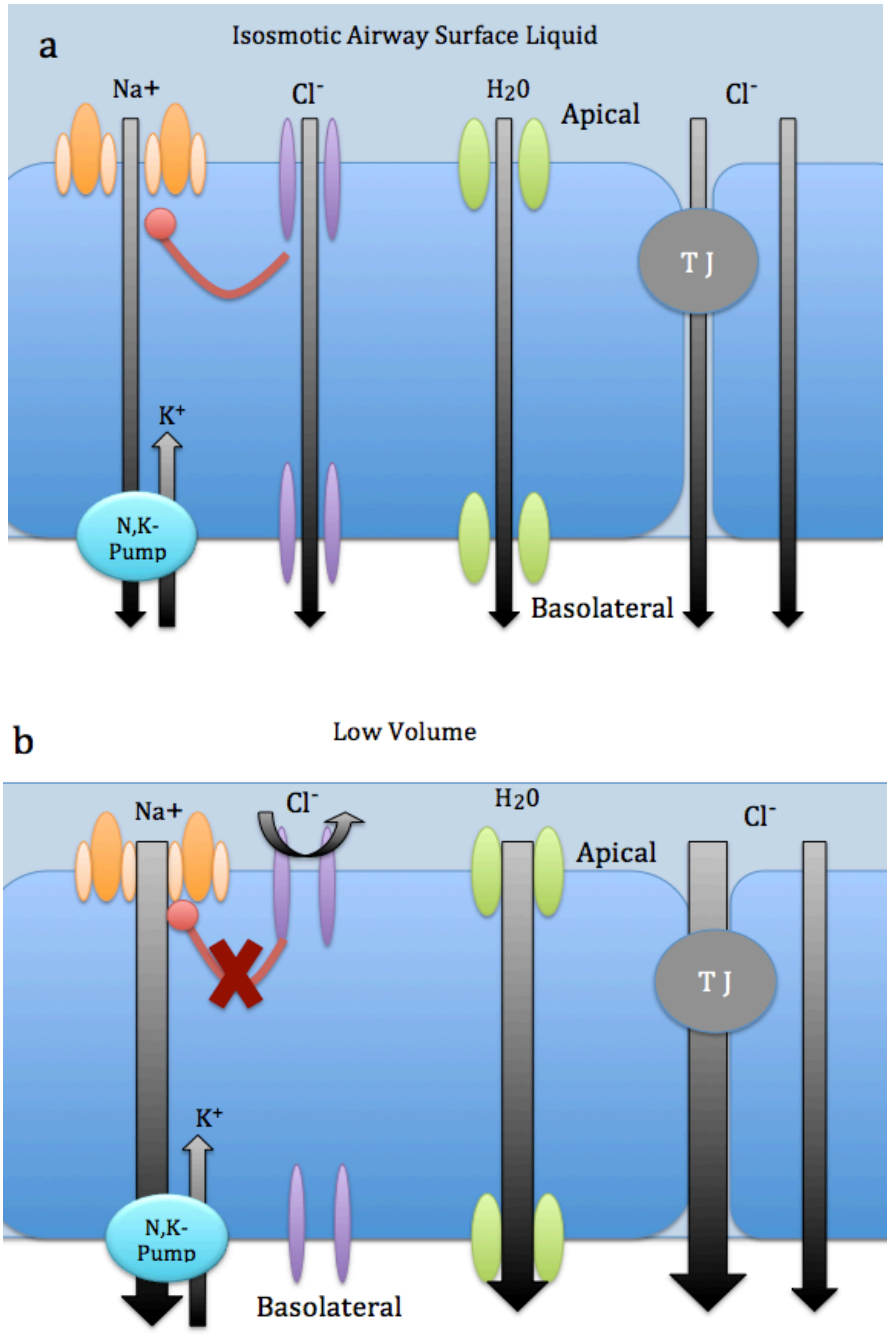

Figure 19: Model of the Low Volume Hypothesis. In Figure 19a, a normally functioning lung epithelial cell while 19b is a lung epithelial cell with defective CFTR channels following the pulmonary disease progression model of the low volume hypothesis.

manipulated for analysis of isolated conditions for a disease. For example, the mechanism of regulation of ENaC by the CFTR channel has been studied in detail using cultured cells. In past cell culture studies, such as the 1998 paper by Matsui et al from Boucher's group and Zabner et al from Welsh's group in the same year, both measured sodium and chloride concentrations in human airway epithelial cells. Matsui et al reported values of 140 mM of sodium and 130 mM of chloride in both, Zabner et al observed values of 50 and 100 mM of sodium and 37 and 90 mM of chloride for normal and CF cells, respectively.

A closer look into each study's protocol suggests some of this discrepancy may be a result of significant variation in the cell culture techniques between and among groups. For example, for a time the Welsh group may have used cells that were not ciliated or well differentiated. Those cells had a low amiloride sensitive short circuit current ($Isc_{(amil)}$), which is ion flux across the epithelia without ENaC activity due to inhibition from amiloride (Jiang et al., 1993; Smith et al., 1994). Later, cell cultures in the 1998 Zabner paper (also Welsh group) demonstrated a high $Isc_{(amil)}$ and found an increased liquid absorption in normal compared to CF epithelia TER as depicted in Figure 20. In addition, Zabner reported transepithelial electrical resistance for all epithelial as ≧ 800 Ω x cm2 which is uncharacteristically high compared with other studies. For example, Calu-3 cells in culture traditionally have achieved a transepithelial resistance of approximately 400 Ω x cm2, while the resistance in Matsui et al was reported as > 300 Ω x cm2 (Stewart et al., 2012). A difference in the transepithelial resistance (TER)

across studies is important in this debate, as TER is indicative of the level of resistance to passive ion flow through tight junctions. A higher resistance means less leaking of ions and fluid between cells, which could be characteristic of an abnormally tight or confluent monolayer that may be attributed to multi-layered growth or increased expression of tight junction proteins (Pohl et al., 2009). The abnormally high TER in cells cultured by Zabner, part of the Welsh group, likely inhibit the paracellular ion flow essential to the low volume model proposed by Boucher's group. This lessens the strength of evidence presented in the Zabner paper.

This highlights a key difference between the characteristics of the two different cell cultures. While Zabner et al focused on well- differentiated cultures with high $Isc_{(amil)}$ values, Matsui et al strived for epithelial that were somewhat leaky as is seen in intact airway tissue (Zeuthen, 1981; Jeffery, 1988; Boucher, 1994). The cultures in Matsui et al, 1998 were described as having a ciliary length of ~ 7 μm, an interciliary distance of ~ 1 μm to ensure a high density of cilia per cell, 60-80% of the cultures consisted of ciliated cells, a ciliary beat of ~ 10 Hz, and vectorial mucus transport of 40 μm/s. Thus, Matsui was attentive to the ciliary components and monolayer characteristics to mimic in vivo conditions closely. This increased the strength of evidence presented by Matsui but of course makes careful and reliable comparison difficult.

Conclusions from cell culture data sets: If you evaluate findings presented by both groups when they were just using cultured cells, and you assume all measures are

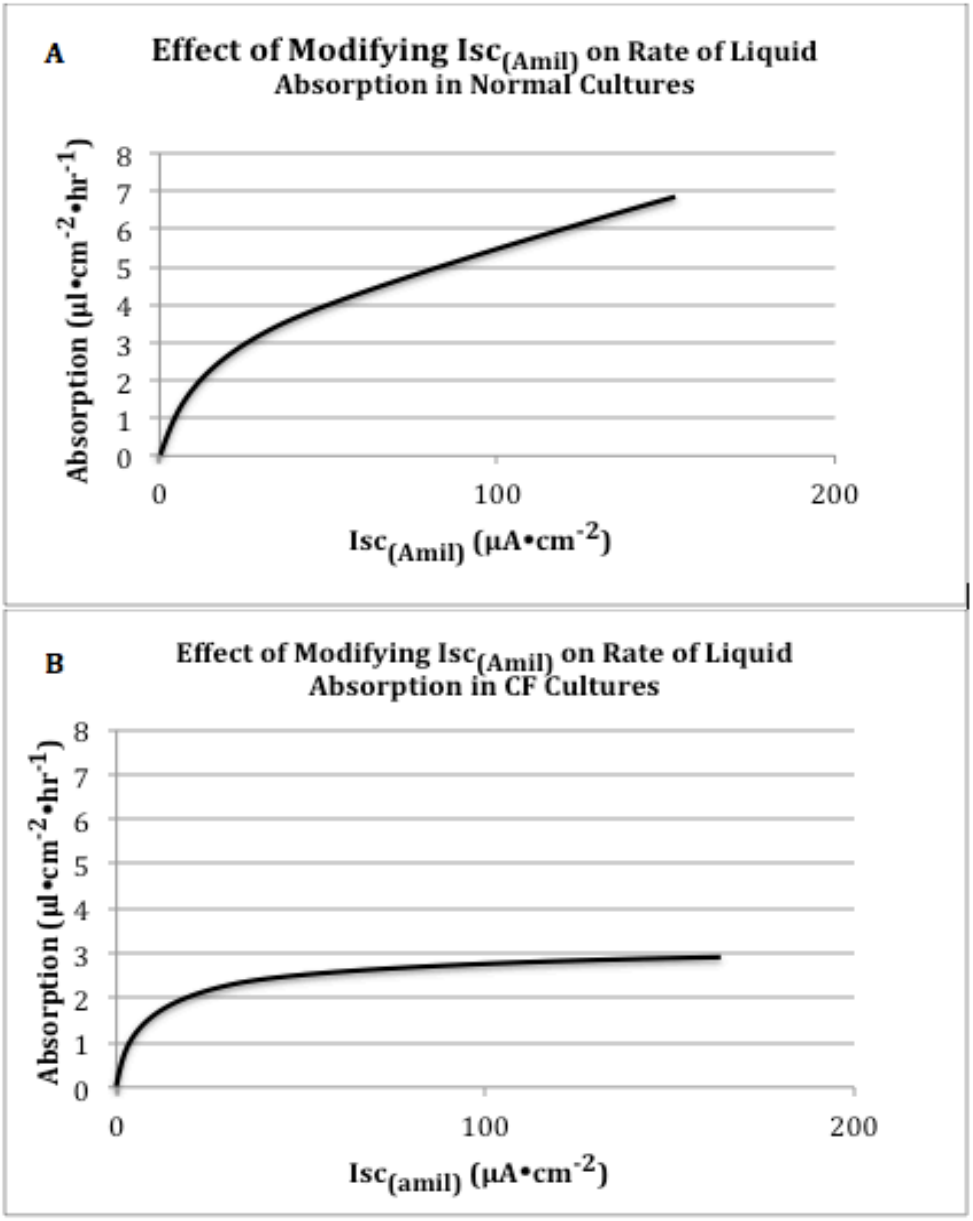

Figure 20: Effect of Modifying $Isc_{(Amil)}$ on Rate of Liquid Absorption in Normal and CF Cultures. Liquid absorption in normal cultures increased as $Isc_{(amil)}$ increased and in CF cell cultures as $Isc_{(amil)}$ increased, liquid absorption initially increased and then plateaued signaling a defect in liquid absorption in cells lacking CFTR channels.

equally valid, pooled data supports the Welsh group's position of increased salt in the ASL of CF airways. On the other hand, our analysis supports that increased TER undermines Zabner's findings, as seen in Figure 20. If you throw out that data, in the pooled set variation then eliminates any statistical significance. Thus our conservative conclusion from the cell culture studies alone is that a case has not been made to support the change in salt concentration proposed by the "high salt" hypothesis.

