ASSOCIATION OF GENETIC VARIATION AND EXERCISE CAPACITY WITH CLINICAL INDICATORS OF DISEASE IN CYSTIC FIBROSIS

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Abstract

Introduction

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), which results in ion dysregulation and mucous buildup, most notably in the lungs. Previously a childhood disease, advancement in treatment options has greatly improved clinical course leading to longer lifespans. This progress has created a need for more sensitive clinical measures and personalized medicine options. Exploring genetic modifiers of disease along with response to exercise would provide valuable and unique information, contributing to better, more personalized assessment and management of disease.

Purpose

The purpose of this dissertation is two-fold. The first aim was to explore the impact of genetic variation at amino acid 663 of the sodium channel epithelial 1 alpha gene (SCNN1A) on clinical features of CF. In Study One it was hypothesized that subjects with at least one copy of the gain-of-function variant T663 (AT/TT) would have poorer clinical outcomes than those homozygous for the wild-type variant A663 (AA). In studies Two and Three the primary aim was to examine the clinical value of the six-minute walk test (6MWT) and one-minute sit-to-stand test (1STS) in the management of CF. It was hypothesized that response to the 6MWT and 1STS would be strongly correlated and measures from each test would be associated with clinical outcomes of disease.

Methods

Thirty-five CF subjects were enrolled and all had at least one copy of the F508del mutation. Buccal swabs were collected and samples were analyzed for genetic variation at position 663 of the SCNN1A gene (AA, AT/TT). The 6MWT and 1STS were performed. Continuous monitoring of heart rate (HR) and peripheral blood oxygen saturation (SpO_2) was taken during exercise testing. The desaturation-distance ratio (DDR) was calculated using SpO_2 measures and six-minute walk distance (6MWD). Medical charts were reviewed for pulmonary function and indicators of disease status. Depending on distribution of data, independent Student's t-test or Mann Whitney U test was used to compare means. Correlations were performed using Spearman test and Fisher's exact test was used to analyze categorical variables. Cox regression was used to assess days to

pulmonary exacerbation. All data are presented as mean ± standard deviation unless otherwise noted. Significance was set at 0.05.

Results

There were no statistically significant differences in clinical outcomes between the AA and AT/TT genotypes. Clinically relevant observations in regards to lung function over time and exercise performance were noted and warrant further research. Further, 6MWD and 1STS repetitions were significantly correlated but neither outcome correlated with measures of pulmonary function. However, DDR was significantly correlated with several measures of pulmonary function, suggesting it is a better indicator of lung function than 6MWD alone. Additionally, those who desaturated during the 1STS (change in SpO_2 > 4% from rest) had significantly lower lung function compared to those who did not. Neither 6MWD nor 1STS repetitions was associated with pulmonary exacerbation during follow-up. Those who experienced a pulmonary exacerbation during follow-up had significantly greater DDR compared to those who did not have an exacerbation.

Conclusions

Variation at position 663 of the SCNN1A gene may modify pulmonary disease in patients with CF, though further research is needed. Additionally, the 6MWT and 1STS show promise in providing unique and meaningful information about CF disease and, used in conjunction with typical 6MWT outcomes, DDR may be a helpful tool in evaluating exercise capacity in patients with CF.

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List of Abbreviations

1STS	One-minute sit-to-stand test
6MWD	Six-minute walk distance
6MWT	Six-minute walk test
AIx	Augmentation index
AMP	Adenosine monophosphate
ANO1	Anoctamin 1
ANO2	Anoctamin 2
ASIC	Acid-sensing ion channels
ASL	Airway surface liquid
ATP	Adenosine triphosphate
β_2AR	Beta-2-adrenergic receptor
BAL	Bronchoalveolar lavage
BMI	Body-mass index
CaCC	Calcium dependent chloride channel
cAMP	Cyclic adenosine monophosphate
CAP	Channel-activating protease
CF	Cystic Fibrosis
CFRD	CF-related diabetes
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
COPD	Chronic Obstructive Lung Disease
CPET	Cardiopulmonary exercise test
CVD	Cardiovascular disease
DA	Desaturation area
DDR	Desaturation-distance ratio
DL_{CO}	Diffusing capacity of the lungs for carbon monoxide
ENaC	Epithelial Sodium Channel
F508del	Primary genetic mutation causing Cystic Fibrosis
$FEF_{25-75\%}$	Forced expiratory flow 25 to 75% of forced vital capacity
FEV_1	Forced expiratory volume in one second
FEV_1/FVC	Ratio of forced expiratory volume in one second to forced vital capacity
FVC	Forced vital capacity
HR	Heart rate

IL-1β	Interleukin 1 beta
LMM	Linear mixed model
LV	Left ventricle of heart
MAPK	Mitogen-activated protein kinases
MDR	Multi-drug resistant
mRNA	Messenger ribonucleic acid
NBD1	Nuclear binding domain one
NBD2	Nuclear binding domain two
NF_kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKCC1	$Na^+K^+2Cl^-$ cotransporter
PCL	Periciliary Layer
PHA1	Pseudohypoaldosteronism TypeI
PIP_2	Phosphatidylinositol 4,5 bisphoaphate
PIP_3	Phosphatidylinositol 3,4,5, triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
RV	Right ventricular of heart
SCNN1A	Sodium channel epithelial 1 alpha gene
SCNN1B	Sodium channel epithelial 1 beta gene
SCNN1G	Sodium channel epithelial 1 gamma gene
SGK1	Serum glucocorticoid regulated kinase 1
SLC26	Solute Carrier – 26
SNP	Single nucleotide polymorphism
SPLUNC1	Short-palate lung and nasal epithelial clone 1
SpO_2	Peripheral blood oxygen saturation
STAS	Sulphate transporter and anti-sigma factor antagonist
SV	Stroke volume
TMEM16A	Transmembrane member 16A
TMEM16B	Transmembrane member 16B
TNF-α	Tumor necrosis factor alpha
VCO_2	Volume of exhaled carbon dioxide
VE/VCO_2	Ventilatory efficiency

Chapter 1: Introduction

Cystic Fibrosis (CF) is an autosomal recessive disease that occurs in approximately 1 in 3,400 live births in those of Northern European descent [1] and is the most lethal genetic disease among Caucasians [2]. CF arises from a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes the CFTR protein [3-5]. The CFTR protein functions primarily as a chloride channel and is found in specialized epithelial tissue throughout the body, including the lungs, pancreas, and gastrointestinal tract [6, 7]. There are over 2,000 identified CF-causing mutations of CFTR with the most common involving a deletion of phenylalanine at residue 508 (F508del), which causes degradation of mutant CFTR in the endoplasmic reticulum [8, 9]. With effectively no CFTR translocated to the plasma membrane cellular processes, including ion regulation, innate defense, and inflammatory signaling, are severely disrupted. This leads to one of the hallmark feature of CF; lung disease associated with sticky mucous build-up in the respiratory tract that leads to chronic bacterial infection and pulmonary exacerbation [10].

Airway epithelial are pseudostratified ciliated cells that encourage movement of mucus and fluid across the respiratory tract and aid in providing basic defense against bacterial infection [11]. In addition to chloride transport, CFTR acts as an inhibitor of the epithelial sodium channel (ENaC), helping to regulate sodium absorption into the cell [12, 13]. In CF, this inhibition of ENaC is absent, resulting hyper-absorption of sodium into the epithelia [14, 15]. Hyper-absorption of sodium has been shown to cause CF-like disease in transgenic mice overexpressing ENaC, with depleted airway surface liquid (ASL) volumes, increased mucous amount and viscosity, and decreased fluid clearance being experienced [16]. This inadequate hydration of the lung causes impairments in mucociliary clearance leading to recurrent infections, chronic inflammation, airway obstruction, and eventual respiratory failure.

ENaC is a heterologous protein containing an alpha, beta and gamma subunit [17, 18]. Although all three subunits are important for optimal protein function, the alpha subunit exhibits the greatest attenuation in channel activity as demonstrated in alpha-ENaC-knockout mice who die of respiratory distress within 40 hours of birth due to their inability to clear amniotic fluid from their lungs [19]. In the lungs, ENaC is located on the apical membrane of both type-I and type-II alveolar cells and is the rate-limiting step in epithelial sodium absorption [20]. A key player in lung fluid clearance, disruption in the amount of ENaC in mice using RNA interference technology was shown to reduce basal lung fluid clearance by 30% [21, 22]. Although ENaC is a principal player in CF, there is minimal research focused on how genetic variation of the channel may impact clinical outcomes and disease progression.

Genetic mutation in ENaC is associated with several disease states including Pseudohypoaldosteronism Type I (PHA1), which causes impaired salt reabsorption in the distal nephron of the kidney and subsequent hypotension [23] and Liddle Syndrome, a gain-of-function mutation that causes salt-sensitive hypertension due to increased open channel probability [24]. Mutations in ENaC have also been linked to CF-like disease, with several reported cases of rare ENaC variants identified in patients with CF-like disease that present with no CFTR mutations [25-28]. Further, the mouse model of CF lung pathology is achieved by over-expression of the beta subunit of ENaC [29]. A common single nucleotide polymorphism (SNP) in ENaC involves a G → A at position 663 of the SCNN1A gene, which encodes for the alpha subunit. This SNP results in an alanine (A663) to threonine (T663) substitution. Cell studies have demonstrated the T663 variant has greater channel activity with higher rates of sodium absorption and fluid clearance [30-32]. Clinically, some studies, but not all, have found that individuals who possess the T663 variant have greater incidence of high blood pressure [33, 34] and differ in lung function and exercise response compared to those who do not possess the variant [35, 36]. However, it is unclear how this SNP may impact clinical outcomes in those with CF. One small study observed that CF patients with at least one copy of T663 had significantly lower body weight, body mass index, and baseline pulmonary function than those homozygous for A663 [37]. This study was limited by its cross-sectional design and restricted examination of clinical parameters. It may be that the presence of the more active T663 variant leads to heightened sodium hyper-absorption in the CF lung, further exacerbating and accelerating disease processes. Given these suggestive, but limited findings, it is of interest to better understand how genetic variation in the alpha subunit of ENaC impacts disease phenotype in CF.

With improvements in screening techniques and medical care, along with pharmacological advancements the average lifespan of CF patients continues to extend. This has led to more years with minimal lung disease progression and development of more extra-pulmonary complications, creating an urgency to adopt more sensitive and holistic clinical tests. Aerobic capacity is a strong and independent predictor of all-cause and disease-specific mortality [38]. This observation has been reproduced using a wide variety of testing methods, supporting the robustness of this finding. CF patients have impaired aerobic [39] and anaerobic capacity [40], with exercise capacity being correlated with lung function in some studies. Peak aerobic capacity is a strong predictor of survival in CF patients [41] and is an outcome used in predicting mortality in CF patients awaiting lung transplant [42]. It has greater prognostic value overtime than the commonly used clinical marker

of lung function decline [43]. Previous research has demonstrated that certain classes of CFTR mutations are associated with lower aerobic capacity and more severe clinical presentation, showing a link between genotype and clinical outcome [44]. Although maximal cardiopulmonary exercise testing (CPET) is the gold-standard for measuring aerobic capacity, it is time consuming and requires special equipment and personnel. Submaximal field tests, such as the six minute walk test (6MWT) and one-minute sit-to-stand test (1STS), have been correlated with maximal exercise tests in healthy and CF patients [45-47] and do not require any special equipment. Results from submaximal tests can provide useful clinical data beyond that obtained from lung function tests, especially in younger, healthier CF patients. Previous research, looking at exercise testing in pediatric and adult CF patients have suggested that outcomes from exercise testing, notably peripheral blood oxygen saturation (SpO_2) , can provide useful clinical information in addition to pulmonary function testing [48-52]. As exercise testing is a known prognostic in CF, providing insight into disease beyond traditional clinical measures, it is of interest to further explore exercise response and its association with clinical outcomes in patients with CF.

Given the continued advancements in the management of CF, disease presentation and course are vastly different from that described in the literature just decades ago. Greater sophistication in pharmacological interventions, along with improved screening techniques has resulted in longer lifespan and more years of non-progressive lung disease. These exciting developments in the field have created the need to develop more personalized and sensitive clinical assessment and management options. This dissertation aims to explore the association of genetic variation and exercise response with clinical outcomes in CF to better understand how these variables could improve monitoring and medical care provided to patients.

Aims and Hypotheses

Study One: Impact of Genetic Variation of Alpha ENaC on Clinical Presentation in Cystic Fibrosis

Specific Aim: Assess differences in clinical outcomes between those homozygous for the A663 allele (AA) of the SCNN1A gene and those with at least one copy of the T663 allele (AT/TT) in CF subjects.

Hypothesis: Subjects with at least one copy of the more active T663 variant (AT/TT) will have poorer clinical outcomes than those who are homozygous for the A663 allele (AA), as heightened ENaC activity would enhance sodium hyper-absorption and accelerate lung dehydration.

Study Two: Correlation between Six-Minute Walk Test and One Minute Sit-to-Stand Test and Clinical Outcomes in Cystic Fibrosis

Specific Aim One: Examine the relationship between physiological parameters and outcomes collected during the 6MWT to those collected during the 1STS in CF subjects.

Hypothesis: Physiological parameters and outcomes collected during the 6MWT will be positively correlated with those of the 1STS.

Specific Aim Two: Assess the association of the 6MWT and 1STS with pulmonary exacerbation during follow-up.

Hypothesis: Outcomes from both the 6MWT and 1STS will be associated with pulmonary exacerbation during follow-up.

Study Three: Evaluation of the Desaturation-Distance Ratio in Cystic Fibrosis Patients

Specific Aim: Evaluate the association of the desaturation-distance ratio (DDR) to clinical measures in CF subjects.

Hypothesis: DDR will be significantly associated with clinical measures in CF subjects.

Outline of Dissertation

Chapter one provides an overview of the dissertation and introduces specific aims and hypotheses.

Chapter two provides an in-depth review of the current literature regarding pathophysiology and exercise testing in CF. Information from cell, animal, and human studies is discussed.

Chapter three reports the findings of Study One, which examines the association of genetic variation in the SCNN1A gene and clinical outcomes in patients with CF.

Chapter four reports the findings of Study Two, which correlates and compares outcomes obtained from the 6MWT and 1STS. The association of these measures with clinical outcomes is also explored.

Chapter five reports the findings of Study Three, which examines the association of DDR, a unique index obtained from measurements taken during the 6MWT, with clinical outcomes.

Chapter six discusses study limitations.

Chapter seven provides an overall summary of relevant findings, concluding remarks, and future directions in the study of genetic modifiers of disease and exercise testing in CF.

Chapter eight provides a list of all references used in the dissertation.

Chapter nine provides a copy of study consent, parental permission, assent, and data collection form.

Chapter 2: Review of Literature

Overview of Cystic Fibrosis

First described in 1938 [53], Cystic Fibrosis (CF) is an autosomal recessive genetic disease that upsets chloride and sodium balance in epithelial tissue, most notably in the lungs, due to dysfunctional Cystic Fibrosis transmembrane regulator (CFTR) protein [6, 54]. Mutation of CFTR can result in a build-up of mucus in epithelial tissues which increases risk of infection, promotes inflammation, leads to nutrient malabsorption, and causes male infertility and pancreatic insufficiency [55]. CF is recognized as the most common fatal genetic disease among those of Northern European decent [1, 56], with an estimated occurrence of 1 in every 3,200 – 3,400 live births [57, 58]. Little was known about the genetics of CF until the 1980's, when a series of papers was published describing the CF gene and its protein product [4, 5, 59]. Using a variety of techniques, including gene walking and jumping, the CF gene was mapped to chromosome 7q31 and a length of DNA encoding a membrane bound protein, CFTR, was described. Additionally, in this series of papers, the deletion of a phenylalanine at position 508 (F508del), the most prominent mutation of the CFTR gene was first identified and reported in 70% of sample subjects. This series of work provided essential beginnings to understanding the complex genetic, biochemical, and pathophysiological elements of CF.

There are currently over 2,000 identified mutations of the CFTR gene, with the clinical importance not known for the vast majority [60]. With this large number of mutations, Welsh and Smith proposed a class system to organize CFTR mutations based on functional defect [61]. Originally four classes were proposed but as understanding of mutations has increased two additional categories have been added for a total of six mutation classes.

Class I Mutations

Class 1 mutations are nonsense mutations that result in a premature stop codon that disrupts translation and causes rapid degradation of messenger RNA (mRNA) [61]. Rapid degradation of mRNA results in little to no detectable quantities of CFTR protein and cell studies have demonstrated that transcription levels are consistently less than 10% of wild-type levels [62]. The G542X, which is the second most common CF mutation, and the W1282X, the most common mutation found in Ashkenazi Jews, are part of this mutation class [63, 64]. Because there is no functional CFTR protein present at the apical membrane, chloride ions become impermeable to the membrane and sodium is hyper-absorbed [16], greatly upsetting fluid and electrolyte transport across the epithelia [9, 63]. This disruption in fluid clearance results in a build-up of mucus that clogs the epithelial tissue [65] and dehydrates the lungs [54] creating an environment prone to

bacterial infection [66], obstruction of secretions [6], and malabsorption of nutrients [67]. This class of mutations is associated with the most severe clinical phenotype. The hallmark feature of CF is chronic respiratory ailment due to recurring pulmonary bacterial infection and exacerbation, which causes permanent pulmonary damage [68]. Within the respiratory tract, cilia are compressed by mucus build-up and their ability to clear particles is extinguished [69], creating a breeding group for bacteria, notably *pseudomonas aeruginosa* [70]. Furthermore, pancreatic insufficiency, a condition that disrupts proper food digestion due to lack of pancreatic digestive enzymes, is very common in patients with class I mutations [71]. Another common clinical feature of this mutation class is the presence of meconium ileus, a fecal obstruction of the bowels due to inadequate fetal pancreatic enzyme production and sticky mucus throughout the gastrointestinal tract [72]. Mucus build-up in the gastrointestinal tract can also lead to chronic microbial infection, with CF patients having diminished growth of healthy gut bacteria [73]. Finally, the vast majority of males (98%) and, to a lesser extent, females (50%) demonstrate infertility [74].

Class II Mutations

Class II mutations include those that result in CFTR misprocessing, as seen in F508del and N1303K [61], and results in the absence of functional protein at the apical membrane, leading to chloride impermeability [63, 64]. With no functional CFTR protein localized to the apical membrane, the protein is unable to perform its job as a chloride channel, resulting in multi-system mucus build-up and secretion obstruction [75]. Although the pathway by which CFTR protein is not localized to the apical membrane differs between Class I and Class II mutations, the clinical outcomes are similar if not identical [76]. As such, Class II mutations demonstrate chronic pulmonary infection, reduced pulmonary function, high rates of pancreatic insufficiency, malabsorption of nutrients, and infertility [77]. It is important to note that clinical symptoms and severity can vary greatly between patients, even with the same mutation, suggesting that additional factors such as modifier genes, epigenetics, and environment impact CF phenotype [78, 79]. In 2015 the first CFTR modulator therapy specifically for individuals with two copies of the F508del mutation was approved for use. The combination treatment of ivacaftor, a CFTR potentiator that works by increasing channel open probability, and lumacaftor, a CFTR corrector that helps to rescue CFTR from degradation and bring it to the cell surface, has been shown to improve lung function and body mass index (BMI) while also reducing incidence of pulmonary exacerbation [80, 81]. In 2018 an additional CFTR modulator treatment was approved for use in those with two copies of the F508del mutation. Similar to ivacaftor-lumacaftor, ivacaftor-tezacaftor is a potentiator-corrector combination therapy that works by increasing the number and function of

CFTR at the cell surface. Ivacaftor-tezacaftor has been shown to be somewhat more effective than ivacaftor-lumacaftor in improving lung function and reducing incidence of pulmonary exacerbation, with less reported adverse effects [82, 83].

Class III Mutations

Class III mutations produce a mature and stable protein, however channel gating is severally disrupted [61]. The third most common CF mutation, G551D, is part of this group [64]. Although functioning of the CFTR protein is reduced, Class III mutations do have CFTR present at the apical membrane and therefore do not share the same timeline of symptom onset and severity as seen in Class I and II mutations [84]. This is demonstrated by lower rates of meconium ileus in G551D mutation compared to W1282X (class 1) and F508del (class 2) [85] and trends showing later onset of pancreatic insufficiency in G551D mutation [86]. However, bacterial infection and chronic inflammation have been demonstrated in mouse models of G551D [87, 88]. The CFTR potentiator Ivacaftor is a FDA-approved treatment option for those with the G551D mutation and has been shown to increase pulmonary function and other clinical outcomes [89]. In the future clinical characteristics of this mutation may resemble milder forms of CF. Clinical severity in Class III mutations are dependent on type of channel gating disruption, underscoring the difficulty of assigning stringent phenotypes to an entire class of mutations [90].

Class IV Mutations

Mutations in Class IV are generally located in DNA regions corresponding to transmembrane segments and cause issues with conductance and gating [61, 91]. Mutations included in this class are R117H, which reduces channel activity, and R334W, which disrupts anion flow by altering the current-voltage relationship of CFTR [64]. As Class IV mutations encode a stable protein that properly responds to stimuli and, albeit at a lower rate than wild-type, conducts chloride across the membrane, the clinical phenotype of this class of mutations is much less severe than the previous three classes [76]. Age of diagnosis is higher in these mutations, with some individuals having such mild symptoms as to not be diagnosed until late into adulthood [92, 93]. Additionally, rates of meconium ileus and pancreatic insufficiency are much lower in R334W when compared to F508del (Class II) [92].

Class V Mutations

Suggested by Zielenski and Tsui in 1995, Class V mutations have reduced protein synthesis and lesser amounts of functional CFTR protein compared to wild-type [91]. Mutations in this class include those found in the promoter region and amino acid substitutions, such as A455E. This

mutation class is associated with mild forms of CF with functional CFTR protein, capable of chloride movement across the membrane, which is properly produced and localized to the membrane [94]. Patients with A455E mutation are diagnosed at a later age, have better pulmonary function, and have higher rates of pancreatic sufficiency compared to patients with F508del [94, 95].

Class VI Mutations

Class VI was first described in 1999 and includes mutations that impact protein stability through acceleration of protein degradation [96]. The mutation Q1412X, is included in this class and is properly processed and transported within the cell and is fully functional but unstable with a decreased half-life. As mutations in this class produce functional CFTR protein that is localized to the membrane, albeit for a shorter time interval than wild-type, the phenotype associated with this class is mild [64].

Pathophysiology of Cystic Fibrosis

Patients with CF are born with relatively normal lungs [97, 98] but begin experiencing chronic pulmonary bacterial infections at a young age which continues throughout life [99, 100]. This causes chronic inflammation that eventually leads to permanent pulmonary damage and respiratory failure, which accounts for over 90% of CF-related deaths [101]. Airway epithelial are pseudostratified ciliated cells that encourage movement of mucus and fluid across the respiratory tract and aid in providing basic defense against bacterial infection [11]. The airway surface liquid (ASL) serves a defensive role by providing a medium through which cilia can beat while also trapping invaders to be expelled during fluid clearance [102, 103]. Maintenance of ASL volume at optimal height (~7μm) is imperative to proper cilia and respiratory functioning and is maintained through balance between sodium absorption and chloride secretion [14, 54, 101, 104].

The fluid clearance mechanism in CF is disrupted due to defective CFTR protein, which impacts ion regulation in two ways [54]. First, in normal cells, CFTR acts as an inhibitor of the epithelial sodium channel (ENaC), helping to regulate sodium absorption into the cell [12, 13]. In CF, this inhibition of ENaC is absent, resulting in hyper-absorption of sodium into the epithelia [14, 15]. Hyper-absorption of sodium has been shown to cause CF-like pulmonary disease in transgenic mice overexpressing the beta subunit of ENaC, with mice having depleted ASL volumes, increased viscosity and volume of mucus, and decreased fluid clearance [16]. These findings strongly support the contention that the basic physiological defect of CF is caused by ion

imbalance and disrupted fluid clearance. In addition to sodium hyper-absorption, the cAMP dependent chloride secretion via CFTR is lost, resulting in impermeability of chloride to the apical membrane [9, 105]. The inability to maintain ion balance quickly depletes the ASL and creates a dehydrated cell surface that negatively impacts cilia movement [15, 106]. In this dehydrated environment the mucus layer becomes thick, containing a greater percent of solids than normal cells as seen in samples from murine nasal septal mucosa [107]. Furthermore, with the depletion of the ASL, the mucus layer interacts with cell surfaces, forming adhesions and eventually plugs surfaces [54, 101]. The mucus layer is further thickened as mucin secretion continues from goblet cells. As the mucus layer thickens, oxygen concentration near the cell surface decreases, creating a hypoxic environment that is hospitable to a variety of bacterial infections [108].

