CANDIDATE GENE STUDY OF FAMILIAL PULMONARY FIBROSIS

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Rachel Marie Sutton

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This thesis was presented

by

Rachel Marie Sutton

It was defended on

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and approved by

Jonathan K. Alder, PhD, Assistant Professor, Division of Pulmonary Allergy, and Critical Care Medicine, School of Medicine, University of Pittsburgh

Robin E. Grubs, MS, PhD, LCGC, Associate Professor of Human Genetics, Director, Genetic Counseling Program, Graduate School of Public Health, University of Pittsburgh

Ryan L. Minster, PhD, MSIS, Assistant Professor, Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh

Thesis Director: Daniel J. Kass, MD, Director, Dorothy P. and Richard P. Simmons Center for Interstitial Lung Disease (ILD), Associate Professor of Medicine, Division of Pulmonary Allergy, and Critical Care Medicine, School of Medicine, University of Pittsburgh

Daniel Kass, MD

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ABSTRACT

Rationale: Idiopathic Pulmonary Fibrosis (IPF) is a progressively fatal interstitial lung disease (ILD) with no known cure. Pathogenic variants in the telomere maintenance and surfactant pathways have been implicated in both familial and sporadic IPF, although a significant fraction of familial IPF cases remain uncharacterized.

Methods: A panel of patients with clinical diagnoses of IPF were selected for whole genome sequencing (WGS) at the Simmons Center for Interstitial Lung Disease of UPMC. Selected patients had comorbid hematologic malignancies or family histories suggestive of familial disease. 25X WGS was performed and all candidate variants were verified by bi-directional sanger sequencing. Protein alignment was done for all candidate variants to determine the phylogenetic conservation. A three-generation pedigree was constructed for participants and included ages and health status of all family members, if known. Targeted questions related to associated malignancies of IPF and telomere mediated disease were additionally ascertained.

Results: Six patients underwent WGS, two patients have pending results. Patients included three women and three men ranging in age from 60 to 83 years old. Five patients had a family history consistent with familial pulmonary fibrosis and one patient had a comorbid hematologic malignancy (a myelodysplastic syndrome). Of the four patients sequenced, two patients were found to have exonic variants in the telomere maintenance genes *RTEL1* and *TERT*. Two of the four patients who were familial by report had no known exonic variants suggesting the possibility

of noncoding variants, potentially novel genes, or shared environmental exposures. The two patients, with results pending, have family histories that are consistent with familial disease and are concerning for telomere-related co-morbidities.

Conclusions: Future studies will require confirmation of these new variants through functional studies or testing segregation in affected families. As genetic variants associated with IPF continue to be identified and characterized, genetic counseling is likely to have an increasing place in the management of IPF patients and their families. Furthermore, studies like these will contribute to the growing body of literature and further impact the field of public health by improving clinical guidelines on the use of genetics in IPF management.

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PREFACE

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1.0 INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a type of idiopathic interstitial pneumonia (IIP) which results in irreversible scarring of the lungs.[1] This progressive lung disease often results in death 3-5 years following a diagnosis, which is typically in the sixth or seventh decade of life.[2,3] In many cases, the cause of IPF is unknown, however there are several known risk factors. Smoking, environmental, and occupation exposures are thought to contribute, although the risks are not empirically quantified. In addition to modifiable lifestyle factors, one of the most underappreciated risk factors in IPF is family history. Up to 20% of individuals have familial pulmonary fibrosis, meaning they report having at least one other family member with the disease.[4]

The disease course of IPF is complicated in many patients due to the presence of multiple co-morbidities. [5-8] Co-morbidities not only affect treatment options, but confound studies aimed at deciphering the effectiveness of interventions.[9,10] Overall, treatment options are limited, some interventions try to treat the symptoms, others aim to prevent further lung fibrosis. Two FDA-approved medications attempt to limit fibrosis to the lung; however, these do not alleviate any symptoms for patients.[11,12] Further, some patients choose to be evaluated for, and undergo, a lung transplant. Lung transplants may prolong survival, but many patients suffer post-transplant complications.[13-15] In addition, many patients are excluded from lung transplants due to age, co-morbidities, and other factors. One of those factors is a pathogenic variant in a gene associated with IPF.

Pathogenic variants in genes have been established in up to 20% of families. Pathogenic variants have also been found in approximately 11% of patients with reportedly sporadic IPF.[16] The genes that have been implicated in IPF are found in the surfactant and telomere biology

pathway. In the telomere pathway, patients have shorter telomeres, this drives the progression of the disease.[17] This may be due to pathogenic variants in genes in the telomere pathway. As telomeres shorten in subsequent generations, different, and more severe manifestations of the disease appear, which is termed genetic anticipation.[18,19] For those patients who choose to undergo a lung transplant, if they have shorter telomeres (<10th percentile) or a pathogenic variant in an implicated gene, they may be a risk for post-transplant complications.[13-15]

The overall goal of this study is to characterize and better understand the genetics of IPF. This study aims to identify candidate genes in IPF and examine the segregation in families in hopes to add to the growing knowledge regarding the genetics of IPF. By constructing and analyzing pedigrees of affected probands, it will aid in confirming segregation within families, and to establish other related malignancies or associated phenotypic features in the family.

The specific aims of this study are as follows:

- Identify and characterize possible pathogenic variants that can be implicated in IPF in probands affected with IPF to identify candidate genes
- Ascertain the family history of the proband in order to construct a pedigree. The pedigrees can then be analyzed for possible inheritance patterns and genetic anticipation of the disease in family members. This will help establish affected and unaffected relatives on the pedigree for whom samples could be obtained to test the segregation of possible pathogenic variants.

This study will be the first of its kind at the Simmons Center for Interstitial Lung Disease at UPMC and will help expand the understanding of the role of genetics in IPF. Additionally, the information generated from this research study has the potential to aid in the development of guidelines for genetic counseling in IPF patients. In turn, future IPF patients and their family

members could benefit from such knowledge and expertise. As more genetic information becomes known about IPF and genetic testing of asymptomatic individuals becomes clinically available, it will become important to ensure that individuals who decide to undergo testing are properly informed of the risks, benefits, and limitations of genetic testing. This is especially imperative in the case of IPF as it has no known long-lasting cure and the possibility of anticipation across multiple generations. We anticipate that this information will inform research toward directing personalized therapies for these individuals.

2.0 LITERATURE REVIEW

2.1 OVERVIEW OF IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is the most common of several forms of interstitial lung disease (ILD). IPF ultimately leads to progressive breathlessness, respiratory failure, and death with a median survival of 4 years from the time of diagnosis.[20] IPF is characterized histologically by the presence of the so-called usual interstitial pneumonia (UIP) pattern. UIP is part of the class of idiopathic interstitial pneumonias (IIPs). IIP's are histologic patterns that are characterized by varying degrees of inflammation and fibrosis of the lung parenchyma, the portion of the lung functioning to maintain gas exchange.[1] Other IIP's include: nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphocytic interstitial pneumonia (LIP).[1] The diagnosis of ILD is challenging as many of the forms of ILD have similar clinical presentations and overlapping histologic patterns on biopsy. Thus an accurate diagnosis is crucial for management and treatment.[21] The American Thoracic Society and the European Respiratory Society have jointly published criteria used to establish the diagnosis of IPF. The diagnosis, based on the current consensus guidelines, requires the following: (1) exclusion of other known causes of ILD and (2) the presence of usual interstitial pneumonia (UIP) pattern by surgical lung biopsy. Alternatively, (3) patients may also be diagnosed with IPF without a surgical lung biopsy if the high-resolution computed tomography (HRCT) demonstrates the UIP pattern.[20]

The incidence (defined as the number of new cases per year) of IPF ranges from 4.6 per 100,000 person-years to 17.43 per 100,000 person-years, as reported from the United States and the United Kingdom.[22,23] Similarly, prevalence (the number of total cases per year) varies from 2.9 per 100,000 to 63 per 100,000, reported from studies in Finland, the United States and Japan.[22,24,25]

IPF predominantly occurs in individuals over the age of 60, with a median survival of 3-5 years. The European IPF registry (erIPFreg) and insights-IPF registry in Germany have reported between 63% and 77% of patients are male.[2,3] Individuals with IPF may present with a variety of symptoms. A study outlining characteristics of the European IPF registry (erIPFreg) documented around 90% presented with dyspnea, 70% with fatigue, and 50% with a dry cough.[3] Upon examination, many individuals have "velcro-type" crackles, which has shown to be a predictor of UIP pattern on HRCT.[26] Clubbing of the fingers has also been recorded in up to 50% of patients with IPF[27].