Cell culture properties that authentically replicate *in vivo* conditions are difficult to develop, characterize, and maintain throughout passages. Variables to consider in cell culture are excess differentiation into goblet cells and ciliated epithelial cells, the expression of proteins indicative of adhesion and tight junction such as E-Cadherin, ciliary length and beat frequency, and the ratio of ciliated cells to goblet cells (Stewart et al., 2012; Wanner et al., 1996; Widdicombe, 1997). Questions involving multiple contributing factors such as mucus, airway surface liquid, the innate immune system, and other non-epithelial airway cells cannot be addressed within the scope of cell culture studies.

Thus, to identify if elevated salt concentration in the ASL of CF patients is the underlying event leading up to chronic lung infections and eventually respiratory complications ending in the death of the patient, *in vivo* measurements are given far more weight and thus were examined with this in mind.

Manifestations of Cystic Fibrosis Symptoms in Murine Models

Comparatively, the *in vivo* murine model has innumerable applications in research across the sciences. The murine model's popularity stems from the ease with which transgenic mice strains can be generated through crossbreeding well-established strains. However, the limitations of a murine model exhibiting a specific mutation to study a disease, such as cystic fibrosis, must not be ignored.

By the year 2007, eleven murine models of cystic fibrosis had been generated and studied (Snouwaert et al., 1992; Clarke et al., 1992; Kent et al., 1997; Dorin et al., 1992a; Dorin et al., 1992b; Ratcliff et al., 1993; O'Neal et al., 1993; Hasty et al., 1995; Rozmahel et al., 1996; van Doornick, 1995; Colledge et al., 1995; Zeiher et al., 1995; Dickinson et al., 2002). The first cystic fibrosis murine model was a knockout mouse generated by the Boucher group in 1992 (Snouwaert et al., 1992; Clarke et al., 1992). It had a very low survival rate with less than 5% of the animals surviving until adulthood (Clarke et al., 1992). This appeared to be a result of severe gastrointestinal impairment, yet the airways showed no symptoms of CF. Additional genetic manipulations then led to the generation of a ΔF508 CFTR murine model (van Doornick et al., 1995). Ultimately, various recognizable CF symptoms were found in most of the various mouse models generated, and these were seen across the affected organ systems in CF mice.

The gastrointestinal system exhibits the most cystic fibrosis complications across the different murine models. Several CF murine models have been reported to demonstrate a failure to thrive along with mucus accumulation and intestinal obstruction (Snouwaert et al., 1992). Obstruction of the murine intestines was similar to that presented by

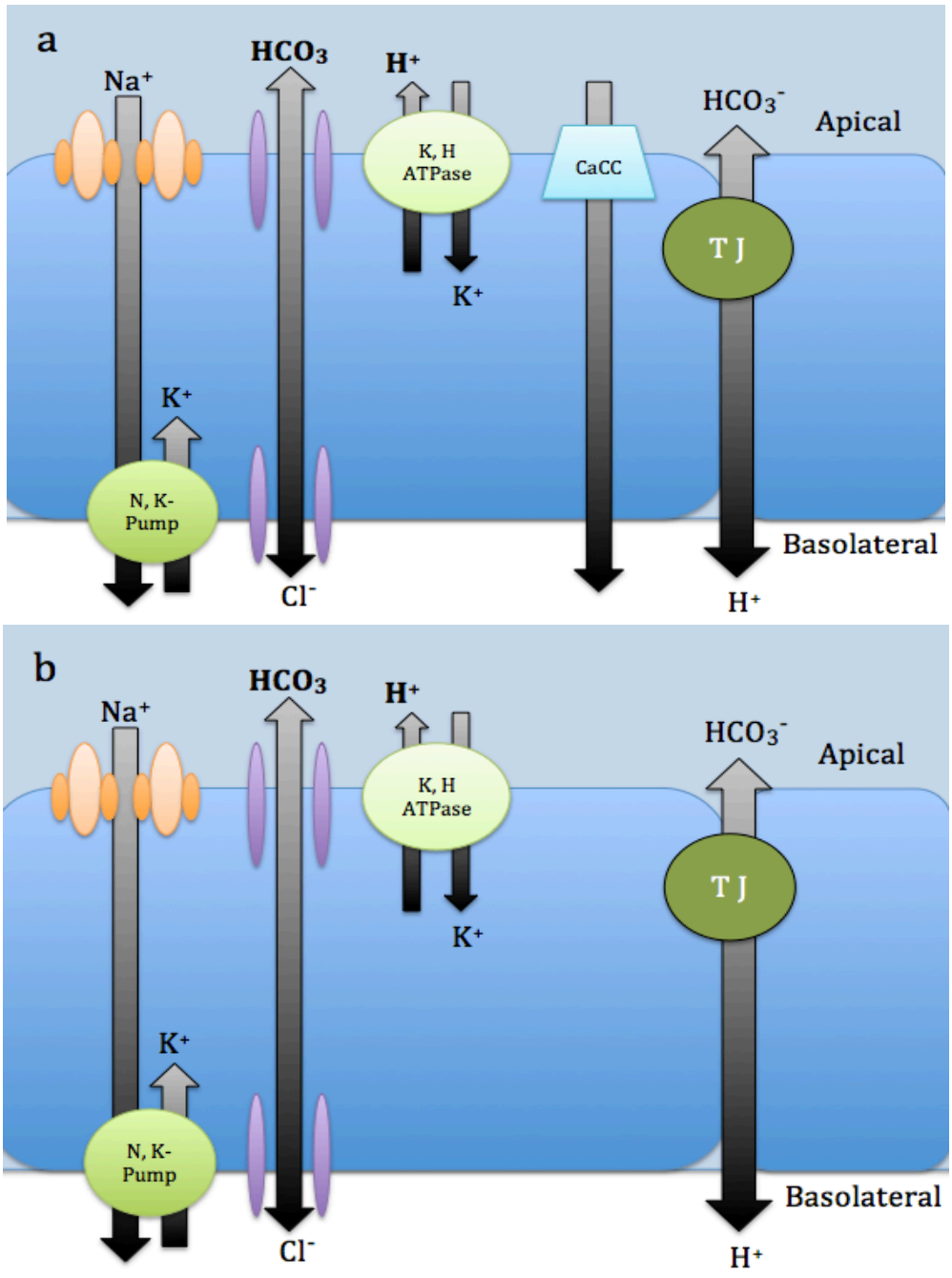

Figure 21: Model of Airway Epithelial Cell of CF Murine Model and Human Subject. In Figure 21a, a lung epithelial cell of a CF murine model while 21b is a human lung epithelial cell with defective CFTR channels to illustrate the differences in the two ASL of CF airways.

meconium ileus, which is typically experienced by CF patients. Models also presented with familiar pancreatic symptoms. Typically the blockage of small pancreatic ducts was seen, but less impact overall on pancreatic function (Ratcliff et al., 1993).

Unfortunately, since 1993, all studies have repeatedly shown that the CFTR-null murine models are an ineffective form of examining pulmonary effects of cystic fibrosis. Mice do not develop any of the typical characteristics of lung disease such as peptides secreted by airway epithelial cells and inflammation from neutrophil and macrophage activity (Colledge et al., 1995; O'Neal et al., 1993; Zeiher et al., 1995). In order for inflammation and decreased bacterial clearance to be seen, mice had to be repeatably exposed to unusually high numbers of bacteria (Davidson et al., 1995).

A range of Na+ and Cl- concentrations, as well as ASL volumetric depths, in murine ASL measurements have been reported, yet no significant differences have been found. This lack of difference between normal and CFTR-null mice can be accounted for by the novel finding that mice express an alternate pathway in the airway; Ca-activated Cl- channels replace the cAMP-dependent CFTR chloride channels lost in CF (Grubb et al., 1994a; Grubb et al., 1994b; Figure 19). Yet, surprisingly, this unusual situation sheds some light on our debate.

Conclusions from murine model studies: Increased activity of Ca2+-activated chloride channels in CFTR-null mice have been demonstrated (Grubb et al, 1994a; Grubb et al, 1994b). We can assume that in this animal model a dysfunctional CFTR is expressed

alongside a viable alternative Cl^- path (do not need a paracellular one) and yet it does not express key features of pulmonary disease. This refutes the low volume hypothesis model as presented by the Boucher group (Figure 19b). If their mechanism is accurate, it would lead to a loss of ASL volume and thus infections in the mouse airway. However, this is not the case.

Yet to be conservative, following a careful analysis of ASL Na+ and Cl- concentrations reported in both murine models and human airway epithelial cell cultures, important downfalls of each model for cystic fibrosis research have been identified and ruled out findings obtained by these models due to the limitations described.

Sodium and Chloride Concentrations in ASL of Normal and Cystic Fibrosis Human Subjects – Analysis of the 'high salt' hypothesis

In vivo measurements of sodium and chloride concentrations in the in vivo airway surface liquid of normal and cystic fibrosis human subjects are then identified as the most reliable source of data for which a conclusion of the accuracy of the high salt hypothesis can be drawn.

With regard to Na+ concentration, values obtained from 4 different studies indicated a normal range of 82 – 103 mM. There was no significant difference found between the reported normal sodium concentrations across studies. Thus, the Na^+ concentration in the ASL of patients without cystic fibrosis can vary within an approximate range of 80 to 100 mM depending on the person. Corresponding ASL Na^+ concentrations were

reported in patients with cystic fibrosis by Joris et al. in 1993, Knowles et al. in 1997, and Hull et al. in 1998. The described range of ASL Na^+ concentrations in CF patients was similar to the range of sodium in normal subjects; 82mM to 121 mM of sodium. An independent samples *t*-test determined that there was no significant difference between human *in vivo* ASL Na^+ concentrations in normal and CF subjects.

Subsequently, the concentration of chloride ions in the airway surface liquid of both normal and cystic fibrosis patients measured *in vivo* underwent a separate meta-analysis.

Overall, there are five studies that report normal Cl^- ASL concentrations: Gilljam et al., 1989; Joris et al., 1993; Knowles et al., 1997; Hull et al., 1998; Jayaraman et al., 2001. The range Cl^- ASL concentrations in normal subjects was 78 mM to 108 mM, with no significant difference between the five data points shown through a Grubbs' outlier test. Thus, chloride concentrations in subjects without CF range from approximately 70 mM to 110 mM, depending on the individual. Four of the aforementioned papers report corresponding Cl^- ASL measurements in CF subjects: Gilljam et al., 1989; Joris et al., 1993; Knowles et al., 1997; Hull et al., 1998. Chloride measurements in CF subjects were between 75 mM and 170 mM. To determine if normal concentrations of chloride are significantly different from those of cystic fibrosis patients, an independent samples *t*-test was performed, which indicated no significant difference. Thus, these data suggest that the ASL Cl^- concentration is not increased in cystic fibrosis, as previously

thought.

