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The role of telomeres in pulmonary fibrosis and its effects on choices of treatment

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Thesis

**THE ROLE OF TELOMERES IN PULMONARY FIBROSIS
AND
ITS EFFECTS ON CHOICE OF TREATMENT**

by

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an aggressive disease with no known origin. Scar tissue continues to build up in the lungs diminishing pulmonary function. Without successful treatment, patients with IPF have a mean survival of 5 years after diagnosis. Though the exact etiology of IPF is unknown, studies have established a role of telomeres in 25% or more of familial or sporadic cases. Mutations in the genes that encode for the components of telomerase have been implicated as the cause of the telomere dysfunction seen in these cases. The mutations examined below include TERT, TERC, RTEL1, TINF2, and PARN.

With this link in mind, the available and the researched treatment plans are discussed. Of the discussed therapies, the researched treatment options that target the telomere-specific cases of IPF are a synthetic sex hormone (danazol), stem cell therapy, and a small molecule activator (GRN510). The remaining treatment plans discussed target either the direct cause of symptoms or the symptoms themselves. These studied options include N-acetylcysteine, prednisone, azathioprine, pirfenidone, nintedanib (with

and without statins), and low-dose inhaled carbon monoxide. The treatment options with the favored positive results are danazol and nintedanib.

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LIST OF ABBREVIATIONS

| | |
|-------|---|
| ADSC | Adipose Stem Cell |
| AECs | Alveolar Epithelial Cells |
| BP | Base Pairs |
| BWH | Brigham and Women's Hospital |
| CO | Carbon Monoxide |
| CTD | Connective Tissue Disease |
| ECM | Extracellular Matrix |
| FEV1 | Forced Expiratory Volume after 1 second |
| FGF | Fibroblast Growth Factor |
| FPF | Familial Pulmonary Fibrosis |
| FVC | Forced Vital Capacity |
| GERD | Gastroesophageal Reflux Disease |
| HRCT | High-resolution Computed Tomography |
| IPF | Idiopathic Pulmonary Fibrosis |
| KB | Kilobases |
| MCV | Mean Corpuscular Volume |
| MSC | Mesenchymal Stem Cell |
| NHLBI | National Heart, Lung, and Blood Institute |
| PDGF | Platelet-Derived Growth Factor |
| RTEL1 | Regulator of Telomere Elongation 1 |
| RTK | Receptor Tyrosine Kinase |

SCNTSomatic Cell Nuclear Transfer
TB Tuberculosis
TGF β Transforming Growth Factor Beta
TINF2..... TRF1-Interacting Nuclear Factor 2
TRFL..... Terminal Restriction Fragment Length
VEGF Vascular Endothelial Growth Factor

INTRODUCTION

Telomeres: Structure and Function in Cell Regulation

Human cells contain their DNA in structures called chromosomes in the nucleus of the cell. These chromosomes consist of very tightly coiled DNA strands wrapped around histones, the proteins that provide structure support. When the cell is dividing, the chromosomes unwind in order for the DNA to be replicated for the new forming cell. A combination of four chemical bases (adenine (A), cytosine (C), guanine (G), and thymine (T)) is paired off to create the double stranded structure of DNA. The sequence of these bases (the variant parts of nucleotides) determines what information is encoded in the DNA. Since DNA contains the genetic material that serves as a template for which all of an organism is built and maintained, it is necessary to have the exact same DNA sequence in almost every cell of the human body. During the process of normal DNA replication, DNA strands are shortened from the ends inward due to the organization of the genetic material and the replication machinery (i.e. DNA and RNA polymerases) of the cell (Figure 1). Thus, the presence of non-coding genetic material (or material that does not contain any information necessary for the creation or maintenance of the organism) at the ends of DNA strands is needed to protect the remainder of genetic material from degradation. The non-coding material at the ends of DNA are called telomeres.