2.1.1 Pathogenesis

The cause of IPF is not always known, though there are a number of environmental, occupational, and genetic risk factors which may contribute to the disease. In addition, up to 20% of cases of IPF are characterized as "familial," meaning more than 2 first-degree relatives in the family have IPF.

2.1.2 Treatment

Management and treatment options are limited and consequently, the median survival of patients with IPF is 3-5 years. Along with IPF there are several co-morbidities which negatively

impact the prognosis, such as cardiovascular disease and diabetes.[28] Medications can be taken to slow the rate of deterioration in the lung which may slow the progression of the disease, however they do not restore lung function already lost to fibrosis nor help alleviate symptoms for patients. The only cure for IPF is lung transplantation. However, the candidacy of these individuals choosing to undergo a single or double lung transplant is compromised with age and comorbidities.

2.2 IPF RISK FACTORS

There are a number of risk factors contributing to both the development of IPF and mortality from IPF. Smoking is considered one of the largest risk factors for IPF. Information collected from the EMPIRE Registry (European MultiPartner IPF Registry) found 53% of patients to be current or former smokers.[29] A case-control study examining cigarette smoking as a risk factor for IPF found that those who had smoked at some point in their lives, including both current and former smokers, had a 60% increased risk for developing IPF.[30] Finally, a retrospective study done by Karkkainen et. al, found current smokers to have a younger age of diagnosis than former or non-smokers, 58.1 versus 71.7, respectively.[31]

2.2.1 Environmental

Occupational and environmental exposures are also thought to be contributory toward the development of IPF. Such reported exposures include: asbestos, metal dusts, raising birds, wood dusts, solvents, and hairdressing.[5,32,33] Although, pulmonary fibrosis due to asbestos exposure has its own diagnosis, asbestosis.[34] A case-control study done in Egypt reported men working in the

chemical and wood-working industry had a higher risk for IPF.[35] In addition, a study in Southern Europe found that farmers, vets, gardeners, and workers in the steel industry had an increased risk for IPF, this risk increased depending on the length of time with said exposure.[36]

2.2.2 Genetics

An often underappreciated risk factor for the development of pulmonary fibrosis is genetics and family history. Many studies have shown that patients who have a family history of pulmonary fibrosis, in parents or siblings, have a younger age of diagnosis than sporadic cases, or those without a family history.[4,37] A case-control study done in Mexico at the National Institute of Respiratory Diseases found that 20% of patients with IPF had a parent or siblings also diagnosed with IPF.[4] In Finland, a study was done to examine how many patients with a diagnosis of IPF also had a family member with a similar disease. The results from a questionnaire sent to IPF patients suggested that 88 of 675 (~13%) patients reported an affected family member.[24] In addition, this study looked at medical records for 17 of these families and found that affected family members had an earlier age of onset compared to sporadic patients (61.9 years versus 65.3 years, respectively).[24]

2.3 IPF PROGNOSTIC FACTORS

2.3.1 Acute Exacerbations in IPF

Many studies have attempted to elucidate acute exacerbations in IPF—episodes of rapid deterioration, which result in decreased pulmonary function and are frequently fatal.[38] Acute exacerbations are seen more often in those with more advanced disease. There have been few therapeutic studies of acute exacerbations, and the field has struggled to define exactly what constitutes an acute exacerbation. A study by Collard et. al, proposed diagnostic criteria for acute exacerbations of IPF, of which are used by many clinical trials.[39] Several studies have reported onset of acute exacerbations following a diagnostic procedure, such as a surgical lung biopsy or bronchoalveolar lavage. A retrospective study from January of 1990 to September of 2003 found 23 of 147 patients with IPF were admitted to the hospital for acute exacerbation.[40] Of those, 11 met the proposed criteria for an acute exacerbation.[40] Three of 11 patients developed acute exacerbation immediately following a surgical lung biopsy or bronchoalveolar lavage, five developed acute exacerbation 3-60 months following the diagnosis of IPF by surgical lung biopsy.[40] Contrary to smoking being a perceived risk factor for IPF, Song et. al found that patients who had never smoked more often had acute exacerbations.[38] This study additionally reported the median survival time after an acute exacerbation was 2.2. months.[38] Intubation due to an acute exacerbation carries a nearly 100% mortality rate.[41]

2.3.2 Comorbidities in IPF

A number of comorbidities have also been shown to impact mortality rates on patients with IPF. The German INSIGHTS-IPF registry reported patients to have the following comorbidities: cardiovascular disease, diabetes, pulmonary hypertension, emphysema, and reflux.[5] Other studies have also reported the incidence of lung cancer, sleep-related breathing disorders and psychological health concerns in patients with IPF.[6-8] Many patients often present or develop multiple comorbidities, further impacting their mortality rate. A study examining a database of IPF patients found that close to 90% of patients had at least one comorbidity, with 30.5% having between 4 and 7 comorbidities.[28] Of importance, this study found cardiovascular disease and lung cancer to have a statistically significant negative impact on survival, whereas GERD had a statistically significant positive impact on survival.[28] A retrospective study done by Alakhras et. al at Mayo Clinic, evaluated a person's BMI and mortality from IPF. Their study found that those with a higher BMI had increased survival compared to those with a lower BMI, a 91% 1-year survival rate as compared to 76% 1-year survival rate, respectively.[42]

2.4 MANAGEMENT AND TREATMENT OF IPF

Due to the severity and poor prognosis in IPF, management of the disease focuses on monitoring the course of IPF through pulmonary function tests. Treatment options remain limited and only serve to manage symptoms and slow the progression of lung fibrosis. The presence of multiple comorbidities can create additional challenges in the management of IPF since they can impact the progression of IPF.

2.4.1 Pulmonary Function Tests

A number of tests are used to assess the severity of IPF. These tests are often done as a baseline test when a patient is diagnosed with IPF and further completed at regular intervals to measure and evaluate the disease course. These tests are also useful in monitoring response to therapies the patient may be undergoing.[43] Physiologic measurements in IPF are those evaluating the function of the lung. A broad group of these tests is pulmonary function tests (PFT's), most commonly tested are forced vital capacity (FVC), diffusing capacity of the lung for carbon monoxide (DLCO), total lung capacity (TLC), and alveolar-arterial oxygen difference in partial pressures ($P(A\text{-}a)_{O2}$).[20,44] FVC is the maximum amount of air exhaled following a maximal inhalation. Declines in FVC have been shown to be associated with increased mortality.[45,46] A study of 179 patients with IIP found that at baseline, low levels of FVC, DLCO, PA02 were associated with a worse prognosis, particularly if the patient was an older male.[46] While these PFT's can be beneficial in assessing and helping to manage IPF, additional comorbidities impact the effectiveness of these tests on predicting disease course. FVC is the best measure at predicting prognosis in IPF.[47]

2.4.2 Six-Minute Walk Test

The six-minute walk test (6MWT) is another measurement taken in patients with IPF to assess the exercise capacity of the lung. This test is often used because it assesses the everyday activity level of the patient. It is employed clinically, especially in lung transplant to assess patient's "readiness" for transplant.[48,49] A double-blind placebo-controlled patient population from the INSPIRE trial found the 6MWT to be a predictor of mortality, such that those with a distance

<250 meters had a two-fold increased rate of mortality in a year.[50] A study utilizing data from UNOS of individuals with IPF waiting for a lung transplant found that those with a 6MWT distance greater than 305 meters survived longer than those whose distance was less than 305 meters.[51] The 6MWT is also utilized in many clinical trials to assess the effectiveness of a medication or drug by comparing 6MWT distances before, during, and after use.[49] Though the 6MWT is used in many studies, there are limitations to the interpretation due to confounding variables.[9,10]

2.4.3 Pulmonary Rehabilitation

In addition to lung function tests and pharmacologic management, pulmonary rehabilitation is often undertaken by many IPF patients. A study by Gaunaurd *et al*., compared patients with IPF undergoing a 3-month pulmonary rehabilitation program to patients with IPF with no organized exercise. The study found that pulmonary rehabilitation helped manage the symptoms brought on by the disease with consistent exercise, however this benefit did not continue long-term when regular exercise stopped.[52] The management of symptoms in that study was assessed using the St George Respiratory Questionnaire for IPF (SGRQ-I), which showed that changes in the score reflected positively on an improvement in quality of life.[52]