A highly anaerobic environment in the airways puts CF patients at an increased risk of infection, ultimately leading to exacerbation of pulmonary symptoms and permanent lung damage. Two mechanisms describe why CF patients are at increased infection risk 1) the lung is unable to clear mucus and move infection out of the lung [98] and 2) several bacteria are able to move through mucus and adapt to the anaerobic environment [108], which is not true of immune cells which are unable to effectively move or function in the thick mucus [109]. Because of the unique respiratory environment in CF, a limited range of pathogens are generally cultured including *pseudomonas aeruginosa* and *staphylococcus aureus* [110]. The primary bacterial infection associated with CF is *pseudomonas aeruginosa*, an infection that proves difficult to eradicate because of its unique mucoid formation that protects the bacteria from immune attack [111, 112]. Bacterial infection has been shown to begin early in life [100, 113] with the lungs eventually becoming chronically infected which results in overproduction of inflammatory markers, exacerbation of respiratory symptoms, permanent lung damage, and eventual respiratory failure for the majority of patients [98, 112].

In response to infection, the immune system mounts an attack to clear the pathogen. However, in CF patients the inflammatory response is dysfunctional, ineffective, and harmful with excessive inflammation and inability to terminate the inflammatory response [55, 114]. Cell cultures have demonstrated over-production of inflammatory cytokines in CF cells compared to normal cells that have been infected with *pseudomonas aeruginosa* [115]. A multitude of studies have demonstrated increased inflammatory markers in bronchoalveolar lavage (BAL) in patients with [116, 117] and without positive culture [99, 100]. Differences in findings may be due to lack of study power (with some studies having only 6 people per group), antibiotic use in some but not

all subjects, and inconsistency in inflammatory analysis techniques across studies. To help provide insight into the debate, animal models have provided valuable information on inflammatory patterns in CF. A study by Guilbault et al. (2005) using CFTR knockout mice found that mice injected with *pseudomonas aeruginosa* had greater neutrophil and lower lymphocyte response compared to wild-type mice, confirming results found in human studies that CFTR defect is associated with a hyper-inflammatory response [118]. Additionally, Kent et al. (1997), showed that even in the absence of bacterial infection, CF mice reported elevated neutrophil activation, consistent with a heightened inflammatory response regardless of infection [119]. These results are supported by another mouse model, where CF mice reported excessive neutrophil activation even with sterile lungs, suggesting that an amplified inflammatory response is an inherent trait of CF disease [16]. Upregulation of inflammatory pathways, notably $NF_{\kappa}B$, contribute to permanent lung damage by activating transcription factors involved in airway plugging, decreased ciliary beating, structural damage to airways, increased sputum production, and further activation of inflammatory markers [120]. Permanent lung damage caused by excessive activation of inflammatory mediators results in progression of disease and ultimate respiratory failure.

In summary, the basic pathophysiologic defect in CF is dysregulation of ion transport and fluid clearance across respiratory epithelia. Hyper-absorption of sodium and impermeability of chloride ions across the apical membrane lead to depletion of the ASL and development of a thick mucus layer. This mucus layer forms adhesions with the cell surface, and creates an anaerobic environment where bacteria, but not immune cells, can reside. This bacterial infection exacerbates the inflammatory response, leading to persistent hyper-inflammation that eventually causes lung cell damage and death. The associated anatomy and physiology underlying these mechanisms are discussed in greater detail below.

Airway Anatomy and Histology

The primary role of the respiratory system is to bring oxygen into the body while expelling carbon dioxide and other waste products. The continuous alveolar surface lining the airways allows the lung to maintain a sterile environment necessary for optimal function [121]. Anatomically, the pulmonary system is divided into two zones: the upper zone which includes the nose, pharynx, and larynx and the lower zone which includes the trachea, bronchi, bronchioles, alveolar ducts and alveoli [122, 123]. Functionally, the pulmonary system is divided into the conducting zone, which carries air into the lungs and the respiratory zone, where gas exchange takes place [122].

Moving down the pulmonary system, the airways become smaller and the structural and functional characteristics alter.

Beginning at the trachea, approximately 21 –23 airway generations are produced through repeated dichotomous branching [124]. The conducting zone includes the trachea (generation 0), cartilaginous bronchi (through generation 10), and terminal bronchioles (through generation 16) [125]. No gas exchange occurs in the conducting zone so the volume of air present is known as ventilatory dead space, which on average represents 150 mL in adults [122]. In children, ventilatory dead space has been shown to have a linear relationship with height and, as would be expected, varies greatly by age [126, 127]. The conducting zone is responsible for sterilizing and humidifying inhaled air and for identifying and removing pathogens [125]. The large, cartilaginous airways (trachea and bronchi) are lined by ciliated pseudostratified columnar epithelium and contain various cell types. The dominant cell type in the lung is the ciliated cell accounting for 50-80% of epithelial lining [128, 129]. Ciliated cells contain hair-like structures that project from the apical cell surface into the periciliary layer (PCL) [129]. Atop the PCL sits a mucus layer that is responsible for trapping particles and defending against infection. Together these layers make up the ASL [15]. Cilia beat in a coordinated manner and are responsible for propelling the ASL along the respiratory tract and play an important role in mucociliary clearance [129].

Goblet cells are columnar epithelial cells scattered between ciliated cells. They produce and secrete mucins which play a role in innate immunity [130]. In healthy airways the conducting airways contain approximately 20% goblet cells but in diseased states, including CF, goblet cell hyperplasia is evident with increased secretion of mucins [107]. Submucosal glands are found through generation 10 of the airways and contain serous (60%) and mucous (40%) cells and play an integral role in mucociliary clearance [131]. Serous cells secrete a mix of anti-inflammatory and antioxidant compounds that play a vital role in immunity by helping to defend against pathogens while the mucous cells secrete mucins [130, 131]. Particles and pathogens are trapped in the mucus layer which is then expelled by the synchronized beating of respiratory cilia. CFTR is most highly expressed in serous cells. Also present are basal cells, which are progenitor cells that produce new epithelial and goblet cells [132]. To a much lesser extent brush cells, chemosensory cells responsible for monitoring the composition of the mucosal lining fluid [133] and neuroepithelial cells, whose exact function is yet to be elucidated, are also expressed [132].

The anatomical and functional properties of the smaller distal airways differ distinctly from the larger conducting airways.

The respiratory zone begins at generation 17 as the terminal bronchioles give rise to respiratory bronchioles, which are sparse with alveoli, the air-filled sacs where gas exchange occurs. The respiratory zone serves as a transition space between the conducting airways and the alveoli [122, 125]. As the airways narrow the epithelium transitions from pseudostratified in the conducting airways to simple columnar epithelium in the respiratory bronchioles [124]. The respiratory bronchioles are void of submucosal glands and goblet cells [125] and instead contain club cells, secretory cells responsible for producing a surfactant like fluid that contains enzymes and proteins to neutralize toxins in the ASL [124, 132, 134].

Respiratory bronchioles divide into alveolar ducts (generations 20-22), which end at the alveolar sac (generation 23). Gas exchange occurs within the alveoli. Dead space within the alveoli is present on surfaces that receive ventilation but lack adequate perfusion from the pulmonary artery, known as alveolar dead space [135]. In healthy airways, alveolar dead space is functionally negligible but in certain disease conditions, such as heart failure and acute respiratory distress, increased alveolar dead space can occur due to shunt, hemodynamic impairment, and/or an exaggerated mismatch in the ratio of ventilation to diffusion. In healthy lungs the structure of the alveoli are optimized for gas exchange [135, 136]. The alveolar epithelium is composed of only two cell types: simple squamous alveolar epithelia Type I cells and cuboidal alveolar epithelia Type II cells [124]. Type I cells line the alveoli as a thin sheet and sit adjacent to the capillary network to allow for efficient gas exchange. Additionally, because they contain tight junctions, Type I cells help preserve oncotic pressure [124, 132, 137]. Type II alveolar cells can function as stem cells from which Type I alveolar cells can arise to allow for repair of damaged tissue [137]. Type II cells are not directly involved in gas exchange but secrete pulmonary surfactant, a fluid that contains lipids and proteins that defend against pathogens and allow for expansion of alveoli while also preventing alveolar collapse during respiration [132, 138]. Type I and Type II cells maintain a delicate and necessary barrier between the air-filled alveoli and the fluid-filled interstitial space. However, movement of water and ions across this barrier is necessary to allow for maintenance and regulation of the ASL, which plays an imperative role in lung function and immune defense.

Lung epithelia are exposed to an exuberant amount of toxins, pathogens, and irritants every day and serve as the first line of defense against these invaders. Mucus traps these particles and moves up the respiratory tract, either by ciliary beating or coughing, to the pharynx where the mucus can be swallowed [20]. This mucus clearance is the primary defense mechanism protecting the airway from invading particles [15]. Effective mucus production and clearance is necessary to ensure sterility and proper function of the respiratory system. Cilia are bathed in the PCL which provides a medium through which the cilia can beat optimally. Although mucus layer height varies by species and technique used, the PCL is estimated to be between 5 and 8 μm deep [139]. The interaction between the PCL and mucus layers is described in a "gel-on-brush" model [140], where the PCL contains membrane-spanning tethered structures including mucins and mucopolysaccharides [139, 141] that form a mesh, referred to as the PCL brush, which prevents secreted mucins and pathogens from the mucus layer to enter the PCL [140]. Further, through inter-molecular repulsion and maintenance of osmotic pressures, the PCL brush stabilizes the two-layers to allow for optimal mucus clearance.

The ASL is composed of a multitude of compounds including salt, water, antimicrobial peptides and proteins, antioxidants, proteases, protease inhibitors, and mucins, notably the polymeric mucin 5B and mucin 5AC [124, 142]. The complex organization of the ASL allows for optimal hydration and lubrication of the airway epithelia while also allowing for the trapping and removal of pathogens [141]. Maintenance of the ASL is essential for proper mucus clearance and the composition of ASL can alter in response to infection, irritants, and disease [103, 143]. Defects in the mechanisms responsible for lung fluid balance and regulation can alter solute concentration in the ASL to cause water to be extracted out of the ASL and effectively dehydrate the airway surface, as is seen in CF [107, 140, 143]. This, along with continued secretions from goblet cells, causes an increase in the percent solids content in the ASL which creates a thick, sticky mucus. The sticky mucus in dehydrated airways results in adhesion of mucins to the airway surface and compression of the PCL and cilia causing disruption in mucus clearance, as seen in CF [107] and Chronic Obstructive Pulmonary Disease (COPD) [144].

Lung Fluid Balance and Regulation

As previously stated, the primary function of the human lung is gas exchange which occurs via diffusion along the alveolar epithelium between alveolar gas and adjacent capillaries. Fick's first law of diffusion states that the rate of diffusion is directly related to surface area and inversely related to membrane thickness of the blood-gas barrier [145]. The human lung is well-designed

for efficient gas exchange as the surface area of the alveolar epithelium is ~150 m^2, representing 99% of the surface area of the lung [146]. Additionally, the blood-gas barrier is kept thin with the capillaries and alveolar epithelium being single-cell-layer structures that are able to maintain structural integrity while not impeding gas diffusion. Interstitial fluid surrounds capillaries and lung parenchyma and contains a plethora of substances including ions, sugars, and hormones [147]. For proper gas exchange to occur it is imperative that the amount of interstitial fluid is maintained outside the alveolar space as not to inhibit gas diffusion or flood the alveolus. Excessive inflammation, as seen in acute lung injury, congestive heart failure, and other inflammatory lung diseases, act to make the endothelial and epithelial lining of the lungs more porous which can result in pulmonary edema and respiratory distress [65, 125, 148].

Integrity of the airway epithelium ensures a necessary barrier between the air-filled respiratory system and the fluid-filled interstitium to allow for normal lung function. The airway epithelium must also allow for the transport of solutes and water to ensure lung fluid balance, which is achieved through resoprtive and secretory fluid transport [149]. Movement of ions and water across the airway epithelium via transcellular and paracellular routes serves to regulate ASL composition and volume [125, 150]. To ensure efficient gas exchange, mucociliary clearance, and pathogen defense maintenance of ASL volume (~5-8μm) is critical for lung health and is achieved through active ion transport [134, 151-153]. The driving force of ASL volume is solute mass, with water moving paracellularly in response to transport of sodium and chloride ions [154]. The primary ions responsible for ASL volume are sodium and chloride [101, 123], with potassium and bicarbonate playing lesser, regulatory roles. The primary structures involved in the vectoral movement of sodium and chloride across the airway epithelium include the epithelial sodium channel (ENaC), CFTR, calcium activated chloride channels (CaCC), and solute carrier-26 family of anion exchangers (SLC26).

Main Regulators of Ion Transport in Lung

A primary characteristic of CF is salty sweat, with sweat test results still being used as a gold standard diagnostic. Prior to identification of CFTR and understanding of CF pathophysiology, it was noted that those with CF had abnormal sodium and chloride transport [14, 155-157]. Epithelial cells are polarized and contain a basolateral and apical membrane [158, 159]. The basolateral membrane is in contact with the interstitium and is responsible for nutrient uptake and disposal of waste. The apical membrane is in contact with the external environment, providing a barrier to pathogens [160, 161]. This polarity allows for vectoral movement of ions and fluid across the

epithelia via paracellular and transcellular mechanisms [162-164]. Similar to ion and fluid regulation mechanisms in the kidney, absorption and secretion of sodium and chloride ions regulates hydration of the airway epithelium [165, 166]. Active absorption of sodium through ENaC creates an electrochemical gradient that allows for the passive movement of chloride across the basolateral membrane and the absorption of water through osmosis [101, 123]. Secretion of chloride across the apical membrane is controlled primarily by CFTR [167]. Active transport of chloride produces paracellular movement of sodium which causes secretion of water via osmosis [168-170].

Regulators of Chloride Secretion

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

The CFTR protein is a chloride channel that is found on the apical membrane of epithelial cells and is responsible for regulation of ion balance contributing to ASL volume and luminal pH which plays an essential role in innate defense [3, 123, 171, 172]. In a series of papers published in 1989 the gene encoding CFTR was mapped to 7q31.2 [3-5]. The CFTR gene spans a length of approximately 189 kb [173], contains 27 exons and encodes a 1480 amino acid protein product, referred to as the CFTR protein [174]. The CFTR gene is expressed in specialized epithelial tissue, notably the respiratory tract, pancreas, and gastrointestinal tract and has also been found on non-epithelial cells including cardiac myocytes and skeletal muscle [75, 175-178].

Defect in the CFTR gene results in dysfunctional CFTR protein to cause CF [9, 179]. In non-CF cells, CFTR is processed by the endoplasmic reticulum where it is glycosylated before being sent to the Golgi apparatus to complete maturation [180]. CFTR is then translocated to the plasma membrane to allow for regulation of ion absorption and secretion. However, in CF cells containing the F508del, mutant CFTR is flagged by the endoplasmic reticulum and degraded resulting in no CFTR being translocated to the plasma membrane [9, 181]. There is some evidence to suggest the possibility of some F508del CFTR making it through posttranslational processing to translocate to the plasma membrane [61]. However, to positively impact ASL and mucociliarly clearance delivery of functional CFTR to at least 25% of airway epithelial cells appears necessary [182]. This has extensive consequences for cell function as CFTR is involved in ion regulation, innate defense, and inflammatory signaling [124]. Notably, absence of CFTR results in uninhibited ENaC resulting in sodium hyper-absorption, a hallmark feature of CF pathophysiology [12, 183].

CFTR is a member of the ATP-binding cassette superfamily of transporters, which all contain a hydrophobic domain embedded in the membrane and a hydrophilic domain on which an adenosine triphosphate (ATP) binding site is located [184]. This family of transporters is responsible for translocating substances across membranes but the CFTR protein is the only transporter in this family to act as an ion channel [185, 186]. The protein is composed of two halves, each containing six hydrophobic transmembrane α-helices, to form a pore through which ions can pass [172, 179]. Additionally, CFTR contains two nuclear binding domains (NBD1 and NBD2), at which ATP binding occurs but is only hydrolyzed at the NBD2 domain [179, 187]. Binding of ATP at the NBDs determines channel gating and is thought to result in dimerization of NBD1 and NBD2 to allow for channel opening and hydrolysis of ATP at NBD2 to cause channel closing [188]. The NBD1 domain has been noted to contain more sites of CF-causing mutation, including the F508del mutation [187, 189]. The two halves are connected by a charged R-domain which contains 10 phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC), which are important in opening the CFTR channel [171, 190]. As such, CFTR is a cyclic adenosine monophosphate (cAMP) dependent channel where an unphosphorylated R-domain acts as an inhibitor to channel opening [191]. Additionally, AMP kinase has been shown to inhibit channel activity, although this is not well understood.

In the lung CFTR is expressed in ciliated airway epithelial cells [192] and serous cells of the submucosal glands [175]. It conducts anions in preference of nitrate > bromide ≥ chloride > iodide > fluroide and also conducts bicarbonate at a rate 1/5 of chloride which is important for maintaining ASL pH [193-195]. Notably, acidification of the airways appears to be an inherent characteristic of CF and is associated with impaired antibacterial and antimicrobial activity [196, 197]. Secretion of chloride across the apical membrane requires the active accumulation of chloride at the basolateral membrane, which occurs as the $Na^+K^+2Cl^-$ cotransporter (NKCC1) utilizes the sodium gradient created by Na^+/K^+-ATPase to collect chloride ions [124]. Additionally, in serous cells, 50-70% of cAMP-dependent chloride accumulation in the basolateral membrane occurs by chloride-bicarbonate exchange that is mediated by CFTR [198-200]. Secretion of chloride across the apical membrane then drives the passive movement of sodium, using the paracellular cation-selective mechanism. Water then follows sodium via osmosis. Movement of chloride ions via CFTR is determined by 1.) channel open probability (controlled by the PKA pathway) 2.) CFTR density and 3.) single channel conductance. Although to a much lesser extent, chloride is also secreted across the apical membrane via CaCC channels and SLC26, discussed in further detail below.

Calcium Dependent Chloride Channels (CaCC)

Although chloride movement across the apical membrane is primarily driven by CFTR in human airways, calcium-dependent chloride channels (CaCC) are also present. CaCCs are expressed in a wide variety of tissues and play a role in many physiological functions including blood pressure regulation and fluid secretion [201]. A few CaCCs have been identified in human tissue, most notably Anoctamin 1 and 2 (ANO1, ANO2) referred to as transmembrane member 16A and 16B (TMEM16A and TMEM16B) in Xenopus, respectively [202, 203]. It is also thought that other CaCCs are present and yet-to-be-identified [204]. In response to an increase in intracellular calcium, ATP is released and binds to $P2Y_2$ receptors, activating CaCCs to cause chloride secretion [205, 206]. $P2Y_2$ purinoceptors interact with extracellular nucleotides to help regulate ion transport and contribute to maintenance of ASL volume [15, 207]. Denufosol, an inhaled treatment option in CF, is a $P2Y_2$ agonist that improves mucociliary clearance and ciliary beat frequency through increased chloride (and subsequent water) secretion. It has been shown to increase pulmonary function in patients with CF [208, 209]. Phasic sheer stress, caused by tidal breathing, coughing, or exposure to bacteria signals the airway epithelia to release ATP in a dose-dependent manner. ANO1 and ANO2 have a homodimer structure [210] and contain eight putative transmembrane domains and intracellular N- and C-termini [211]. A pore located between transmembrane domains 5 and 6 transports chloride and other anions [201, 212]. TMEM16A has been shown to play a vital role in mucous transport and clearance, with TMEM16A null mice experiencing accumulation of mucous [213] and failure of mucociliary clearance in response to cholinergic stimulation [214]. Although the interaction between each is not well understood, chloride flow through CaCCs appears to be regulated by a variety of mechanisms including permeant anions, intracellular calcium levels, and membrane voltage [201]. Cruz-Rangel et al. (2015) suggests that both membrane potential and extracellular chloride concentration determine gating speed of CaCCs (slow v. fast) [215]. Regulation of CaCCs occurs most notably through alternative splicing but post-translational glycosylation and phosphorylation also modifies CaCC activity. The effect of CaCC activity on basal rates of fluid secretion appears to be short lived but does impart enough of an effect to increase mucus transport in CF airway cultures [103].

CFTR is a negative regulator of CaCCs, with some evidence to suggest that CaCCs are upregulated in CF airways [216] and under pro-inflammatory conditions [203]. Activated CFTR appears to interact with TMEM16A to cause inhibition of chloride secretion through CaCCs [217]. Under

basal conditions it appears that CaCCs contribution to chloride secretion is minimal, although it may be upregulated in disease states.

Solute Carrier – 26 Family of Anion Exchangers (SLC26)

The Solute Carrier – 26 family of anion exchangers (SLC26) is composed of 10 proteins that contain a yet-to-be-identified transmembrane domain that is flanked by C- and N- termini and are responsible for transporting a variety of anions including bicarbonate and chloride [218]. The C-terminus contains a regulatory region, referred to as the sulphate transporter and anti-sigma factor antagonist (STAS), which regulates cell expression and protein-protein interaction. Defects in protein structure result in tissue-specific disease including defects in transepithelial bicarbonate and chloride movement [123]. In the airways, SLC26A4 and SLC26A9 have been shown to be expressed in the apical membrane [124]. SLC26A9 specifically has been shown to play an important role in lung fluid balance under basal conditions and pro-inflammatory conditions [219, 220] by acting as a chloride channel. CFTR has been shown to regulate SLC26A9, with F508del CFTR preventing SLC26A9 activity, which may occur via interaction of the STAS and R-domain of CFTR [220, 221]. Further, Ko et al. (2004) demonstrated that SLC26A9 activity substantially increased open probability of CFTR further supporting interaction between the proteins [222]. However, further research is needed to fully elucidate the mechanism. SLC26A4 appears to play less of a role in ion balance under basal conditions but has been shown to be upregulated under pro-inflammatory conditions, a hallmark characteristic in CF disease.

Regulators of Sodium Absorption

Epithelial Sodium Channel (ENaC)

The ENaC/DEG superfamily of ion channels is composed of ENaC and acid-sensing ion channels (ASIC) and is involved in a variety of functions including regulation of extracellular fluid volume and ion balance and thus play an important role in ASL hydration [223, 224]. ENaC is expressed on the apical membrane of polarized epithelial cells throughout the body, is selective for sodium, and is inhibited by amiloride [225]. A rudimentary description of ENaC structure first appeared in 1993 with the cloning of ENaC from rat distal colon [226]. More detailed channel architecture was described shortly after, revealing ENaC to contain short cytoplasmic N- and C-termini and two transmembrane domains that are connected by a large extracellular loop [18, 227]. ENaC is most commonly described as a heterotrimer with three distinct subunits: alpha, beta, and gamma, present in a 1:1:1 ratio [18, 223, 228]. Some have reported a heterotetrametric configuration with either the addition of another alpha [229, 230] or a separate delta subunit [231], although these are

not necessary for channel function. Each main subunit (alpha, beta, and gamma) contributes to channel function in a unique way and all three are needed for optimal channel function. That being said, the alpha subunit is independently functional, being able to insert into the membrane and conduct current in isolation of the other subunits [18]. Each subunit is encoded by a different sodium channel epithelial 1 gene (SCNN1): SCNN1A (alpha), SCNN1B (beta), SCNN1G (gamma).

Active transport of sodium across the alveolar epithelium is essential in maintaining lung hydration. ENaC is the rate-limiting step in the process [20]. Sodium is transported into the cell across the apical membrane and pumped out across the basolateral membrane into the interstitial fluid via Na^+/K^+-ATPase to cause osmotic water transport [18, 124]. ENaC is present in the distal nephron of the kidney and is responsible for the reabsorption of sodium, playing a vital role in blood pressure regulation [165]. Pseudohypoaldosteronism Type I (PHA1), in an autosomal dominant disease that results from a loss-of-function mutation in either an ENaC subunit (alpha, beta, or gamma) or in the gene encoding the mineralocorticoid receptor (NR3C2) [232]. PHA1 is characterized by hypotension due to impaired salt reabsorption in the distal nephron, and increased mucociliary clearance in the lungs [23, 233]. Liddle syndrome is a monogenic, salt-sensitive hypertension that is caused by mutation in either the beta or gamma subunit of ENaC which results in impaired ubiquination and increased open probability of the channel resulting in channel gain-of-function [24]. This manifests as hypertension, hypokalemia, and reduced aldosterone secretion. In the lung, ENaC functions in mucociliarly clearance and maintenance of ASL volume. Specifically, the alpha subunit appears to be necessary for proper lung function and survival. Mice with complete knockout of alpha ENaC are unable to clear amniotic fluid from their lungs and die of respiratory distress within 40 hours of birth [19]. It has been shown that reduced expression of alpha ENaC, but not other subunits, increases incidence of pulmonary edema [234] and alpha ENaC is necessary for recovery of high-altitude edema [235]. Further, overexpression of the beta subunit in mice results in CF-like lung pathology and mice overexpressing all three subunits die within 24 hours after birth [29].