Figure 16.20

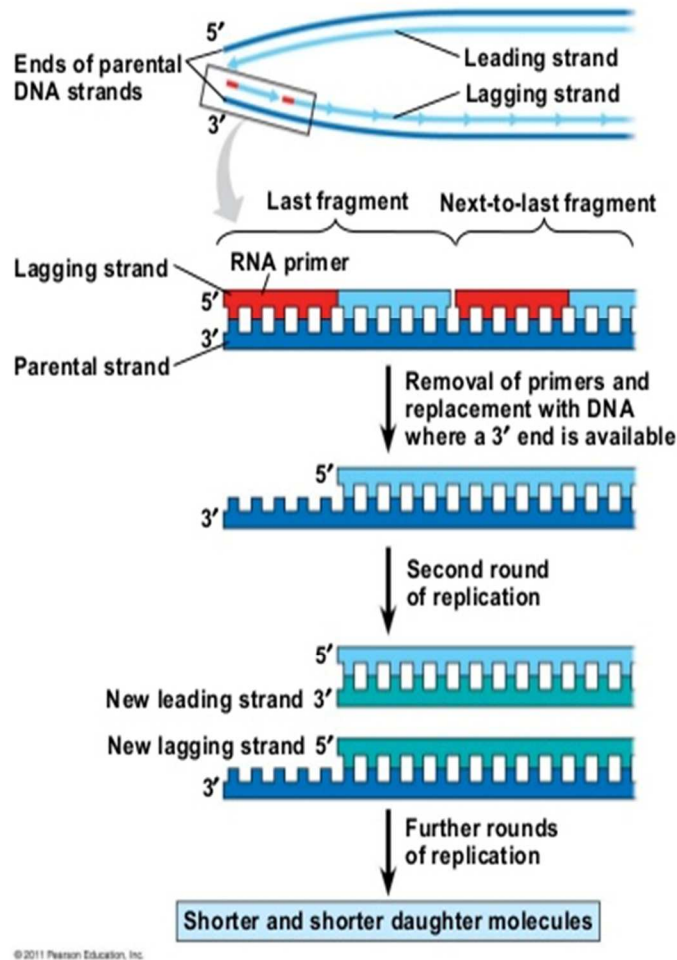


Figure 1—The DNA Replication Problem: DNA is replicated by separating the two parent strands and then adding complementary daughter ones to each strand. Because the parent strands are separated, one parent strand is oriented in a 5' to 3' direction (leading strand) and the other is oriented in a 3' to 5' direction (lagging strand) at the time of replication. As a result of DNA polymerase's limitations to only add nucleotides to a 3' end of DNA sequence and in a 5' to 3' direction, the lagging strand is replicated in pieces (known as Okazaki fragments). RNA primers are continuously added to the lagging strand as the parental strands are further separated and DNA polymerase adds DNA nucleotides to 3' end of the primers. These primers are later removed and replaced with DNA nucleotides where there are 3' ends of DNA. When the RNA primers are removed, there would exist a section of parental-strand DNA left unmatched at the 5' end of the daughter strand and, subsequently, removed in every consequent replication. (Adapted from "Boundless Biology", 2013 (5))

Telomeres are repeated sequences of the nucleic acid base pairs TTAGGG. Telomerases are enzymes that regenerate and restore telomeres (Figure 3). While they are expressed and active in certain cell lines (germ cells, hematopoietic cells), when they are inappropriately activated in somatic cell lines, immortalization and malignancy can result. Telomerase consists of an RNA subunit, known as TERC (TR), and a catalytic protein that has reverse transcriptase ability, TERT (Figure 2). These subunits are required to be functional in order for telomeres to be maintained with every cell division. In addition to the subunits that make up the enzyme, a shelterin complex associated to telomeres is made up of six proteins: TRF1, TRF2, POT1, TIN2, TPP1, and Rap1 (Figure 2). These subunits come together to surround the repeated telomere sequence, TTAGGG, to shield it from being recognized by the DNA repair machinery, which would process the telomere as damaged DNA. ¹

With every mitotic event (cell division), there is a natural, physiological shortening of telomeres. In addition to this, telomeres are also shortened in human cells through the “wear and tear” events of aging (which includes damage done by oxygen radicals and environmental toxins and exposures). Once the telomeres have reached a length threshold, healthy cells will respond by undergoing apoptosis or entering senescence. If the function of telomerases is not regulated, telomeres can repeatedly be elongated and a cell can continue to divide beyond the normal (or healthy) amount. When this occurs, these cells are labeled “cancerous.”