2.4.4 Medications

There are currently two FDA-approved drugs for the treatment of IPF: pirfenidone and nintedanib. These were approved in October of 2014 and are antifibrotic agents. Prior to their approval, two randomized CAPACITY trials (004 and 006) were used to confirm that pirfenidone reduced lung deterioration, as shown from the Phase 2 study. The study assessed the efficacy of

pirfenidone through evaluating FVC. Study 004 found a reduced decline in FVC in those taking pirfendione compared to the placebo group after 72 weeks.[11] Post-approval, another study used data from the CAPACITY and ASCEND trials to compare survival (in years) when using pirfenidone versus supportive care (i.e. supplemental oxygen, pulmonary rehab, etc.). Their results showed approximately a two-and-a-half-year increase in survival with pirfenidone compared to supportive care.[12] The efficacy of nintedanib has also been shown through a reduction in decline of FVC. Patients who completed the Phase III INPULSIS trial were eligible for the open-label INPULSIS extension comparing nintedanib versus a placebo. The study compared those with >50% and <50% predicted FVC at the start of the study and the results showed both had a similar decline in FVC.[53] Patients with <50% predicted FVC have significant deterioration to the lungs, however these results showed nintedanib is beneficial despite how significant the presence of lung damage.[53]

2.4.5 Supplemental Oxygen

Many patients have disease progression requiring the use of supplemental oxygen to improve quality of life in daily activities. Supplemental oxygen helps alleviate dyspnea and hypoxia.[54] Much research on the use of oxygen in patients with interstitial lung diseases is extrapolated from studies involving COPD. Although, a retrospective study was done comparing use of oxygen in COPD and ILD by measuring the change in oxygen saturation (SpO_2) throughout the 6MWT. The results showed ILD patients had a larger change in oxygen saturation and lower levels of SpO_2, suggesting data from COPD patients may not be as applicable as previously thought.[55] A number of studies and surveys have been conducted to better understand patient perceptions and challenges of using supplemental oxygen. Data from these studies have shown

many individuals experience (or are afraid of experiencing) issues with malfunctioning equipment and difficulty traveling with supplemental oxygen[54,56,57]. In addition, many individuals describe the psychosocial impact of being on oxygen, particularly in feeling stigmatized for needing supplemental oxygen, as well as being unable to hide their illness.[54] Overall, supplemental oxygen provides many benefits to patients and improves their quality of life, with some unfortunate drawbacks.

2.4.6 Quality of Life in IPF

Given the poor prognosis in IPF, assessing a patient's quality of life and mental health is an important area of investigation. A number of studies have assessed patients' quality of life through health-related quality of life (HRQL) surveys.[58,59] Swirgris *et al*., developed an IPF specific HRQL, called a tool to assess quality of life in IPF (ATAQ-IPF).[60] This survey incorporates results from the 6MWT and PFT testing as well as results on over 200 questions. These questions incorporate information from all aspects of their life and are organized into domains. These domains range from symptoms to finances and relationships.[60] In assessing the answers to questions in the study sample, results found a significant correlation in HRQL with measures of FVC%, DLCO%, and 6MWD for eight, nine, and five the 13 domains.[60] There was also significantly greater ATAQ-IPF scores in nine of the 13 domains for patients who required supplemental oxygen compared to those who do not.[60] Data from an Australian IPF registry utilized the SGRQ to assess quality of life in IPF. They found that dyspnea, cough, and depression most significantly contributed to the quality of life in IPF.[61]

2.4.7 Lung Transplant

For some patients with IPF, lung transplants may be a treatment option as a means to extend their lifespan. The International Society for Heart and Lung Transplantation has specific guidelines for listing a patient for lung transplantation with IPF. They are as following: histologic or radiographic evidence of UIP and any of the following: A DLCO <39% predicted, a 10% or greater decrement in FVC during 6 months of follow up, a decrease in pulse oximetry <88% during a 6-minute walk test and honeycombing on HRCT (fibrosis score >2).[48] Ultimately, deciding to be listed for a lung transplant also relies on other factors, such as quality of life, projected clinical course, and risks of lung transplantation.[48] Data from The Registry of the International Society for Heart and Lung Transplantation reports that from January of 1995 to June of 2011 23.2% of all lung transplants were done due to IPF, accounting for the second highest number of lung transplants, second to COPD and emphysema.[62] Of those done for IPF, about 56% were single lung transplants and 44% were double lung transplants.[62] A retrospective study done to assess survival in patients receiving either a single lung transplant or double lung transplant for IPF found that survival was better in double lung transplants than single lung transplants, 8.34 years versus 7.37 years, respectfully.[63] One study aimed at comparing survival time following a lung transplant compared to remaining on the wait list. This study found that despite the high risk of mortality immediately following a lung transplant, after one year the survival rate post-transplant was better than remaining on the wait list.[64] Several limitations from that study should be considered, in that everyone on the wait list and those who received transplants were likely not equally as healthy or unhealthy. Many individuals with IPF have a number of comorbidities which affect the prognosis. In addition, quantifying any gained longevity following a lung transplant is difficult due to a

myriad of factors. Further complicating prognosis following a lung transplant is certain genetic factors mediating the disease progression (discussed in the next section).

2.5 GENETICS OF IPF

Genetics is thought to play a large role in pulmonary fibrosis with studies reporting up to 20% of cases of IPF having a family history of lung disease.[65] Familial pulmonary fibrosis, which is used to describe those with two or more family members with pulmonary fibrosis, provides clues for the genetic underpinnings of IPF. A twin study case report from 1950 reported monozygotic twins who both developed IPF close to the age of 50 and had not lived together since childhood, thus reducing the likelihood of shared environmental exposures significantly impacting the occurrence of IPF.[66] Although many genes have been implicated in familial IPF, the mechanism behind the disease most commonly involves the telomeres and telomeric shortening. Many of these implicated genes exhibit autosomal dominant inheritance, often consistent with genetic anticipation.

2.5.1 Genes and Inheritance of IPF

Both rare and common variants have been identified through family studies in sporadic and familial IPF. Rare variants are defined as those with a minor allele frequency less than 0.1%, common variants are those with minor allele frequency >5%. Rare variants have been identified in genes in the telomere pathway (*TERT*, *TERC*, *DKC1*, *TINF2*, *RTEL1*, NAF1 and *PARN*) and in surfactant production (*SFTPC*, *SFTPA1*, *SFTPA2,* and *ABCA3*).[67-69] Common variants have also

been associated with familial and sporadic IPF, although the pathogenic mechanism is unknown and their usefulness in the clinic is limited by their high allele frequency. The most significant common variant is found in the promoter of MUC5B.[70] Additional variants have been identified in *TERT, TERC,* and OBCF1.[71]

The inheritance of pulmonary fibrosis is not clearly established, though most family histories tend to show an autosomal dominant mode of inheritance. Autosomal recessive and x-linked inheritance have also been suggested. A study of 111 families with pulmonary fibrosis conducted by Steele *et al*., at three sites in the United States, found the pedigrees supported an autosomal dominant mode of inheritance.[72] Further, segregation in 30 families reported by Marshall *et al*., in the United Kingdom supported an autosomal dominant mode of inheritance, although autosomal recessive could not be ruled out without further genetic testing.[37]

One of the genes implicated in IPF is *DKC1*, a gene located on the X chromosome, and is consistent with x-linked inheritance. In addition to causing IPF, pathogenic variants in *DKC1* can cause Dyskeratosis Congenita (DC), a childhood onset disorder characterized by oral leukoplakia, nail dystrophy, and hyperpigmentation of the skin.[73] In addition, those with DC often have bone marrow failure, pulmonary fibrosis, and increased cancer risks.[73] Other genes have also been implicated in DC, resulting in autosomal dominant and autosomal recessive inheritance. Pathogenic variants in *hTR* (*TERC*) have been associated with autosomal dominant DC, as shown by three pedigrees reported in a study done at Hammersmith Hospital in Iowa.[74] It is thought that x-linked DC is more severe and onset is earlier than in autosomal dominant DC.[75] Mechanistically, this is because *DKC1* binds to *hTR* and is necessary for stability and telomerase synthesis, thus, patients with pathogenic variants in *DKC1* have lower levels of *hTR* resulting in insufficient telomerase to maintain the length of telomeres.[76]