Transepithelial sodium absorption is elevated in CF airway epithelia, with CF nasal epithelial cells demonstrating enhanced open probability two to three times greater than non-CF cells [236]. Excessive saltiness is a hallmark feature of CF and its significance in disease was noted before the identification of CFTR and ENaC. Knowles and colleagues noted in 1981 that CF patients had increased nasal potential difference and showed a stronger inhibitory response to amiloride (an

inhibitor of ENaC) compared to controls, suggesting excessive sodium absorption in CF airway epithelium [237]. Further it was demonstrated that nasal potential differences in CF neonates were greater than control neonates and inhibition due to amiloride was greater in CF, suggesting an innate defect in epithelial sodium transport [157]. Quinton noted in 1983 that excised sweat ducts from CF patients demonstrated impermeability to chloride, which resulted in salty sweat [238].

Due to the large extracellular domain and asymmetric configuration of subunits, ENaC is able to sense and respond to a variety of stimuli including pH, mechanical forces, proteases, ions, acidic phospholipids and palmitoylation to allow for multiple regulatory pathways [124, 223, 225]. The extracellular region of each subunit contains a cysteine rich area that is made up of three distinct domains: knuckle, finger, and thumb whose interactions define channel activity [239]. ENaC activity is impacted by 1. channel number, 2. channel open probability, and 3. degree of channel recruitment. Unlike other members of the ENaC/DEG family, ENaC is constitutively active and is regulated by both intra- and extra-cellular factors [224]. For ENaC to be mature and maximally activated it must undergo proteolytic processing with immature, unprocessed ENaC channels described as "near-silent" channels that can be activated in response to proteolysis [240].

Regulation of ENaC by CFTR

CFTR serves as an inhibitor of ENaC, although the precise mechanisms have yet to be elucidated. ENaC is expressed on the apical membrane of epithelial cells and is responsible for transport of sodium across the apical membrane [223]. It plays an important role in the pathophysiology of CF. In the airways, activation of CFTR is associated with inhibition of ENaC [183, 241-244] although this is not the case in sweat ducts where CFTR increases ENaC activity [245]. Early studies demonstrated regulation is achieved through alteration of PKA-mediated channel open probability [241, 243], which is thought to occur via proteolytic cleavage of extracellular loops in the alpha and gamma subunits (cleaved = high open probability) [246]. The proportion of cleaved (active) ENaC activity has been shown to be higher in cells from CF compared to non-CF airways [247]. Further, Rubenstein et al. (2011) demonstrated that expression of wild-type CFTR in Xenopus oocytes regulated ENaC surface expression whereas F508del CFTR did not [248]. The results from this study suggest that regulation of ENaC by CFTR impacts expression and trafficking of ENaC, with wild-type CFTR demonstrating reduced ENaC expression compared to F508del CFTR. Further, Kunzelman et al. (1997), used transformed Xenopus oocytes to demonstrate protein-protein interaction between the alpha subunit of ENaC and the R-domain of CFTR [244]. These findings suggest that downregulation of ENaC by CFTR is due to interaction

of the C terminus of the alpha subunit with the R domain of CFTR. However, Ji et al. (2000) demonstrated that the carboxyl-terminus of the beta subunit was required for regulation of ENaC by CFTR, with the amino terminus of the gamma subunit also playing an important role [249]. Others have suggested that ENaC is sensitive to chloride concentration which can inhibit ENaC gating by 1.) direct inhibition and 2.) enhancing ENaC self-inhibition [250, 251]. Chloride residues have been identified on both beta and gamma subunits and serve as the predominant inhibitory sites [252]. It is likely that multiple mechanisms influence the regulation of ENaC by CFTR and their interactions are yet-to-be realized.

Regulation of ENaC by Proteolysis and Self-Inhibition

Both alpha and gamma subunits contain inhibitory peptide tracts that, when cleaved, allow for channel activation [253]. Proteolysis of these inhibitory tracts by serine proteases activate the channel by altering the interaction of the thumb and finger domains to allow for greater open probability and greater activation of near-silent channels [246, 253, 254]. Chronic inflammation can promote hyper-absoprtion of sodium by excessive cleaving, and thus activation of ENaC. Indeed, increased proteolysis of the alpha subunit has been shown in CF patients to cause activation of near-silent ENaC channels and increased sodium absorption [255]. This is consistent with findings that CF nasal epithelia have higher rates of amiloride-sensitive sodium reabsorption than non-CF epithelia. Absence of proteolytic processing has been shown to dramatically reduce channel activity and enhance sodium self-inhibition, a negative feedback where channel activity is reduced in the presence of high intracellular sodium concentration [256].

ENaC demonstrates sodium self-inhibition, where the open probability is inhibited in the presence of high extracellular sodium [256, 257]. Sodium binding sites are present in the extracellular region and are sensitive to proteolytic processes as evidenced by the amelioration of sodium self-inhibition in ENaC channels that have been cleaved [258, 259]. Mutations in the thumb domain of alpha are associated with disruption in sodium self-inhibition with most mutations enhancing self-inhibition, causing decreased channel function. One study found that deletion of the thumb domain in alpha or gamma subunits results in reduced sodium self-inhibition response, leading to increased open probability of the channel [239]. Additionally, the protein short-palate lung and nasal epithelial clone 1 (SPLUNC1), which is found in the airway lumen, has been shown to be an allosteric modulator of ENaC, inhibiting channel function by binding to the beta subunit [260]. This induces dissociation of the subunits and tags the alpha and gamma subunits for lysosomal degradation.

Regulation of ENaC by Beta-2-Adrenergic Receptor (β_2AR)

The Beta-2-Adrenergic Receptor (β_2AR) subtype is found in the smooth muscle of the respiratory system [261, 262] and is responsible for smooth muscle relaxation, resulting in airway dilation and reduction in airflow resistance [263]. Additionally, β_2ARs mediate fluid clearance in the lung by regulating active sodium transport into epithelial cells to maintain lung fluid balance [264]. Once bound to an endogenous (i.e. epinephrine) or exogenous (i.e. albuterol) agent binding of G_s protein causes a stimulatory response. In this pathway, after dissociation of G_α from the receptor and the $G_{\beta\gamma}$ dimer, the βγ-subunit interacts with the enzyme adenylyl cyclase, which catalyzes the conversion of ATP to cAMP and pyrophosphate, effectively increasing the intracellular concentration of cAMP [265, 266]. In this pathway, cAMP is responsible for activating protein kinase A (PKA) by causing dissociation of PKA's regulatory and catalytic subunits [267, 268]. Increased PKA in turn increases ENaC activity via increases in channel open probability [243] and/or channel number [264]. Further, overexpression of β_2ARs has been show to accelerate fluid clearance [269], supporting the regulatory role of β_2ARs on ENaC.

Regulation of ENaC by Inflammatory Pathways

Chronic and excessive inflammation, as seen in CF, can cause severe and permanent damage to lung tissue. Additionally, increased cytokine levels are associated with pulmonary edema and acute respiratory distress [270]. ENaC has been shown to be regulated by a variety of cytokines including tumor necrosis factor alpha (TNF-α), which has been shown to decrease alpha and gamma subunit mRNA levels while also reducing channel current [271]. Interleukin 1 beta (IL-1β) has been shown to directly influence ENaC by downregulating mRNA expression, membrane protein, and channel current, potentially through a p38 mitogen-activated protein kinase (MAPK) dependent pathway [272]. One proposed mechanism for inhibition of ENaC in response to cytokines is the disruption of the serum glucocorticoid regulated kinase 1 (SGK1) pathway. The ubiquitin E3 ligase Nedd4-2 inhibits ENaC expression by binding to PP_xY motifs in ENaC, initiating retrieval of the channel from the plasma membrane to be sent to lysosomes or proteasomes [165]. This regulatory process is dependent on the SGK1 pathway, which has been shown to directly activate ENaC and is inhibited by the inflammatory pathway nuclear factor kappa-light-chain-enhancer of activated B cells (NF_kB) [273]. Nedd4-2 knockout mice show pathological sodium transport due to sodium hyper-absorption, with one study demonstrating salt-sensitive hypertension [274] and another describing CF-like lung disease [275]. Oxidative stress has also been shown to inhibit alpha ENaC activity with one study demonstrating a dose-

dependent reduction in alpha ENaC transcription in response to hydrogen peroxide through inhibition of the promotor region [276]. Bacterial infection and chronic inflammation are hallmark features of CF and a cornerstone of disease physiology. Bacterial infection has been shown to suppress alpha ENaC expression with mice infected with the bacteria *Pseudomonas aeruginosa*, a common and clinically concerning infection in CF, having reduced ENaC activity compared to wild-type mice [271]. It appears that an inflammatory or oxidative environment, regardless of specific compound, has a direct effect on ENaC function [277].

In addition to extracellular regulators, ENaC has been shown to be regulated by a set of intracellular factors. The mechanical force of shear stress, achieved in the lung through breathing, has been shown to activate ENaC channels in a dose-dependent manner by increasing channel open probability [278]. Additionally, the acidic phosphatidylinositol 4,5 bisphoaphate (PIP_2) and phosphatidylinositol 3,4,5, triphosphate (PIP_3) have been shown to regulate ENaC by increasing channel open probability [279]. This is achieved by directly binding to the channel to cause a conformational change at the channel gate. Further, the beta and gamma subunits have been shown to contain Cys-palmitoylated residues, a reversible post-translational modification involving the attachment of a palmitate to cytoplasmic Cys residues to cause alteration in protein conformation and interaction [280]. Absence of Cys-palmitoylated sites show reduced channel activity through the mechanisms of enhanced sodium self-inhibition and reduced channel open probability. Due to the large number of extra- and intra-cellular factors that impact ENaC activity, regulation of the channel is complex and likely redundant and further work is needed to better understand how these factors interact to influence channel activity.

Pharmacological Targets of ENaC

As ENaC plays a central role in the pathophysiology of CF and is independent of CFTR, there has been much interest in ENaC as a therapeutic target. Previous attempts to effectively inhibit ENaC in CF have been unsuccessful [281]. Original efforts focused on using amiloride, a pyrazinoyl guanidine compound, and its derivatives to directly inhibit ENaC but due to its short half-life and rapid clearance from the lung it proved to be an ineffective therapeutic option [282, 283]. Additionally, because ENaC is present throughout the body (most notably in the kidney), a multitude of off-target effects have been reported including hyperkalemia, disruption of water transport, and arrhythmia [284, 285].

Indirect inhibition through targeting regulators of ENaC, primarily channel-activating protease (CAP) inhibitors, has been little more successful. CAP inhibitors interact with proteases, including prostasin and furin, to interfere with proteolysis of ENaC subunits to inhibit ENaC [286, 287]. Phase II clinical trials showed that patients receiving the drug camostat, a serine protease inhibitor, had reduced sodium transport as measured by transepithelial nasal potential difference assays, though adverse events were reported [286].

Even with set-backs in the development of ENaC inhibitors, ENaC continues to be perceived as a worthy therapeutic target in the management of CF, especially since a CFTR-independent intervention would benefit all CF patients, regardless of CFTR mutation class. Several ongoing preclinical trials continue to pursue direct and indirect inhibition of ENaC. These early trials have shown promise in demonstrating greater lung specificity, limited off-target interactions, and longer effect time [284, 288]. Although encouraging, additional time and research is needed to develop viable pharmacological ENaC inhibitors that can effectively be used in the management of CF.

Functional Polymorphisms in ENaC

Individuals with the same disease-causing mutation of CF can have very different clinical presentation and disease course. Although some of this variation in phenotype may be explained by environmental factors, such as medication regiment and treatment adherence [289], the vast continuum of CF phenotype suggests a complex, integrated role of modifier genes [290, 291]. As ENaC plays a central role in lung function and CF pathophysiology functional polymorphisms in this channel, specifically the alpha subunit, likely impact disease status and outcome.

Several studies have shown association of rare polymorphisms in ENaC with CF-like disease, even in the absence of CFTR mutation [25-28]. These findings support the contention that genetic variation in ENaC may contribute to symptoms and progression of CF lung disease. Conversely, a small study examining genetic modifiers contributing to long-term non-progressive lung disease in CF found that 80% of subjects had rare ENaC variants even though they were all homozygous for the F508del mutation, one of the more severe CF-causing mutations [292]. However, these studies looked at rare or never described ENaC polymorphisms and do not necessarily provide insight into clinical variation of disease in the broader CF community. Examination of known, common polymorphisms of ENaC would provide this information. As such, it is of interest to

explore the effect that a single nucleotide polymorphism (SNP) at amino acid 663 has on clinical outcome in patients with CF.

The SCNN1A gene encodes the alpha subunit of ENaC and is located at 12p13.31 [293]. It contains 14 exons and is expressed throughout the body, most heavily in the kidney and lung [294]. Located in exon 13 of the SCNN1A gene, a region that is not well conserved across species, a G → A SNP at amino acid 663 results in an alanine to threonine substitution [31]. Cell studies demonstrate A663T to be a gain-of-function polymorphism with increased channel activity due to greater surface expression, suggesting the polymorphism impacts cell trafficking and activation of near-silent channels [30-32]. Further, it has been associated with hypertension, although this appears to be population dependent [33, 34]. Reports of allele frequency varies between populations. The frequency of the A663 allele was found to be 0.58-0.64 in Japanese subjects [34], 0.15 in African American subjects [33], and 0.49 in Caucasian subjects [295]. Additionally, one study reported a frequency of the A663 variant in a sample of homozygous F508del patients to be 70.8% [28].

Variation at amino acid 663 has been shown to impact lung function in healthy individuals with one study demonstrating a greater percent increase in lung diffusion, suggesting higher lung fluid clearance, and greater decrease in systemic vascular resistance in response to exercise in those homozygous for the A663 variant (AA) [35]. Additionally, in response to albuterol, a β_2AR agonist, those homozygous for A663 (AA) had a greater reduction in exhaled sodium, a marker of lung fluid clearance, compared to those with at least one copy of T663 (AT/TT), which showed no change in exhaled sodium in response to albuterol [36]. Taken together, these results are contrary to what would be expected given results from culture studies indicating that T663 is more active than A663 and therefore it would be expected that those with the T663 would have greater lung fluid clearance.

However, the A663T polymorphism may impact those with CF in a manner different than healthy individuals, as suggested in a small study which found individuals with at least one copy of T663 (AT/TT) had significantly lower body weight, body mass index, and baseline pulmonary function than those homozygous for A663 (AA) [37]. It was postulated that poorer clinical measures in the T group (AT/TT) may be due to more active ENaC to cause a drier lung and subsequently reduce mucus clearance. Given the suggestive but inconclusive findings in the literature it is of interest

to better understand how genetic variation in the alpha subunit of ENaC impacts disease phenotype in CF patients.

Exercise in Cystic Fibrosis

The hallmark feature of CF is lung disease associated with sticky mucous build-up in the respiratory tract which leads to chronic bacterial infection and eventual respiratory failure [10]. However, due to the ubiquitous nature of CFTR, several other organs are also negatively impacted including the pancreas [296], gastrointestinal tract [297], and cardiovascular system [298]. As the lifespan of CF patients continues to improve [299] extra-pulmonary complications have become more clinically significant, creating an urgency to adopt more holistic clinical tests.

Impairment of Cardiovascular Function in CF

Cardiovascular function is attenuated in CF, with reported right ventricular (RV) [300, 301] and left ventricular (LV) [302, 303] dysfunction, increased vessel stiffness [304], and impaired cardiac response to exercise [52, 304, 305]. Cardiovascular disruption appears to be an inherent characteristic of CF disease, as opposed to a by-product of pulmonary distress [300, 306]. Recent data has suggested that cardiac output is impaired in patients with CF at rest and exercise, even in those with mild lung disease [52, 305, 307, 308]. This may be due to abnormal cardiac function [302, 303, 306] and/or impaired stroke volume (SV) [307, 309] that is seen in CF patients. Cardiac CFTR has been shown to be involved in regulation of contraction rate and contractility, with CF mice demonstrating increased fractional shortening, decreased contraction time, and decreased diastolic function [310-312]. Increased myocardial contractility and contraction rate may lead to undesirable cardiac remodeling such as pathological ventricular hypertrophy [311]. Those with CF have been shown to have dysfunction of the RV [300, 301, 306, 313] and LV [302, 303] along with impairment of central and peripheral hemodynamics [304, 305], which all contribute to lower SV via decreased preload and increased afterload, effectively attenuating cardiac output.

Sympathetic control of vascular tone is mediated through agonist binding of α and β adrenergic receptors [314]. In healthy individuals, the normal exercise response (increases in heart rate, cardiac output and blood pressure) occur due to a dose-dependent increase in epinephrine, a β_2AR substrate [315, 316] and attenuation of sympathetic vasoconstrictor response through decreased sensitivity of αARs [317, 318]. Binding of norepinephrine to αARs results in calcium influx to cause vasoconstriction [319, 320], while binding of epinephrine to β_2AR results in calcium movement out of the cell to cause vasodilation [321]. Dysfunction of sympathetic response have

been described in CF patients, with attenuated sensitivity to β_2AR stimulation and heightened reactivity to αAR stimulation being reported [322, 323]. This imbalance of sympathetic tone has been shown to lead to attenuated vasodilatory response to β_2AR stimulation in CF patients [308, 324, 325] and likely contributes to diminished vascular reactivity.

CF patients have been shown to have decreased arterial compliance, decreased arterial lumen diameter, decreased blood pressure, and endothelial dysfunction [304, 310, 311, 326-329]. Although complete understanding of the mechanisms behind these observations have yet to be elucidated, there is evidence to suggest that CFTR mutation results in autonomic dysregulation, endothelial/smooth muscle dysfunction, and augmented inflammatory state may play a role in vascular function in CF patients. Systemic inflammation and presence of reactive oxygen species is recognized as a contributor to cardiovascular disease (CVD), with increased apoptosis and tissue damage occurring in this pathological state [330]. In a study by Hull et al. (2013), which examined augmentation index (AIx, a measure of arterial stiffness) before and after two weeks of antibiotic treatment in adults with CF, AIx and C-reactive protein were lower after treatment suggesting that systemic inflammation may impact vascular function [327]. Another study found that greater inflammation was correlated with poorer pulmonary function and higher pulmonary artery pressure in children and adults with CF [331]. As systemic inflammation is recognized as a conditional risk factor for CVD [332], it seems reasonable to consider chronic inflammation as a mechanism explaining vascular function in CF. However, at this point in time there is not enough evidence to confidently state that chronic inflammation leads to vascular dysfunction in CF. These cardiovascular aspects of disease are noteworthy and directly impact long-term, overall patient health. However, current clinical evaluation does not address this aspect of disease. Exercise testing is an important clinical tool as it allows a holistic assessment of the cardiopulmonary system and provides information on how well body systems function together.

Exercise Testing in CF

The positive, far-reaching effects of exercise are well described in the literature and the importance of regular physical activity has been promoted since ancient times [333]. Exercise has been shown to be an important part of management of CF disease, with individuals who partake in regular physical activity demonstrating improved aerobic capacity and respiratory muscle endurance, reduced rates of pulmonary function decline, healthier anthropometric measures, better measures of lipid and glucose metabolism, reduced rates of antibiotic treatment, and greater quality of life

[334-337]. Exercise has been shown to aid in sputum clearance with regular activity associated with greater ease and amount of sputum clearance [338-340].

Maximal uptake and utilization of oxygen (VO_{2max}) is a strong and independent predictor of death and lung transplant in patients with CF and provides meaningfully and exclusive information not encompassed in pulmonary function testing [41, 43, 341-343]. In children with mild-to-moderate CF disease, greater aerobic capacity was associated with longer time free of pulmonary exacerbation requiring hospitalization [344]. This is significant as pulmonary exacerbations result in permanent lung damage and are a marker of worsening pulmonary disease. One large, multi-center study found that those with the highest VO_2 and ventilatory efficiency (VE/VCO_2, a measure of how well the lungs are able to meet metabolic demand) at peak exercise had the greatest survival after 10 years and determined who is at highest risk for event [342]. Additionally, VE/VCO_2 was associated with death/lung transplant, showing that additional measures special to exercise testing may provide insight into disease progression and prognosis [341]. This study also identified a high-risk disease group characterized by poor lung function, reduced exercise capacity, and poor nutrition status. In a study of 95 children that stratified F508del heterozygotes by class of their second CFTR mutation aerobic and anaerobic capacity differed significantly between classes with those with Class I or II mutation (the most severe) having lower aerobic and anaerobic capacity than those in the Classes II, IV or V (less severe) [44]. This observation occurred even though no differences in forced expiratory volume in one second (FEV_1-%predicted) were appreciated between the groups, suggesting that functional measurements provide insight into body functions different than that of traditional pulmonary function testing.

Exercise testing is a powerful clinical measure as it provides unique assessment of the integration of multiple body systems including pulmonary, cardiovascular, skeletal muscle, and metabolic that cannot be measured through assessment of each system independently [345]. Aerobic capacity (as measured by both maximal and sub-maximal efforts) is a significant and independent predictor of morbidity and mortality in both healthy and diseased states, including CF [38, 41] and exercise testing is now recognized as clinically useful for a variety of diseases including heart failure, pulmonary hypertension, interstitial lung disease, and CF [346]. As such, annual exercise testing in CF patients is recommended in the United Kingdom, Australia, and New Zealand [337, 347]. Although a variety of exercise tests exist, the gold-standard maximal, incremental cardiopulmonary exercise test (CPET), performed on either a bike or treadmill involves the measurement of breathed gases to determine VO_{2max}, carbon dioxide production (VCO2), along

with other ventilatory measures [345]. Attenuated aerobic capacity can occur due to reduction in maximal heart rate, maximal stroke volume, and/or maximal arterial oxygen concentration or an increase in resting mixed venous oxygen content. As mentioned previously, dysregulation of the cardiovascular system appears to be an inherent piece of CF disease, with lower stroke volume and stiff vessels being observed. Further, individuals with CF have greater dead space than healthy individuals due to damaged lung tissue, which becomes more pronounced in response to exercise. Oxygen exchange is not supported in areas of dead space, thus those with CF, especially those with more progressive disease, have reduced ability to increase alveolar ventilation [348]. As such, the cost of exercise is greater for those with CF as they must work harder (higher ventilation rate) to make-up for inefficient gas-exchange. These physiological features of CF are evidenced by the noted lower aerobic and anaerobic capacity compared to non-CF individuals [349-351].

Although CPET is the gold-standard to determine exercise capacity, the equipment, time, and staff required do not make it feasible for most clinics [352]. As CF patients are prone to infection, special and extensive precautions are taken as part of infection control. As such, performing CPET on CF patients would require disposal of the pneumotachographs and special cleaning of the sampling lines used for ventilatory gas analyses, adding additional costs and time [348]. To accommodate these restrictions, field tests can be utilized to measure aerobic capacity without the need for trained staff and special equipment [353]. The six-minute walk test (6MWT) and one-minute sit-to-stand test (1STS) are field tests that require minimal equipment, are easy to administer, and are safe to perform over a range of disease severity.

The 6MWT is a well-used, practical exercise test that only requires a 30-meter hallway to perform [354]. The 6MWT is routinely used to measure response to medical intervention, assess functional capacity, and determine eligibility for transplant. Outcomes of the 6MWT (six-minute walk distance, 6MWD) have been shown to be strongly and significantly correlated with VO_{2max} in a variety of patients, including CF [355-357]. Further, test results have been shown to be valid and reliable in CF [358, 359], and have also been shown to be correlated with radiological measures of lung disease [360]. 6MWD has also been shown to have prognostic ability, with reduced distance covered associated with lung transplant and death over 7- [49] and 12-year periods [51]. Additionally, one study in CF youth found that 6MWD predicted risk of hospitalization over a five-year period, with patients with greater 6MWD having reduced risk of hospitalization [48]. Of note, desaturation during 6MWT has also been demonstrated to be a clinically useful outcome in patients with CF, even in the absence of 6MWD impairment [51, 361]. The 6MWT is an easy to

administer, clinically meaningful test that can be used in the CF population to assess functional capacity and exercise tolerance.

Although the 6MWT requires minimal equipment, some clinics may lack a 30-meter hallway in which to administer the test. Again, as CF protocol requires stringent infection control procedures, a functional capacity test that can be performed in the same space as a clinical exam is desirable. The 1STS requires only the use of a standard, armless chair and can be performed in a clinic room or at a patient's home. The 1STS involves repetitions of alternating sitting to standing for one-minute with the primary outcome being number of sit-stand repetitions [46]. Outcomes from the 1STS have been correlated with 6MWD in healthy individuals [362] and with VO_{2max} in COPD and CF patients [46, 47]. The 1STS is a useful measure of functional capacity that can be utilized in a clinic room or at a patient's home to provide unique insight into disease state.

Exercise capacity is a powerful measurement in healthy and diseased populations and is a known prognostic in patients with CF. The outcomes measured by exercise testing provide meaningful clinical information into disease state by providing insight into whole body function that goes beyond pulmonary function testing. As such, exercise testing is a relevant and important tool in the evaluation of CF.

Chapter 3: Study One - Impact of Genetic Variation of Alpha ENaC on Clinical Presentation in Cystic Fibrosis

Synopsis

Background: Cystic Fibrosis (CF) patients demonstrate extensive variability in clinical presentation and course even in the presence of the same disease causing genotype, suggesting the role of modifier genes. In CF, the epithelial sodium channel (ENaC) is over active, resulting in sodium hyper-absorption and dehydration of the airways. As ENaC plays a crucial role in CF lung disease the purpose of this study was to examine the association of genetic variation in the alpha subunit of ENaC with clinical features of the disease.