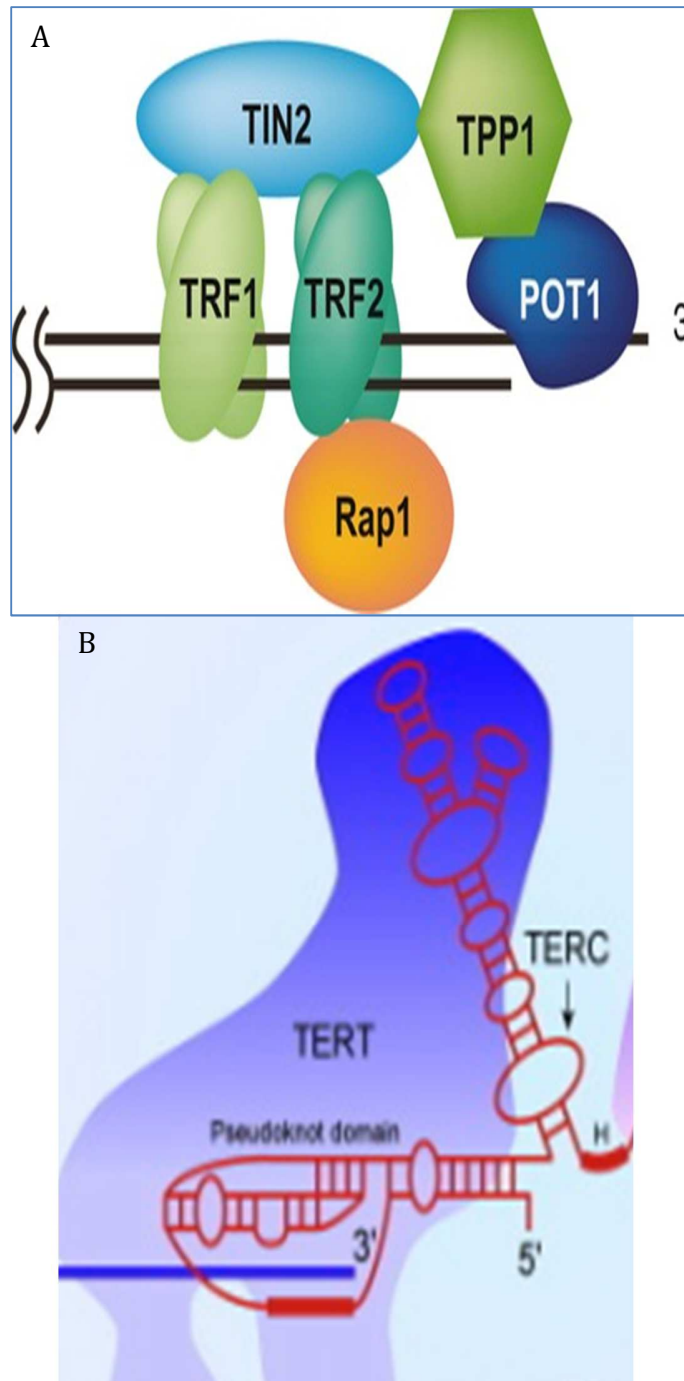


Figure 2 – Telomerase Structure: A) The shelterin complex is composed of the 6 proteins shown. The function of the complex is to protect the telomere from being read as a mistake during DNA replication.² B) The subunits TERT and TERC make up the telomerase enzyme, which functions to create and regenerate the telomere. (Adapted from A) Ishikawa, Fuyuki, 2013 (4) & B) Kinwan, M. and Dokan, I., 2009 (3))