With the exception of Gilljam et al. in 1989, the studies that measured human *in vivo* ASL salt concentrations used varying forms of filter paper to collect ASL through a bronchoscope from the distal trachea or bronchi, which has been argued to have its drawbacks as well. Protocols utilizing the placement of a filter paper to the airway can wick fluid from areas outside the paper's diameter and likely from inside the cell, since the removal of fluid will cause a shift in fluid to the apical surface. However, all three studies used this methodology, which limits the concern that differences in reported findings between studies are caused by this technique. It should be noted that, although it is unlikely to explain cross-study variations, filter paper collection may artificially increase or decrease salt concentrations measured in ASL.

The technique used by Gilljam et al. in 1989 to collect ASL salt concentration measurements, and the manner in which error was reported, explains the larger margin of error relative to those of the other 3 human *in vivo* studies. Gilljam et al. aspirated ASL from the bronchial surface through a bronchoscope into a collection vial, while the other studies used filter paper collection techniques similar to each other. Aspiration of the very thin layer of liquid lining the lungs allows for significant evaporation of fluid as it ascends the bronchoscope, resulting in a higher salt concentration by default. In addition, error measures were reported as one standard deviation instead of as a standard error measure (SEM) as done in Joris et al in 1993, Knowles et al in 1997, and Hull et al in 1998. The error of ASL chloride concentrations was reduced from 79 mM for

CF patients and 54 mM for normal subjects to 26.3 mM and 18 mM respectively, by calculating the SEM using the reported sample size and standard deviation. These dissimilarities in error calculation and collection technique could lead to the discrepancies noted.

The finding of no difference between CF and normal human Na^+ and Cl^- ASL concentrations disagrees with the findings of Joris et al. in 1993 and Gilljam et al in 1989. CF subjects from the 1993 Joris paper were found to have approximately 45% more Na^+ in ASL than their normal counterparts (Joris et al., 1993). However, the sample size for normal subjects was seventeen compared to only three CF subjects. Additionally, the three CF subjects included a 6 month old undergoing surgery to remedy a collapsed lung, and a 16 year old and 81 year old hospitalized for elective surgical procedures. By the age of 5, 60% of people with CF have symptoms of lung disease (Peterson et al., 1981). Therefore, it is likely that due to the age of the older CF subjects, at least two-thirds of the CF patient sample in the 1993 Joris paper had lung infections or inflammation. Between studies, there were not only differences in CF subjects measured but also in normal subjects. Gilljam et al. measured subjects who did not have CF, but were diagnosed with chronic bronchitis. The significant difference found in Na^+ and Cl^- concentrations by Joris et al. and Gilljam et al., is likely due to the limited and skewed range between CF subjects' ASL measurements and the wider range of unhealthy normal subjects.

Conclusions from human subjects studies: Through analysis of available data from both the Welsh group of the University of Iowa who support the high salt hypothesis and Boucher group of the University of North Carolina at Chapel Hill who support the low volume hypothesis, available evidence suggests that neither Na^+ nor Cl^- concentrations are statistically higher in the ASL of cystic fibrosis vs. normal patients. The high salt hypothesis model described in Figure 18b is therefore unlikely to be an accurate depiction of CF on the cellular level in the airways.

Height of Airway Surface Liquid in Normal and Cystic Fibrosis Models – Analysis of the 'low volume' hypothesis

Airway surface liquid depth is the widely measured and defining variable of the low volume model for CF lung disease proposed by the Boucher group (Figure 19). For years, the low volume hypothesis has claimed that the characteristic lung infections of cystic fibrosis initiate from a decrease in airway surface liquid depth. Water is hypothesized to move into the cell via osmosis following an increased absorption of Na^+ through an un-inhibited ENaC channel, thus reducing the thickness of the ASL. Eventually, the loss of water is thought to lower ASL below the height of the cilia (7 μm), resulting in ciliary collapse, loss of mucus transport, and disruption of normal bacterial clearance. The standing mucus then becomes a breeding ground for bacteria such as *pseudomonas aeruginosa*.

In order to determine the soundness this hypothesis published ASL depths obtained from normal and CF models were gathered. Normal ASL depths measured by Matsui et

al. in 1998, Tarran et al. in 2001, Song et al. in 2009, Chen et al. in 2010, and Harvey et al. in 2011 varied from 4 µl to to 20 µl. In comparison, ASL depths of CF models ranged from 4.5 µl to 10 µl. The highest values for both normal and cystic fibrosis ASL depth were significant outlier's were found indicated by a Grubbs' test. Both outliers were observed by Matsui et al., 1998. Despite the significant outliers, an independent samples *t*-test found no significant difference in ASL depth measured in normal and CF models.

Variations in the methodology carried out by Matsui et al. in 1998 to measure ASL depth could result in the increased values observed. Matsui and colleagues added 50 µl of media to the 1 cm^2 culture samples. In comparison, Song et al. added only 20 µl of media and Tarran et al. aspirated off excess fluid leaving only 10 µl on the culture samples. An excess of 30 µl of fluid covering the surface is a sufficient difference and likely lead to the 13 - 17 µl gap in ASL depth between Matsui et al and the other four studies.

Variations in ASL depth are seen across normal models as well. These measurements are different, in spite of being from normal models, due to the use of varying cell culture techniques, murine models, and airway diameter of samples collected. As cell culture and murine models limitations have been discussed previously, the effect of airway diameter on ASL depth will be highlighted. In 2003, Song et al. measured ASL depth in normal subjects as airway diameter decreased, which elucidated a linear relationship

between the two variables: as airway diameter decreases the ASL depth decreases in order to maintain uninterrupted air flow. These changes in ASL depth in relation to airway diameter have not been reported as being controlled for in previous tissue or *in vivo* studies. Thus, is the observed decrease in CF ASL depth reported by many studies seen in intact tissue or CF subjects?

The more recent porcine model may help to answer this question. In Chen et al., CFTR-null porcine models were investigated and ASL depths were measured in CF and normal newborn pigs. There was no significant difference between the normal and CF ASL depths (Chen et al., 2010). Additionally, imaging of the samples from both normal and CFTR-null pigs showed erect and bent cilia that correlated with topographically changing ASL heights. The varying state of cilia, from bent to erect, is similar to a topographical map of a landscape encompassing mountains and valleys. The airway surface is not a single layer of one height, but a landscape with highs and lows, which is in agreement with Widdicombe, 2002.

Even though the basal ASL height may be similar, a recent study has suggested that the height of the ASL is not set at any specific value, but rather, it changes in response to the airway environment. In that study, Song et al. measured ASL depth in *ex vivo* tissue samples of human and porcine trachea harvested within 12 hours of excision. Since at the time of this study, no CF porcine models had been generated, to investigate the transient ASL depth changes in cystic fibrosis, a CFTR inhibitor was used to mimick the loss of CFTR function. Under CF-like conditions, ASL fluid was not

increased in the presence of secretory stimuli, as it was in tissue with unhindered CFTR activity. These findings by Song et al. in 2009, suggested a transient change in ASL depth in response to extracellular conditions was lost in cystic fibrosis.

Consequently, a second look into how the ASL depth of subjects with cystic fibrosis responds under different agonists and inhibitors compared to normal subjects should be considered. Transient increases in the depth of the ASL were observed when exposed to the CFTR agonist, forskolin, or ENaC inhibitor, amiloride (Song et al., 2009). Because the use of molecular compounds to achieve CFTR inhibition and mimic CF may not result in findings comparable to *in vivo* CF models, we suggest future work focus on using the ΔF508 porcine model developed by the Welsh group (Ostedgaard et al., 2011). *In vivo* measurements of transient ASL depth in this model, when exposed to forskolin and amiloride, will be necessary.

The experiment would incorporate common techniques, as well as those deemed more reliable: Pigs should be anesthetized using ketamine (20 mg/kg, IM) and xylazine (2 mg/kg, IM) while being maintained with propofol (2 mg/kg, IV) in a humidified chamber (100% relative humidity, 25-30 ℃ (Pezzulo et al., 2012). A mid-line neck incision would be made, resulting in a small window into the anterior tracheal wall, as previously described. Fluorescent indicator is then deposited 2-3 minutes prior to measurements into the trachea through the mouth and tracheal window, which will be covered with plastic wrap and tissue adhesive (Jayaraman et al., 2001). At separate times, 100 μM amiloride or 20 μM forskolin and 100 μM IBMX will be sprayed onto the airway surface

to be measured (Song et al, 2009). Changes in ASL depth will be measured using 40X objective lens (Jayaraman et al, 2001). The findings of this study will either support those of Song et al. in 2009 by demonstrating that the ASL depth of CF pigs do not increase when treated with amiloride or forskolin, or they will disagree. A lack of difference between the changes of ASL depth in CF and normal porcine models will demonstrate a disagreement with previous work. Determining whether transient changes in ASL depth are absent in CF is important to finally support or refute ASL depth as an underlying mechanism for CF lung disease.

The Cause of Cystic Fibrosis Lung Disease: High Salt or Low Volume Hypothesis

After critical analyses of the data from the Boucher group of the University of North Carolina who support the low volume hypothesis and the Welsh group of the University of Iowa who have proposed the high salt hypothesis, as well as data from various independent groups, the most likely conclusion is that neither hypothesis is correct, as suggested by *in vivo* human or porcine studies. These studies strongly suggest that airway surface liquid does not have an increased salt concentration in cystic fibrosis, nor is the depth of the airway surface liquid lowered or depleted in the disease.

As neither of the major hypotheses of cystic fibrosis lung disease appear to be supported, it begs the question: what is the underlying cause of pulmonary infection in cystic fibrosis?

The current focus of some groups researching the underlying cause of lung disease in

cystic fibrosis has shifted to differences in pH between the ASL of normal individuals and of CF subjects. This shift from the concentrations of Cl^- and Na^+ in ASL to pH is a direct consequence of the finding that CFTR not only transports Cl^- ions but bicarbonate as well (Welsh and Smith, 2001). In fact, a study done utilizing a virtual gland apparatus has shown that approximately 50% of the anion secretion in the airway is bicarbonate, making this now very significant area of research (Irokawa et al., 2004).

Increased levels of bicarbonate in the ASL of CF subjects could alter its pH, which would have broad-sweeping effects across organ systems that have previously not been able to be tied to a simple defect in Cl^- conductance (Quinton, 1999). Over several studies, the Verkman group initialy reported finding no significant change in pH between genotypes, but in 2004 when submucosal gland fluid from human and porcine airways was examined, a significant difference between the pH of ASL from CF and normal models was found. The new hypothesis then is: A lack of functional CFTR chloride channels results in a decrease in bicarbonate ions in the ASL of cystic fibrosis patients, thereby increasing the acidity of the airway surface liquid and thus resulting in a decrease in the bactericidal activity of the innate immune system.