Methods: Thirty-five subjects were enrolled in the study. All subjects had at least one copy of the F508del mutation. Buccal swabs were collected and samples were analyzed for genetic variation at position 663 of the SCNN1A gene (AA, AT/TT). The six-minute walk test (6MWT) and one-minute sit-to-stand test (1STS) were performed to assess functional capacity. Medical charts were reviewed for resting vitals, pulmonary function, and indicators of disease status.

Results: Twenty-one subjects were in the A group (AA) and 14 subjects were in the T group (AT/TT). No statistically significant differences were found between clinical outcomes of interest and genotype group. We did note clinically relevant observations suggesting the A group may have better preserved lung FEV_1%-predicted overtime and better performance on exercise tests, though further study is needed.

Conclusions: Variation at position 663 of the SCNN1A gene may modify pulmonary disease in patients with CF. Additional research is needed to determine the magnitude of the effect and the complexities of how this variation impacts disease course and presentation.

Keywords: F508del, epithelial sodium channel, SCNN1A, single nucleotide polymorphism, genetic variation, pulmonary function

Funding: None

Introduction

Hydrated airways are an essential component of proper and efficient lung function, requiring tight regulation of lung fluid balance. To ensure efficient gas exchange, mucociliary clearance, and pathogen defense maintenance of airway surface liquid (ASL) volume is critical for lung health [134, 151-153]. Airway epithelia closely regulate ASL volume by way of ion-mediated transport [104]. Human airway epithelia contain epithelial sodium channels (ENaC) to mediate sodium absorption into the cell and express cystic fibrosis transmembrane regulator (CFTR), to secrete chloride out of the cell [153, 226, 363, 364]. Balance between sodium absorption and chloride secretion results in efficient fluid clearance across the membrane which allows ASL height to be maintained [14, 54]

Cystic Fibrosis (CF) is an autosomal recessive genetic disease and is recognized as the most common fatal genetic disease among those of Northern European decent [1, 56], with an estimated occurrence of 1 in every 3,200-3,400 live births [57, 58]. CF is caused by mutation of the CFTR gene which causes misfolding of the CFTR protein [3, 9]. In the lung CFTR is expressed in ciliated airway epithelial cells [192], serous cells of the submucosal glands [175], and pulmonary alveolar Type 1 [365] and Type 2 [366] cells. In addition to being the primary driver of chloride movement in the cell, CFTR also regulates a variety of other cell functions including inhibiting ENaC [171, 172]. The most common CF causing mutation is deletion of a phenylalanine at position 508 of the CFTR gene (F508del) which results in the absence of functional protein at the apical membrane [63, 64]. With no functional CFTR protein, chloride ions become impermeable to the membrane and sodium is hyper-absorbed through an uninhibited ENaC [16], greatly upsetting fluid and electrolyte transport across the epithelia [9, 63]. This disruption in fluid clearance results in a build-up of mucus that clogs epithelial tissue [65] and dehydrates the lungs [54], creating an environment prone to bacterial infection [66]. This in turn causes chronic inflammation that eventually leads to permanent pulmonary damage and respiratory failure, which account for over 90% of CF related deaths [153]. There are more than 2,000 identified mutations of the CFTR gene that demonstrate a wide range of clinical presentations [60]. Even so, clinical symptoms and severity can vary greatly between patients with the same disease-causing CFTR mutation, suggesting that additional factors such as modifier genes, epigenetics, and environment may impact CF phenotype [78, 79].

ENaC is an amiloride-sensitive sodium channel that is expressed on the apical membrane of polarized epithelial cells throughout the body and is the rate-limiting step in epithelial sodium

absorption [225]. It is most commonly described as a heterotrimer with three main subunits (alpha, beta, and gamma) that are present in a 1:1:1 ratio [18, 223, 279]. Some have reported a heterotetrametric configuration with either the addition of another alpha [229] or a separate delta subunit [231], although these additional subunits are not necessary for channel function. Each subunit (alpha, beta, and gamma) contributes to channel function in a unique way and all are needed for optimal channel function. The alpha subunit, encoded by the SCNN1A gene, has been shown to be independently functional, being able to insert into the membrane and conduct current in isolation of the other subunits [18]. The alpha subunit appears to be necessary for proper lung function and survival, as evidenced by alpha-ENaC knockout mice being unable to clear amniotic fluid from their lungs and dying of respiratory distress within 40 hours of birth [19]. Additionally, previous work has shown that reduced expression of alpha ENaC, but not other subunits, increases incidence of pulmonary edema [234].

Polymorphisms in ENaC have been shown to be associated with CF-like disease, even in the absence of CFTR mutation, suggesting the importance of genetic variation in ENaC in relation to pulmonary disease [25-28]. In a small sample of CF patients homozygous for the F508del mutation who had long-term non-progressive disease, 80% of patients had rare variants of ENaC [292]. Additionally, overexpression of the beta subunit of ENaC is used to model CF-like lung pathology in mice and overexpression of all ENaC subunits results in death within 24 hours [29]. These findings support the role that genetic variation in ENaC have in propagating symptoms of CF. Located in exon 13 of the SCNN1A gene a G → A at amino acid 663 results in an alanine to threonine substitution. Cell studies demonstrate A663T to be a gain-of-function polymorphism with increased channel activity due to greater surface expression [30, 31] and some in-vivo studies have suggested the T663 variant is associated with high blood pressure and reduced lung function in otherwise healthy individuals [33, 35].

Previous studies on genetic variation in ENaC in CF have focused on identifying rare variants or variants that have not been previously described so do not necessarily provide insight into clinical variation of disease within the broader CF community. As such, it is of interest to explore what effect a common, gain-of-function single nucleotide polymorphism (SNP) has on clinical outcomes in patients with CF. The purpose of the present study was to assess differences in clinical characteristics and markers of disease between those homozygous for the A663 allele (AA) and those with at least one copy of the T663 allele (AT/TT). As greater ENaC activity would contribute to greater lung fluid clearance and accelerated lung dehydration in CF it is hypothesized that those

with at least one copy of the more active T663 variant (AT/TT) would have poorer clinical presentation than CF patients who are homozygous for the A663 variant (AA).

Methods

Study Population

This study was approved by the Institutional Review Board at the University of Minnesota (IRB#00000972). Consent was obtained by all subjects aged 18 years and older and parental permission and assent were obtained for all subjects 7 – 17 years prior to study procedures. Subjects were recruited via listserv email that is maintained by the Minnesota Cystic Fibrosis Center. Individuals were eligible for study participation if they had a confirmed diagnosis of CF by chloride sweat test (>60 mmol) and/or genetic testing, had at least one copy of the F508del mutation, and were between the ages of 7 and 64 years. Patients were excluded if they had a history of cardiovascular or inflammatory disease, had previously had a lung transplant, or had experienced a change in medication or antibiotic use within the four weeks prior to study participation.

Clinical Markers

Subject medical records were queried for demographics, anthropometrics, pulmonary function, hospitalization, antibiotic use, pulmonary exacerbation, bacterial infection of the respiratory tract and bacterial drug-resistance. Lung function was assessed during regularly scheduled clinic visits according to American Thoracic Society guidelines and standards [367]. Forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC), and forced expiratory flow 25 to 75% of FVC ($FEF_{25-75\%}$) were recorded. All results are expressed in %-predicted.

Antibiotic use is expressed as days of treatment. Days of treatment was calculated by summing the days of treatment for each antibiotic prescribed (if two antibiotics were prescribed on the same day this counted as two days of treatment). Pulmonary exacerbation was determined based on provider notes and discharge diagnosis, regardless of antibiotic prescription. Hospitalizations were included if they related to pulmonary symptoms of disease and were recorded as a count (number of instances per patient) and total days. Cultures were assessed for bacterial growth and drug resistance, when available, as well as for *pseudomonas aeruginosa* and *staphylococcus aureus*. Bacterial infection was defined as chronic if at least 50% of cultures were positive in the last year. Bacterial infection

was defined as multi-drug resistant (MDR) if a bacterial strain was resistant to at least one antibiotic from three or more antibiotic categories [368].

Genetic Analysis

Buccal swabs were collected for assessment of amino acid at position 663 of the SCNN1A gene. The inside of each cheek was swabbed and the swab was immediately placed in a stabilizing buffer (50mM Tris, pH 8.0; 50mM EDTA; 25mM Sucrose; 100mM NaCl; 1 % SDS) and kept at room temperature until analysis. Samples were evaluated by the University of Arizona Genetics Core Laboratory using a Taqman SNP assay for rs#2228576. Initial DNA quantification was performed using PicoGreen (Life Technologies). Pre-validated primers and probe sets for TaqMan Allelic Discrimination Assay were obtained from Thermo Fisher Scientific. Using TaqMan Universal PCR Master Mix, No AmpEraseR UNG (Thermo Fisher Scientific), reactions were run with 10ng of DNA and 1X Assay Mix. Real-Time PCR (Applied Biosystems, Model: 7900) was performed with cycling conditions set to 95oC for 10 minutes followed by fifty cycles of 92oC for 15 seconds and 60oC for 1 minute. Genotyper software (SDS system, version 2.3) was used to analyze samples. Subjects were grouped according to amino acid at position 663 of SCNN1A. Due to low sample size in the TT group (n=2), those with at least one copy of the T allele (AT/TT) were included in the T group. Those homozygous for the A allele (AA) represent the A group.

Functional Capacity Testing

Subjects performed the six-minute walk test (6MWT) and 1-minute sit-to-stand (1STS) at time of enrollment. The order of tests was randomized and patients were given enough rest time between tests to return to resting vitals before starting the next test.

Six-Minute Walk Test

The 6MWT was performed according to American Thoracic Society guidelines [369]. Using standardized language, subjects were instructed to walk as far as possible during the test time, which took place in an enclosed, 30-meter hallway. They were assured they could stop at any time during the test and could stop the test at any time. Subjects were given standard encouragements during the test. Test procedures were demonstrated by the researcher and an opportunity to practice directions was given prior to the start of the test to reduce learning effect. Heart rate (HR) and peripheral blood oxygen saturation (SpO_2) were evaluated at rest, during, and immediately post-test via a blue-tooth enabled finger probe (Masimo MightStat, Masimo Corp., Irvine, CA, USA).

One-Minute Sit-to-Stand Test

The 1STS was performed according to previously described methods using a standard 48cm armless chair [370]. The subject started in a seated position with knees and hips flexed as close to 90^{o} as possible with feet hip width apart and hands on hips. Subjects were instructed to perform as many self-paced sit-to-stand repetitions as possible during the test. To be counted as a repetition on the stand portion, knees had to be entirely straightened, and the buttocks had to make contact with the chair on the sit portion. Test procedures were demonstrated by the researcher and an opportunity to practice a few repetitions was given prior to the start of the test to reduce learning effect. HR and SpO_2 were evaluated at rest and immediately post-test.

Statistical Analysis

Statistical analyses were performed using SPSS (Version 23, IBM Corp., Armonk, NY). Continuous variables were assessed for normality using Shapiro-Wilk test. For normally distributed data, Student's t-test was performed to compare means between genotype groups, and Mann Whitney U test was used to compare means for non-normally distributed data. All continuous data are presented as mean±standard deviation unless otherwise noted. Fisher's exact test was used to analyze categorical variables. All categorical data is presented as frequency (%) Significance was set at 0.05.

To model repeated measures of pulmonary function (FVC%-predicted, FEV_1%-predicted, FEV_1/FVC%, and $FEF_{25-75\%}$%-predicted), linear mixed models (LMM) were constructed using individual subjects and age as random factors and ENaC group as the fixed factor. This approach was selected to account for the variability in number of measurements per subject, time between measurements, and clustering of individual subject data. Restricted maximum likelihood method was used to estimate model parameters and covariance type was set to unstructured. The estimated fixed effects, 95% confidence interval, and p-value for ENaC group are presented. Percentiles were calculated for age at time of pulmonary function testing and the lower and upper 10% of age at time of pulmonary function testing was trimmed for analysis. This was done to account for the lack of data from multiple patients at the extremes of our inclusion ages. Thus the LMM represents 75% of the available pulmonary data and 83% of patients (age range: 13.12 - 41.98 years).

Results

Thirty-five participants were enrolled in the study (19 females, 16 males; mean age = 30.2±13.1 years) and all were included in analysis. Of these, 27 completed the 6MWT to standard (6 were

unable due to clinic space restraints, 2 due to illness on day of visit) and 22 completed the 1STS test (8 were pediatric patients, 3 due to clinic space restraints, 2 due to illness on day of visit). Subject characteristics are summarized in Table 1. Subjects were relatively healthy with a mean FEV1%-predicted of 84.3±22.7%-predicted and body-mass index (BMI) of 22.9±4.5 kg/m^2 on the day of study visit. All subjects identified as non-Hispanic Caucasian. Twenty subjects (57.1%) were homozygous for the F508del mutation in CFTR and 20 subjects (57.1%) were on CFTR modulator therapy at time of enrollment. All but one subject had pancreatic insufficiency and 15 (42.9%) had CF-related Diabetes (CFRD). Twenty-nine (82.9%) subjects had confirmed history of *pseudomonas aeruginosa*, with 22 subjects (62.9%) having at least one positive culture for *pseudomonas aeruginosa* in the previous two years.

Twenty-one subjects were homozygous for the A allele at amino acid 663 of the SCNN1A gene while 14 subjects had at least one copy of the T allele (AT, n=10; TT, n=2) (Table 2). In this sample, the A663 variant was more common than the T663 variant (77.1% v. 22.9%). Clinical outcomes were compared between groups and are depicted in Tables 3. There were no statistically significant differences between the A and T group on the clinical outcomes of interest. However, there were some clinically relevant findings worth noting. First, the A group demonstrated better exercise performance on both the 6MWT (580.8±85.8 v. 554.6±59.1, p=0.39), and completed more repetitions during the 1STS (57.7±14.5 v. 50.3±9.0, p=0.19) compared to the T group. This is worth noting as the minimal clinical important difference for each test has been found to be 30 meters and 3-5 repetitions, respectively [371]. Further, the T group had a non-significant but higher percentage of females (71.4% v. 42.9%) and lower frequency of CFRD (35.7% v. 52.4%) compared to the A group. The A group had greater days of antibiotic treatment in the year of enrollment (468.9±327.3 v. 350.9±261.9), number of hospitalizations (0.43±0.6 v. 0.07±0.3), and number of hospital days (3.5±7.9 v. 0.36±1.3) compared to the T group, but again these differences did not reach significance ($p > 0.05$). There was no significant differences in frequency of bacterial infection or MDR infection between groups.

Pulmonary data for all subjects, divided by ENaC group, are depicted in Figure 1. Results from the LMM are depicted in Table 4. No statistically significant differences in pulmonary function overtime were seen between groups. It is worth noting that the A group had an FEV_1%-predicted estimate of 6.02±1.05% and $FEF_{25-75\%}$%-predicted estimate of 7.85±12.21%, which are clinically relevant spreads in pulmonary function overtime and warrants further research. As evidenced by the large confidence intervals, the variability in pulmonary function measures may have been too

great to detect differences between groups with the sample size. Larger study is needed to better define and understand these initial findings.

Discussion

There were no statistically significant differences in clinical outcomes of interest between those who had at least one copy of threonine (AT/TT) at position 663 of the SCNN1A gene compared to those with two copies of alanine (AA). Not statistically, but potentially clinically relevant, it was observed that 1) over time, those in the A group may maintain better pulmonary function than those in the T group and 2) those in the A group had better exercise performance on both the 6MWT and 1STS. Taken together, these findings provide preliminary data to suggest genetic variation at position 663 of the SCNN1A may modify pulmonary disease in CF. Further study is warranted to expand on these initial findings.

In the lung, the maintenance and regulation of ASL volume is imperative to proper respiratory functioning and is achieved by way of ion-mediated transport [54, 104]. Human airway epithelia express ENaC to mediate sodium absorption [153, 226, 364] and CFTR to mediate chloride secretion [363], which together work to maintain ion balance and allow for efficient fluid clearance and maintenance of ASL volume [14, 54]. In CF, this fluid clearance mechanism is disrupted due to defective CFTR protein, which results in sodium hyper-absorption through ENaC [15, 54]. As ENaC plays a central role in lung function and CF pathophysiology, functional polymorphisms in this channel may impact disease presentation and status.

ENaC is a constitutively active channel and is regulated by both intra- and extra-cellular factors, including inhibition by CFTR [250]. Channel activity is impacted by channel number, channel open probability, and degree of channel recruitment. For ENaC to be maximally activated it must undergo proteolytic processing. Unprocessed ENaC channels are described as "near-silent" channels located in the plasma membrane and are not active until they have undergone proteolysis [240]. The SCNN1A gene encodes the alpha subunit of ENaC and is expressed throughout the body, most heavily in the kidney and lung. Located in exon 13 of the SCNN1A gene, a G→A SNP at amino acid 663 results in an alanine to threonine substitution. Cell studies demonstrate A663T to be a gain-of-function polymorphism with increased channel activity due to greater surface expression, likely due to activation of near-silent channels [30-32].

Results from cell studies suggest that presence of T663 would cause greater sodium absorption into the cell, leading to ion disequilibrium and disease. Indeed, it has been observed that those with T663 have a higher risk of hypertension [33], although others have found no difference in risk between groups [34, 372]. A meta-analysis examining the role of T663 in hypertension found that in North American samples (Caucasian and African American), T663 was associated with greater risk of hypertension but not in Southeast Asian samples, suggesting the impact of this polymorphism on disease is population dependent [8].

These sparse and inconclusive results are mirrored by those found when examining the impact of the A663T polymorphism in lung function and CF disease. In healthy individuals, variation at amino acid 663 has been shown to impact lung function, with one study demonstrating a greater percent increase in lung diffusion (marker of lung fluid clearance) and greater decrease in systemic vascular resistance in response to exercise in those homozygous for A663 [35]. Additionally, in response to albuterol, those homozygous for A663 had a greater reduction in exhaled sodium, a marker of lung fluid clearance, compared to those with at least one copy of T663, which showed no change in exhaled sodium in response to albuterol [36]. Taken together, these results suggest that A663, not T663, is associated with greater lung fluid clearance in healthy individuals, opposite of findings observed in cell studies. As CF lungs differ considerably from non-CF lungs, the A663T polymorphism may impact those with CF in a manner different than healthy individuals. One small study found that CF individuals in the T group had significantly lower body weight, body mass index, and baseline pulmonary function (FVC%-predicted, FEV_1%-predicted, and FEF_{50}%-predicted) than those in the A group, suggesting that A663 contributes to better health in CF subjects [37]. There were not any difference in body weight or BMI between groups in this sample but did observe better, though not statistically significant, pulmonary function across time in the A group. Additionally, this study found no difference between the groups in response to maximal exercise testing, similar to the current findings of functional capacity. Though in our sample the difference in performance between the two groups may be clinically relevant even though statistical significance was not demonstrated, supporting the need to expanded research on the topic.

It was observed a discrepancy in allele frequency between this sample and those found in previous studies. Reports of allele frequency is known to vary between populations with the frequency of the A663 allele reported 0.58-0.64 in Asian subjects [34], 0.15 in African American subjects [33], and 0.49 in Caucasian subjects [295]. These previously reported frequencies are smaller than the current observed frequency of 77.1%, which is similar to other reports in CF patients [28, 37]. The

frequency of the TT genotype seems especially sparse in light of previous reports, suggesting that there may be a connection between mutations causing CF and variation at position 663, although there does not appear to be anything in the literature to support this reasoning. It may be that individuals homozygous for the T variant experience a more severe disease progression that was not observe in this study due to survivor bias. Further research is needed to better examine and understand the mechanisms behind this observation.

This study has several limitations. First, patients self-identified, which may have resulted in a selection bias. This could have led to healthier patients being more likely to participate in the study, which included functional capacity testing, than those with more severe disease. Second, the sample size was smaller than desired, especially given the spread in the data, causing the analysis to be under powered to detect any differences in pulmonary function between groups that might actually exist. Further, there was an inability to fully examine differences between each genotype (AA, AT, TT) due to only having two subjects with the TT genotype. It may be that heterozygotes have different clinical profiles than their homozygous counterparts. Further research is needed to better address this question.

Conclusion

There was no detectable differences in clinical markers of CF disease between A663 and T663 of the SCNN1A gene. Though not statistically significant, clinically relevant observations of potentially preserved lung function overtime and better exercise performance were noted and warrants further research. The results suggest that variation at position 663 of the SCNN1A gene may modify pulmonary disease in patients with CF, though further research is needed to better understand the magnitude of effect and the complexities of how this variation impacts disease course and presentation. Additionally, the allelic frequency of T663 was well below that reported in previous studies in non-CF populations, suggesting that distribution of allelic frequency may differ between CF and non-CF populations. Further research is needed to explore these questions.

Table Legend

Table 1. Subject Clinical Characteristics

Table 2. Allele frequency at position 663 of SCNN1A gene

Table 3. Comparison of Clinical Outcomes between Genotypes

Table 4: Estimated Fixed Effect of AA Genotype on Measures of Pulmonary Function as determined by linear mixed models

Table 1: Subject Clinical Characteristics

Variable	Mean ± SD	Range
Age (years)	30.2 ± 13.1	7.0 – 60.4
Height (cm)	164.4 ± 14.4	125.6 - 185.4
Weight (kg)	63.6 ± 19.3	23.8 – 104.0
BMI (kg/m^2)	22.9 ± 4.5	15.1 - 37.0
Diagnosis Age (months)	3.2 ± 8.5	0 - 41.5
Resting SpO_2 (%)	97.0 ± 1.6	93 – 100
Resting HR (beats per minute)	86.2 ± 12.6	58- 122
Systolic Blood Pressure (mmHg)	118.8 ± 13.4	90 – 146
Diastolic Blood Pressure (mmHg)	76.4 ± 8.1	62- 100
6MWD (m)	570.1 ± 75.9	405-766
1STS repetitions	54.8 ± 12.9	23-82
Annual of antibiotic treatment (days)	421.7 ± 304.5	0 – 1097
Annual exacerbations (count)	1.0 ± 1.2	0-6
Annual hospitalizations (count)	0.29 ± 0.52	0 – 2
Annual hospital days	2.26 ± 6.20	0 – 35
	Frequency (%)	
Female	19 (54.3)	
F508del homozygous	20 (57.1)	
CFRD	15 (42.9)	
pseudomonas aeruginosa (previous two years)	22 (62.9)	
staphylococcus aureus (previous two years)	22 (62.9)	
Chronic *pseudomonas aeruginosa*	15 (42.9)	
Chronic *staphylococcus aureus*	14 (40.0)	
MDR *pseudomonas aeruginosa*	14 (60.0)	
MDR *staphylococcus aureus*	5 (14.3)	
Modulator Therapy	20 (57.1)	

BMI = body mass index; SpO_2 = peripheral blood oxygen saturation; HR = heart rate; 6MWD = six minute walk distance; 1STS = one-minute sit-to-stand test; CFRD = Cystic Fibrosis related diabetes; MDR = multi-drug resistant

Table 2: Allele frequency at position 663 of SCNN1A gene

Genotype	**Frequency (%)**	**Allele**	**Allele frequency (%)**
AA	21 (60.0)	A	54 (77.1)
AT	12 (34.0)	T	16 (22.9)
TT	2 (5.7)	Total	70 (100)