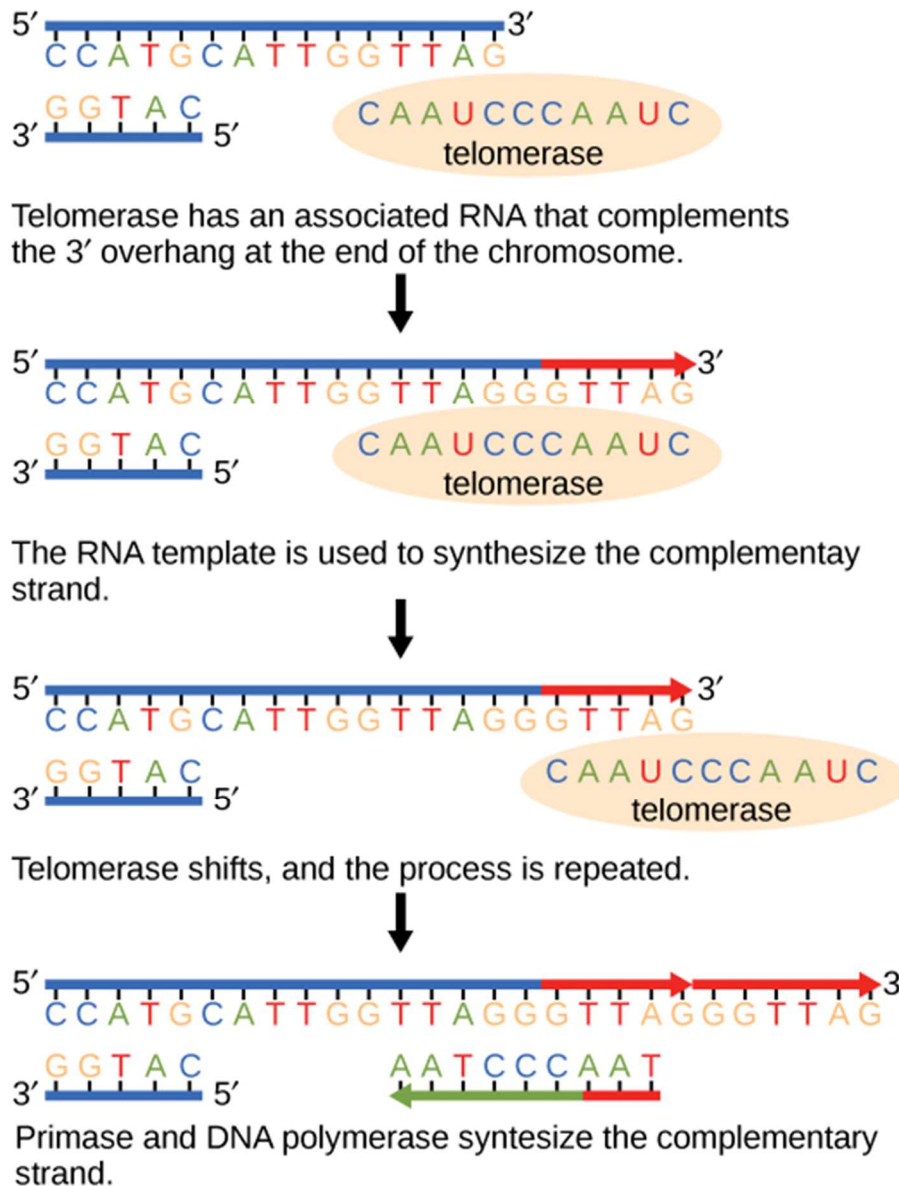


Figure 3—Telomerase Activity: Telomerase uses an RNA component to attach to the DNA overhang and synthesizes a complement to its own repetitive sequence in a 5' to 3' direction before relocating and repeating. As the enzyme synthesizes further down, DNA polymerase adds to the sequence in a 5' to 3' direction. (Adapted from Lecture Presentations from Campbell Biology, 9th Edition, 2011 (2))