2.5.2 Telomeres

Telomeres are structures located at the ends of chromosomes, made up of repeated DNA sequences. In humans, the telomere DNA is 2-20 kb long, with repeats of the sequence TTAGGG.[77] Telomeres provide several functions in cells, but their primary role is suppressing a DNA damage response and subsequent end-to-end fusion. Telomeres shorten each time a cell replicates. The "end-replication problem", a term coined by Watson in 1972, exists because there is a 3' overhang of single stranded telomeric DNA on the lagging strand, which cannot be completely synthesized by DNA polymerase.[78] Incomplete synthesis of the 3' end of DNA leads to shortening of the chromosome during the following round of replication. To circumvent this, an enzyme, telomerase, provides the *de novo* addition of nucleotides to the ends of chromosomes to allow replication to proceed to the end of the chromosome. Telomerase is an RNA-dependent DNA polymerase composed of human telomerase RNA (hTR, also known as *TERC*) and human telomerase reverse transcriptase (hTERT). These components allow for replication of the telomere DNA on the 3' end by using hTR as the template and hTERT to reverse-transcribe the sequence.[79] Telomerase and the shelterin complex, a complex of 6 proteins that coat the telomere, control the length of telomeric DNA. The shelterin complex includes the following proteins: TRF1, TRF2, POT1, RAP1, TIN2 and TPP1.[80] TRF1 and TRF2 bind to the double stranded TTAGGG repeats to inhibit telomere elongation through recruitment of the other 4 proteins in the shelterin complex.[81] POT1 binds single-stranded telomeric DNA and is regulated by TPP1[82]. TIN2 can bind both TRF1 and TRF2, while RAP1 is recruited by TRF2.[82,83]

2.5.3 Short Telomeres in IPF

Although a number of pathogenic variants have been implicated in the telomere pathway, it is the resulting telomere shortening that appears to be the primary contributor to the manifestations of the disease. A study examining familial and sporadic IPF stratified individuals whom had a *TERT* or *TERC* pathogenic variant. Telomeres were <10th percentile in all probands who had a *TERT* or *TERC* pathogenic variant.[84] Of those with no pathogenic variants, telomeres <10th percentile were found in 25% and 37% of individuals with sporadic and familial IPF, respectively.[84] Another study utilized probands and their family members enrolled in the Vanderbilt Familial Pulmonary Fibrosis Registry and found that affected individuals who have a pathogenic variant in a telomerase also had significantly shorter telomeres than asymptomatic carriers of the same pathogenic variant.[17] In this study, asymptomatic carriers were about 11 years younger than the probands at their time of diagnosis, which is consistent with the mechanism of shorter telomeres driving disease.[17]

A further sequalae of telomere-mediated IPF is significant co-morbidities, due to the shortening. Bone-marrow failure can manifest in patients with IPF, or separately in individuals who have short telomeres. Pathogenic variants in *TERT* and *TERC* have been found in patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[85,86] These were found in patients lacking the characteristic skin findings of Dyskeratosis Congenita (DC), given DC is a bone-marrow failure syndrome as well. An additional co-morbidity previously established in telomere-mediated disease is gastrointestinal concerns. A sample of 38 individuals ranging in age from 15 months to 34 years found that 16% had an evaluation by a gastroenterologist. In these six individuals, four had pathogenic variants in *DKC1, TERT, or TR*.[87] Presentations varied in these

individuals, some had difficulty swallowing, esophageal stenosis, abdominal pain, and a colectomy.[87]

2.5.4 Anticipation in telomere-mediated disease

One of the strongest observations linking telomere length to disease pathogenesis is the occurrence of genetic anticipation in families with variants in telomere-maintenance genes. Genetic anticipation occurs when successive generations have earlier ages of onset of disease and have a more severe presentation. This was identified in a study of eight families with *TERC* pathogenic variants in which parents were affected with symptoms of DC between 36 and 61 years of age, whereas their children showed symptoms around 14.5 years of age.[18] Telomere length of these individuals was studied to assess if that contributed to the anticipation, and it was found that children had statistically significant shorter telomere length than their parents indicating that shortened telomere length may contribute to anticipation.[18] Telomere-mediated disease provides the only example of genetic anticipation in which the disease phenotype can change in each subsequent generation.[88] In a family with a known *TERT* pathogenic variant, a pedigree analysis showed genetic anticipation in the family. In this three-generation pedigree, it was found that individuals in subsequent generations developed premature graying at younger ages (age 20's vs age 9), liver and lung fibrosis, and aplastic anemia.[19]

2.5.5 Lung transplants and telomeres

Lung transplant is the only clear life-extending therapy for patients with IPF. However, recent data suggest that post-transplant complications may arise due to shortened telomere length

or the presence of a pathogenic variant implicated in IPF.[13-15] An observational cohort study followed 82 individuals pre- and post-lung transplant who were divided into two groups based on telomere length. A total of 26 individuals had telomere length <10th percentile and 56 individuals had telomere length >10th percentile.[13] This study found that the rate of death was higher in individuals with telomere length <10th percentile (54% vs 18%).[13] In addition to decreased survival time, these individuals also had an increased risk of infections and higher rates of allograft dysfunction.[13] Eight subjects with telomerase pathogenic variants were evaluated for post-transplant outcomes through a study conducted at Johns Hopkins. The results from this study described that patients with telomerase pathogenic variants are at a higher likelihood of having post-transplant complications such as: minimal/moderate rejection, infectious complications, hematologic complications, and medication-related toxicities.[14] A recent study conducted on lung transplant recipients at the University of Pittsburgh and Johns Hopkins found that recipients with short telomeres have an increased risk for CMV infection—a very serious complication of lung transplant and a major risk for chronic allograft dysfunction (CLAD).[15] Ultimately, data from this study and others has suggested telomere length may be useful in stratifying post-transplant risks for patients as well as helping to personalize their treatment.[15]

2.5.6 Current Understanding of the Use of Genetic Information

Currently, and despite the significant evidence supporting a role for genetics in the pathogenesis of disease, there are no guidelines regarding genetic testing in patients with pulmonary fibrosis.[20] The majority of the knowledge generated regarding the genetics is from research studies, which are often through active efforts aimed at better characterizing this disease

through the collaboration of many large centers. Despite this, cohorts of family member participation are small, making the characterization of rare variants difficult.

Historically, physicians who treat patients with IPF have never considered the impact of genetics on decision-making for treatment options because the results were not "actionable." That is, patients with clearly familial disease were treated the same as patients with so-called "sporadic" disease. In fact, many IPF providers would pursue a "Don't ask, don't tell" policy, since genetics could negatively impact a decision for transplant.[89] However, given the insight provided from genetic studies, a clinical shift is happening to personalize care for patients through precision medicine initiatives. This is quite evident for patients undergoing lung transplants. As mentioned in the preceding subsection (2.4.5), there are poor outcomes post-transplant for patients with short telomeres or pathogenic variants in telomere-related genes.[13-15] The evidence from these studies indicate that bone marrow failure following lung transplant, probably the consequence of anti-rejection toxicity on particularly vulnerable hematopoietic cells with short telomeres, is to blame for the poor outcomes of these patients following transplant.[13-15] A proof-of-concept trial is currently underway at the University of Pittsburgh Medical Center (UPMC) to address the risk of bone marrow failure following lung transplant for patients with short telomere pulmonary fibrosis. The treatment will include lung transplant followed by allogeneic bone marrow transplant *from the donor* at 4 months following the transplant.[90] The bone marrow will then recognize the lung as *self* and may allow for significant reduction or even elimination of the anti-rejection meds in these patients. Patients have had success with this new protocol who have end-stage lung disease.[91]

Furthermore, researchers have mentioned the importance of referring early for lung transplants, as historically patients were in their 70's, and now many centers do not perform lung transplants in patients who are older due to the increased risk for comorbidities in these

patients.[92,93] One study argued that patients who have short telomeres should be identified for earlier transplant evaluation.[93] The preference for many centers and physicians is to transplant younger individuals, as they are considered healthier and likely have less comorbidities. It is possible that the younger patients with pulmonary fibrosis who are more often referred for lung transplant, may be the population enriched with short telomeres. Not only because they may have been clinically tested for short telomeres and referred earlier as studies suggest, but also because patients who have shorter telomeres or pathogenic variants develop the disease at a younger age. Overall, this suggests that genetic testing and measurement of telomere length may be particularly important in this population in the context of lung transplant.