Table 3: Comparison of Clinical Outcomes between Genotypes

Variable	Genotype	Mean ± SD	Range	p-value
Age (years)	AA	31.9 ±12.2	9.1 - 60.4	0.36
	AT/TT	27.7 ± 14.4	7.0 – 45.0	
Height (cm)	AA	167.4 ± 10.9	139.0 - 185.4	0.17
	AT/TT	159.9 ± 17.90	125.6 - 182.2	
Weight (kg)	AA	65.6 ± 14.1	39.1 - 96.6	0.26
	AT/TT	60.6 ± 25.6	23.8 – 104.0	
BMI (kg/m^2)	AA	23.16 ± 3.07	18.8 - 28.2	0.38
	AT/TT	22.61 ± 6.25	15.1 - 37.0	
Resting SpO_2 (%)	AA	96.7 ± 1.6	93 – 100	0.08
	AT/TT	97.5 ± 1.5	94 – 99	
Resting HR (beats per minute)	AA	84.8 ± 14.6	58 – 103	0.21
	AT/TT	88.4 ± 9.0	75 – 100	
Systolic Blood Pressure (mmHg)	AA	118.3 ± 15.3	90 - 146	0.40
	AT/TT	119.6 ± 10.5	104 -143	
Diastolic Blood Pressure (mmHg)	AA	76.8 ± 8.4	67 – 100	0.50
	AT/TT	75.9 ± 8.1	62 - 98	
6MWD (m)	AA	580.8 ± 85.8	405-766	0.39
	AT/TT	554.6 ± 59.1	469-640	
1STS repetitions	AA	57.7 ± 14.5	23-82	0.19
	AT/TT	50.3 ± 9.0	34-62	
Days of antibiotic treatment (annual)	AA	468.9 ± 327.3	0- 940	0.13
	AT/TT	350.9 ± 261.9	0 - 730	
Number of exacerbations (annual)	AA	1.00 ± 0.95	0- 3	0.27
	AT/TT	1.00 ± 1.57	0 - 6	
Number of hospitalizations (annual)	AA	0.43 ± 0.60	0 - 1	0.06
	AT/TT	0.07 ± 0.27	0 - 1	
Hospital days (annual)	AA	3.52 ± 7.78	0 - 10	0.06
	AT/TT	0.36 ± 1.34	0 - 5	
		Frequency		
Female	AA	9 (42.9)		0.17
	AT/TT	10 (71.4)		
F508del homozygous	AA	12 (57.1)		1.00
	AT/TT	8 (57.1)		
CFRD	AA	11 (52.4)		0.32
	AT/TT	5 (35.7)		
pseudomonas aeruginosa (previous two years)	AA	13 (61.9)		0.59
	AT/TT	9 (64.3)		
staphylococcus aureus (previous two years)	AA	11 (52.4)		0.11
	AT/TT	11 (78.6)		
Chronic *pseudomonas aeruginosa*	AA	9 (42.9)		0.47
	AT/TT	7 (50.0)		
Chronic *staphylococcus aureus*	AA	7 (33.3)		0.26
	AT/TT	8 (57.1)		
MDR *pseudomonas aeruginosa*	AA	7 (33.3)		0.32
	AT/TT	7 (50.0)		
MDR *staphylococcus aureus*	AA	4 (19.0)		0.15
	AT/TT	1 (7.1)		
Modulator Therapy	AA	11 (52.4)		0.27
	AT/TT	8 (57.1)		

BMI = body mass index; SpO_2 = peripheral blood oxygen saturation; HR = heart rate; 6MWD = six minute walk distance; 1STS = one-minute sit-to-stand test; CFRD = Cystic Fibrosis related diabetes; MDR = multi-drug resistant

Table 4: Estimated Fixed Effect of AA Genotype on Measures of Pulmonary Function as determined by linear mixed models

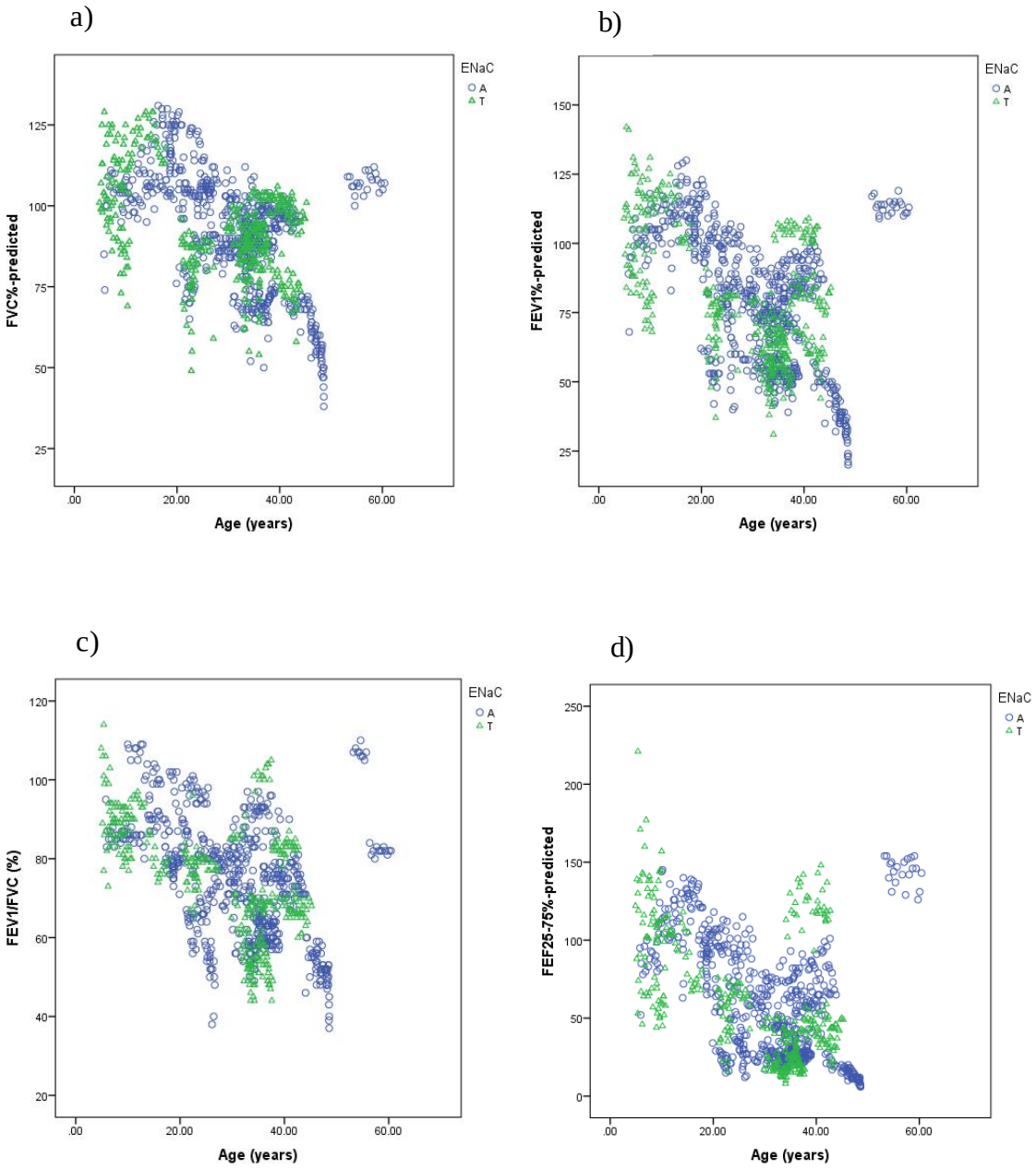
Variable	**Estimate**	**SE**	**95% CI**	**p-value**
FVC%-predicted	3.62	5.91	-8.51 – 15.75	0.55
FEV_1%-predicted	6.02	1.05	-8.24 – 20.27	0.40
FEV_1/FVC%	-2.53	9.83	-22.75 – 17.69	0.80
$FEF_{25-75\%}$%-predicted	7.85	12.21	-17.23 – 32.93	0.53

This model used the middle 75% of pulmonary data which represented 83% of subjects (ages: 13.17 – 41.98)

Figure Legend

Figure 1: Measures of pulmonary function across time by genotype group. Scatter plots of a) FVC%-predicted, b) FEV_1%-predicted, c) FEV_1/FVC-%, and d) $FEF_{25-75\%}$%-predicted are depicted with each data point representing pulmonary function parameter at given age in years. Pulmonary function was collected during a regularly scheduled clinic visit and adhered to American Thoracic Society testing standards. Blue open circles represent the A group and green open triangles represent the T group.

Figure 1: Measures of pulmonary function across time by genotype group



Chapter 4: Study Two - Correlation between Six-Minute Walk Test and One Minute Sit-to-Stand and Clinical Outcomes in Cystic Fibrosis

Synopsis

Background: Advancements in screening and therapy options has greatly improved clinical course and extended lifespan for patients with Cystic Fibrosis (CF). This has created a need for more sensitive and dynamic clinical measures to better assess disease state than those traditionally used. Exercise testing provides valuable and unique information about disease status in CF.

Methods: Twenty-three subjects were enrolled in the study. All subjects had at least one copy of the F508del mutation. Subjects completed the six-minute walk test (6MWT) and one-minute sit-to-stand test (1STS). Heart rate (HR) and peripheral blood oxygen saturation (SpO_2) were measured continuously. Dyspnea was measured upon test completion. Test performance, HR, SpO_2, and dyspnea score were correlated between tests. Medical charts were reviewed for indicators of disease. Subjects were followed for at least 120-days and performance on exercise tests were evaluated for association with pulmonary exacerbation and hospitalization during follow-up.

Results: Six minute walk distance (6MWD) and 1STS repetitions were significantly correlated ($r=0.573$, $p=0.002$) but neither outcome correlated with measures of pulmonary function ($p>0.05$). SpO_2 and post-test dyspnea score were significantly correlated between tests ($r=0.458$, $p=0.02$; $r=0.433$, $p=0.047$, respectively). Those who desaturated during the 1STS (change in SpO_2 > 4% from rest) had significantly lower FEV_1%-predicted (80.4±19.4% v. 98.0±14.9%, $p=0.05$) and $FEF_{25-75\%}$%-predicted 57.7±32.5 v. 92.8±41.4%, $p=0.04$) compared to those who did not. Neither 6MWD nor 1STS repetitions was associated with pulmonary exacerbation during follow-up.

Conclusions: 6MWD and 1STS repetitions were significantly correlated in a sample of clinically stable CF patients. The results suggest that the 1STS may be a useful exercise test that can easily be incorporated into routine clinical care.

Keywords: F508del, exercise, 6MWT, 1STS, pulmonary function, desaturation

Funding: None

Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disease that upsets chloride and sodium balance in epithelial tissue, most notably in the lungs, due to dysfunctional Cystic Fibrosis transmembrane conductance regulator (CFTR) protein [6, 54]. CF is recognized as the most common fatal genetic disease among those of Northern European decent [1, 56], with an estimated occurrence of 1 in every 3,200-3,400 live births [57, 58]. The hallmark feature of CF is the development of lung disease with substantial mucus build-up in the respiratory tract, leading to chronic bacterial infection and eventual respiratory failure [10]. Other organs where CFTR is present are also negatively impacted, including the pancreas [296], gastrointestinal tract [297], and cardiovascular system [373].

Due to improved screening techniques and advancements in medical care the lifespan of CF patients continues to extend [299]. Because of this extra-pulmonary complications have become more clinically significant, in particular cardiovascular disruption appears to be an inherent characteristic of CF disease [300, 306]. Right and left ventricular dysfunction, arterial stiffness and impaired exercise response have all been reported in CF patients and are known to negatively impact long-term health [52, 304, 305] However, current clinical evaluation does not address this critical aspect of disease progression, prompting the need for additional, more holistic clinical measures. Exercise testing is a powerful clinical measure as it provides unique assessment of multiple body systems including pulmonary, cardiovascular, skeletal muscle, and metabolic [345].

Exercise testing is now recognized as clinically useful for a variety of diseases including heart failure, pulmonary hypertension, interstitial lung disease, and CF [346]. Aerobic capacity, as measured in exercise testing by both maximal and sub-maximal efforts, is a significant and independent predictor of morbidity and mortality in both healthy and diseased states, including CF [38, 41]. As such, annual exercise testing in CF patients is now recommended in the United Kingdom, Australia, and New Zealand [347]. The gold-standard for exercise testing is a maximal, incremental cardiopulmonary exercise test (CPET) performed on either a bike or treadmill while collecting exhaled gases to determine peak oxygen uptake (VO_{2max}), exhaled carbon dioxide (VCO_2), ventilatory efficiency (VE/VCO_2), along with other ventilatory measures [345] VO_{2max} is the primary outcome measure obtained during CPET and represents the body's ability to utilize oxygen during exercise. VO_{2max} is a strong and independent predictor of death and lung transplant in patients with CF and provides valuable disease information beyond standard pulmonary function testing [41, 43, 341-343]. In children with mild-to-moderate CF disease, higher aerobic capacity is

associated with greater freedom from pulmonary exacerbation requiring hospitalization [344]. One large, multi-center study found that those with the highest VO_{2max} and VE/VCO_2, a measure taken during CPET of how well the lungs are able to meet metabolic demand, at peak exercise had the greatest survival after ten years and successfully predicted who was at highest risk for an adverse event [342].

Even though CPET is the preferred method for exercise testing, the equipment, time, and staff required to regularly perform CPET is oftentimes not feasible for most clinics [352]. To accommodate for this limitation, field testing provides a low-cost, efficient alternative for obtaining estimates of aerobic capacity [374]. The six-minute walk test (6MWT) and one-minute sit-to-stand test (1STS) are validated field tests that require minimal equipment, are easy to administer, and have been shown as safe to perform over a wide range of disease severities [375, 376].

The 6MWT is a well-utilized and practical exercise test that has been shown to be valid and reliable in the CF population [354, 356, 358]. The 6MWT is routinely used to measure exercise response to medical interventions, assess functional capacity, and determine eligibility for lung transplant. Six-minute walk distance (6MWD) has been shown to have prognostic ability, with reduced distance covered associated with lung transplant and death over 7 [49] and 12 years [51] in CF patients. One study in CF youth found that 6MWD predicted risk of hospitalization over a five-year period, with greater 6MWD associated with reduced risk of hospitalization [48]. Of note, desaturation during the 6MWT has been demonstrated to be a clinically useful outcome in patients with CF, even in the absence of 6MWD impairment [51, 361]. The 6MWT is an easy-to-administer, clinically meaningful test that can be used in the CF population to assess functional capacity and exercise tolerance.

Although the 6MWT requires minimal equipment, some clinics may lack a hallway in which to administer the test. As CF protocol requires stringent infection control procedures, a functional capacity test that can be performed in the same space as a clinical exam is desirable. The 1STS involves repetitions of alternating sitting to standing and requires only the use of a standard, armless chair and can be performed in a clinic room or at a patient's home [46, 377] . Outcomes from the 1STS have been correlated with 6MWD in healthy individuals and in COPD [362, 377] as well as with VO_{2max} in COPD and CF patients [46, 47]. However, no previous studies have correlated response to 1STS and 6MWT in patients with CF. As the 1STS can be utilized in a clinic room or at a patient's home, it may be a more practical test to employ in certain settings.

The purpose of this study was to examine the correlation of physiological parameters and outcomes collected during the 6MWT to those of the 1STS in a CF population. Further, the study aimed to assess the association of the 6MWT and 1STS with days to pulmonary exacerbation requiring antibiotic treatment. It was hypothesized that outcomes of the 6MWT would be positively correlated with those of the 1STS and that both measures from the 6MWT and 1STS would be associated with time to exacerbation.

Methods

Subjects

This protocol was approved by the University of Minnesota Institutional Review Board (IRB#00000972). Written informed consent was provided by all subjects. Inclusion criteria were as follows: 1.) confirmed diagnosis of CF by sweat test and/or genetic evaluation, 2.) at least one copy of the F508del mutation, 3.) percent predicted forced expiratory volume in one second (FEV_1%-predicted) greater than 40% predicted. Exclusion criteria included: 1.) exacerbation or change to medication in the four weeks prior to enrollment, 2.) no history of cardiovascular or inflammatory disease, and 3.) illness or injury on day of visit.

All patients performed the 6MWT and 1STS at time of enrollment, which took place on the same day as a regularly scheduled outpatient clinic visit where routine spirometry and anthropometric procedures were performed. Exercise test administration was randomized for each patient with adequate rest time between efforts. Historical and post-visit pulmonary function and anthropometric data was collected from the medical record along with relevant clinical information (genetic mutation, presence of comorbidities, bacterial colonization, exacerbation, use of modulator medication, and antibiotic treatment). Chronic *pseudomonas aeruginosa* colonization was defined as at least 50% of sputum samples in the previous year [378]. Patients were followed for at least 120-days after date of enrollment for documented pulmonary exacerbation, antibiotic prescription, hospitalization, and pulmonary function testing.

Pulmonary Exacerbation

Days to exacerbation were recorded during the follow-up period. Presence of exacerbation was defined based on provider notes and prescription of antibiotic(s). Follow-up time ranged from 120 days to 300 days.

Lung Function

Lung function was assessed during regularly scheduled clinic visits according to American Thoracic Society guidelines and standards [367]. Forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC), and forced expiratory flow 25 to 75% of FVC ($FEF_{25-75\%}$) were recorded. All results are expressed in %-predicted.

Six-Minute Walk Test

The 6MWT was performed according to American Thoracic Society guidelines [354]. Using standardized language, subjects were instructed to walk as far as possible during the test time, which took place in an enclosed, 30-meter hallway. They were assured they could rest at any time during the test and could end the test at any time. Subjects were given standard encouragements during the test. The protocol was demonstrated by the researcher, and an opportunity to practice the test procedure was provided to reduce learning effect. Heart rate (HR) and peripheral blood oxygen saturation (SpO_2) were collected at rest, during exercise, and immediately post-test via a blue-tooth enabled finger probe (Masimo MightStat, Masimo Corp., Irvine, CA, USA). Desaturation was defined as change in SpO_2 greater than 4% from rest [360, 379]. Perception of dyspnea was evaluated using the modified Borg scale at the beginning and end of the test.

One-Minute Sit-to-Stand Test

The 1STS was performed according to previously described methods using a standard 48cm armless chair [370, 371]. The subject started in a seated position with knees and hips flexed as close to 90^o as possible with feet hip-width apart and hands on hips. Subjects were instructed to perform as many self-paced sit-to-stand repetitions as possible during the test. To be counted as a repetition, knees had to be entirely straightened during the stand portion; with the buttocks making contact with the chair on the sit portion. The protocol was demonstrated by the researcher, and an opportunity to practice the test procedure was provided to reduce learning effect. HR and SpO_2 were collected at rest and immediately post-test. Desaturation was defined as change in SpO_2 greater than 4% from rest to immediately post-test. Perception of dyspnea was evaluated using the modified Borg scale at the beginning and end of the test.

Statistical Analysis

Statistical analysis was performed using SPSS (Version 23, IBM Corp., Armonk, NY). Data normality was assessed by Shapiro-Wilk test and comparisons of means was performed using

Student's independent t-test or Mann-Whitney U, depending on data normality. Continuous variables are presented as mean ± SD and categorical variables are presented as absolute frequency (%). Correlations were performed using Spearman test. Statistical significance was set at $p < 0.05$.

Univariate Cox proportional hazards regression were determined with days to exacerbation as the dependent variable and the following independent variables: age, body mass index (BMI), sex, chronic *pseudomonas aeruginosa* colonization, Cystic Fibrosis Related Diabetes (CFRD), 1STS repetitions, 6MWD, FVC%-predicted, FEV_1%-predicted, FEV_1/FVC%-, and $FEF_{25-75\%}$%-predicted.

To determine the association of 6MWD and 1STS repetitions with exacerbation during the follow-up period, relative risk and 95% confidence interval were calculated. Subjects were categorized as either above or below the median value for 6MWD and 1STS repetitions.

Results

A sub-sample from Study One was used to complete this study. Twenty-three subjects were enrolled in this study (10 male, 13 female, mean age = 35.5±9.3 years) and all subjects identified as non-Hispanic Caucasian. Demographic and clinical characteristics are presented in Table 1. Thirteen subjects (56.5%) were homozygous for F508del mutation, with the remaining subjects being F508del heterozygotes. Due to technical difficulties not all HR and SpO_2 data were collected on all subjects at all time-points therefore the sample size (n) is reported for test results that do not include the entire sample. The subjects were relatively healthy CF patients with a mean BMI of 23.87±3.39kg/m^2, mean FEV_1%-predicted of 82.7±19.6% and mean FVC%-predicted of 94.8±12.3%. All but one subject had pancreatic insufficiency, with 12 (52.2%) having Cystic Fibrosis Related Diabetes (CFRD) and 14 (60.9%) with chronic *pseudomonas aeruginosa* colonization. Fourteen (60.8%) subjects were on modulator therapy at time of testing. There was no significant difference in walk distance between females and males (559.8m, 103.1 v. 567.8m, 59.5 p=0.82), but females did achieve significantly more repetitions during the 1STS (59.7, 9.8 v. 48.5, 14.2 p = 0.036).

As seen in Figure 1, 6MWD and 1STS repetitions were significantly correlated ($r = 0.573$, $p = 0.02$). Table 2 summarizes correlation coefficients for 6MWD, 1STS repetitions, and measures of pulmonary function. Neither 6MWD nor 1STS was correlated with measures of pulmonary function (FVC, FEV_1, FEV_1/FVC, and $FEF_{25-75\%}$, $p>0.05$). SpO_2 ($r = 0.458$, $p = 0.02$) and post-test

dyspnea score (r = 0.433, p = 0.047, n = 22) were significantly correlated between tests, however post-HR was not (r = 0.198, p = 0.201, n = 20). Post-test dyspnea score was significantly higher after the 1STS compared to the 6MWT (Figure 2; 3.8 ± 1.7 v. 2.9 ± 1.3, p = 0.03). No differences in 6MWD or 1STS repetitions were found between homozygous F508del, CFRD, or modulator therapy (p > 0.05). Those with chronic *pseudomonas aeruginosa* colonization had significantly greater 6MWD (595.0±72.9m v 524.4±72.0m, p=0.02) and tended to have greater 1STS repetitions (58.5±8.6 v. 50.1±16.3, p = 0.06) compared to those without chronic *pseudomonas aeruginosa* colonization.

Differences in measures of pulmonary function did not differ between those who desaturated and those who did not during the 6MWT (Table 3, p > 0.05). However, those who desaturated during the 1STS had significantly lower FEV_1%-predicted (80.4±19.4% v. 98.0±14.9%, p = 0.05) and $FEF_{25-75\%}$%-predicted (57.7±32.5% v. 92.8±41.4%, p = 0.04) compared to those who did not (Figure 3).

Twelve individuals experienced a pulmonary exacerbation during follow-up. Univariate Cox proportional hazards were calculated using the predictors of age, BMI, sex, chronic *pseudomonas aeruginosa* colonization, CFRD, 1STS repetitions, 6MWD, FVC%-predicted, FEV_1%-predicted, FEV_1/FVC%-predicted, and $FEF_{25-75\%}$%-predicted to explain days to exacerbation. Univariate analysis demonstrated $FEF_{25-75\%}$%-predicted to be the only variable significantly associated with days to exacerbation (p = 0.02). The median distance walked during the 6MWT was 582.5m and the median STS repetitions was 59. There was no increase in risk of exacerbation during follow-up for those who performed below the median values for either the 6MWT (risk = 1.5, 95%CI = 0.68 - 3.42) or 1STS (risk = 0.78, 95% CI = 0.35 - 1.7).

Discussion

It was observed that 6MWD was significantly correlated with 1STS repetitions but neither was correlated with measures of pulmonary function in a sample of clinically stable CF subjects. Further, it was observed that those who desaturated during the 1STS, but not the 6MWT, had significantly lower FEV_1- %predicted and $FEF_{25-75\%}$%-predicted compared to those who did not desaturate during testing. These findings support the use of the 1STS in the CF population to assess functional capacity when the equipment and/or space for CPET or 6MWT is unavailable. These results further contribute to the literature that exercise testing in CF patients is a valuable tool to be

utilized in clinic as it provides a full assessment of disease beyond that captured in lung function testing alone.

The classic marker of morbidity and mortality in CF is FEV_1%-predicted and this measure is the primary driver of clinical decision making. However, improvements in early intervention and therapy options have slowed pulmonary decline, meaning this outcome may no longer be sensitive enough to indicate disease progression, especially as individuals live longer and develop more extra-pulmonary co-morbidities. Exercise testing has been shown to be a reliable and sensitive measure in CF, with exercise parameters demonstrating prognostic and interventional ability, even when FEV_1 is unaffected [41, 43, 48]. One study, in a sample of relatively healthy pediatric CF patients, found that VO_{2max} as measured by CPET, but not FEV_1%-predicted, was significantly correlated with structural damage as assessed by Bhalla score using High Resolution Computed Tomography [380]. Further, over a two-year period, Bhalla score and VO_{2max} decreased significantly, while FEV_1%-predicted remained stable. Rosenfeld and colleagues (2001) found that reports of decreased exercise tolerance had a greater association with pulmonary exacerbation (OR = 22.4) compared to classic markers including change in sputum appearance (OR = 11.4) and decline in FEV_1%-predicted (OR = 2.7) [381]. These findings confirm that exercise testing provides a unique opportunity to examine the complex, interacting systems of the pulmonary, cardiovascular, metabolic, and muscular systems in a way that system-specific tests do not and therefore should be included in routine clinical evaluation.

CPET is the gold standard for exercise testing and provides the most complete information about exercise parameters and limitations to exercise performance. However, CPET requires the use of expensive, space-consuming equipment and trained professionals to complete testing. Further, the stringent infection prevention protocol in CF requires disposal and extensive cleaning of equipment beyond normal requirements, resulting in additional expense and logistic complexity. Therefore, submaximal field testing lends itself well to assessing functional capacity in CF patients. Indeed, the 6MWT has been shown to be a valid and reliable measure in CF [356, 358] and has been shown to be significantly correlated with CPET [50]. Further, it is known to have significant prognostic ability, with shorter distance covered associated with hospitalization, antibiotic use, lung transplant and death [48, 49, 51]. Although the 6MWT is a well-used test, it requires a dedicated space to perform and not all clinics have an unobstructed, long hallway to perform the test. As such, it is of interest to explore exercise testing that may be done in a clinic room or at home with already available space and equipment. The 1STS mimics movements performed during daily activities

and can be used to measure lower limb muscle function and functional capacity [382, 383]. The 1STS has been most explored in COPD, where it has been shown to be associated with quadriceps muscle force, quality of life, and mortality [46]. In CF, Radtke and colleagues have demonstrated that the exercise response to 1STS was significantly correlated with the exercise response to CPET and measures of quality of life [47, 382]. These findings were confirmed by Gruet et al. (2016) who also demonstrated that desaturation during 1STS was significantly related to desaturation during CPET, demonstrating its ability as a surrogate measure for CPET [50]. Further, the 1STS lends itself well to repeated testing and may be useful to track daily changes in exercise tolerance to allow for better early identification and treatment of pulmonary exacerbation.