While unregulated elongation of telomeres is linked to various cancers, accelerated shortening of telomeres has been found to increase the risk of many pathophysiological states. This accelerated shortening can be a result of environmental exposures from diet and lifestyle choices, such as smoking or alcohol misuse.¹ The environmental causes of this shortening lead to an increased need for cell replacement, as they are damaging the affected tissue. This need for new cells means undergoing more cell divisions, which leads to increased rate of telomere attrition (shortening).¹ In addition to environmental exposures, there exist genetic predispositions to rapid shortening of telomeres and this process has been associated with several inherited disorders of rapid, premature aging.¹ Mutations in the genes that code for the components of the telomerase enzyme, TERT and TERC, as well as the genes that code for those genes' regulation, can be inherited. These mutations result in a dysfunctional enzyme.¹ Older adults with shorter telomeres have been found to be at increased risk of coronary artery disease, myocardial infarctions, and osteoporosis than peers with longer telomeres.¹

Idiopathic Pulmonary Fibrosis

Pulmonary fibrosis, a disease in which dense and rigid scar tissue builds up in lung tissue making the lungs deficient in their function (Figure 4), is said to be idiopathic when the cause of the pathology is unknown.⁶ “Idiopathic pulmonary fibrosis (IPF), the most common form of the idiopathic interstitial pneumonias, is a chronic, progressive, irreversible, and usually lethal lung disease of unknown cause.”⁷ IPF is thought to be the result of excessive and unprovoked production of inflammatory mediators by alveolar macrophages. These inflammatory mediators activate fibroblasts to produce dense collagen deposits and stimulate the hyperplasia of type II pneumocytes causing the characteristic histopathologic findings necessary for diagnosis of this disease.⁷

The pathogenesis of the disease varies in timeline among patients. While IPF may progress slowly in some, it also may cause a quick decline in a patient’s condition. Affecting between 4.6 and 16.3 in 100,000 people every year, with incidence rising, IPF has been found in mostly middle-aged and elderly adults. Cases of IPF are more common in men than in women with a ratio of incidence of 1.5-1.7 to 1.⁴ Progression of the disease may lead to other issues such as a collapsed lung, lung infections, blood clots in the lung, and lung cancer.⁶ In IPF patients, the most common cause of death is respiratory failure, with other causes including pulmonary hypertension, heart failure, pulmonary embolism, pneumonia, and lung cancer.⁶ Factors that may increase the risk of developing IPF include cigarette smoking, viral infections (Epstein-Barr virus, influenza A virus, hepatitis

C virus, HIV, and herpes virus 6, family history of IPF, and gastroesophageal reflux disease (GERD).⁶

The primary symptom of IPF is shortness of breath. Initially, this dyspnea will occur only during exercise, but it will likely become a symptom at rest, as well. The other most common symptom is a dry, hacking cough with no signs of improvement.⁶ As a result of a “relatively slow clinical course,” patients often seek medical treatment after months to years of initial symptoms, when they have chronic and progressively worsening exertional dyspnea (difficulty breathing) and coughing. Other possible symptoms include: rapid and shallow breathing, gradual and unintended weight loss, fatigue or malaise, aching muscles and joints, and clubbing.⁶