As telomere length and genetic variants in pulmonary fibrosis now appear, based on high quality evidence, to impact the clinical outcomes of patients, especially following transplant, it is clear that genetic testing may have a role in the assessment of patients. This does, of course, come with several caveats: first, genetic testing can potentially bias providers away from a potentially life-saving transplant. Second, similar to at-risk patients for non-curable diseases such as Huntington's disease, genetic testing needs to be considered very carefully in this population. Such information can cause significant psychosocial sequelae in young people and may be used to deny coverage for insurance. Clinical genetic testing in pulmonary fibrosis is in its infancy. All these issues need to be considered very carefully as the field evolves and genetic information becomes a standard of care in patients.

3.0 MANUSCRIPT

3.1 BACKGROUND

Idiopathic pulmonary fibrosis (IPF) is a form of interstitial lung disease (ILD) characterized by dyspnea, and histologically, by the presence of usual interstitial pneumonia pattern.[1] This progressive lung disease ultimately leads to respiratory failure in the sixth or seventh decade of life.[2,3] Following a diagnosis, the median survival is 3-5 years due to the limited management and treatment options. The term idiopathic is used due to the unknown cause of IPF, although a number of risk factors and genetic changes contribute toward the risk of IPF.

3.1.1 Risk Factors

A number of environmental, occupational, and genetic risk factors have been identified in IPF. Of these factors, smoking is considered to be the largest preventable risk factor. Many studies suggest more than 50% of patients with IPF are current or former smokers.[29,30] Environmental and occupation exposures have also been established, though the empirical risk, while not as well quantified, is thought to be increased with longer exposure times.[5,32,33,36] Lastly, the role of genetics is rapidly evolving. Around 20% of individuals report a family history of IPF.[4,37] These individuals have what is considered "familial" IPF, meaning they have one or more affected first-degree relatives.

3.1.2 Prognostic Factors

Further complicating the disease course in IPF are acute exacerbations and comorbidities. Acute exacerbations are episodes of rapid deterioration often following a diagnostic procedure, which ultimately result in decreased survival time.[38,40] For many individuals, comorbidities may have a negative impact on survival, such as cardiovascular disease and lung cancer.[5-8] Almost all patients report at least one comorbidity, though many report numerous.[28] In addition to the impact comorbidities have on survival, they also result in challenges related to managing IPF symptoms and predicting disease course.[28,42]

3.1.3 Management

The disease course of IPF is often monitored through a variety of pulmonary function tests. Two in particular, forced vital capacity (FVC) and the six-minute walk test (6MWT), are commonly used in clinical trials and research studies as a means to determine the effectiveness of an intervention.[20,43,44] Although, the effectiveness of these measures is unclear due to the presence of confounding variables in many situations.[9,10]

Pulmonary rehabilitation, supplemental oxygen, and medications are interventions used to mediate the disease course of pulmonary fibrosis. Pirfenidone and Nintedanib are two FDA-approved drugs which may help slow the progression of lung fibrosis, but do not restore lung function lost to fibrosis or increase lifespan.[11,12,53] These medications additionally have a myriad of side effects and do not help patients with the symptoms associated with IPF. Other patients choose to undergo a single or double lung transplant to extend their lifespan.[62,63] Co-morbidities

and genetic factors complicate the indications and prognosis for a lung transplant (discussed in section 3.1.4.1)

3.1.4 Genetics of IPF

In many familial IPF cases, as well as some sporadic cases, pathogenic variants in genes have been identified. These genes are located in the telomere maintenance or surfactant pathways. In the telomere maintenance pathway, the mechanism behind genetic pathogenic variants is thought to be well-understood, in that the shortening of telomeres is the driver of the disease.[17] Telomeres are located at the ends of chromosomes and are maintained by telomerase, composed of telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT), which catalyzes de novo addition of telomere DNA to prevent loss of essential DNA.[79] The majority of genes implicated in IPF have been shown to have autosomal dominant inheritance.[72] The shortening of telomeres appears to be associated with the genetic anticipation seen with IPF.[18] As telomeres shorten in each successive generation, there is earlier age of onset of disease as well as changes in presentation of the disease.[18] For example, younger generations may have premature graying, liver fibrosis, and aplastic anemia, which may not be something older generations of the family manifest.[19] In addition to genetic anticipation, there are further co-morbidities associated with telomere-mediated IPF. Other bone-marrow failure disease, such as Myelodysplastic Syndrome (MDS) or Acute Myeloid Leukemia (AML) have been found in patients with short telomeres, as well as patients with pathogenic variants in *TERT* and *TERC.*[86,87] In addition, gastrointestinal concerns have been reported in patients with short telomeres, including esophageal stenosis and dysphagia.[87]

3.1.4.1 Complications of IPF Genetics in Lung Transplant

Perhaps one of the most important implications of genetic findings is in those patients considering a lung transplant. Patients who have shortened telomeres (<10th percentile) or a pathogenic variant in the telomere pathway are at risk for a number of post-transplant complications.[13] Such complications may include an increased chance of rejection, infections, hematologic complications, CMV infections, and other clinical morbidities.[13-15] Knowing a patient's telomere length and genetic status may help personalize treatment, particularly in assessing the risk for post-transplant complications.[15]

3.1.5 Goals of the Study

Currently, the clinical guidelines set forth by the American Thoracic Society do not have recommendations that genetic testing be undertaken in any patient with IPF. Given the important implications this knowledge may have on a patient's treatment and outcome, this goal of this study is to better understand and characterize genetic variants in IPF and their segregation in families. Clinical information from the patient and the family history were ascertained. A blood sample was obtained from the proband for whole genome sequencing (WGS). The WGS data was analyzed for rare variants in genes implicated in the telomere or surfactant pathway. In addition, samples from affected and unaffected family members were obtained from those willing and able to test for segregation of rare variants in families.

3.2 METHODS

3.2.1 Study Population

The study population included individuals who were treated for pulmonary fibrosis at the Dorothy P. and Richard P. Simmons Center for Interstitial Lung Disease. This project was approved under two different Institutional Review Board protocols: the Genomics and Proteomics of Idiopathic Pulmonary Fibrosis (GAP) Study and the Familial IPF Genetics Study. For the GAP study, clinical history was extracted from the participant's medical chart. Participants were eligible for the genetic study if their lung disease was suggestive of familial IPF and noted by their physician in their medical chart. For the familial IPF genetic study, the proband was consented and a pedigree was taken. In the event the proband was unavailable, a pedigree was constructed from information obtained from their medical records. To facilitate family member participation, probands were given a family letter that they could distribute to their family members who may be interested in participating in the study. Because of the high mortality of this disease, patients who were previously consented are still included in this study even if they are now deceased. For these individuals, pedigrees were ascertained via medical record review.

3.2.2 Whole Genome Sequencing and Variant Classification.

Whole genome sequencing was carried out at the University of Pittsburgh Medical Center Genome Center on blood obtained from the proband from each family. For sequencing, Illumina 150 bp paired-end sequencing on NovaSeq 6000 was done with an average coverage of 25x across

the entire genome. This CLIA certified sequencing center utilizes the DRAGEN Germline V2 Pipeline for initial read alignment and variant calls.

A variant call file (VCF) of every variant in each individual was generated. Variants of interest were mapped using the NCBI RefSeq coordinates using ANNOVAR (December 2018), and allele frequencies from various populations were extracted from gnomAD (https://gnomad.broadinstitute.org, December 2018) in addition to medically relevant variants and their phenotypes as reported in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, December 2018).

Variants from the genes associated with interstitial lung disease or telomere biology were extracted and annotated including 1000 bp 5′ and 3′ of the transcriptional start and stop sites, respectively. These were: *TERT, TERC, RTEL1, PARN, TINF2, NAF1, DKC1, CTC1, OBCF1, TEN1, SFTPC1, SFTPA1, SFTPA2, and ABCA3*. Analysis focused on coding variants or variants proximal to the splice donor or acceptor sites.