In Vivo Measurements of ASL pH in Normal and Cystic Fibrosis Human Subjects

Differences in the pH of ASL from normal and cystic fibrosis models have been reported in several studies over the years (Jayaraman et al., 2001; Coakley et al., 2003; Song et al., 2006; Cho et al., 2011; Pezzulo et al., 2013). The most reproducible and compelling differences, due to similarity in disease progression, are found in the porcine model and

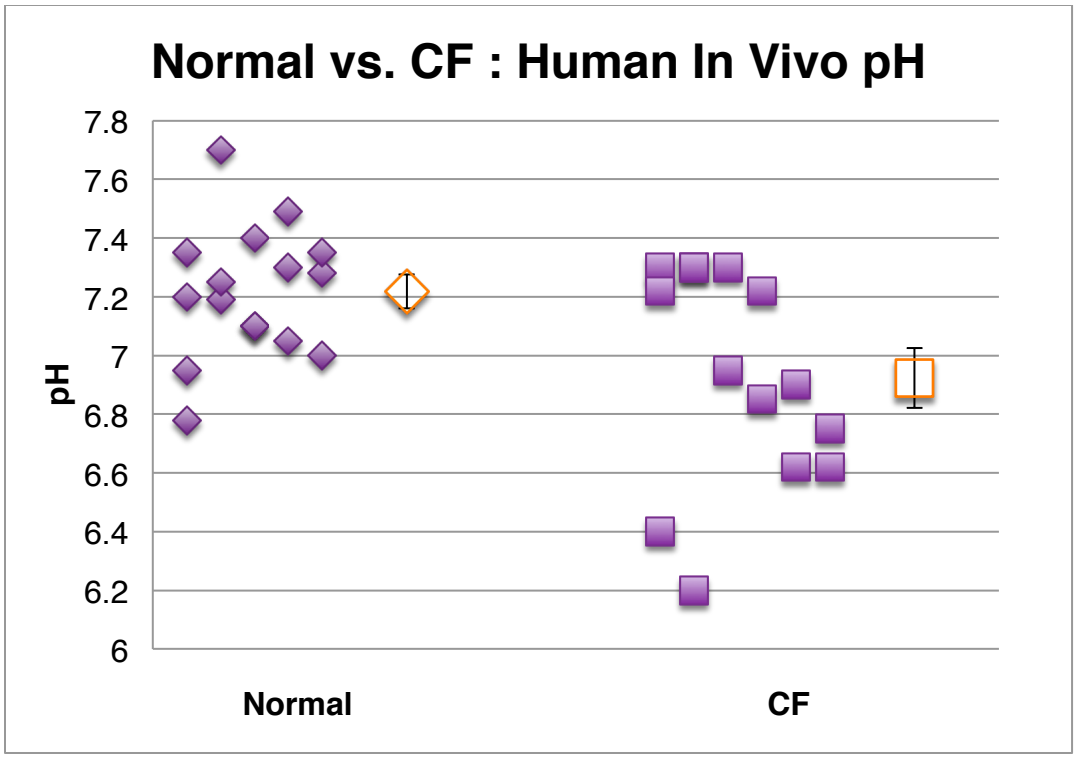

Figure 22: Normal vs. CF: Human *In Vivo* pH. Compilation of ASL pH values from several articles (listed below) for both normal and CF human subjects with a calculated average value shown by the correlating larger marker to the right of the individual data points. The mean pH value of ASL in CF patients is 6.92 while the mean of normal subjects is 7.22. An independent samples t-test resulted in a p-value of 0.00595. (Data from Jayaraman et al., 2001; Cho et al., 2011; and Song et al., 2006)

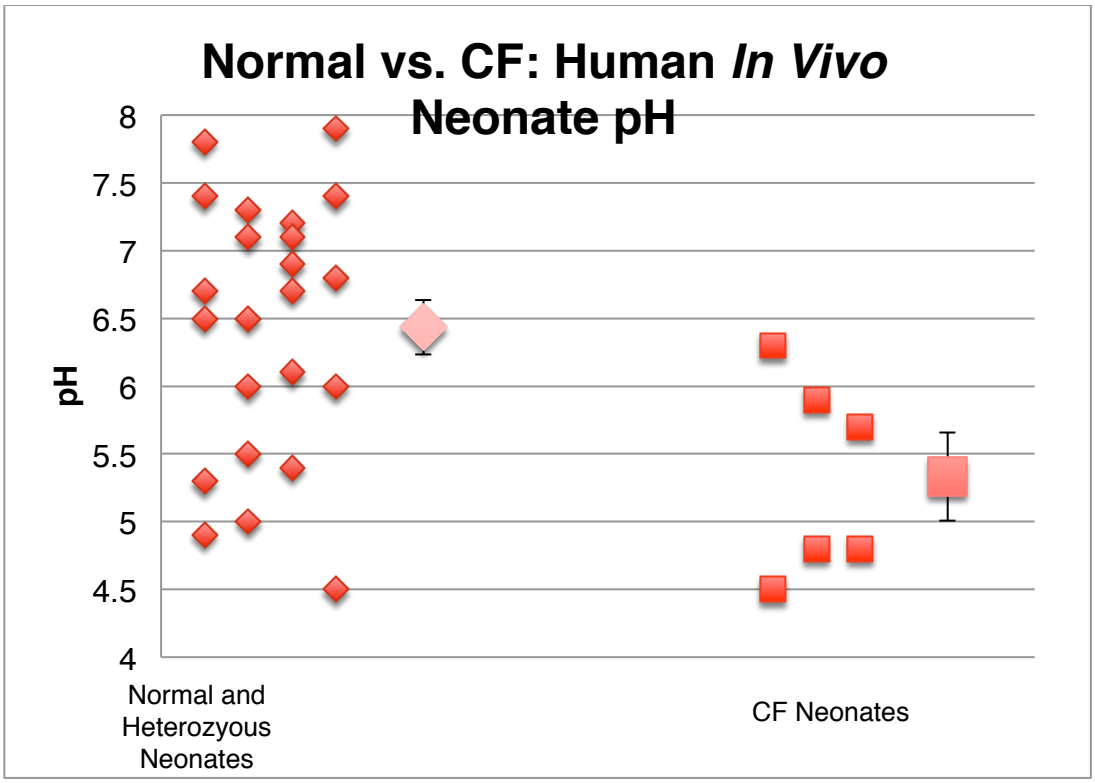

Figure 23: Normal vs. CF: Human *In Vivo* Neonate pH. Measurements of human neonatal ASL pH *in vivo* from normal subjects and subjects heterozygous for a CFTR mutation in comparison to neonates homozygous for CFTR mutations (same or different). In an independent samples t-test, a p-value of 0.00746 was caluculated. (Raw data from Abou Alaiwa et al., 2014)

in vivo human studies. In Figure 22, *in vivo* ASL pH measurements for normal human subjects and CF subjects are shown with the mean value for each group, indicated by the large corresponding marker. Mean measurements for human subjects with CF and those of normal individuals were reported in Jayaraman et al., 2001 while Song et al., 2006 and Cho et al., 2011, reported individual pH measurements. The mean pH for normal subjects was 7.22, with a standard deviation of 0.22, while the mean pH for subjects with CF was 6.92, with a standard deviation of 0.35. An independent sample t-test showed a significant difference between the ASL pH of normal and CF patients. In 2014, Abou Alaiwa et al. measured ASL pH in normal and CF carriers to compare to CF neonates who were homozygous for CFTR mutations. In neonates, the difference between normal/non-affected CF and CF subjects is more pronounced, as shown in Figure 23. Although there is a larger sample size of “normal” subjects compared to CF neonates, a significant difference was found in an independent samples student’s t-test. Thus, a difference in pH most likely is present even at birth, before inflammation and exposure to bacterial pathogens that could result in infections and alter findings, are involved.

Thus, a lower pH in those with cystic fibrosis at birth and throughout later stages of life is highly suggestive as an underlying cause of lung infections and pulmonary complications associated with cystic fibrosis.

Future Directions for Research Involving the Relationship of Decreased pH to CF Lung Disease

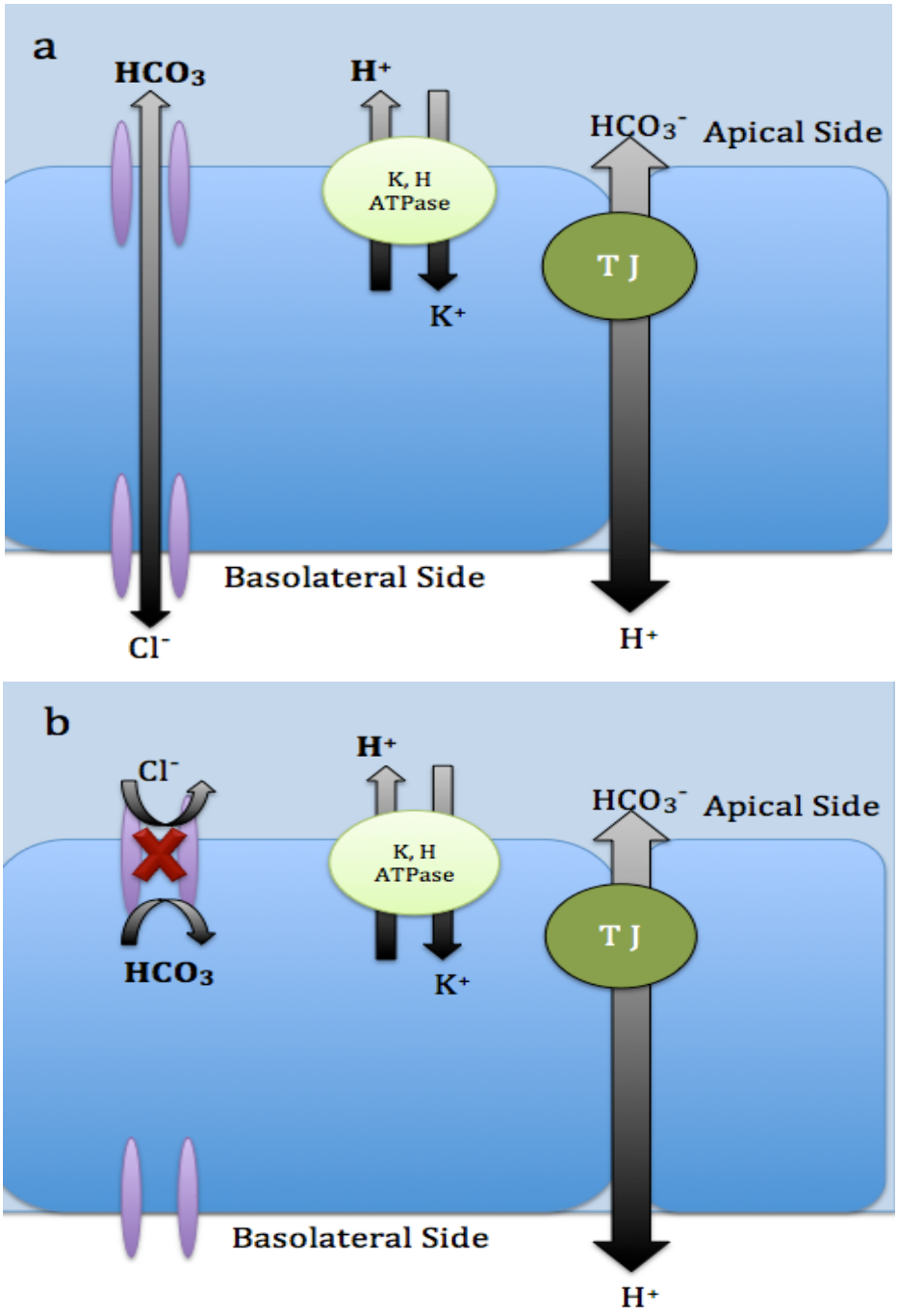

Figure 24: Revised Model of CF Human Airway Epithelial Cell. In Figure 24a, a normally functions human lung epithelial cell, while 24b is a human lung epithelial cell with defective CFTR channel to illustrate the differences in the two systems when bicarbonate is considered.