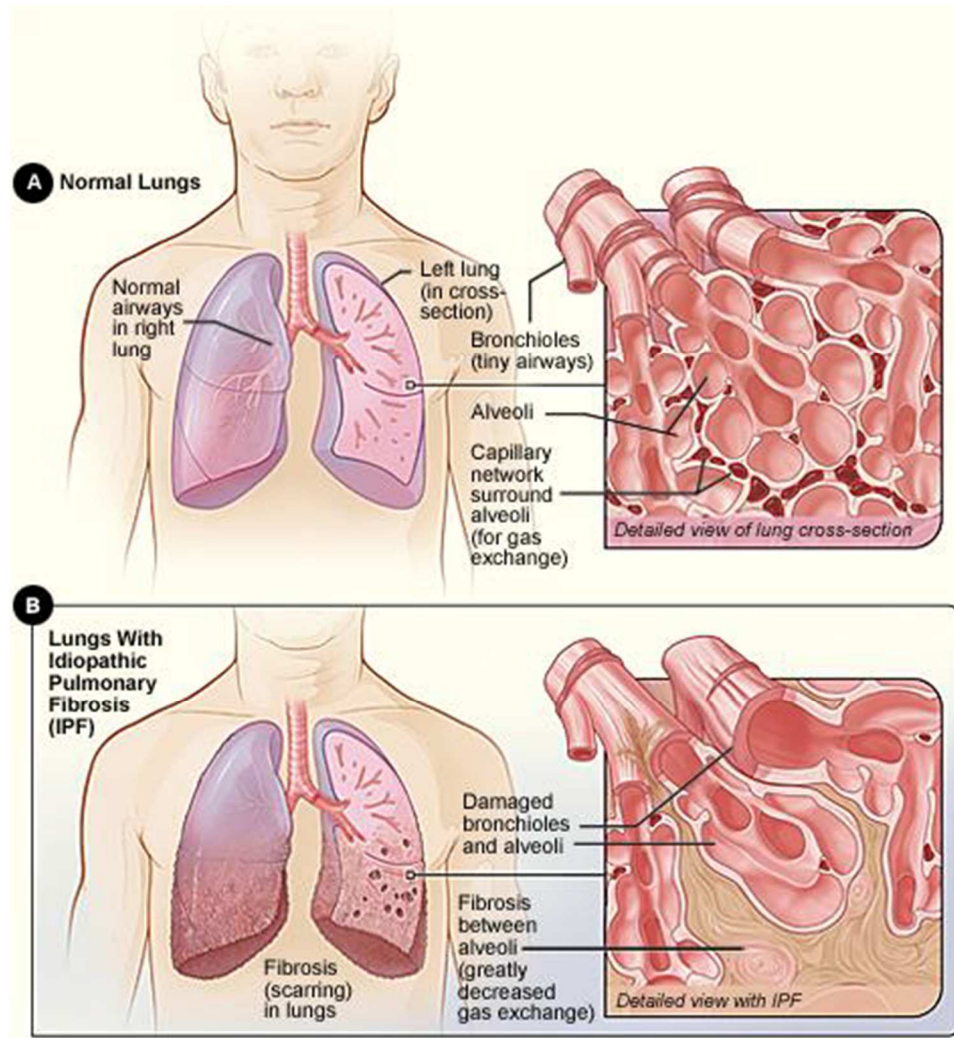


Figure 4-Anatomy of Normal Lungs vs. Lungs with IPF:

- A) Normal lungs have an extensive network of expansive, elastic alveoli. This network allows for adequate gas exchange and distribution of oxygen to peripheral tissues.
- B) Lungs of patients with IPF have less alveoli, which have been replaced with constricting and rigid scar tissue. This scar tissue, made up of collagen, restricts the expansion of lung tissue and prevents sufficient inhalation and gas exchange.

(Adapted from NHLBI: Idiopathic Pulmonary Fibrosis, 2013 (6))

Diagnosis of IPF is difficult since it presents similarly to other lung diseases. A combination of several diagnostic exams is used to confirm the pathology, which includes a chest X-ray, a high-resolution computed tomography (HRCT) scan, spirometry exam, an arterial blood gas exam, a skin test for tuberculosis, exercise testing, and a lung biopsy. An X-ray is done to check for scar tissue. A shadow might be seen in the X-ray that indicated the presence of scarring, though many patients with IPF have normal X-rays at the time of diagnosis.⁶ An HRCT scan provides a higher quality image than an X-ray and can show scar tissue as well as the extent of lung damage. It is more likely to help a physician detect IPF at an earlier stage.⁶ Spirometry is used to measure the amount of air that one can breathe out after a deep breath (Figure 5). A patient with IPF would not be able to breathe out as much air due to the lung scar tissue (Figure 6).⁶ An arterial blood gas test is taken to measure the levels of oxygen and carbon dioxide in the blood, which could tell physicians if the patient's lungs are performing gas exchange as they should. This would be deficient in a patient with IPF.⁶ A skin test for tuberculosis (TB) is done to rule out TB as the cause of symptoms. If positive, a lump would form where the injection was done on the forearm 48 to 72 after the test. An exercise test will show how well gas exchange occurs when the body is active. Dyspnea is likely to be exacerbated by activity in a patient with IPF.⁶ Finally, a lung biopsy is the most concrete method to diagnose IPF.⁶ A lung biopsy would consist of taking a sample of lung tissue from the patient and putting it under the microscope to check for signs of scar tissue. Once