3.2.3 Sanger Sequence Confirmation

Each potential variant identified in WGS was confirmed by PCR amplification using oligonucleotides flanking the exon containing the variant of interest (see Table 1). PCR amplicons were purified over silica columns and sent for Sanger sequencing and Genewiz (South Plainfield, NJ). Sequence traces were aligned using Sequencher or SnapGene. PCR was carried out using phusion polymerase with 50ng template DNA under standard conditions. Some PCRs included 1 M Betaine has a PCR adjuvant.

Table 1. *TERT* and *RTEL1* forward and reverse primers

Gene	Forward Primer	Reverse Primer
TERT	5' -GCATTCATGCACGCACACAGGCAC- 3'	3' – CACTCACTCAGGCCTCAGACTC- 5'
RTEL1	5' -GGCAGGATGGGAGTTTCCTG- 3'	3' -CCGCCAGAGAACCAAAGTGA- 5'

3.2.4 Pedigree Ascertainment

Pedigrees were ascertained from probands who consented and were able to provide family history information. Pedigrees were drawn using standard nomenclature, as outlined by the National Society of Genetic Counselors.[94] A number of questions were asked of the proband, as stated in the Interview Guide for Pedigree (Appendix B). Pedigrees are not provided in this document to preserve confidentiality. The author may be contacted for further information regarding the information contained in the pedigrees (Appendix C).

3.2.5 Protein Alignment

Amino acid sequences from the corresponding RefSeq genes were obtained from the NCBI (https://www.ncbi.nlm.nih.gov, February 2019) and aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, February 2019).

3.3 RESULTS

The participants in this study were six patients diagnosed with IPF. These patients were known to have familial disease or concurrent hematologic malignancies. Four of the patients have results back from whole genome sequencing (WGS), two patients have results pending however, their pedigrees have been analyzed given the classical presentation of familial IPF. The results from WGS were filtered to variants with <1% allele frequency in the gnomAD database. Further, the results were analyzed for non-synonymous exonic variants in the genes listed in section 3.2.2. If no non-synonymous exonic variants were found, then rare intronic variants were examined in the genes listed in section 3.2.2. The results from the six patients are described in the following subsections.

3.3.1 Patient 1

Patient 1 is a deceased 83-year-old female, diagnosed with IPF at the age of 78. She had a history of smoking. A pedigree was ascertained from her medical chart and of her five siblings, one had a diagnosis of IPF and another had a myeloproliferative disorder. Results from WGS found no rare exonic variants in the genes evaluated.

3.3.2 Patient 2

Patient 2 is a male in his early 70's, diagnosed with familial IPF three years prior. He was a former smoker and reported a number of environmental exposures. A pedigree was collected from the patient which describes a sibling with IPF, their mother had emphysema and lung cancer.

Results from WGS for this patient found no rare exonic variants. This patient did have an intronic variant in *DKC1*, with an allele frequency of 0.0003 (7 of 10,655 sequenced individuals) (https://gnomad.broadinstitute.org, April 15, 2019). This intronic variant is located 1,025 bases upstream of the second exon.

3.3.3 Patient 3

Patient 3 is a deceased, 67-year-old male with a complex medical history of idiopathic pulmonary fibrosis (IPF) and a myelodysplastic syndrome (MDS). He was diagnosed with IPF one year prior to his death when a CT scan showed usual interstitial pneumonia. Pathology showed atypical adenomatous hyperplasia which can increase the risk of lung cancer in patients with IPF. At age 67, he was diagnosed with an MDS. A bone marrow chromosome analysis showed a complex karyotype consisting of numerous numerical and structural abnormalities. Double minute chromosomes and heterogenous staining regions are present, indicating gene amplification has likely occurred. This karyotype also confirmed the MDS was transforming to AML. Additional information in his medical record indicated he had exposures of asbestos, benzene, lead, radiation, and petroleum products due to his occupation. No family history of the disease was identified.

Results from WGS for this patient identified a rare exonic variant in *RTEL1*. The variant has an allele frequency of 0.0005 (76 carriers of 139,870 sequenced individuals) (https://gnomad.broadinstitute.org, April 15, 2019). This coding DNA change, *RTEL1*(NM_001283009.1):c.1955T>C, was confirmed via Sanger sequencing. The trace generated from SnapGene is shown in Figure 1, which shows the patient is heterozygous for this change. The amino acid change is *RTEL1*(NP_001269938.1):p.M652T. Methionine (M) is a non-polar amino acid, and threonine (T) is a polar amino acid. By using the UCSC Genome Browser

track “Vertebrate Multiz Alignment & Conservation (100 Species)”, this amino acid is relatively well conserved down to fish, with a few exceptions (https://genome.ucsc.edu/cgi-bin/hgGateway, April 15, 2019). As shown in Figure 2, one of those exceptions is in chicken, where the amino acid is tryptophan. However, tryptophan (W) is also a non-polar amino acid like methionine. This variant has not been reported in the literature. Though, it has been reported by a lab on ClinVar. With a two-star rating, this was classified as a variant of uncertain significance (VUS).

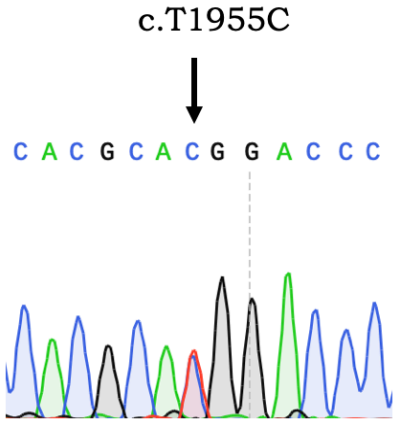

Figure 1. *RTEL1* Trace

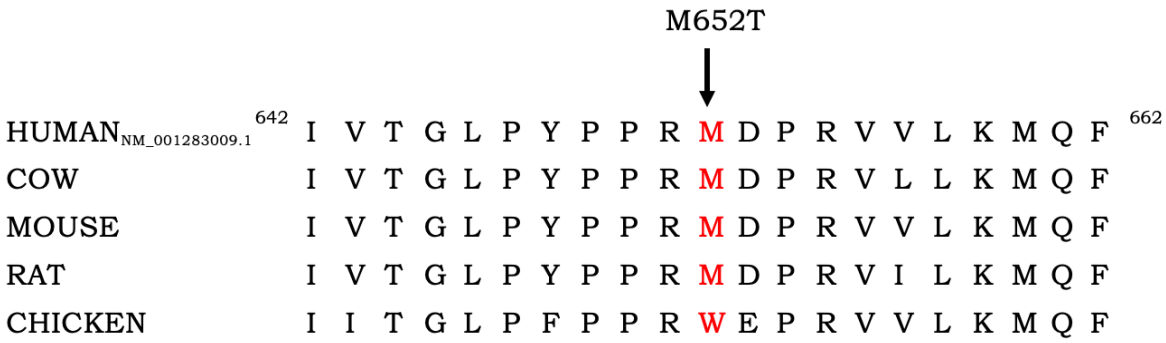

Figure 2. *RTEL1* ClustalW Alignment

3.3.4 Patient 4

Patient 4 is a male in his late 60's, diagnosed with familial idiopathic pulmonary fibrosis (IPF) one year ago. His medical history is notable for GERD. In addition, he reported a number of exposures, including chemicals, fertilizers, and asbestos. A pedigree was collected and shows the proband had a sibling with IPF who underwent lung transplantation.