It is important to the future method for treating cystic fibrosis to understand how a more acidic ASL pH affects the functions of the airway surface liquid and how this change in pH is created. There are a few key experiments that would greatly enlighten the cystic fibrosis research field, which will be described here.

Most importantly, a model that is as similar as possible to the disease pathology experienced in humans is needed. Recent advances, in this aspect, have been made by the Welsh group at the University of Iowa with the development of the $CFTR^{\Delta F508/\Delta F508}$ porcine model that are homozygous for the most common CF mutation (Ostedgaard et al., 2011). To further characterize this model, this study suggests an in-depth look at the pH of the airway surface liquid in the nasal cavity and distal trachea at 8-15 hours after birth (neonates), 8 weeks old (adolescence), 16 weeks old (beginning of adulthood), and 24 weeks old when full adulthood is reached (Chen et al., 2010; Swindle et al., 2011). This is necessary in order to compare with the findings of Abou Alaiwa et al. (2014) that suggest the difference in pH between normal and CF subjects lessens over the course of a patient's life and to establish reliable pH values obtained at 37°C.

The procedure for this experiment might go as follows: Pigs are anesthetized according to the same protocol described previously for the transient ASL depth measurements, however it is key that the humidified chamber be at 37°C, as pH will increase in acidity as the temperature drops (Pezzulo et al., 2013). A simple reference electrode could be

used to measure nasal pH, while a dual-excitation wavelength pH indicator (Molecular Probes Inc.) could be used for tracheal pH measurements (Abou Alaiwa et al., 2014; Jayaraman et al., 2001). A tracheal window is made and the indicator applied as described in the previous ASL depth experiments (Jayaraman et al., 2001). A 20X objective lens is used to measure pH via fluorescent microscopy. If the pH of ASL in CF porcine models decreases in acidity with age, the initial defect in function that permits bacterial colonization is likely due the more acidic pH at birth, but at later stages, other components may exacerberate the condition and become more prevalent.

Conclusion

After an in depth analysis of the two conflicting hypotheses concerning the difference in the airway surface liquid and cystic fibrosis lung disease, this study proposes that neither the high salt hypothesis or the low volume hypothesis is accurate. According to recent research, the data more likely support a significant decrease, and thus acidity, in the pH of the airway surface liquid of CF patients.

The proposed experiments will further the study of airway surface liquid and etiology of CF lung disease as well as evaluate pH changes and whether transient ASL depth changes is what is defective in response to CFTR stimulation and ENaC inhibition. The lack of passive transport of bicarbonate ions seems to be a significant change in cystic fibrosis airway and may be an important future direction of CF research and treatment.

APPENDICES

Appendix A

Extended Methods

Cell Culture

- The University of Iowa

Epithelial cells were acquired from participants, isolated, grown in a humidified environment on collagen-coated, semi-permeable membranes at 37°C with a maintained air composition of 5% CO_2. Cell types used throughout the years were nasal, tracheal, and bronchial epithelial cells from both non-CF and CF participants. Smith, Karp, and Welsh used a 1:1 media mixture of Dulbecco.t ASL depth chanll medium and Hamed throughout the :F12) with 10 10 sed throughout the years were nasal, tracheal, and bronchial epithelial cells from both non-CF and CFreptomycin. In their 1994 study, a transepithelial resistance ≧ 900 stx cm^2 was reported for all epithelia (Smith et al., 1994).

In a 1996 study by Smith et al., primary cultures were established in a medium of 100 mU/ml penicillin with 100 0 tablished in a medium of 100 mU/ml penicillin with 10g/ml gentamicin, 40 µ 40 micin, 40 , 40 1010established , 125 cg/ml ceftazidime, and 2 µ andcfluconizole for 2 zole for 2 nd 2 0 tablished in a medium of 100 mU/ml penicillin with 100 lial celbasolateral side was replaced a total of five times with antibiotic-free media while the apical surface was rinsed with antibiotic-free phosphate-buffered Ringer's solution three times. The changing of media continued every 2-3 days until the

cells were used approximately 8-45 days after initial placement on membranes. The transepithelial resistance was >500 ibx cm^2 for all epithelia studied (Smith et al., 1996).

A 1:1 mixture of DME:F12 with 2% Ultroser G is generally used for cell culture studies now, including CF pig cultures, with cells studied a minimum of 14 days after they were placed on the membranes. A transepithelial resistance ≧ 800 gx cm^2 was the only reported resistance and was done for the measurement of liquid absorption and Cl- flux in the 1998 study by Zabner et al. (Zabner et al., 1998).

- University of North Carolina at Chapel Hill

Matsui et al. and later studies collected epithelial cells from the main stem or lobar bronchi of excess donor tissue at the time of lung transplantation and then seeded the cells on culture dishes in LHC Basal Media with multiple supplements. At the first passage of cells, the media was switched to a 50:50 mixture of LHC Basal Media and DMEMH. When confluency was reached, the media was replaced on the basolateral side while the apical surface was rinsed with phosphate buffered saline (PBS). Fully differentiated cells were used within 2 − 4 weeks after reaching confluence and while maintaining rotational mucus transport (Matsui et al., 1998).

Starting in a second study in 1998 by Matsui et al., bronchial epithelial cells were collected from both non-CF and CF participant tissue specimens and cultured in two separate manners. Planar cultures were grown on membranes in modified Bronchial Epithelial Cell Growth medium (BEGM) at the air-liquid interface in an incubator for

approximately four weeks when full differentiation occurred. Cells grown by means of planar culture techniques had transepithelial resistances > 300 Ω x cm^2. For tubular cultures, cells were placed in the lumen of small hollow tubes and placed in BEGM media for about 2 weeks until studied (Matsui et al., 1998).

ASL Collection and Measurement

- The University of Iowa

Airway surface liquid spiked with $^3H_20$ and ^{22}Na or ^{36}Cl was collected from nasal, tracheal, and bronchial epithelial cells in a 1998 study by Zabner et al.. In a humidified chamber, epithelial cells with approximately 500 lls in a 1998 study by Zabner ong other^4 cpm of $^3H_20$ and ^{22}Na or ^{36}Cl were sealed and supplied with $^3H_20$ labeled water for humidification. After a 48 hour incubation period, the apical surface was rinsed for 1 second with 100 tain cells until use which occurred 7 − 10 days after seedinged Bronchia$^3H_20$:^{22}Na and $^3H_20$:^{36}Cl in the collected ASL (Zabner et al., 1998).

In 2012, Lorentzen et al. anesthetized CF pig model specimens and accessed the trachea via cauterization to collect ASL samples. A probe with 2 nl mannitol with 300 ith 300 0 model specimens and accessed the trachea via cauterization to collect ASL samples. A probe with h 100 h hof media and then through liquid scintillation to determine rns blue dye (Lorentzen et al., 2012). Another method for ASL collection, to measure antimicrobial proteins in the CF pig model, stimulated tracheal secretion via methacholine and an incision in the anterior tracheal wall was made to swab for ASL

fluid. This technique reportedly collected approximately 10 − 20 µl of ASL when centrifuged. To measure [Na^+] and [K^+] in ASL, Pezzulo et al. fused thin lens paper and parafilm together via 70-90s in a 205℃ oven. The strips were washed three times with double distilled water and dried overnight in a 40℃ oven. After weighed, strips were placed onto the posterior tracheal surface for 15s and then weighed again, dried overnight, and added to 1 mL flame photometer standard solution to measure [Na^+] and [K^+] by a flame photometer (Pezzulo et al., 2012).

Nasal ASL was collected from human subjects by means of filter paper in 1996 by Smith et al.. A nose clip was applied to subjectssbjects by means of filter paper in 1996 by Smith nd then weighed again, dried overnight, and added to 1 mL flame phoral oil (Smith et al., 1996). Currently, micro-sampling probes are applied to the nasal turbinates for 30 es for 30 sampling probes are applied to the nasal turbinates for 30 by Smith nd then weighed again, dri. Similarly, About Alaiwa et al. measured nasal pH using a probe on the caudal aspect of the turbinate until a stable reading was established for 15 seconds (Abou Alaiwa et al., 2014).

- The University of North Carolina at Chapel Hill

Matsui et al. studied tracheobronchial epithelial ASL transport in a 1998 study. Cells were stained with a 10 uM calcein/AM for 15 minutes. Mucus was removed by a 10 mM dithiothreitol wash for 5 minutes followed by PBS. ELIZA verified efficacy of technique by analyzing mucus content. ASL was labelled through the addition of fluorescent latex

microspheres and/or with dextrans with Texax Red or to DMNB caged-fluorescein in 20 n 20 in 20 n in 2epifluorescence microscopy or scanning confocal microscopy was used in measurement of rotational mucus transport rate using the movement of photo-activated caged-fluorescein dextrans (Matsui et al., 1998).

Both planar and tubular human bronchial epithelial culture concentrations of Na^+ and Cl^- in ASl were measured by Matsui et al.. Planar cultures were rinsed three times with PBS, 200 re concentrations of Nacopy or scanning caken, and then the cultures were placed in a humidified incubator. After 24 hours, a constant-bore pipette was placed to the cell surface via a hole drilled into the top of the lid and a sample drawn. A stable measurement for 15s was required for the Cl^- selective electrode to obtain a $[Cl^-]$ in the planar culture. In the tubular culture, the biofiber was plugged at one end while the other was connected to a constant-bore capillary tube that contained Krebs Bicarbonate Ringer (KBR). The biofiber was then placed in KBR for 5 or 24 hours in an incubator at which time the luminal contents were removed under water-saturated mineral oil. To measure the sample, a capillary tube was used to draw up the contents. Samples from both planar and tubular cultures were placed on filter paper and the osmolality was measured using a vapor pressure osmometer. The length of the liquid in the capillary tube was utilized to measure the volume of the sample. Na^+ concentration was measured by flame emission spectroscopy for both planar and tubular samples, while amperometric titration was used for $[Cl^-]$ in tubular cultures (Matsui et al., 1998).