For the 6MWT, subjects walked similar distances to those previously reported in CF patients with similar pulmonary function [51, 360], which is shorter than previous reports of the mean 6MWD of healthy participants between 602 and 667 meters [362, 384, 385]. This sample performed more 1STS repetitions than has been reported in COPD patients [371] but was similar to what has been reported in CF patients [47]. The 6MWT and 1STS have been shown to be significantly correlated in healthy [362] and COPD [371, 377] samples, but their relationship has not previously been assessed in CF patients. It was observed that 6MWD and 1STS repetitions were significantly correlated with one another. SpO_2 and dyspnea were significantly correlated between tests, but HR response was greater during 6MWT, suggesting a higher reliance on cardiovascular function during this test. Individuals with CF have been shown to have attenuated cardiac function in response to exercise, with lower stroke volume and cardiac output compared to healthy individuals [305]. As such, performing the 6MWT appears to be more taxing on the cardiovascular system and therefore may have elicited more compensatory mechanisms (i.e. increased HR) than the 1STS. Additionally, dyspnea scores after the 1STS, although correlated with those during the 6MWT, were significantly higher than dyspnea scores following the 6MWT. As the 1STS is a measure of lower limb function, which has shown to be impaired in CF, this finding suggests that stress to the muscular system is perceived as more demanding to relatively healthy CF patients compared to cardiopulmonary stress.

The 1STS mimics activities performed in daily life (climbing stairs, short sprints, etc.) that stress the anaerobic system. Anaerobic metabolism has been shown to be impaired in CF individuals [350, 386] and anaerobic capacity has been shown to be related to pulmonary function in some reports [387]. There was not a significant correlation between the 6MWT or 1STS with measures of pulmonary function, similar to previous reports [350, 358, 388], suggesting that exercise

performance was not limited by pulmonary factors in the sample. Additionally, there was not a significant association between occurrence of pulmonary exacerbation and performance on 6MWT or 1STS or an increased risk of exacerbation in those who performed below the median value for either test. These results are somewhat dissimilar to previous findings that reported the prognostic ability of the 6MWT in CF patients. Martin et al. (2013) observed an increased risk of lung transplant and death for those who had the shortest 6MWD at 12-year follow-up [51]. These findings were replicated Flores et al. (2016) at a 7-year follow-up [49] and supported by another study which found 6MWD predicted risk of hospitalization over a five-year period in CF youth [48]. This study has important differences when compared to these previous studies and may explain the differences in findings. First, the follow-up time in this study was much shorter than previous studies. Second, the outcome of interest was acute pulmonary exacerbation, not severe disease events. Third, the sample was overall healthier and more homogenous than previously reported samples. In the sample, pulmonary function does not appear to be a limiting factor in performance on the 6MWT or 1STS as indicated by the lack of correlation between test performance and pulmonary function. As drop in FEV_1%-predicted is a major driver in the diagnosis of pulmonary exacerbation, it may be that in relatively healthy CF patients the lack of correlation between functional capacity and pulmonary function explains the lack of association between exacerbation and performance. To better comprehend the physiology behind these findings further research is needed to understand the limiting factors in exercise performance in CF. Desaturation during the 1STS was found to be significantly associated with desaturation during CPET, supporting the role of 1STS desaturation as a surrogate marker for CPET saturation status [50]. Those who desaturated during the 1STS, but not the 6MWT, had significantly lower FEV_1%-predicted, congruent with previous findings. These findings support the clinical usefulness of the 1STS in those with CF.

This study had several limitations. Firstly, a control group was not included by which to compare the results. Previous work on the 6MWT and 1STS has provided some reference values by which to compare this sample [362, 385]. However, these previously reported values do not necessarily correspond to the anthropometric or disease status of this sample. Secondly, the sample size was small and relatively healthy, limiting the generalizability and depth of conclusions that can draw from the data. Finally, individual differences in limb length, height, body mass, and daily physical activity levels, which may impact performance on 6MWT and 1STS, were not accounted for in analysis. Previous reports are inconclusive on whether these anthropometrics impact outcomes [47, 50, 389]. Further research is needed to better understand these interactions.

Both the 1STS and 6MWT have been shown to be sensitive to intervention and changes in disease status, with minimal clinical importance of 3-5 repetitions and 30-meters being reported, respectively [371, 382]. These findings further support the sensitivity and usefulness of these tests in clinical practice. An association between pulmonary exacerbation during follow-up and performance on 6MWT or 1STS was not observed in this study. However, both tests have been shown to provide clinically meaningful information outside of traditionally used clinical measures and further support the notion that exercise testing should be incorporated into monitoring of CF patients. The 1STS lends itself well to routine clinical testing as it can be performed in a clinic room using already available equipment and provides information beyond resting measures. Further, it can be performed at home and be used as a daily measure to assess reduced exercise tolerance, an important marker of pulmonary exacerbation. The results of this study support the use and clinical importance of the 6MWT and 1STS in CF patients.

Conclusion

The main finding from this study was 6MWD and 1STS repetitions were significantly correlated in a sample of relatively healthy CF patients. There was not an association between functional capacity testing and pulmonary exacerbation during follow-up. However, is was observed that those who desaturated during the 1STS had significantly lower FEV_1%-predicted, suggesting the usefulness of this test during routine clinical care.

Table Legend

Table 1. Subject Clinical Characteristics and Resting Values

Table 2. Correlations between 6MWT, 1STS and measures of pulmonary function.

Table 3. Performance and Physiological Response to 6MWT and 1STS

Table 1: Subject Clinical Characteristics and Resting Values

Variable	Female (n=13)	Male (n=10)	p-value	All
Age (years)	33.5 ± 8.1	38.2 ± 10.5	0.245	35.5 ± 9.3
Weight (kg)	61.1 ± 12.9	78.1 ± 12.7	0.003*	68.5 ± 15.2
Height (cm)	164.2 ± 9.2	174.1 ± 6.5	0.005*	168.5 ± 9.4
BMI (kg/m^2)	22.5 ± 3.0	25.7 ± 3.1	0.012*	23.9 ± 3.4
Systolic Blood Pressure (mmHg)	117.2 ± 10.5	127.2 ± 11.7	0.040*	121.5 ± 11.9
Diastolic Blood Pressure (mmHg)	75.0 ± 7.9	81.5 ± 8.1	0.012*	77.8 ± 8.5
Resting HR (beats per minute)	78.1 ± 9.0	82.5 ± 11.6	0.314	80.0 ± 10.2
Resting SpO_2	97.5 ± 1.3	96.4 ± 1.0	0.040*	97.0 ± 1.2
FEV_1%-predicted	79.7 ± 15.4	86.7 ± 24.3	0.407	82.7 ± 19.6
FVC%-predicted	92.8 ± 10.7	97.4 ± 14.3	0.391	94.8 ± 12.3
FEV_1/FVC%-predicted	71.7 ± 9.9	71.1 ± 11.3	0.894	71.4 ± 10.3
$FEF_{25-75\%}$%-predicted	59.7 ± 9.8	72.5 ± 43.9	0.258	62.8 ± 35.4
6MWD (m)	567.8 ± 59.5	559.8 ± 103.1	0.816	564.3 ± 79.3
1STS repetitions	59.7 ± 9.8	48.5 ± 14.2	0.036*	54.8 ± 12.9
Variable				**Frequency (%)**
Female				13 (56.5)
Homozygous F508del				13 (56.5)
Modulator Therapy				14 (60.8)
CFRD				12 (52.2)
Chronic *pseudomonas aeruginosa*				14 (60.9)

BMI = body mass index; HR = heart rate; SpO_2 = peripheral blood oxygen saturation; FEV_1 = Forced expiratory flow in one second; FVC = forced vital capacity; $FEF_{25-75\%}$ = Forced expiratory flow at 25-75%; 6MWD = six-minute walk distance; 1STS = one-minute sit-to-stand test; CFRD = Cystic Fibrosis related diabetes
Continuous data presented as mean ± standard deviation
*Denotes significance at $p < 0.05$

Table 2: Correlations between 6MWT, 1STS and measures of pulmonary function

Variable	6MWD	p-value	1STS Repetitions	p-value
6MWD	-	-	0.573	0.002*
1STS repetitions	0.573	0.002*	-	-
FVC%-predicted	0.101	0.323	0.206	0.173
FEV_1%-predicted	0.061	0.390	0.012	0.478
FEV_1/FVC%-predicted	0.117	0.297	-0.042	0.425
$FEF_{25-75\%}$%-predicted	0.085	0.350	-0.086	0.348

6MWD = six minute walk distance; 1STS = one-minute sit-to-stand; FEV_1 = Forced expiratory flow in one second; FVC = forced vital capacity; $FEF_{25-75\%}$ = Forced exiratory flow at 25-75%
Data presented are correlation coefficients obtain from Spearman correlation test.
*Denotes statistical significance at $p < 0.05$

Table 3: Performance and Physiological Response to 6MWT and 1STS

Variable	Mean ± SD
6MWT	
6MWD (m)	564.3 ± 79.3
Post-SpO_2	95.2 ± 2.1
1-min post-SpO_2	97.1 ± 1.4
Delta SpO_2	-4.3 ± 2.8
Post-HR	116.6 ± 15.8
1-min post-HR	98.3 ± 11.0
Post-Borg	2.9 ± 1.3
1-min post-Borg	2.0 ± 1.2
1STS	
1STS Repetitions	54.8 ± 12.9
Post-SpO_2	94.8 ± 2.8
1-min post-SpO_2	96.8 ± 1.3
Delta SpO_2	-2.3 ± 2.8
Post-HR	113.7 ± 9.2
1-min post-HR	95.0 ± 11.4
Post-Borg	3.8 ± 1.7
1-min post-Borg	2.2 ± 1.5
Variable	**Frequency**
6MWT – Desaturation	7 (30.4)
1STS – Desaturation	4 (17.4)
Any Desaturation	11 (47.8)

6MWD = six minute walk distance; SpO_2 = peripheral blood oxygen saturation
HR = heart rate; 1STS = one-minute sit-to-stand; 6MWT = six minute walk test

Figure Legend

Figure 1: Correlation of 6MWD and STS repetitions. Subjects completed 6MWT and 1STS on the same day of testing. Performance measures of 6MWD and STS repetitions were found to be significantly correlated using Spearman test ($r = 0.573$, $p = 0.02$).

Figure 2: Comparison of perceived dyspnea score immediately post-exercise test. Subjects completed 6MWT and 1STS on the same day. Measures of perceived dyspnea score using the Modified Borg Scale were collected at the end of each test. Bars represent mean dyspnea score as reported using modified Borg Scale. Error bars represent standard mean error.

Figure 3. Pulmonary function parameters and desaturation during exercise. Measures of SpO_2 were collected immediately upon ceassation of the 1STS. Desaturation was defined as change in SpO_2 > 4% from resting to end-of-exercise. Pulmonary function compared between subjects who did and did not desaturate during the 1STS. Bars represent mean value and error bars represent standard error mean.

Figure 1: Correlation of 6MWD and 1STS repetitions.

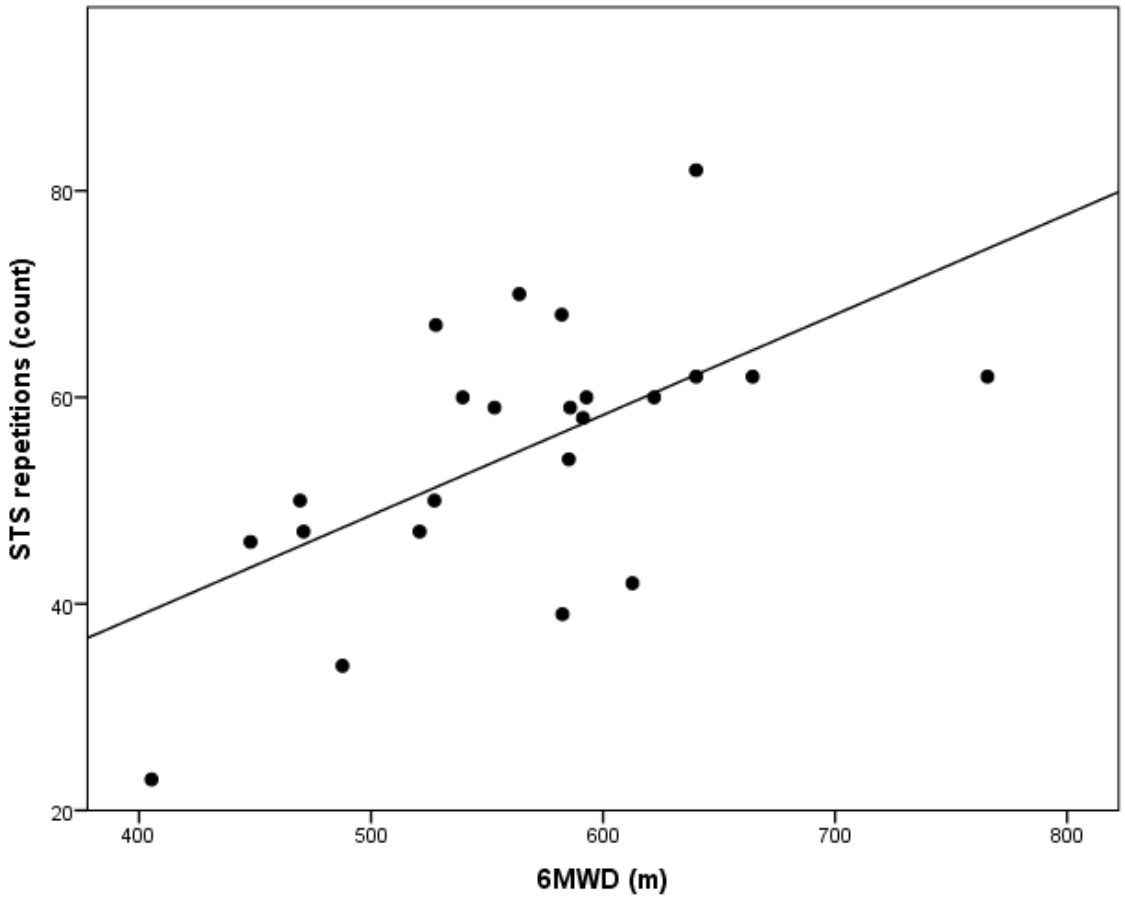
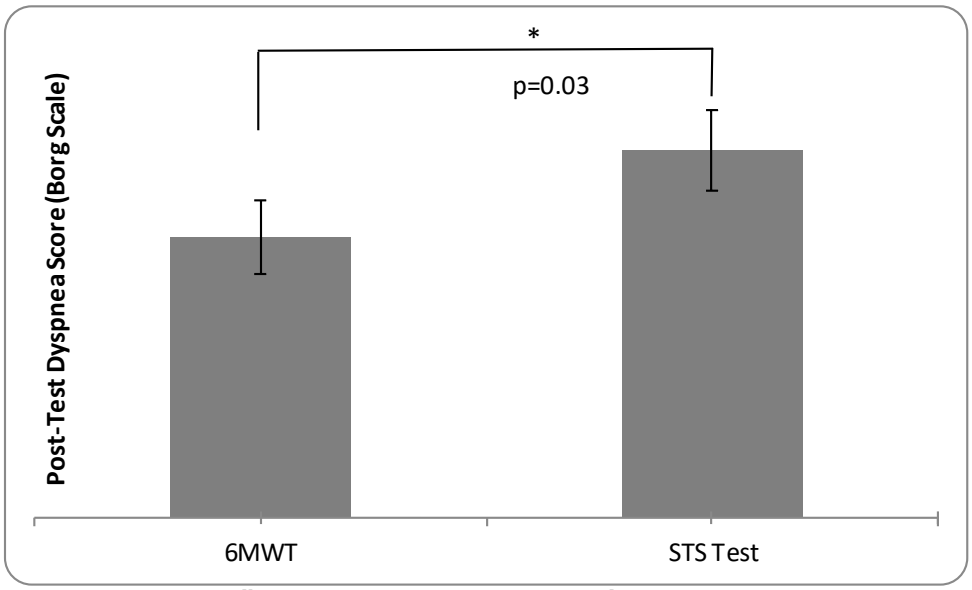
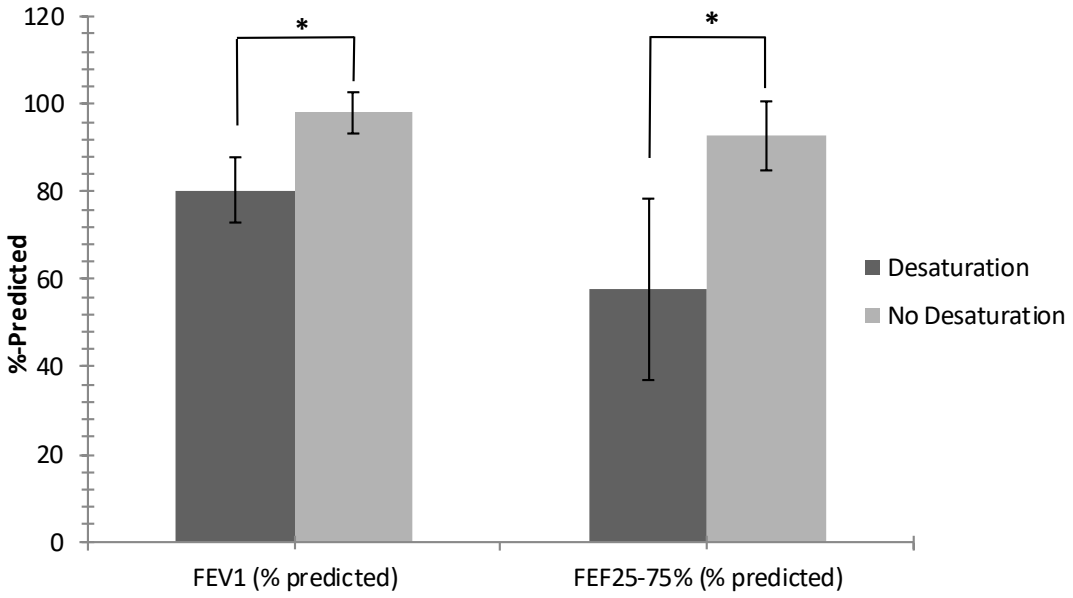


Figure 2: Comparison of perceived dyspnea score immediately post-exercise



6MWT = six-minute walk test; STS = one-minute sit-to-stand
* denotes signifigance ($p < 0.05$)

Figure 3: Pulmonary function parameters and desaturation during exercise



FEV_1 = Forced expiratory volume in one second; $FEF_{25-75\%}$ = Forced expirtory flow 25-75%
* denotes signifigance ($p < 0.05$)

Chapter 5: Study Three - Evaluation of the Desaturation-Distance Ratio in Cystic Fibrosis Patients

Synopsis

Background: The six-minute walk test (6MWT) is a well-validated clinical exercise test used to monitor a variety of patient populations. Traditional performance measures obtained during the 6MWT have been significantly correlated with hospitalization, time to lung transplant, and death in patients with Cystic Fibrosis (CF). Use of additional measures derived from the 6MWT may provide valuable clinical information beyond traditional measures. The purpose of this study was to examine the clinical utility of the desaturation-distance ratio (DDR) in patients with CF.

Methods: Twenty subjects were enrolled in the study. All subjects had at least one copy of the F508del mutation. Subjects completed the six-minute walk test (6MWT) with heart rate (HR) and peripheral blood oxygen saturation (SpO_2) being measured continuously. Two version of DDR were calculated (DDR_{Total} and DDR_{Simple}) using the ratio of the sum of desaturation during the 6MWT and distance covered (6MWD). Subjects were followed for at least 120-days and medical charts were reviewed for indicators of disease. Performance on exercise tests were evaluated for association with pulmonary exacerbation and hospitalization during the follow-up.

Results: The average 6MWD was 568.0±79.7m, average DDR_{total} was 2.93±0.85 and average DDR_{simple} was 0.045±0.02. Both measures of $DDR_{(total\ and\ simple)}$ provided greater correlation with classic clinical measures than either 6MWD or SpO_2 alone, though DDR_{total} correlated better with measures of pulmonary function and 6MWD than DDR_{Simple}, suggesting that DDR_{total} is a better indicator of lung function and functional capacity than DDR_{simple} in those with CF. Those who experienced a pulmonary exacerbation during follow-up had significantly higher $DDR_{(total\ and\ simple)}$ than those who did not have a pulmonary exacerbation during follow-up. There was no difference in 6MWD between those who did and did not have a pulmonary exacerbation.

Conclusions: DDR was significantly correlated with clinical measures of CF disease and may be useful when evaluating functional capacity in the CF population, especially when used in conjunction with typical 6MWT measures.

Keywords: F508del, exercise, six-minute walk test, DDR, pulmonary function, desaturation

Funding: None

Introduction

Cystic fibrosis (CF) is one of the most common autosomal recessive genetic disorders among Caucasians [56]. It is multi-system disease that is primarily characterized by chronic airway infection and progressive respiratory disease, with approximately 90% of CF patients dying from respiratory failure [390]. Improvements in disease identification, organization of care, therapy regiments, and medication options have led to vast improvements in clinical outcomes, increasing the median age of survival from approximately 10 years in the 1960s to over 40 years today [391]. With improving survival rates, emergence of non-lung co-morbidities not previously associated with CF are emerging. Notably, cardiovascular function has been shown to be attenuated in those with CF with increased vessel stiffness and cardiac dilation being noted [300, 301, 373, 392]. These new disease trends have prompted a need for sensitive and dynamic clinical outcomes that are able to assess multiple organ systems and their interactions to best measure disease severity [345].

Exercise capacity, as determined by maximal cardiopulmonary exercise testing (CPET), measures the functionality and coordination of the pulmonary, cardiovascular, and muscular systems to provide key insight in (dys)function of each system and their interactions that may not be apparent at rest [38, 345, 347]. CPET has been shown to be a significant independent predictor of morbidity and mortality in CF patients and provides meaningful information on disease severity and clinical outcomes [41, 43, 341-343]. As such, some countries recommend annual exercise testing for CF patients [337].

Although maximal CPET testing is the gold standard and provides valuable information, the equipment and staff needed to perform these tests may exclude facilities from being able to administer these tests [352]. The six-minute walk test (6MWT) is a simple and inexpensive field test that is designed to be administered in a variety of settings with minimal training [369]. The 6MWT has been shown to have prognostic value in a variety of disease populations, including CF. Martin et al. (2013) demonstrated, in a large diverse sample, that six-minute walk distance (6MWD) and desaturation during the 6MWT were indicative of increased risk of lung transplant or death [51]. Further, a study examining 6MWT in youths found that 6MWD, but not FEV_1, had a strong negative correlation with hospitalization days and that greater 6MWD reduced the risk of hospitalization over a five-year period [48]. The 6MWT is a useful tool in measuring disease status in patients with CF.

In addition to exercise capacity, desaturation during exercise has been shown to be an important indicator of disease severity. In those with idiopathic interstitial pneumonia, desaturation during exercise testing was a better predictor of mortality than exercise capacity alone [393]. Even without differences in 6MWD, one study found that CF patients who desaturated during 6MWT had greater clinical severity scores and bacterial infections than those who did not desaturate [360]. Further, greater desaturation during the 6MWT was associated with greater odds of lung transplant or death in a seven-year follow-up of CF patients [49]. The 6MWT demonstrates clinical relevance in characterizing the progression of CF disease, with outcomes obtained from the 6MWT providing insight into disease severity separate from pulmonary function testing.

As 6MWD and desaturation during exercise have been shown to be important indicators of disease, a measurement that takes both these variables into account may prove more useful than either alone. The desaturation-distance ratio (DDR) is a measurement that has been used in COPD and non-CF lung disease and encompasses both 6MWD and desaturation during exercise [394-396]. Previous work has shown its usefulness as a clinical outcome in providing additional information about exercise response than either 6MWD or desaturation alone. However, no work has been done to examine the usefulness of DDR in CF. The purpose of this study was to evaluate whether DDR provides additional benefit in the functional assessment of CF patients.

Methods

Subjects

All study procedures were approved by the Institutional Review Board at the University of Minnesota (IRB#00000972). Subjects were recruited via email from a listserv maintained by the Minnesota Cystic Fibrosis Center. All subjects provided written informed consent. Patients were eligible for participation if they had a confirmed diagnosis of CF by sweat test and/or genetic evaluation and had at least one copy of the F508del mutation, had a forced expiratory volume (FEV_1) greater than 40% predicted, and had no history of cardiovascular or inflammatory disease. Patients were excluded if they had experienced an exacerbation or change to medication in the four weeks prior to enrollment or were sick/injured the day of visit.