Results from WGS for this patient identified a rare exonic variant in *TERT*. The variant has an allele frequency of 0.0004 (48 carriers of 140,174 sequenced individuals) (https://gnomad.broadinstitute.org, April 15, 2019). The coding DNA change, *TERT*(NM_198253.2):c.3257G>A was confirmed via Sanger sequencing. The trace generated from SnapGene for this variant, shown in Figure 3, confirms the patient is heterozygous for this change. The amino acid change is *TERT*(NP_937983.2):p.R1086H. Arginine (R) and histidine (H) are both non-polar positive amino acids. By using the UCSC Genome Browser track "Vertebrate Multiz Alignment & Conservation (100 Species)", this amino acid is not well conserved (https://genome.ucsc.edu/cgi-bin/hgGateway, April 15, 2019). Figure 4 shows a sample of species, exhibiting the poor conservation. Serine (S), as in mouse and rat, is a polar amino acid. Lysine (K) is a non-polar positive amino acid as well. Although this amino acid is not conserved through many species, a different change has been reported in the literature at this base (p.R1086C) for an individual with usual interstitial pneumonia with connective tissue disease.[16]

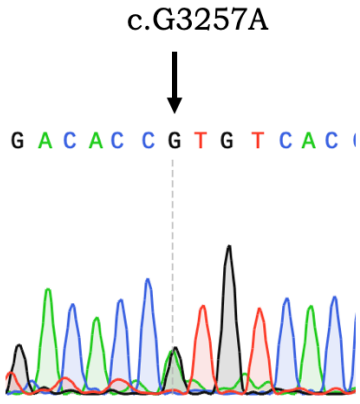

Figure 3. *TERT* Trace

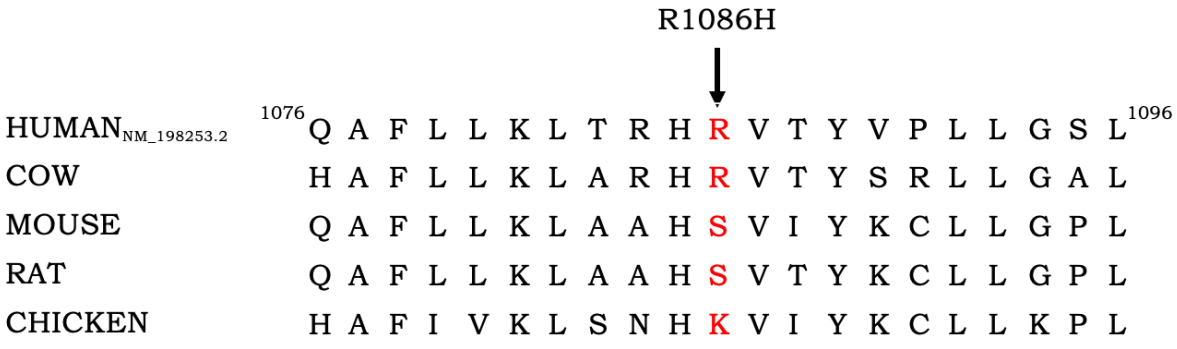

Figure 4. *TERT* ClustalW Alignment

3.3.5 Patient 5

Patient 5 is a woman in her 60's who has several siblings that have been diagnosed with IPF. A pedigree was collected and shows a number of autoimmune comorbidities amongst the siblings. A parent also had a diagnosis of IPF.

Whole genome sequencing is currently pending for individuals in this family.

3.3.6 Patient 6

Patient 6 is a woman diagnosed with IPF in her 50's who has had IPF for three years. A pedigree was collected, one sibling has an autoimmune disease. Another sibling has IPF with additional comorbidities, some of which include COPD and cancer. Lung cancer is also prevalent in older generations of family members.

Whole genome sequencing is currently pending for this individual.

3.4 DISCUSSION

In this study, we characterized a small sample of sporadic and familial IPF probands in hopes of characterizing genes implicated in IPF. In turn, this characterization will help us better understand the genetics of IPF and how this information may impact patients, their family members, and their course of treatment. Whole genome sequencing was undertaken in these individuals with sporadic and familial IPF. The results for this small cohort of patients identified a rare *RTEL1* variant in a patient with sporadic IPF and MDS, and a rare *TERT* variant in a patient

with familial IPF. Two familial IPF patients were found to have no rare, exonic variants in genes previously associated with telomere or surfactant biology. These results do not rule out that there is not a genetic cause for those two familial IPF patients, as it is possible they have a rare variant in a gene not yet known to be associated with IPF. An alternative cause for those patients could be shared environmental factors between the siblings leading to the disease.

RTEL1 and *TERT* are genes known to be implicated in IPF. *RTEL1* was first implicated in Dyskeratosis Congenita and autosomal recessive Hoyeraal-Hreidarsson Syndrome (HS), a severe bone-marrow failure syndrome, but has since been implicated in a number of familial IPF kindreds.[95-97] As in Patient 3, who has an *RTEL1* variant, recent studies have shown an association between *RTEL1* pathogenic variants in patients with bone marrow failure, though this association is premature and not well defined.[98,99] All of these syndromes are characterized by short telomeres, leading to the disease phenotype. This is because *RTEL1* encodes a helicase and functions to unwind the secondary structure of DNA at the telomere.[100] Similarly, pathogenic variants in *TERT* cause shortening of the telomeres. *TERT* encodes a reverse transcriptase and is important for extending the telomeres using an RNA template. In both of these genes, through different mechanisms, we see shortened telomeres which drive the clinical phenotype of IPF.

These results further support the difficulty in characterizing the genetics of the disease, particularly in identifying variants in both familial and sporadic patients. In addition, tests of segregation or functional assays are necessary to determine the pathogenicity of identified variants. Currently, only functional assays exist for *TERT* and *TERC*. There is no functional assay for *RTEL1*[101], so in this study, tests of segregation would be recommended in Patient 3, who had a variant in *RTEL1*. However, the majority of this patient population is in their sixth or seventh

decade of life, meaning many of the patients' parents and even siblings are deceased. This makes it difficult to test for segregation in these families.

Using whole genome sequencing was an effective approach in analyzing the genetics of IPF for this study given its decreasing cost. This test can identify rare variants in both intronic, exonic, and splice variants which could result in this disease. In addition, this test will enable us to possibly identify new genes associated with IPF, for those individuals whom a rare variant was not identified. This would not be possible if a panel test was used, as that test would only look for genes currently associated with IPF and would not look for intronic or splice variants.

As more research better elucidates the genetic underpinnings of this disease and testing continues to enter the clinical arena, it will be imperative to have a plan in place to discuss genetic testing and this disease with patients and families. This disease does have incomplete penetrance, and variable manifestations because of genetic anticipation due to the shortening of telomeres.[102] Given all of these aspects, ethical questions arise as to whether to test asymptomatic relatives. Even if a relative is found to carry a pathogenic variant, there are currently no guidelines recommending any surveillance or management. Additionally, a person who carries a pathogenic variant may not develop this disease due to incomplete penetrance.[18] On the other hand, in patients with short telomeres we may see genetic anticipation in families, meaning subsequent generations are manifesting extra-pulmonary symptoms, which may be more severe, and at earlier ages.[18,19] Lastly, identification of a pathogenic variant associated with shortened telomeres has implications for management outcomes, thus this information may be beneficial for precision medicine initiatives.

3.4.1 Study Limitations

In this study, a limitation is the size. This cohort only included six probands with sporadic or familial disease who underwent WGS to look for rare variants in the telomere or surfactant pathway. This sample size is not representative of all patients with either sporadic or familial disease.

3.4.2 Future Directions

In subsequent studies, it will be important to ascertain family member participation to track segregation of identified variants in family to determine their pathogenicity. Alternatively, functional assays could be used to determine pathogenicity, should they exist for the given gene. In some cases, family member segregation can additionally be used to identify new genes associated with IPF. The use of whole-genome sequencing allows for the investigation of intronic variants, when no rare exonic variant is found. Future studies could determine if intronic variants affect the splicing patterns of the gene.

3.5 CONCLUSION

In this study we ascertained six pedigrees for individuals with IPF who have familial or sporadic disease. Four of these individuals had whole genome sequencing in which two individuals had rare variants in telomere pathway genes, *RTEL1* and *TERT*. Two familial patients had no exonic variants identified, although this does not rule out the possibility of a gene not yet associated

with IPF or environmental exposures leading to IPF. Additionally, the two patients who do not yet have results from sequencing have pedigrees consistent with familial IPF and further concerns of telomere-mediated disease due to co-morbidities identified through the pedigree analysis.

This study utilized whole-genome sequencing and was successful in identifying rare, exonic variants in two of the four patients. The data analysis focused on exonic variants and splice variants, although intronic variants can also contribute to disease. Further analysis of intronic variants are warranted and have been found, particularly when no exonic variants are disease-causing.[103] While not undertaken in this study, whole-genome sequencing can be used to measure telomere length, which would be important information to gather for these patients, due to the mechanism of telomere shortening in both familial and sporadic IPF.[104]

Currently, clinical genetic testing is not the standard of care for IPF, however therapies are being developed based on the genetic status of a patient. These therapies can help personalize care and treatment, as well as limit risks and morbidity associated with lung transplant, which is the only life extending treatment. Further, these results may suggest genetic testing could yield relevant information in all patients with IPF, not just those with familial disease. This study, and others, have shown that more than 10% of individuals with sporadic IPF may have pathogenic variants in genes implicated in IPF. In some cases, it is possible reportedly sporadic IPF patients have other family members with this disease or associated malignancies but are unaware of these associations. It is important for physicians to ask appropriate family history questions to elucidate possible familial disease.