Chloride concentrations of anesthetized wild-type and $Cftr^{tm1Unc}$ CF mice (mixed strain) were measured by a micro-dialysis probe placed onto the nasal septum. The probed nostril was then sealed with a quick-drying silicon and ASL [Cl^-] was calculated using the zero net flux method. Approximately, 3-5 known solutions of Cl^- were supplied through probe at 0.5 net flux method. Approximately, 3-5 known solutions of Cltum. The probed nostril was chloridometer analyzed samples collected (Tarran et al., 2001).

Normal mouse ASL depth was measured in a strain of normal mice. C57BL/6 female mice were killed with Nembutal overdose and whole tracheas were removed from the carina to the cricoid cartilage. Each end was connected to air-filled capillary tubes and then extended to normal between-cartilage lengths. The capillaries were attached to a ventilator that provided the equivalent of 80 breath/min of room air and the whole set-up was placed in a chamber filled with PBS. X-ray images were taken of the vertical trachea prior to the treatment delivery and at 3 minute intervals up to a total of 18 minutes. Ten images were taken at each of the seven time points. ASL depth was computationally calculated by tracing the airway/ASL interface and ASL/tissue interface, then taking the mean distance between the two for each sample (Jayaraman et al., 2001).

In a 1997 paper by Knowles et al., normal and CF subjects' nasal and bronchial ASL concentrations of Na^+, Cl^-, and K^+ were measured. Filter paper was washed in double-distilled deionized water, dried overnight, and weighed right before used. The end of the filter paper was held in a "V" shape against the inferior turbinate for 20s in the nostril. In

the bronchial sample collection, 5 filter papers were held in forceps. All filter papers were weighed 15s after removal for both nasal and bronchial collection. For 90 seconds following collection, the paper was weighed and the data extrapolated to find the weight at time zero. To measure ion concentrations, filter papers and pans were placed in acid-washed vials along with 1 ml of double-distilled deionized water and placed in shaker for 24 hours. At that time, the mixture was transferred to a volumetric tube and 0.5 ml of 0.3 N HNO3 was added. The filter paper was wrung out several times, removed, and then the vial was filled to 2.0 ml with 0.2 N HNO3. A digital chloridometer was used to measure [Cl^-] and while [Na^+]and [K^+] were measured using a flame photometer (Knowles et al., 1997).

- Independent Laboratories

Joris et al. measured ASl ion concentrations in airways of subjects in the following categories; normal, sustained airway irritation (long-term intubation) , acute airway infection (>24 hr intubation with signs of infection), CF, and severe asthmatic patients after intubation. A fiberoptic bronchoscope was used to access the distal trachea and proximal stem bronchus and then a catheter with an ash less filter paper was held to mucosa for less than 5s and immediately immersed in water-saturated mineral oil at −20°C. To analyze [Cl^-], [Na^+], and [K^+] the samples were thawed and the papers compressed with a hemostat in the oil immersion. The fluid was collected by a micro-capillary pipette and 75 d was pl volumes were placed on thin film substrates with nickel grids alongside standards. All samples were desiccated for energy dispersive x-ray analysis (Joris et al., 1993).

Hull et al. utilized a similar method as stated above with infants diagnosed with CF. A catheter containing a nitrocellulose membrane was held to tracheal or nasal mucosa for 10s and then centrifuged to obtain samples for energy dispersive x-ray analysis (Hull et al., 1998).

In a 2003 paper by Kotaru et al., ASL from atopic asthmatic subjects was collected in the same manner as Knowles et al. previously mentioned above. The ASL samples from non-CF subjects were measured via freezing-point depression (Kotaru et al., 2003).

Animal Models

Mouse:

CF mouse models have not been found to display lung complications symptomatic in humans and therefore are typically not used in research involving the respiratory system. However, there is a CF-like mouse model that has been designed by the low volume hypothesis group at the University of North Carolina at Chapel Hill. Na^+ absorption rates were measured in transgenic mice with each of the three subunits of ENaC individually over expressed in an airway-specific manner (Mall et al., 2004).

Pig:

The CF pig model is the newest model and was designed by the high salt hypothesis group at the University of Iowa. All protocols involving the pig model were described previously.

Human Trials

- The University of North Carolina

Donaldson et al. in 2006 studied the effects of a 7% NaCl aerosol four times daily on 24 CF patients over the course of two weeks. Half the patients were given amiloride before treatment (1 mg/ml in 4.5 ml of 0.12% NaCl) and the other half a placebo. Changes in forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) along with mucus clearance and quality of life were measured throughout the study (Donaldson et al., 2006).

- Independent Laboratories

Another two week study on the effects of saline on 58 CF patients was done in 1996 prior to that of Donaldson et al.. Patients received a normal dose of 0.9% saline or a hypertonic 6.0% saline solution to inhale twice a day for 2 weeks. A nebulizer was used for dosing to inhale 10ml of the saline solution. Before each dose, 6 puff of salbutamol from a metered dose inhaler was taken. Measures used to track changes were FEV1 and FVC taken at the beginning and end of the study (Eng et al., 1996).

In a 1996 paper by Robinson et al., 12 CF patients were classified into three categories; mild (> 80% predicted FEV1), moderate (40 categories; mild (> 80% predicted FEV1), moderate (40 ice a day for 2 weeks. A nebulizer was used for dosing to inhale via spirometry for FEV1 and FVC and then a standardized dose of albuterol was inhaled. Afterwards, another set of spirometric measurements were taken and the patient then

inhaled a radio aerosol from a computerized breathing circuit for a transmission scan from a gamma camera. Baseline mucus clearance measurements were taken and spirometry values obtained once more. Next, either a 0.9% saline solution, amiloride, or 7.0% saline solution was inhaled followed by another scan, mucus clearance measurement, and spirometry measurements (Robinson et al., 1996).

Appendix B

Original CFTR Drawing

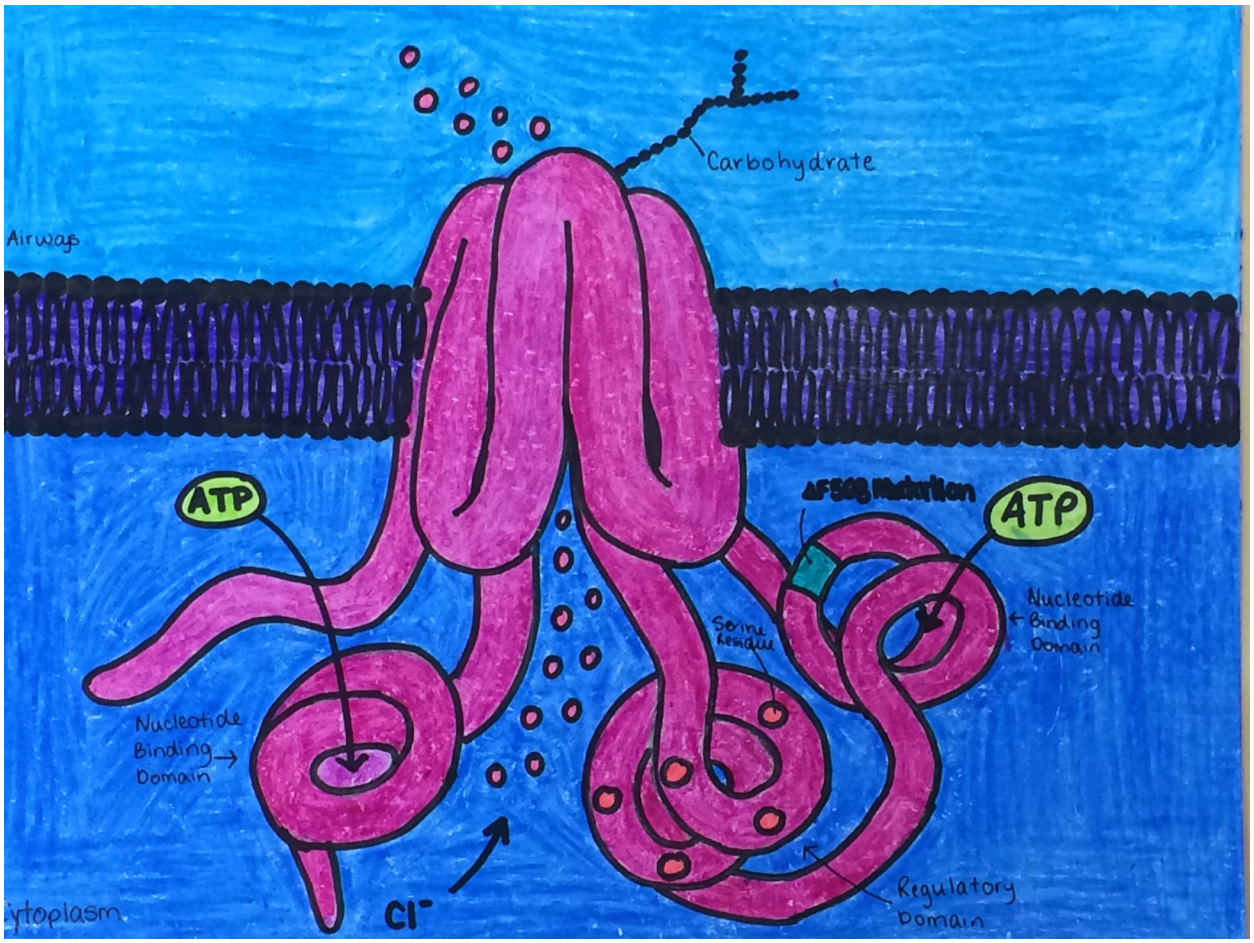

Figure 25: Original CFTR Drawing.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abou Alaiwa MH, Beer AM, Pezzulo AA, Launspach JL, Horan RA, Stoltz DA, Starner TD, Welsh MJ, Zabner J. Neonates with cystic fibrosis have a reduced nasal liquid pH; A small pilot study. *Journal of Cystic Fibrosis*. 2014; epub ahead of print.

Andersen DH. Celiac Syndrome III. *American Journal of Diseases of Children*. 1944; 70: 100 ; 70:

Anguiano A, Oates RD, Amos JA, Dean M, Gerrard B, Stewart C, Maher TA, White MB, Milunsky A. Congenital bilateral absence of the vas deferens. A primarily genital form of cystic fibrosis. *The Journal of American Medical Association*. 1992; 267(13): 1794 − 1797.

Armstrong DS, Grimwood K, Carzino R, Carlin JB, Olinsky A, Phelan PD. Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. *British Medical Journal*. 1995; 310(6994): 1571 − 1572.

Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell*. 1992; 67: 809 − 818.

Berger AL, Ikuma M, Welsh MJ. Normal gating of CFTR requires ATP binding to both nucleotide-binding domains and hydrolysis at the second nucleotide-binding domain. *Proceeding of the National Academy of Sciences of the United States of America*. 2005; 102(2): 455 − 460.