diagnosed, patients with IPF have a median life expectancy of 2.5 to 3.5 years.

Poorer prognosis is positively correlated with age.⁶

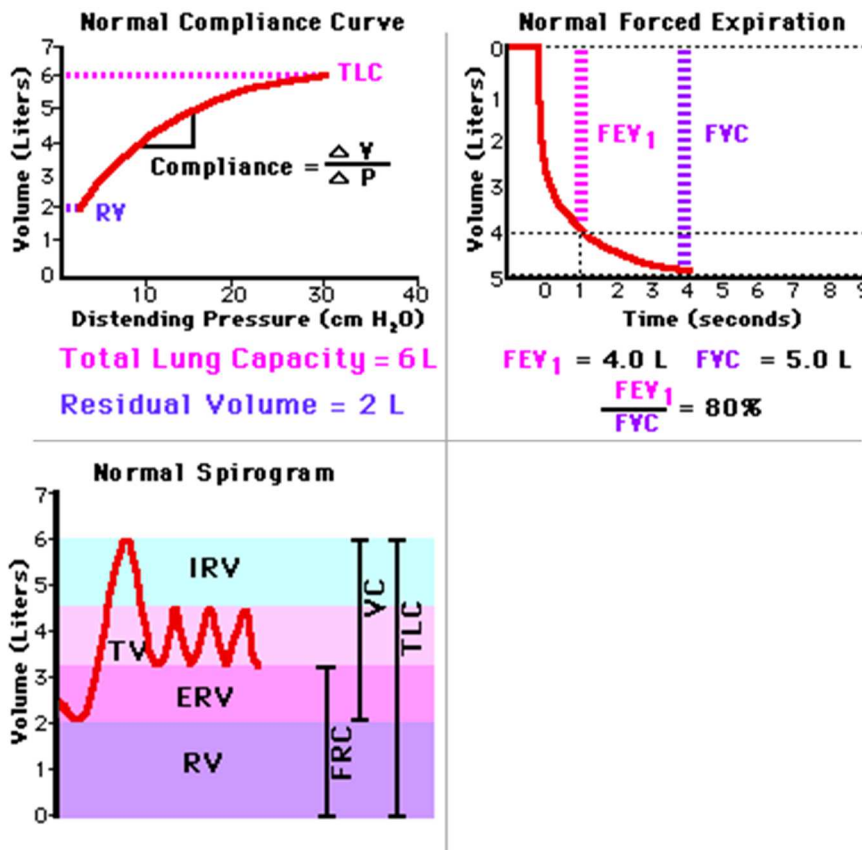


Figure 5-- Spirometry Results of a Normal Lung: The normal compliance curve indicates a normal expansion of lungs. Normal forced expiration curve indicates a normal ratio of forced expiratory volume (FEV1) to forced vital capacity (FVC). FEV1 is the measure of air one can expire from their lungs in one second. FVC is the measure of air one can breathe in and out in one deep breath. A normal spirogram indicates a normal range of volumes of air the lungs breathe in and out. (Adapted from Johns Hopkins School of Medicine: “Lab: Disease States”, 1994 (8))