Six-Minute Walk Test

Twenty-four participants met eligibility criteria and were included in the study. Of those, five did not have analyzable heart rate (HR) and SpO_2 data during the walk test so they were excluded from

calculations for DDR. The 6MWT was performed according to current American Thoracic Society guidelines [354] and was administered by a trained researcher. Patients were instructed to walk as far as possible at their own pace along a 30-meter indoor corridor and were assured they could rest or stop at any time during the test. Test procedures were demonstrated by the researcher and an opportunity to practice directions was given prior to the start of the test to reduce learning effect. Standard phrases were communicated every minute. HR and SpO_2 were evaluated continuously at two-second intervals during the duration of the test and during recovery via a blue-tooth enabled finger probe (Masimo MightStat, Masimo Corp., Irvine, CA, USA). Measures of dyspnea were taken at the beginning and end of the 6MWT using the modified Borg scale [397]. Demographic, anthropometric, and lung function data was collected from patient medical records.

Desaturation Distance Ratio (DDR)

The desaturation area (DA) was calculated as previously described [394, 395]. Briefly, the total area above the SpO_2 curve was calculated by subtracting each SpO_2 reading from 100% and then adding all the differences together. DDR was then calculated by using the equation DDR = DA/6MWD. Two versions of the DDR (DDR_{total} and DDR_{simple}) were calculated based on different methods described in the literature [394, 395]. DDR_{total} used SpO_2 readings taken every two seconds whereas DDR_{simple} calculated DA based on SpO_2 readings taken each minute.

Lung Function

Lung function was assessed during regularly scheduled clinic visits according to American Thoracic Society guidelines and standards [367]. Forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), ratio of forced expiratory volume in one second to forced vital capacity (FEV_1/FVC), and forced expiratory flow 25 to 75% of FVC ($FEF_{25-75\%}$) were recorded. All results are expressed in %-predicted.

Pulmonary Exacerbation

Days to exacerbation were recorded during the follow-up period. Presence of exacerbation was defined based on provider notes and prescription of antibiotic(s). Follow-up time ranged from 120 days to 300 days.

Statistical Analysis

All statistical analyses were performed using SPSS Statistics for Windows, Version 23 (IBM Corporation, Armonk, NY, USA). All quantitative data is expressed as mean±SD or counts and

percentages, where appropriate. Student's independent t-test was performed to compare outcome means between significant markers of disease. Correlations between 6MWT, DDR, and pulmonary function were assessed using Spearman's correlation coefficient. Significance was set at 0.05.

Univariate Cox proportional hazards regression were determined with days to exacerbation as the dependent variable and the following independent variables: DDR_{Total}, DDR_{Simple}, 6MWD, FVC%-predicted, FEV_1%-predicted, FEV_1/FVC%, and $FEF_{25-75\%}$%-predicted.

Results

Subjects used in this study represent a sub-sample from Study One. Twenty-four participants (11 males, 13 females) completed study procedures and were included in analysis, however due to technical difficulties during data collection DDR values were not able to be calculated for five participants. Sample characteristics are described in Table 1. Subjects had relatively healthy lungs with an average FEV_1-%predicted of 82.0±19.5% and average FVC-%predicted of 94.5±12.1%. Ten participants had an FEV_1-%predicted below 80%, indicative of mild obstructive lung disease. All participants had at least one copy of the F508del mutation, with fourteen participants being F508del homozygous. All but one subject were pancreatic insufficient and 13 subjects had CF-Related Diabetes (CFRD) (54.2%).

The average 6MWD for all participants was 568.0±79.7m with a range of 405-766 meters. Distance covered did not differ significantly between FEV_1-%predicted status (below 80%predicted: 563.1±83.5 meters v. above 80%predicted: 571.5±79.8 meters, $p = 0.4$), number of F508del copies (homozygous: 552.8±84.0 v. heterozygous: 589.2±71.9 meters, p = 0.14), or presence of CFRD (present: 549.65±77.5 v. absent: 589.7±80.2 meters, p = 0.11). All subjects completed the 6MWT without stopping and no subject required supplemental oxygen. 6MWD correlated significantly with measures of heart rate response but not measures of SpO_2 or pulmonary function (Table 2).

DDR_{total} was calculated for nineteen participants and DDR_{simple} was calculated for twenty participants where satisfactory data was collected. Average DDR_{total} was 2.93±0.85 and average DDR_{simple} 0.045±0.02. Results from Spearman analysis are depicted in Table 2. DDR_{total}, but not DDR_{simple}, was significantly correlated with FEV_1%-predicted (r= -0.401, p = 0.045 and r = -0.301, p = 0.099, respectively) and FVC%-predicted (r = -0.412, p = 0.047; r = 0.003, p = 0.496). Both measures were significantly correlated with FEV_1/FVC-% (r = -0.412, p = 0.040; r = -0.539, p = 0.007). Neither were correlated with FEF_{25-75}%-predicted (r = -0.372, p = 0.059 and r = -0.335, p =

0.075, respectively). These findings suggest that DDR_{total} is a better indicator of lung function than DDR_{simple} in those with CF. Interestingly, DDR_{simple} correlated with more measures of SpO_2 than DDR_{total}. $DDR_{total,}$ but not DDR_{simple}, was correlated with 6MWD ($r = -0.438$, $p = 0.030$ and $r = -0.222$, $p = 0.173$, respectively). Resting HR and SpO_2 were not significantly correlated with measures of DDR, 6MWD or pulmonary function, however HR response during exercise was correlated with 6MWD. Dyspnea as measured by Borg scale was not significantly correlated with DDR_{total}, DDR_{simple}, 6MWD, or measures of pulmonary function.

Eleven individuals experienced a pulmonary exacerbation during follow-up. Univariate Cox proportional hazards were calculated using the predictors DDR_{Total}, DDR_{Simple}, 6MWD, FVC%-predicted, FEV_1%-predicted, FEV_1/FVC%, and $FEF_{25-75\%}$%-predicted to explain days to exacerbation. None of the measures of pulmonary function, DDR measures, or 6MWD were significantly associated with days to exacerbation. Those who experienced an exacerbation during follow-up had significantly higher $DDR_{(total\ and\ simple)}$ (1.62±0.41 v. 1.24±0.36, p=0.03; 5.36±1.55 v. 4.03±0.95 p=0.02, respectively). There was no difference in 6MWD between those who did and did not experience an exacerbation during follow-up (554.5±95.8 v 589.3±64.2 p= 0.19, respectively).

Discussion

Previous research has demonstrated the clinical importance of exercise testing in patients with CF and current recommendations call for annual exercise testing of CF patients [43, 337, 347]. As CF impacts several organ systems including pulmonary, cardiovascular, metabolic, and muscular, exercise testing provides unique insight into the (dys)function of these systems [345]. The 6MWT is an inexpensive, simple submaximal exercise test that can be administered to patients, even those with severe disease, and has been shown to be associated with clinical outcomes in several patient populations, including CF [51, 342, 398]. As great variation in clinical presentation and disease severity is demonstrated in CF, additional measures derived from the 6MWT may prove useful in accurately characterizing functional capacity in this patient population. In this study the correlation of DDR with pulmonary function in patients with CF was evaluated. It was found that DDR_{total} was better correlated with measures of pulmonary function than DDR_{simple} and neither was correlated with HR response during exercise. Conversely, 6MWD was significantly correlated with HR response during exercise but not pulmonary function or SpO_2, suggesting that DDR and 6MWD provide insight into related but separate components of exercise response. Both measures of

$DDR_{(total\ and\ simple)}$ provided greater correlation with classic clinical measures than either 6MWD or SpO_2 alone and may be a useful measure of exercise capacity in the CF population.

Both 6MWD and desaturation during exercise have independently been shown to have prognostic value in lung diseases [399, 400], including CF [48, 51]. Creating a metric that combines these two variables may improve insight into disease state and provide a more complete view of exercise response. DDR was first used in patients with interstitial lung disease where it was found that DDR, 6MWD, and SpO_2 measures all significantly correlated with measures of pulmonary function [394]. However, DDR showed a stronger correlation with pulmonary function, notably diffusing capacity of the lungs for carbon monoxide (DL_{CO}), compared to 6MWD and SpO_2 alone, suggesting that DDR provides additional clinical information than either measure alone. Similar to the current findings, the authors noted that 6MWD was not correlated with measures of SpO_2, suggesting that 6MWD and SpO_2 data provide insight into different components of exercise response. Follow-up studies completed in COPD patients support initial findings on DDR, showing stronger correlation of DDR with measures of pulmonary function than 6MWD alone [395, 396].

In contrast to previous reports, there was not a correlation between 6MWD and pulmonary function observed in this study. This may be due to the relatively healthy nature of the sample population compared the other studies. Fujimoto et al. (2017) found that in patients who had greater obstructive lung disease as defined by $FEV_1/FVC\% < 70\%$, 6MWD was significantly correlated with FEV_1%-predicted, however this was not the case for patients with a $FEV_1/FVC\% > 70\%$, where 6MWD and pulmonary function measures were not correlated [395]. Results of the correlation between 6MWD and FEV_1%-predicted, the most commonly reported pulmonary function measure among CF patients is mixed, with some studies finding significant correlation between 6MWD and FEV_1%-predicted [48, 51] but not others [49, 401]. This may be due to differences in statistical methods, severity of disease, and CF mutation. Additionally, it was found that DDR_{total}, but not DDR_{simple}, was significantly correlated with measures of pulmonary function. This suggests that collecting SpO_2 at one-minute intervals may not be sufficient to calculate a meaningful DDR value in CF patients. This is in contrast to previous research in COPD patients which found that DDR_{total} and DDR_{simple} produced congruent results [395]. Additionally, there was no difference in 6MWD between subjects who experienced a pulmonary exacerbation during follow-up but $DDR_{(total\ and\ simple)}$ were significantly higher in those who experienced a pulmonary exacerbation compared to those who did not, suggesting that DDR measures provide more insight into disease than 6MWD alone.

In this study it was found that DDR, but not 6MWD, correlated with measures of SpO_2, consistent with previous findings [396]. However, not reported previously but of interest, is the finding that DDR correlated with measures of SpO_2 but not measures of HR, whereas 6MWD correlated with all measures of HR but not SpO_2. This finding suggests that different parameters of the 6MWT provide insight into different aspects of exercise response and may help to better understand limiting factors of exercise in CF patients. This is important as cardiovascular function has been shown to be attenuated in those with CF [300, 301] and impaired cardiac response to exercise has been noted [52, 304, 392]. HR response to exercise is an important measure of cardiovascular health, with impairment associated with morbidity and mortality in healthy and diseased populations [402-405]. One study found that heart rate response during the 6MWT was significantly associated with clinical outcomes one-year post pulmonary endarterectomy in those with thromboembolic pulmonary hypertension [406]. This observation suggests that although DDR may provide meaningful insight into SpO_2 response to exercise, it does not provide information on HR response, a clinically relevant outcome. Therefore, DDR may serve as a useful tool for practitioners to assess exercise capacity but should not be used to the exclusion of classic 6MWT outcomes.

Limitations of this study include a small, relatively homogenous sample size, blocking the ability to determine usefulness of DDR across a continuum of disease states. We did not include measures of daily physical activity, which may have impacted performance results and contributed to findings. Additionally, due to the cross-sectional nature of this study no follow-up was achieved to determine prognostic value of DDR. It may be that change in DDR is more indicative of disease status. Finally, as CF disease impacts multiple organ systems collecting data on additional markers of disease, in addition to pulmonary function, would have been beneficial. Future research should examine how well DDR functions as a prognostic for morbidity and mortality in CF.

Conclusion

DDR is significantly correlated with measures of SpO_2 and pulmonary function and, used in conjunction with typical 6MWT outcomes, may be a helpful tool in evaluating exercise capacity in patients with CF.

Table Legend

Table 1. Subject clinical characteristics

Table 2. Spearman correlation coefficients for measures of functional capacity and pulmonary function

Table 1: Subject clinical characteristics

Variable	Mean	Range
Age (years)	35.68 ± 9.14	20.70 – 60.43
Height (cm)	168.35 ± 9.25	152.40 – 185.39
Weight (kg)	68.36 ± 14.87	46.41 – 96.58
BMI (kg/m^2)	23.87 ± 3.31	19.16 – 30.68
Resting SpO_2	97.01 ± 1.19	95 - 100
Resting HR (bpm)	80.06 ± 9.94	61 - 99
Systolic Blood Pressure (mmHg)	121.75 ± 11.72	102-146
Diastolic Blood Pressure (mmHg)	77.67 ± 8.33	67-100
FVC%-predicted	94.5 ± 12.1	72-125
FEV_1%-predicted	82.0 ± 19.5	53-118
FEV_1/FVC%	71.0 ± 10.2	50-85
$FEF_{25-75\%}$%-predicted	61.3 ± 35.4	18-143
6MWD (m)	567.98 ± 79.67	405-766
DDR_{total}	2.93 ± 0.85	1.62 – 4.49
DDR_{simple}	0.045 ± 0.017	0.004 – 0.075
	Frequency (%)	
Female	13 (54.2)	
F508del homozygous	14 (58.3)	
CFRD	13 (54.2)	
Current *pseudomonas aeruginosa*	15 (62.5)	
Current *staphylococcus aureus*	8 (33.3)	
Chronic *pseudomonas aeruginosa*	15 (62.5)	
Chronic *staphylococcus aureus*	7 (29.2)	

BMI = body mass index; SpO_2 = peripheral blood oxygen saturation; HR = heart rate; FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second; $FEF_{25-75\%}$ = forced expiratory flow at 25-75%;
6MWD = six minute walk distance; DDR = desaturation-distance ratio; CFRD = Cystic Fibrosis related diabetes

Table 2: Spearman correlation coefficients for measures of functional capacity and pulmonary function

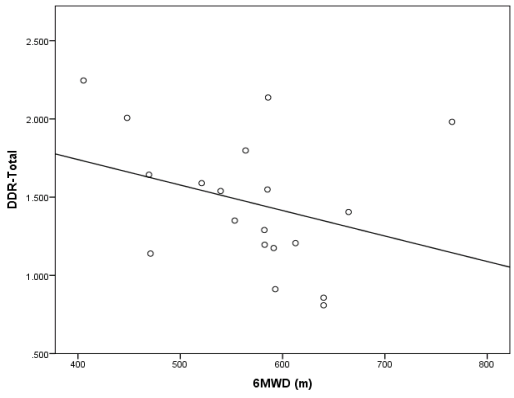
DDR_{total}	*r*	p
FEV_1%-predicted	-0.401	0.045*
FVC%-predicted	-0.396	0.047*
FEV_1/FVC%	-0.412	0.040*
$FEF_{25-75\%}$-predicted	-0.372	0.059
Resting SpO_2	-0.137	0.288
Post-SpO_2	-0.361	0.064
Delta SpO_2	-0.275	0.127
Lowest SpO_2	-0.431	0.033*
Resting HR	0.061	0.401
Post-HR	-0.201	0.212
Change in HR	-0.323	0.096
Highest HR	-0.163	0.259
HR recovery	0.164	0.258
DDR_{simple}		
FEV_1%-predicted	-0.301	0.099
FVC%-predicted	0.003	0.496
FEV_1/FVC%	-0.539	0.007*
$FEF_{25-75\%}$-predicted	-0.335	0.075
Resting SpO_2	-0.306	0.095
Post-SpO_2	-0.759	0.000*
Delta SpO_2	-0.581	0.004*
Lowest SpO_2	-0.759	0.000*
Resting HR	0.173	0.233
Post-HR	0.031	0.449
Change in HR	-0.187	0.222
Highest HR	0.106	0.338
HR recovery	0.340	0.077
6MWD		
FEV_1%-predicted	-0.007	0.487
FVC%-predicted	0.024	0.465
FEV_1/FVC%	0.046	0.416
$FEF_{25-75\%}$-predicted	0.008	0.485
Resting SpO_2	-0.025	0.454
Post-SpO_2	-0.037	0.432
Delta SpO_2	0.016	0.471
Lowest SpO_2	-0.241	0.160
Resting HR	-0.205	0.169
Post-HR	-0.837	0.000*
Change in HR	-0.748	0.000*
Highest HR	0.821	0.000*
HR recovery	-0.551	0.004*
DDR_{total}	-0.438	0.030*
DDR_{simple}	-0.222	0.173

FEV_1 = Forced expiratory flow in one second; FVC = forced vital capacity
$FEF_{25-75\%}$ = Forced exiratory flow at 25-75%; SpO_2= peripheral blood oxygen saturation
HR = heart rate; DDR = desaturation-distance ratio
*Denotes significance at $p < 0.05$

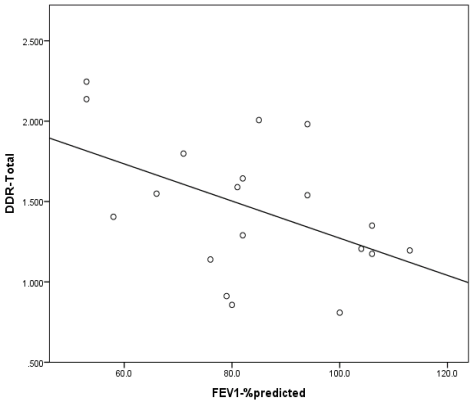
Figure Legend

Figure 1: Correlation between measures of functional capacity and pulmonary function. SpO_2 was continuously measured during the 6MWT and DDR was calculated using the sum of desaturation during the 6MWT and 6MWD. Spearman correlation was used to determine the following relationships: a.) $DDR_{total,}$ was significantly correlated with 6MWD ($r = -0.438$, $p = 0.030$) and b.) FEV_1%-predicted ($r= -0.401$, $p = 0.045$) but c.) 6MWD was not significantly correlated with FEV_1%-predicted ($r = -0.007$, $p = 0.487$).

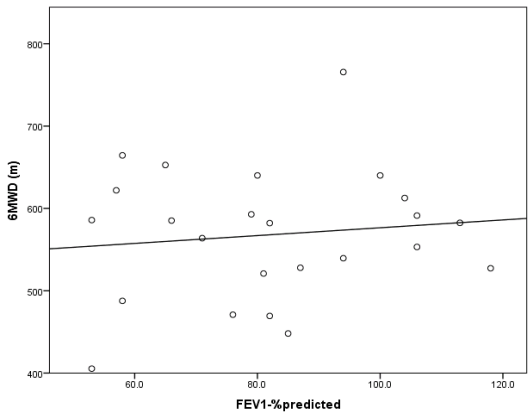
Figure 1: Correlation between measures of functional capacity and pulmonary function.



a)



b)



c)

DDR = Desaturation-distance ratio; 6MWD = six-minute walk distance; FEV_1 = Forced expiratory flow in one second.

Chapter 6: Limitations

This dissertation was limited by several factors, including those inherent to cross-sectional study design and genetic methodology. Notably, the small sample size and uneven genotype distribution in Study One limited statistical power and did not allow comparison between each genotype (AA, AT, TT). As only 2 subjects presented with the TT genotype, two genotypes were collapsed into one group (AA, AT/TT) even though physiological differences may exist between the AT and TT genotypes. Further, the large spread in data given the sample size may have muffled the statistical and clinical significance of the findings. Post-hoc power analysis was performed on the clinical outcomes of antibiotic days (0.22), CFRD (0.16), MDR *pseudomonas aeruginosa* (0.17), 6MWT (0.16), 1STS (0.33) to confirm that the study was not powered well enough to detect statistically significant differences between the A and T groups in these measures. Given this, the required sample size needed to detect a statistically significant difference ($p < 0.05$) was calculated: antibiotic days (n=121/group), CFRD (n=138/group), MDR *pseudomonas aeruginosa* (n=138/group), 6MWT (n=167/group), and 1STS (n=60/group).

Subjects were recruited via listserv email and self-identified to participate in the study. This may have skewed the sample towards healthier, more active participants as sicker, less active patients may have self-selected out of the study, which was advertised as involving exercise. Additionally, more sensitive measures of lung obstruction and damage, such as that available from computed tomography and magnetic resonance imaging scans, were not available. Previous studies have used validated scoring measures, such as Bhalla [407] and Shwachman-Kulczycki [408], to describe lung structure damage in CF patients. These measures would have provided better insight into disease progression, especially in younger patients, than pulmonary function testing. Finally, due to the relatively healthy sample, there were not enough adverse events (hospitalizations, lung transplant, etc.) to determine any differences in these clinically important outcomes. Inclusion of a more clinically diverse sample may have allowed for these comparisons and provided a more complete picture of the association between genetic variation in the SCNN1A gene and exercise response with clinical outcomes in CF.

Exercise testing was only completed once during the study. Learning effects have been reported in regards to exercise testing, with best outcomes being achieved after practice of exercise protocol [409]. Although the American Thoracic Society Guidelines for the 6MWT do not include having the subject perform the test twice, some have found a practice effect with better performance on subsequent 6MWT [410, 411], though others have not observed this [412] suggesting degree of learning effect may be specific to patient population Due to time and space limitations in clinic

only one session of the 6MWT and 1STS were completed for each subject. These may have resulted in exercise testing measurements that did not reflect a subject's best performance. Standard instructions and encouragement were given to each subject along with demonstration of protocol and opportunity to practice prior to data collection. Further, we did not collect any measures of daily physical activity to assess for any training effects that may have been present between groups.

Chapter 7: Conclusion and Future Directions

Conclusion

CF is an autosomal recessive genetic disease that results in dysfunction of the chloride channel CFTR, leading to dysregulation of chloride transport in epithelial tissue throughout the body, including the lung. In addition to chloride transport, CFTR is involved in regulating sodium transport by serving as an inhibitor of ENaC. In the absence of CFTR, ENaC remains uninhibited resulting in hyper-absorption of sodium across the apical membrane and subsequent depletion of the ASL. This depletion of the ASL causes development of sticky mucus that impedes mucociliary clearance and contributes to eventual tissue necrosis and respiratory failure. Although there is no cure for CF, improvements in screening and therapeutic options has extended average lifespan and slowed disease progression. This has prompted the need for clinical tests and interventions that are more personalized and sensitive to achieve optimal outcomes for every patient. The purpose of this dissertation was to examine the association of genetic variation in alpha ENaC and exercise response with clinical outcomes in patients with CF. Overall, the three unique studies presented in this work focus on improving personalized care and monitoring of CF patients.

The **first aim** was to examine the association of genetic variation at position 663 of the SCNN1A gene, which encodes for the alpha subunit of ENaC, with clinical outcomes in CF patients (Chapter 3). It was hypothesized that those with at least one copy of the more active T663 allele (AT/TT) would have poorer clinical outcomes than those homozygous for the A663 allele (AA). This hypothesis was based on the reasoning that greater ENaC activity would further contribute to the sodium hyper-absorption already present in CF, effectively accelerating the mechanism of lung disease. However, the findings did not fully support the hypothesis as there were not any statistically significant differences between the two groups in the primary outcomes of antibiotic use, hospitalization, or presence of co-morbidities. Using LMM a trend across time was observed that those in the A group had greater pulmonary function than those in the T group, though this observation was not statistically significant. Although not statistically significant, those in the A group walked an average of 26.3 meters further on the 6MWT and performed, on average, 8 more repetitions during the 1STS than those in the T group. This is worth noting because previous work has suggested that the minimal clinical significance for the 6MWT is 30 meters and for the 1STS is 3-5 repetitions. So although there was no statistical difference in these measures between groups the differences observed are still large enough to suggest some clinical importance. Due to the large variability in the data and the relatively small sample size the study was not powered high enough to detect any true differences that may exist. Additionally, low numbers in the TT genotype (n=2),

did not allow for comparison of outcomes between the three genotypes (AA, AT, TT) but instead the two genotypes containing the T663 variant (AA, AT/TT) had to be combined. This may have washed out any true, clinically relevant modifying effect that the TT genotype may have on CF disease or heterozygote advantage that may be present. Further, a difference in allele frequency between the study sample and those previously reported was observed. Notably, the frequency of the TT genotype seemed especially low, suggesting that there may be a connection between CF and genetic variation at position 663. The findings from Study One suggests that genetic variation at position 663 of the SCNN1A may modify pulmonary disease in CF, though the effect may not be as great as already appreciated disease modifiers.