Boucher, R.C. Human airway ion transport. Part 1. *American Journal of Respiratory Critical Care Medicine*. 1994a; 150: 271–281.

Brennan AL, Geddes DM, Gyi KM, Baker EH. Clinical importance of cystic fibrosis-related diabetes. *Journal of Cystic Fibrosis*. 2004; 3: 209 − 222.

Canessa CM, Horisberger JD, Rossier BC. Expression cloning of the epithelial sodium channel. *Nature.* 1993; 361:4677ning

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC. Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature*. 1994; 367:463made

Chen JH, Stoltz DA, Karp PH, Ernst SE, Pezzulo AA, Moninger TO, Rector MV, Reznikov LR, Launspach JL, Chaloner K, Zabner J, Welsh MJ. Loss of anion transport without increased sodium absorption characterizes newborn porcine cystic fibrosis airway epithelia. Cell. 2010; 143(6): 911 elia.

Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ, Smith AE. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates CFTR chloride channel. *Cell*. 1991; 66: 1027 − 1036.

Cho DY, Hwang PH, Illek B, Fischer H. Acid and base secretion in freshly excised nasal tissue from cystic fibrosis patients with ΔF508 mutation. *International Forum of Allergy and Rhinology*. 2011; 1(2): 123 – 127.

Clarke LL, Grubb BR, Gabriel SE, Smithies O, Koller BH, Boucher RC. Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science.* 1992; 257:1125–1128.

Coakley RD, Grubb BR, Paradiso AM, Gatzy JT, Johnson LG, Kreda SM, O'Neal WK, Boucher RC. Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. *Proceedngs of the National Academy of Sciences of the USA*. 2003; 100(26): 16083 – 16088.

Colledge WH, Abella BS, Sother KW, Ratcliff R, Jian C, Cheng SH, MacVinish LJ, Anderson JR, Cuthbert AW, and Evan MJ. Generation and characterization of a ΔF508 cystic fibrosis mouse model. Nature Genetics. 1995; 10: 445 − 452.

Davidson DJ, Dorin JR, McLachlan F, Ranaldi V, Lamb D. Doherty C, Govan J, Porteous DJ. Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. Nature Genetics. 1995; 9: 351 − 357.

Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, Thomson SA, Hume DA, Lamb D, Porteous DJ, Dorin JR, Wainwright BJ. Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *EMBO Journal.* 1996; 15: 955–963.

Di Sant'Agnese PA, Powell GF. The eccrine sweat defect in cystic fibrosis of the pancreas (mucoviscidosis). *Annals of the New York Academy of Sciences*. 1962; 93: 555 − 599.

Dickinson P, Smith SN, Webb S, Kilanowski FM, Campbell IJ, Taylor MS, Porteous DJ, Willemsen R, de Jonge HR, Farley R, Alton EW, Dorin JR. The severe G480C cystic fibrosis mutation, when replicated in the mouse, demonstrates mistrafficking, normal survival and organ-specific bioelectrics. *Human Molecular Genetics.* 2002; 11: 243–251.

Dodge JA, Lewis PA, Stanton M, Wilsher J. Cystic fibrosis mortality and survival in the UK: 1947-2003. *European Respiratory Journal*. 2007; 29(3): 522 − 526.

Donaldson SH, Bennett WD, Zeman KL, Knowles MR, Tarran R, Boucher RC. Mucus clearance and lung function in cystic fibrosis with hypertonic saline. *New England Journal of Medicine*. 2006; 354 (3): 241 f Me

Dorin JR, Dickinson P, Emslie E, Clarke AR, Dobbie L, Hooper ML, Halford S, Wainwright BJ, Porteous DJ. Successful targeting of the mouse cystic fibrosis transmembrane conductance regulator gene in embryonal stem cells. *Transgenic Research.* 1992; 1:101–105.

Dorin JR, Dickinson P, Alton EW, Smith SN, Geddes DM, Stevenson BJ, Kimber WL, Fleming S, Clarke AR, Hooper ML. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature.* 1992; 359: 211–215.

Eckman EA, Cotton CU, Kube DM, Davis PB. Dietary changes improve survival of CFTR S489X homozygous mutant mouse. *American Journal of Physiology.* 1995; 269: L625–L630.

Edenborough FP. Women with cystic fibrosis and their potential for reproduction. *Thora*x. 2001; 56: 649 − 655.

Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatric Pulmonology.* 2002; 34: 91-100.

Emrich HM, Stoll E, Friolet B, Colombo JP, Richterich R, Rossi E. Sweat composition in relation to rate of sweating in patients with cystic fibrosis of the pancreas. *Pediatric Research*. 1968; 2: 464 − 478.

Eng PA, Morton J, Douglass JA, Riedler J, Wilson J, Robertson CF. Short-term efficacy of ultrasonically nebulized hypertonic saline in cystic fibrosis. *Pediatric Pulmonology*. 1996; 21: 77 7 nol

Gershman AJ, Mehta AC, Infeld M, Budev MM. Cystic fibrosis in adults: an overview for the internist. *Cleveland Clinical Journal of Medicine*. 2006; 73(12): 1065 urnal

Gilljam H, Ellin A, Strandvik B. Increased bronchial chloride concentration in cystic fibrosis. *Scandinavian Journal of Clinical Laboratory Investigation*. 1989; 49: 121 − 124.

Grubb BR, Paradiso AM, Boucher RC. Anomalies in ion transport in CF mouse tracheal epithelium. *American Journal of Physiology*. 1994a; 267: C293—300.

Grubb BR, Vick RN, Boucher RC. Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice. *American Journal of Physiology*. 1994b; 266: C1478—1483.

Guggino WB, Stanton BA. New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nature Reviews Molecular Cell Biology*. 2006; 7: 426 -436.

Haack A, Aragao GG, Novae MR. Pathophysiology of cystic fibrosis and drugs used in associated digestive tract diseases. *World Journal of Gastroenterology*. 2013; 19(46): 8552 − 8561.

Harvey PR, Tarran R, Garoff S, Myerburg MM. Measurement of the airway surface liquid volume with simple light refraction microscopy. *American Journal of Respiratory Cell Molecular Biology*. 2011; 45: 592 – 599.

Hasty P, O'Neal WK, Liu KQ, Morris AP, Bebok Z, Shumyatsky GB, Jilling T, Sorscher EJ, Bradley A, Beaudet al.. Severe phenotype in mice with termination mutation in exon 2 of cystic fibrosis gene. *Somatic Cell Molecular Genetics.* 1995; 21:177–187.

Hollingsworth MA, Swanson BJ. Mucins in cancer: Protection and control of the cell surface. *Nature Review Cancer*. 2004; 4: 45 − 60.

Hull J, Skinner W, Robertson C, Phelan P. Elemental content of airway surface liquid from infants with cystic fibrosis. *American Journal of Respiratory Critical Care Medicine*. 1998; 157: 10 of

Jayaraman S, Song Y, Verkman AS. Airway surface liquid pH in well-differentiated airway epithelial cell cultures and mouse trachea. *American Journal of Cell Physiology*. 2001; 281: C1504 – C1511.

Jiang C, Finkbeiner WE, Widdicombe JH, McCray PB, Miller SS, Altered fluid transport across airway epithelium in cystic fibrosis. *Science*. 1993; 262: 424 4 thel

Jin R, Hodges CA, Drumm ML, Palmert MR. The cystic fibrosis transmembrane conductance regulator (Cftr) modulates the timing of puberty in mice. *Journal of Medical Genetics*. 2006; 43(6): 1 ; 43

Joris L, Dab I, Quinton PM. Elemental composition of human airway surface fluid in healthy and diseased airways. *American Review of Respiratory Disease*. 1993; 148: 1633 − 1637.

Kent G, Iles R, Bear CE, Huan LJ, Griesenbach U, McKerlie C, Frndova H, Ackerley C, Gosselin D, Radzioch D, O'Brodovich H, Tsui LC, Buchwald M, Tanswell AK. Lung disease in mice with cystic fibrosis. *Journal of Clinical Investigation.* 1997; 100:3060–3069.

Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC. Identification of the cystic fibrosis gene: Genetic analysis. *Science*. 1989; 245: 1073–1080.

Knowles MR, Robinson JM, Wood RE, Pue CA, Mentz WM, Wagner GC, Gatzy JT, Boucher RC. Ion composition of airway surface liquid of patients with cystic fibrosis as compared with normal and disease-control subjects. *Journal of Clinical Investigation*. 1997; 100: 2588 −2595.

Kopito LE, Kosasky HJ, Shwachman H. Water and electrolytes in cervical mucus from patients with cystic fibrosis. *Fertility and Sterility*. 1973; 24:512 − 516.

Koscik RL, Farrell PM, Kosorok MR, Zaremba KM, Laxova A, Lai HC, Douglas JA, Rock MJ, Splaingard ML. Cognitive function of children with cystic fibrosis: deleterious effect of early malnutrition. *Pediatrics*. 2004; 113: 1549 − 1558.

Kosorok MR, Wen-Hsiang W, Farrell PM. The incidence of cystic fibrosis. *Statistics in Medicine.* 1996; 15: 449 − 462.

Kotaru C, Hejal RB, Finigan JH, Coreno AJ, Skowronski ME, Brianas L, McFadden ER. Desiccation and hypertonicity of the airway surface fluid and thermally induced asthma. *Journal of Applied Physiology*. 2003; 94: 227 ed P

Lingueglia E, Voilley N, Waldmann R, Lazdunski M, Barbry P. FEBS Lett 1993;318:95–ingueglia E, V. Physiological basis of cystic fibrosis: a historical perspective. *Physiological Review*s. 1999; 79: S3 - S22.

Lissens W, Mercier B, Tournaye H, Bonduelle M, Férec C, Seneca S, Devroey P, Silber S, Van Steirteghem A, Liebaers I. Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. *Human Reproduction*. 1996; 11(4): 55 − 80.

Lobeck CC, McSherry NR. The ionic composition of pilocarpine induced sweat in relation to gland output during aging and cystic fibrosis. *Modern Problems in Pediatrics*. 1967: 41 − 53.

Lorentzen D, Durairaj L, Pezzulo AA, Nakano Y, Launspach J, Stoltz DA, Zamba G, McCray PB, Zabner J, Welsh MJ, Nauseef WM, BáWM, Bf WM, B Nauseef of the antibacterial precursor thiocyanate in cystic fibrosis airway secretions. *Free Radical Biology and Medicine*. 2011; 50 (9): 1144 4 and M

Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. *Nature Medicine*. 2004; 10: 487 − 493.

Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, Boucher RC. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell*. 1998; 95: 1005 − 1015.

McCray PB, Zabner J, Jia HP, Welsh MJ, Thorne PS. Efficient killing of inhaled bacteria in DeltaF508 mice: role of airway surface liquid composition. *American Journal of Physiology*. 1999; 277(1) L183 – L190.