Anecdotally, patients often show a strong interest in understanding this disease and its impact on their family as well as a desire to participate in research. As genetic information evolves and shifts into clinical practice, it will be imperative that patients are educated about the differences

between clinical and research testing. This education is important for both patients and providers, in terms of genetic education and the role genetics plays in this disease. Finally, this study supports the importance of ascertaining pedigrees and family history information in patients with IPF, as it can provide valuable information as to the manifestations of the disease in families.

4.0 RESEARCH SIGNIFICANCE TO GENETIC COUNSELING AND PUBLIC HEALTH

Although this thesis project studied a small subset of patients with pulmonary fibrosis, the results provide further evidence of the role genetics plays in pulmonary fibrosis, in both familial and sporadic cases. The American Thoracic Society (ATS) has set forth guidelines for the care and management of patients, though these guidelines do not include recommendations for genetic testing in this disease.[20] However, research has demonstrated a relationship between telomere length and lung transplant outcome, suggesting genetic testing and genetic counseling has growing relevancy in this setting.[13-15]

This research has public health implications. In public health, there are three core functions: assessment, policy development, and assurance.[105] Policy development is particularly relevant to this study. Currently, there is no policy to guide genetic testing in these families, despite the preponderance of evidence regarding the implications of genetic information. Speculatively, policies may not exist because genetic testing has not been viewed as medically actionable. This is ethically problematic due to the relationship between shortened telomeres and outcomes post-transplant. Recent studies have suggested considerations for genetic testing, though these are not official statements of ATS.[106,107] In addition to these studies, recent research has suggested it may be time to consider clinical genetic testing for numerous reasons. One of these reasons is because patients want to know if their family members are at risk. Another reason is because of the possibility of modifying transplant protocols to improve the outcome for those undergoing a lung transplant. These alternative approaches aim to reduce morbidity and mortality and have been implicated in those with pathogenic variants in telomere genes or the presence of shortened

telomeres. As more research studies elucidate the genetic underpinnings of this disease, it will be imperative that policies are developed to ensure patients are provided the best possible care, which may include clinical genetic testing.

At the Simmons Center for Interstitial Lung Disease, many patients are quite invested in contributing to research studies that aim to enhance understanding of the disease and the development of therapeutic and treatment approaches. A difficulty with this study was helping participants distinguish between clinical and research testing, ensuring they understood that receiving results was not part of the study. This is a known difficulty in genetic studies and clinical trials, especially in regards to obtaining informed consent.[108,109] In fact, physicians, nurses, and research coordinators have commented that patients frequently ask, "Is this [pulmonary fibrosis] something that my children will get?" Interacting with these patients and ascertaining their family histories, as well as consenting them for the study, has provided valuable insight into the importance of genetic counseling, especially if a patient was to undergo clinical testing. In addition to genetic counselors ensuring the distinction between clinical and research testing is explained, discussing the implications of this testing is essential, for both the patient and their family members. For the patient, it will be important to discuss what this testing is, how testing may impact their care, how IPF is inherited, and the possible impact of testing on insurance (via the genetic information non-discrimination act). For family members, this is an adult-onset condition, with variable penetrance and anticipation, making it difficult to determine their immediate risk. In addition, due to the lack of guidelines for genetic testing, there are no clear recommendation for the clinical management of unaffected carriers of pathogenic variants. Concerns related to insurance discrimination should be addressed when individuals consider predictive genetic testing. Overall, should genetic testing begin to be offered to patients, it will be important that genetic

counselors are available to discuss the many implications this testing may have, as well as the psychosocial concerns that may arise in order to best meet the needs of patients and their family members.

APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVALS

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Kevin Gibson, MD
From: IRB Office
Date: 10/11/2018
IRB#: MOD0610029-21 / IRB0610029
Subject: Genomic and Proteomic Analysis of Disease Progression in Idiopathic Pulmonary Fibrosis

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The University of Pittsburgh Institutional Review Board reviewed and approved the requested modifications by expedited review procedure authorized under 45 CFR 46.110 and 21 CFR 56.110.

Modification Approval Date: 10/11/2018
Expiration Date: 6/19/2019

For studies being conducted in UPMC facilities, no clinical activities that are impacted by the modifications can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

University of Pittsburgh
Institutional Review Board

Human Research Protection Office
3500 Fifth Avenue, Suite 106
Pittsburgh, PA 15213
Tel (412) 383-1480
www.hrpo@pitt.edu

APPROVAL OF SUBMISSION

IRB:	STUDY18070008
PI:	Daniel Kass
Title:	Novel Telomerase Mutations in patients and families with Pulmonary Fibrosis
Funding:	None
Date:	September 19, 2018

On 9/19/2018, the Institutional Review Board reviewed and approved the above referenced study. The study may begin as outlined in the University of Pittsburgh approved application and documents.

Approval Documentation

Review type:	Initial Study
Risk Level:	No greater than minimal risk
Approval Date:	9/19/2018
Expiration Date:	9/18/2019

Determinations:	• Waiver/alteration of the consent process
Approved Documents:	• ITTC Informed Consent

As the Principal Investigator, you are responsible for the conduct of the research and to ensure accurate documentation, protocol compliance, reporting of possibly study-related adverse events and unanticipated problems involving risk to participants or others.

Continuing review (CR) can be submitted by clicking "Create Modification/CR" from the active study at least 5 weeks prior to the expiration date.

If this trial meets the definition of a clinical trial, accrual cannot begin until it has been registered at clinicaltrials.gov and a National Clinical Trial number (NCT) provided. Contact ctgov@pitt.edu with questions.

Clinical research being conducted in an UPMC facility cannot begin until fiscal approval is received from the UPMC Office of Sponsored Programs and Research Support (OSPARS).

If you have any questions, please contact the University of Pittsburgh IRB Coordinator, Amy Fuhrman.

Please take a moment to complete our Satisfaction Survey as we appreciate your feedback.

APPENDIX B: INTERVIEW GUIDE FOR PEDIGREE

STUDY18070008 – IPF GENETIC STUDY – Daniel Kass, PI

Interview Guide to Draw a pedigree:

1. A pedigree is drawn with symbols: circles representing females, squares representing males, diamonds representing unknown sex. Lines are drawn in certain ways to designate relationships (e.g. spouses, siblings, and children). A line is drawn through the symbol if a person is deceased. Under the symbol will be the participant's age, as well as, pertinent health conditions.

2. When taking the participant's (proband's) pedigree, the appropriate symbol will be drawn based on their reported sex. The participant will have a known diagnosis of IPF. The following information will be collected: current age, age of diagnosis, and treatment. The participant will then be asked about their family members. Family members include: children of the participant, siblings of the participant, nieces or nephews of the participant, and the parents of the participant. Questions will include health status and approximate age. Should a health problem be reported in a family member, the participant will be asked when the issue began and what treatment(s) is being used (if known). **Additional follow-up questions may be necessary based on the information the participant provides.**

3. After construction of the pedigree, the participant will be asked their ethnic background (maternal and paternal) and if there are any known consanguineous relationships (spouses related by blood). A few additional questions may be asked depending on the information elicited during construction of the pedigree. These questions are intended to incorporate all family members including extended family not already explicitly drawn on the pedigree. These questions include:
   - Anyone diagnosed with pulmonary fibrosis?
   - Anyone have a bone marrow failure?
   - Anyone with liver cirrhosis?
   - Anyone have a pediatric form of interstitial lung disease?
   - Anyone with early onset osteoporosis?
   - Any other known genetic syndromes in the family?
   - Anyone have infertility?
   - Anyone with premature graying?
   - Anyone with diabetes?
   - Anyone have trouble swallowing?
   - Anyone with autoimmune diseases?
   - Anyone with hypersensitivity pneumonitis?
   - Anyone exposed to environmental toxins?
   - Anyone a smoker?

Should the participant report on any of the above questions in a family member, the affected family member may be drawn on the pedigree, based on the information the proband provides.

APPENDIX C: PEDIGREES

For further information, please contact the author.

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