The **second aim** was to examine the correlation of physiological parameters and performance between the 6MWT and 1STS in patients with CF (Chapter 4). It was hypothesized that physiological parameters and performance outcomes collected during the 6MWT would be positively correlated with those of the 1STS. The findings supported the hypothesis, as 6MWD and 1STS repetitions, the main performance outcome of each, were significantly correlated. Additionally, the study aimed to assess the association of the 6MWT and 1STS with pulmonary exacerbation during follow-up and it was hypothesized that performance outcomes from both the 6MWT and 1STS would be associated with pulmonary exacerbation. These findings did not support the hypothesis as neither 6MWD nor 1STS repetitions was significantly correlated with time to or presence of pulmonary exacerbation. Given the previous literature that 6MWD is significantly correlated with measures of mortality and hospitalization in CF it was surprising that neither measure was associated with pulmonary exacerbation. This may be because the sample had less severe lung disease than those previously reported and prognostic ability may be somewhat dependent on severity of lung disease. Additionally, because functional capacity was assessed at only one time-point change in functional capacity overtime was not able to be evaluated. It may be that in patients with healthier lungs change from baseline functional capacity is more helpful in predicting pulmonary exacerbation than an isolated functional capacity test. Future research is needed to assess this question. The association of desaturation during exercise testing with clinical measures was also assessed and it was found that those who desaturated during the 1STS, but not 6MWT, had significantly lower FEV_1%-predicted and $FEF_{25-75\%}$%-predicted compared to those who did not desaturate. This suggests that although measures of 6MWT and 1STS are correlated the physiological response to each test is unique and incorporating both tests into routine clinical care may be useful.

The **third aim** of this dissertation was to evaluate the association of DDR to measures of pulmonary function in CF subjects (Chapter 5). It was hypothesized that DDR would be significantly associated with measures of pulmonary function in CF subjects and be associated with pulmonary exacerbation during follow-up. The hypothesis was partially supported by the findings. It was observed that both DDR_{total} and DDR_{simple}, were significantly correlated with measures of pulmonary function, though DDR_{total} was more strongly correlated, suggesting this measure is a better indicator of lung function. Even though neither DDR measure was significantly associated with days to exacerbation as it was hypothesized, those who experienced an exacerbation during follow-up had significantly higher $DDR_{(total\ and\ simple)}$ than those who did not report an exacerbation. This was not true of 6MWD, which saw no difference between those who did and did not experience a pulmonary exacerbation during follow-up. These results suggest that DDR_{total} may be a useful supplementary measure in CF patients performing the 6MWT.

This dissertation adds to the current body of literature on clinical monitoring in CF. Although clear evidence that variation at position 663 of the SCNN1A gene is a modifier of CF lung disease was not observed, this work did add important observations to this question that suggest some degree of disease modification may be present. Additionally, it was demonstrated that the 1STS and DDR, measures not currently used clinically in CF, provide relevant information that can be obtained with minimal time and equipment.

Future Directions

The findings of this dissertation highlight the importance of exploring genetic modifiers and clinical utility of functional capacity testing in CF. In regards to Study One, future research should better characterize the frequency of A663 and T663 variants in the CF population by collection data from a wider range of ages, CFTR mutation classes, and disease states. Additionally, it is of interest to examine the mechanisms of this SNP to better understand how this genetic variation impacts disease processes, such as mucociliary clearance and inflammation, in CF. Further, exploring the pharmacological interactions that SNPs may have with commonly used treatments would help to propel personalized medicine regiments. In regards to Study Two, future research should explore the feasibility and usefulness of repeated, at-home measures of the 1STS to monitor CF disease. Additionally, care should be taken to examine the association between changes in easily obtained resting measures, such as HR and SpO_2, and development of pulmonary symptoms. Finally, in regards to Study Three, future research should better examine how well DDR functions as a prognostic for important clinical milestones such as antibiotic use, hospitalization, lung transplant,

and death. As there is currently no cure for CF it is vital that research efforts are given to expanding the knowledge of intricate disease processes and developing purposeful monitoring techniques to ensure that all patients receive the best possible outcomes.

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Chapter 9: Appendices

Appendix A
Patient Recruitment Letter

Greetings,

The University of Minnesota is currently conducting a research study for Cystic Fibrosis Patients age 7-64 years. We are currently collaborating with physicians and nurse practitioners from the Clinics and Surgery Center and Pediatric Specialty Care Discovery Clinic to recruit for this study.

With this research we hope to better understand the clinical impact that genetic variation within a specific cellular channel has in Cystic Fibrosis. If you choose to participate you would complete study procedures in-clinic at your next clinic visit or during a scheduled visit to the Laboratory of Physiological Hygiene and Exercise Science at the University of Minnesota – Twin Cities. These procedures include a cheek swab, submaximal exercise test, and collection of exhaled breath condensate which involves breathing into a tube at rest. These procedures will add 30-45 minutes to your clinic visit. Compensation will be provided.

If you are interested in participating, or simply would like more details about the study before agreeing, please feel free to contact me directly with the information listed below.

Thank you so much for your time!

Best Regards,

Hanan Zavala

Email: zaval013@umn.edu

Appendix B
Minnesota Cystic Fibrosis Center Listserv Recruitment Email

"Cystic Fibrosis Patients Needed for Research Study Examining Genetic Variability and Clinical Outcomes"

We are currently seeking individuals with Cystic Fibrosis caused by del.F508 mutation to take part in a study being conducted at the University of Minnesota looking at genetic variability and clinical outcomes. With this research we hope to better understand how genetic variation within a specific cellular channel impacts clinical outcomes in Cystic Fibrosis. Study procedures include collection of a cheek swab and exhaled breath condensate (which involves breathing into a tube at rest), along with functional capacity tests (which involve completing everyday activities such as walking). These procedures take approximately 30 - 45 minutes to complete and are done the same day as a regularly scheduled clinic visit. Compensation is provided.

If you are interested in participating or would like more details about the study, please email zaval013@umn.edu to determine if you are eligible.

Thank you so much for your time! We look forward to hearing from you.

Appendix C

Study Consent Form
IRB Study Number: 00000972

Title of Research Study: Title of Research Study: Influence of Genetic Variation of αENac in Cystic Fibrosis Patients

Researcher Team Contact Information: Hanan Zavala, M.S.

For questions about research appointments, the research study, research results, or other concerns, call the study team at:

Researcher Name: Hanan Zavala, M.S. Email Address: zaval013@umn.edu	Research Advisor: Eric Snyder, Ph.D Email Address: snyd0180@umn.edu

What is research?
Doctors and researchers are committed to your care and safety. There are important differences between research and treatment plans:

The goal of research is to learn new things in order to help groups of people in the future. Researchers learn things by following the same plan with a number of participants, so they do not usually make changes to the plan for individual research participants. You, as an individual, may or may not be helped by volunteering for a research study.
The goal of treatment is to help you get better or to improve your quality of life. Doctors can make changes to your treatment plan as needed.

Why am I being asked to take part in this research study?
We are asking you to take part in this research study because you have a diagnosis of cystic fibrosis and are being seen in clinic today.

What should I know about a research study?
Someone will explain this research study to you.
Whether or not you take part is up to you.
You can choose not to take part.
You can agree to take part and later change your mind.
Your decision will not be held against you.
You can ask all the questions you want before you decide.

Why is this research being done?
The purpose of this research is to learn more about why certain CF patients have better clinical outcomes than others. We are interested in looking at a specific part of the DNA to see if individual differences in this area are associated with better lung function. We are also interested in seeing how this area of DNA influences aerobic capacity in patients with CF. There are no direct benefits to participating in this study. With this information we hope to improve care for patients in the future by encouraging personalized medical decisions based on an individuals' DNA.

How long will the research last?

Your participation will last only during today's visit (1 visit) and take approximately 30 – 45 minutes to complete. Your electronic medical record will be reviewed and data extracted from clinic visits and hospitalizations up to one year after enrollment into the study.

How many people will be studied?
We expect up to 200 adults will be in this research study and up to 200 children, for a total of up to 400 participants.

What happens if I say "Yes, I want to be in this research"?
Today you will be asked to complete a cheek swab, which involves rubbing the inside of both cheeks with a toothbrush like instrument. Then you may be asked to breathe into a mouthpiece for approximately 10 minutes. Finally, you may be asked to complete three submaximal exercise tests. One test involves walking at a brisk pace for six minutes, one involves stepping up and down on a step for three minutes, and one involves transitioning from sitting and standing for one minute. Information from your medical chart (including results from pulmonary function tests, hospitalizations, and demographic information) will be used, including information from previous clinical visits and clinical visits taking place up to one year after today's date. You will be monitored by a trained researcher during all procedures and procedures will be ceased immediately at any point during data collection if you do not want to continue. All clinical tests, exams, and consultations as ordered by your provider will still be done, even if you do not take part in the research study. Declining to participate does not alter your care during this or any future visits.

What happens if I say "Yes", but I change my mind later?
You can leave the research at any time. Leaving will not be held against you.

If you decide to leave the research, provide verbal notice or written notice to the investigator. Verbal notice can be given to the investigator in person or by contacting them at 952-456-6356. Written notice can be sent to Room 110A Cooke Hall 1900 University Ave SE, Minneapolis, MN 55455 or zaval013@umn.edu so that the investigator can cease collecting further data from the medical record. Your written request does not need to provide a specific reason for withdrawal.

Choosing not to be in this study or to stop being in this study will not result in any penalty to you or loss of benefit to which you are entitled. Meaning, your choice not to be in this study will not negatively affect your right to any present or future medical treatment.

If you withdraw, no more information will be collected from you. When you indicate that you wish to withdraw, the information already collected from you will be used in the study because we will not be able to remove it from the information we have gathered.

What are the risks of being in this study? Is there any way being in this study could be bad for me?
This study involves no greater than minimal risks. You may have some discomfort during the exercise testing as heart and breathing rate will increase and they may feel warm.

Will it cost me anything to participate in this research study?
Taking part in this research study will not lead to any costs to you.

What happens to the information collected for the research?
Efforts will be made to limit the use and disclosure of your personal information, including research study and medical records, to people who have a need to review this information. We cannot promise complete privacy. Organizations that may inspect and copy your information include the

IRB and other representatives of this institution, including those that have responsibilities for monitoring or ensuring compliance. All of the information collected as part of this research study, including genetic information and exercise test results, will only be used for research purposes and will not be included in your medical chart.

Will anyone besides the study team be at my consent meeting?
You may be asked by the study team for your permission for an auditor to observe your consent meeting (or a recording of your consent meeting). Observing the consent meeting is one way that the University of Minnesota makes sure that your rights as a research participant are protected. The auditor is there to observe the consent meeting, which will be carried out by the people on the study team. The auditor will not record any personal (e.g. name, date of birth) or confidential information about you. The auditor will not observe your consent meeting (or a recording of your consent meeting) without your permission ahead of time.

Who do I contact if I have question, concerns or feedback about my experience?
This research has been reviewed and approved by an Institutional Review Board (IRB) within the Human Research Protections Program (HRPP). To share feedback privately with the HRPP about your research experience, call the Research Participants' Advocate Line at 612-625-1650 or go to www.irb.umn.edu/report.html. You are encouraged to contact the HRPP if:

Your questions, concerns, or complaints are not being answered by the research team.
You cannot reach the research team.
You want to talk to someone besides the research team.
You have questions about your rights as a research participant.
You want to get information or provide input about this research.

Will I have a chance to provide feedback after the study is over?
After the study, you might be asked to complete a survey about your experience as a research participant. You do not have to complete the survey if you do not want to. If you do choose to complete the survey, your responses will be anonymous.
If you are not asked to complete a survey after the study is over, but you would like to share feedback, please contact the study team or the Human Research Protection Program (HRPP). See the "Who Can I Talk To?" section of this form for study team and HRPP contact information.

What else do I need to know?
In the event that this research activity results in an injury, treatment will be available, including first aid, emergency treatment and follow-up care as needed. Care for such injuries will be billed in the ordinary manner to you or your insurance company. If you think that you have suffered a research related injury, let the study physicians know right away.

A federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. This law generally will protect you in the following ways:

Health insurance companies and group health plans may not request your genetic information that we get from this research.

Health insurance companies and group health plans may not use your genetic information when making decisions regarding your eligibility or premiums.

Employers with 15 or more employees may not use your genetic information that we get from this research when making a decision to hire, promote, or fire you or when setting the terms of your employment.

Be aware that this federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

Will I be compensated for my participation?
If you agree to take part in this research study, we will pay you $10 for your time and effort.

Use of Identifiable Health Information
We are committed to respect your privacy and to keep your personal information confidential. When choosing to take part in this study, you are giving us the permission to use your personal health information that includes health information in their medical records and information that can identify them. For example, personal health information may include name, address, phone number or social security number. Those persons who get health information may not be required by Federal privacy laws (such as the Privacy Rule) to protect it. Some of those persons may be able to share information with others without your separate permission. Please read the HIPAA Authorization form that we have provided and discussed.

The results of this study may also be used for teaching, publications, or for presentation at scientific meetings. You will not be personally identified in any presentation or publication.

Your signature documents your permission to take part in this research. You will be provided a copy of this signed document.

_____ _____
Signature of Participant Date

_____
Printed Name of Participant

_____ _____
Signature of Person Obtaining Consent Date

_____
Printed Name of Person Obtaining Consent
Your signature documents your permission for the named child to take part in this research.

Note: Investigators are to ensure that individuals who are not parents can demonstrate their legal authority to consent to the child's participation in the research. Contact legal counsel if any questions arise.

Appendix D
Parental Permission Form
IRB Study Number: 00000972

Title of Research Study: Influence of Genetic Variation of αENaC on Clinical Outcomes in Cystic Fibrosis Patients

Investigator: Hanan Zavala, M.S.

Researcher Name: Hanan Zavala, M.S. Email Address: zaval013@umn.edu	Research Advisor: Eric Snyder, Ph.D Email Address: snyd0180@umn.edu

What is research?
Doctors and researchers are committed to your child's care and safety. There are important differences between research and treatment plans:

The goal of research is to learn new things in order to help groups of people in the future. Researchers learn things by following the same plan with a number of participants, so they do not usually make changes to the plan for individual research participants. You, as an individual, may or may not be helped by volunteering for a research study.

The goal of treatment is to help you get better or to improve your quality of life. Doctors can make changes to your treatment plan as needed.

Why am I being asked to take part in this research study?
We are asking you and your child to take part in this research study because you are the parent of a child who has a diagnosis of cystic fibrosis who is being seen in clinic today.

What should I know about being in a research study?
Someone will explain this research study to you.
Whether or not your child takes part is up to you and your child.
You can choose not to have your child take part.
You can agree to take part and later change your mind.
Your decision will not be held against you.
You can ask all the questions you want before you decide.

Who can I talk to?
For questions about research appointments, the research study, research results, or other concerns, call the study team at:

This research has been reviewed and approved by an Institutional Review Board (IRB) within the Human Research Protections Program (HRPP). To share feedback privately with the HRPP about your or your child's research experience, call the Research Participants' Advocate Line at 612-625-1650 or go to www.irb.umn.edu/report.html. You are encouraged to contact the HRPP if:

Your questions, concerns, or complaints are not being answered by the research team.
You cannot reach the research team.
You want to talk to someone besides the research team.
You have questions about your or your child's rights as a research participant.
You want to get information or provide input about this research.

Why is this research being done?
The purpose of this research is to learn more about why certain CF patients have better clinical outcomes than others. We are interested in looking at a specific part of the DNA to see if individual differences in this area are associated with better lung function. We are also interested in seeing how this area of DNA influences aerobic capacity in patients with CF. There are no direct benefits to participating in this study. With this information we hope to improve care for patients in the future by encouraging personalized medical decisions based on an individuals' DNA.

How long will the research last?
Your participation will last only during today's visit (1 visit) and take approximately 30 – 45 minutes to complete. Your child's electronic medical record will be reviewed and data extracted from clinic visits and hospitalizations up to one year after enrollment into the study.

How many children / parents will be studied?
We expect up to 200 children will be in this research study. We also expect up to 200 adult CF patients to be in this research study.

What happens if I say "Yes, I want to be in this research"?
Today your child will be asked to complete a cheek swab, which involves rubbing the inside of both cheeks with a toothbrush like instrument. Then your child may be asked to breathe into a mouthpiece with a plug placed over their nose for approximately 10 minutes. Finally, your child may be asked to complete three submaximal exercise tests. One test involves walking at a brisk pace for six minutes, one involves stepping up and down on a step for three minutes, and one involves transitioning from sitting and standing for one minute. Information from your child's medical chart (including results from pulmonary function tests, hospitalizations, and demographic information) will be used, including information from previous clinical visits and clinical visits taking place up to one year after today's date. Your child will be monitored by a trained researcher during all procedures and procedures will be ceased immediately at any point during data collection if you or your child do not want to continue. All clinical tests, exams, and consultations as ordered by your provider will still be done, even if you do not take part in the research study. Declining to participate does not alter your child's care during this or any future visits.

What happens if I do not want to be in this research?
You and your child may decline to participate and it will not be held against you. Future care and treatment of your child will not be impacted by your decision to participate or not.

What happens if I say "Yes", but I change my mind later?
You and your child can leave the research at any time and it will not be held against you. If you decide to leave the research, provide verbal notice or written notice to the investigator. Verbal notice can be given to the investigator in person or by contacting them at 952-456-6356. Written notice can be sent to Room 110A Cooke Hall 1900 University Ave SE, Minneapolis, MN 55455 or zaval013@umn.edu so that the investigator can cease collecting further data from the medical record. Your written request does not need to provide a specific reason for withdrawal.

Choosing not to be in this study or to stop being in this study will not result in any penalty to you or loss of benefit to which you are entitled. Meaning, your choice not to be in this study will not negatively affect your child's right to any present or future medical treatment.

At any time, you or your child may decide to withdraw from the study. If you withdraw, no more information will be collected from you or your child. When you indicate that you wish to withdraw,

the information already collected from you and your child will be used in the study because they will not be able to remove it from the information they have gathered.

What are the risks? Is there any way being in this study could be bad for me or my child?
This study involves no greater than minimal risks. Your child may have some discomfort during the exercise testing as their heart and breathing rate will increase and they may feel warm. If your child does experience some discomfort, it is anticipated to be minimal.

What happens to the information collected for the research?
Efforts will be made to limit the use and disclosure of you and your child's personal information, including research study records, to people who have a need to review this information. We cannot promise complete secrecy. Organizations that may inspect and copy your information include the IRB and other representatives of this institution. The video recorded conversation is for data analysis purposes only. It will not be used in any presentations or publications. All of the information collected as part of this research study, including genetic information and exercise test results, will only be used for research purposes and will not be included in your medical chart.

To help maintain privacy, your child will be given a unique study ID to identify them. Only one link will exist between this study ID and your child's identifying information. Data will be kept in a locked electronic file which will be stored on a secure University of Minnesota network that is password protected. Data kept on paper files will be stored in locked offices and will not be released without your consent. A copy of the consent will be kept in your child's medical chart.
We will not ask about child abuse, but if your child tells us about child abuse or neglect, we are legally obligated to report it to state authorities.

Will anyone besides the study team be at the consent meeting?
You may be asked by the study team for your permission for an auditor to observe the consent meeting (or a recording of the consent meeting). Observing the consent meeting is one way that the University of Minnesota makes sure that the rights of research participants are protected. The auditor is there to observe the consent meeting, which will be carried out by the people on the study team. The auditor will not record any personal (e.g. name, date of birth) or confidential information about you or your child. The auditor will not observe the consent meeting (or a recording of the consent meeting) without your permission ahead of time.

Will I have a chance to provide feedback after the study is over?
After the study, you might be asked to complete a survey about your child's experience as a research participant. You do not have to complete the survey if you do not want to. If you do choose to complete the survey, your responses will be anonymous.
If you are not asked to complete a survey after the study is over, but you would like to share feedback, please contact the study team or the Human Research Protection Program (HRPP). See the "Who Can I Talk To?" section of this form for study team and HRPP contact information.

What else do I need to know?
In the event that this research activity results in an injury, treatment will be available, including first aid, emergency treatment and follow-up care as needed. Care for such injuries will be billed in the ordinary manner to you or your insurance company. If you think that you have suffered a research related injury, let the study physicians know right away.

A federal law, called the Genetic Information Nondiscrimination Act (GINA), generally makes it illegal for health insurance companies, group health plans, and most employers to discriminate

against you based on your genetic information. This law generally will protect you in the following ways:

Health insurance companies and group health plans may not request your genetic information that we get from this research.

Health insurance companies and group health plans may not use your genetic information when making decisions regarding your eligibility or premiums.

Employers with 15 or more employees may not use your genetic information that we get from this research when making a decision to hire, promote, or fire you or when setting the terms of your employment.

Be aware that this federal law does not protect you against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

Will I be compensated for my participation?
You or your child will be paid $10 for their participation in this study.
If for any reason you and your child do not complete the whole study, you will still receive the full payment.
Your signature documents your permission for you and the named child to take part in this research.

_____
Printed name of child participant

_____ _____
Printed name of parent [ ] or individual legally authorized [ ] Date
to consent for the child to participate

_____ _____
Signature of parent [ ] or individual legally authorized [ ] Date
to consent for the child to participate

_____ _____
Signature of person obtaining consent and assent Date

_____
Printed name of person obtaining consent and assent

Appendix E
Assent Form
IRB Study Number: 00000972

University of Minnesota

Assent to Participate in Research

Title of Research Study: Influence of Genetic Variation of αENaC on Clinical Outcomes in Cystic Fibrosis Patients

Researcher: Hanan Zavala, M.S.

Researcher Name: Hanan Zavala, M.S. Email Address: zaval013@umn.edu	Research Advisor: Eric Snyder, Ph.D Email Address: snyd0180@umn.edu

What is research?
Doctors and researchers are committed to your care and safety. There are important differences between research and treatment plans:

The goal of research is to learn new things in order to help groups of kids in the future. Researchers learn things by asking a question, making a plan, and testing it. The goal of treatment is to help you get better by using medication, therapy, surgery or other things that usually makes kids feel better. Sometimes treatments help make you feel better or get rid of the condition completely. Doctors can make changes to your treatment plan as needed.

Why am I being asked to take part in this research study?
A research study is usually done to find a better way to treat people or to understand how things work. You are being asked to take part in this research study because you have cystic fibrosis and are being seen in clinic today.

What should I know about being in a research study?
You do not have to be in this study if you do not want to do so. It is up to you if you want to participate and if you want to, talk to your parents about any questions or concerns you have about the study. You can choose not to take part now and change your mind later if you want. If you decide you do not want to be in this study, no one will be mad at you. You can ask all the questions you want before you decide.

Why is this research being done?
In this study, I want to find out more about how your DNA impacts how well your lungs work and how hard you have to work during exercise.

How long will the research last?
Your participation will last only during today's visit (1 visit) and take approximately 30 – 45 minutes to complete. Your child's electronic medical record will be reviewed and data extracted from clinic visits and hospitalizations up to one year after enrollment into the study.

What happens if I say "Yes, I want to be in this research"?
If it is okay with you and you agree to join this study, you will be asked to do a cheek swab, which involves rubbing the inside of both cheeks with something that is similar to a toothbrush. Then you may be asked to breathe into a mouthpiece, similar to the one used during pulmonary function

testing, for approximately 10 minutes. Finally, you may be asked to complete three exercise tests. One test involves walking at a brisk pace for six minutes, one involves stepping up and down on a step for three minutes, and one involves going from sitting to standing for one minute. Information from your medical chart will be used, including information from previous clinical visits and clinical visits taking place up to a year after today's date.

Is there any way being in this study could be bad for me?
You may have some discomfort during the exercise testing as you may breathe fast and feel hot.

What happens to the information collected for the research?
The researchers will share your information, including research study records, to only people who have a need to review this information. For example, sometimes researchers need to share information with the University or other people that work in research to make sure the researchers are following the rules. All of the information collected as part of this research study, including genetic information and exercise test results, will only be used for research purposes and will not be included in your medical chart.

What else do I need to know?
If you agree to take part in this research study, the researcher will compensate you $10.

Who can I talk to?
For questions about research appointments, the research study, research results, or other concerns, call the study team at:

This research has been reviewed and approved by an Institutional Review Board (IRB), a group of people that look at the research before it starts. This group is part of the Human Research Protection Program (HRPP). To share concerns privately with the HRPP about your research experience, call the Research Participants' Advocate Line at 612-625-1650 or go to www.irb.umn.edu/report.html. You are encouraged to contact the HRPP if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You cannot reach the research team.
- You want to talk to someone besides the research team or your parents.
- You have questions about your rights as a research participant.
- You want to get information or provide feedback about this research.

Signature Block for Child Assent

_____ _____
Signature of child Date

_____
Printed name of child

_____ _____
Printed name of person obtaining assent Date

_____
Signature of person obtaining assent

Appendix F
Data Collection Form

Subject ID:

Date:

Time:

Gift Card Number:

Medications taken before testing (dose and time):

Genetic Sample ID
Left Right

Exhaled Breath Condensate
Start Time:
End Time:

Six minute walk test
Start time:
End time:

Stopped or paused before 6 minutes? No Yes
Other symptoms at end of exercise: angina dizziness hip, leg, or calf pain
Number of laps:
Total distance walked in 6 minutes:
Predicted distance:___meters
Percent predicted:____%

	Pre	1 min	2 min	3min	4 min	5 min	Post	1 min post	2 min post
HR									
O_2									
RPE		X	X	X	X	X			
Dyspnea		X	X	X	X	X			

1 minute sit-stand test
Number of reps:

Stop during test:

	Pre	Post	1 min post	2 min post
HR				
O_2				
RPE				