Meyerholz DK, Stoltz DA, Pezzulo AA, Welsh MJ. Pathology of gastrointestinal organs in a porcine model of cystic fibrosis. *American Journal of Pathology*. 2010; 176(3): 1377 – 1389.

Morgan KS, Donnelley M, Paganin DM, Fouras A, Yagi N, Suzuki Y, Takeuchi A, Uesugi K, Boucher RC, Parsons DW, Siu KKW. Measuring airway surface liquid depth in *ex vivo* mouse airways by x-ray imaging for the assessment of cystic fibrosis airway therapies. *PLOS One*. 2013; 8 (1): [online].

Morgenroth K, Bolz J. Morphological features of the interaction between mucus and surfactant on the bronchial mucosa. *Respiration*. 1985; 47(3): 225 − 231.

Morral N, Bertranpetit J, Estivill X, Nunes V, Casals T, Giménez J, Reis A, Varon-Mateeva R, Macek M Jr, Kalaydjieva L, Angelicheva D, Dancheva R, Romeo G, Russo MP, Garnerone S, Restagno G, Ferrari M, Magnani C, Claustres M, Desgeorges M, Schwartz M, Schwarz M, Dallapiccola B, Novelli G, Ferec C, de Arce M, Nemeti M, Kere J, Anvret M, Dahl N, Kadasi L. The origin of the major cystic fibrosis mutation (delta F508) in European populations. *Nature Genetics*. 1994; 7(2): 169 − 175.

O'Neal WK, Hasty P, McCray Jr. PB, casey B, Rivera-Perez J, Welsh MJ, Beaudet al., Bradley A. A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Human Molecular Genetics*. 1993; 2: 1561 − 1569.

Oppenheimer EA, Case AL, Esterly JR, Rothberg RM. Cervical mucus in cystic fibrosis: a possible cause of infertility. *American Journal of Obstetrics and Gynecology*. 1970; 108: 673 − 674.

Ostedgaard LS, Meyerholz DK, Chen JH, Pezzulo AA, Karp PH, Rokhlina T, Ernst SE, Hanfland RA, Reznikov LR, Ludwig PS, Rogan MP, Davis GJ, Dohrn CL, Wohlford-Lenane C, Taft PJ, Rector MV, Hornick E, Nassar BS, Samuel M, Zhang Y, Richeter SS, Uc A, Shilyansky J, Prather RS, McCray PB, Zabner J, Welsh MJ, Stoltz DA. The ΔF508 mutation causes CFTR misprocessing and cystic fibrosis-like disease in pigs. *Science Translational Medicine*. 2011; 3(74): 1 – 24.

Panagopoulou P, Maria F, Nikolaou A, Nousia-Arvanitakis S. Prevalence of malnutrition and obesity among cystic fibrosis patients. *Pediatrics International*. 2013; Epub ahead of print [PMID: 24003895 DOI:10.1111/ped.12214]

Park RW, Grand RJ. Gastrointestinal manifestations of cystic fibrosis: a review. *Gastroenterology*. 1981; 81: 1143 − 1161.

Penketh AR, Wise A, Mearns MB, Hodson ME, Batten JC. Cystic fibrosis in adolescents and adults. *Thorax*. 1987; 42(7): 526 − 532.

Peterson T, Høiby N, Mordhorst CH, Lind K, Flensborg EW, Bruun B. Respiratory infections in cystic fibrosis patients caused by virus, chlamydia and mycoplasma - possible synergism with pseudomonas aeruginosa. *Acta Paediatrica Scandinavica*. 1981; 70: 623 − 628.

Pezzulo, AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M, Bánfi B, Horswill AR, Stoltz DA, McCray PB Jr, Welsh MJ, Zabner J. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature*. 2012; 487(7405): 109 − 113.

Ponting CP, Phillips C, Davies KE, Blake DJ. PDZ domains: targeting signalling molecules to sub-membranous sites. *Bioessays.* 1997; 19(6): 469–479.

Plummer A, Wildman M. Duration of intravenous antibiotic therapy in people with cystic fibrosis. *The Cochrane Group*. 2013; [online].

Quinton, PM. Physiological basis of cystic fibrosis: a historical perspective. *Physiological Review*. 1999; 79: S3 – S22.

Ratcliff R, Evans MJ, Cuthbert AW, MacVinish LJ, Foster D, Anderson JR, Colledge WH. Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nature Genetics.* 1993; 4: 35–41.

Reddy MM, Bell CL, Quinton PM. Cystic fibrosis affects specific cell type in sweat gland secretory coil. *American Journal of Physiology*. 1997; 273(2 Pt 1): C426 − 433.

Reis FJ, Damaceno N. [Cystic fibrosis]. *Journal of Pediatrics (Rio J)*. 1998; 74 Suppl 1: S76 - S94.

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al.. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*. 1989; 245: 1066 − 1073.

Robinson M, Regnis JA, Bailey DL, King M, Bautovich GJ, Bye PTP. Effect of hypertonic saline, amiloride, and cough on mucociliary clearance in patients with cystic fibrosis. *American Journal of Respiratory Critical Care Medicine*. 1996; 153: 1503 − 1509.

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB, Zabner J, Prather RS, Welsh MJ. *Science*. 2008; 321(5897): 1837 – 1841.

Rowe SM, Miller S, Sorcher EJ. Mechanisms of disease: cystic fibrosis. *New England Journal of Medicine*. 2005; 352(19): 1992 − 2001.

Rozmahel R, Wilschanski M, Matin A, Plyte S, Oliver M, Auerbach W, Moore A, Forstner J, Durie P, Nadeau J, Bear C, Tsui LC. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nature Genetics.* 1996; 12: 280–287.

Shawker TH, Hubbard VS, Reichart CM, Guerreiro de Matos OM.. Cystic ovaries in cystic fibrosis: an ultrasound and autopsy study. *Journal of Ultrasound Medicin*e. 1983; 2: 439 − 444.

Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Strutts MJ, Milgram SL. An apical PDZ protein anchors the cystic fibrosis transmembrane

conductance regulator to the cytoskeleton. *Journal of Biological Chemistry*. 1998; 273: 19797 − 19801.

Shwachman H, Mahmoodian A. Pilocarpine iontophoresis sweat testing results of seven years' experience. *Bibliotheca Paediatrica*. 1967; 86: 158 − 182.

Smith JJ, Karp PH, Welsh MJ. Defective fluid transport by cystic fibrosis epithelia. *Journal of Clinical Investigation*. 1994; 93: 1307 − 1311.

Smith JJ, Travis SM, Greenberg EP, Welsh MJ. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface liquid. *Cell*. 1996; 85: 229 − 236.

Snouwaert JN, Brigman KK, Latour AM, Malouf NN, Boucher RC, Smithies O, Koller BH. An animal model for cystic fibrosis made by gene targeting. *Science.* 1992; 257:1083–1088.

Song Y, Thiagarajah J, Verkman AS. Sodium and chloride concentrations, pH, and depth of airway surface liquid in distal airways. *Journal of General Physiology*. 2003; 122: 511 – 519.

Song Y, Salinas D, Nielson DW, Verkman AS. Hyperacidity of secreted fluid from submucosal glands in early cystic fibrosis. *American Journal Physiology – Cell Physiology*. 2006; 290: C741 – C749.

Song Y, Namkung W, Nielson DW, Lee JW, Finkbeiner WE, Verkman AS. Airway surface liquid depth measured in *ex vivo* fragments of pig and human trachea: dependence on Na^+ and Cl^- channel function. *American Journal of Lung Cell Molecular Physiology*. 2009; 297(6): L1131 – L1140.

Stewart CE, Torr EE, Jamili NHM, Bosquillon C, and Sayers I. Evaluation of differentiated human bronchial epithelial cell culture systems for asthma research. *Journal of Allergy*. 2012; 2012: 1 − 11.

Stoltz DA, Rokhlina T, Ernst SE, Pezzulo AA, Ostedgaard LS, Karp PH, Samuel MS, Reznikov LR, Rector MV, Gansemer ND, Bouzek DC, Abou Alaiwa MH, Hoegger MJ, Ludwig PS, Taft PJ, Wallen TJ, Wohlford-Lenane C, McMenimen JD, Chen JH, Bogan KL, Adam RJ, Hornick EE, Nelson GA, Hoffman EA, Chang EH, Zabner J, McCray PB, Prather RS, Meyerholz DK, Welsh MJ. Intestinal CFTR expression alleviates meconium ileus in cystic fibrosis pigs. *Journal of Clinical Investigation*. 2013; 123(6): 2685 – 2693.

Stutts MJ, Rossier BC, Boucher RC. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel single channel kinetics. *Journal of Biological Chemistry*. 1997; 272: 14037 − 14040.

Tarran R, Grubb BR, Parsons D, Picher M, Hirsh AJ, Davis CW, Boucher RC. The CF salt controversy: *in vivo* observations and therapeutic approaches. *Molecular Cell*. 2001; 8: 149 − 158.

Tizzano EF, Buchwald M. CFTR expression and organ damage in cystic fibrosis. *Annals of Internal Medicine*. 1995; 123: 305 − 308.

Tizzano EF, Silver MM, Chitayat D, Benichou JC, Buchwald M. Differential cellular expression in cystic fibrosis transmembrane regulator in human reproductive tissues: Clues for the infertility in patients with cystic fibrosis. *American Journal of Pathology*. 1994; 144: 906 − 914.

van Doorninck JH, French PJ, Verbeek E, Peters RH, Morreau H, Bijman J, Scholte BJ. A mouse model for the cystic fibrosis delta F508 mutation. *EMBO Journal.* 1995; 14: 4403–4411.

Wanner A, Salathe M, O'Riordan TG. Mucociliary clearance in the airways [State of the Art]. *American Journal of Respiratory Critical Care Medicine*. 1996; 154: 1868 − 1902.

Wat D. Impact of respiratory viral infections on cystic fibrosis. *Postgraduate Medical Journal*. 2003; 79(930): 201 – 203.

Welsh MJ and Smith JJ. cAMP stimulation of HCO_3^- secretion across airway epithelia. J Pancreas. 2001; 2:291-293.

Widdicombe JH. Airway surface liquid: concepts and measurements. In airway mucus: basic mechanisms and clinical perspectives. (Basel : Birkhauser) 1997; 1 − 17.

Widdicombe JH, Bastacky SJ, Wu DXY, Lee CY. Regulation of the depth and composition of airway surface liquid. *Journal of Anatomy*. 2002; 201(4): 313 − 318.

Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. Reference range data base for serum chemistry and hematology values in laboratory animals. *Journal of Toxicology and Environmental Health*. 1986; 18: 161 − 188.

Zabner J, Smith JJ, Karp PH, Widdicombe JH, Welsh MJ. Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia in vitro. *Molecular Cell*. 1998; 2: 397 − 403.

Zeihar BG, Eichwald E, Zabner J, Smith JJ, Puga AP, McCray Jr. PB, Capecchi MR, Welsh MJ, and Thomas KR. A mouse model for the ΔF508 allele of cystic fibrosis. *Journal of Clinical Investigation*. 1995; 96: 2051 − 2064.