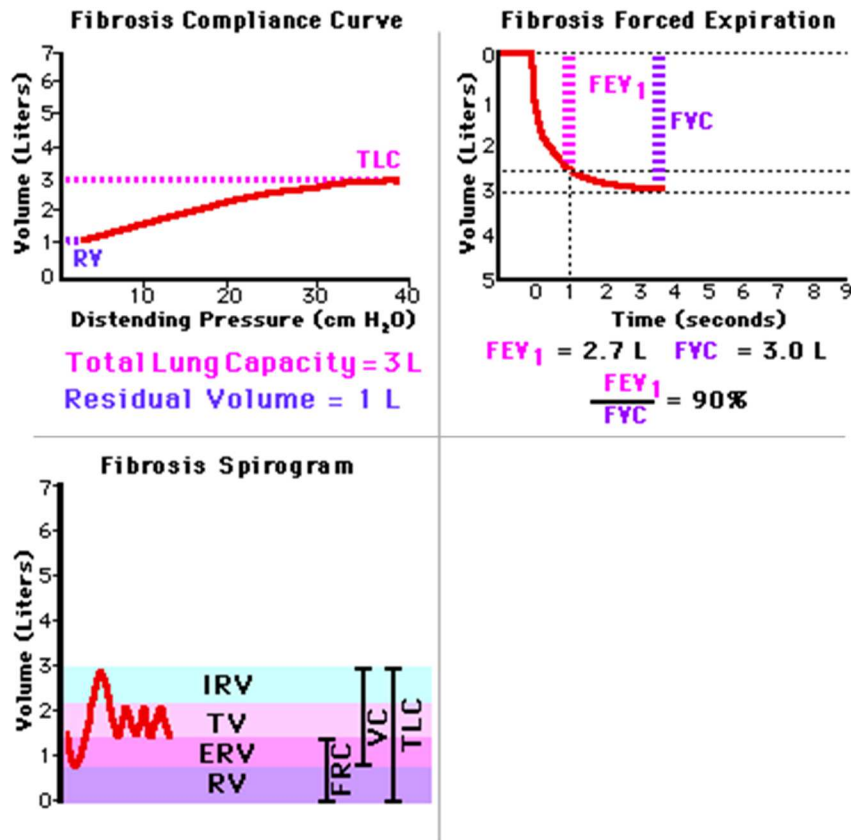


Figure 6—Spirometry Results of a Fibrotic Lung: The fibrosis compliance curve shows a decline in expansion of lung tissue. The fibrosis FEV₁ to FVC ratio is increased, though showing a total decrease in both total volumes. The spirogram of a fibrotic lung is significantly condensed, showing a decrease in volumes of the lung across the board. (Adapted from Johns Hopkins School of Medicine: “Lab: Disease States”, 1994 (8))

Objectives

The objectives of this thesis are as follows:

- 1.** To review the literature available to define the relationship between telomeres and pulmonary fibrosis in humans.
- 2.** To discuss and assess the current therapies available for IPF patients with telomeropathies.

PUBLISHED STUDIES

The Role of Telomeres in the Pathogenesis of Idiopathic Pulmonary Fibrosis:

Idiopathic pulmonary fibrosis has prompted many research studies in efforts to understand its etiology. Among the many avenues researchers have explored for an answer to the cause, genetics continue to be amongst them.

Mutations That Cause Telomere Dysfunction

TERT and TERC

Amongst one of the earliest studies done, Armanios, M. et al. (2007) screened 73 subjects for TERT and TERC mutations. The pedigrees of the six subjects that carried heterozygous TERT mutations (8%) were studied to determine if the incidence of the IPF phenotype followed the pattern of inherited mutations. The pattern was consistent with an autosomal dominant inheritance of the disease in that the mutated allele was present in those affected by IPF and generally absent in those who were not carriers. The mutant carriers that did not present with IPF were significantly younger, supporting the previous indication that the disease was age-dependent. The study further analyzed whether or not the mutations correlated with shorter telomere length. It was found that the mutant carriers, even those that showed no symptoms, had significantly shorter telomeres on average than their non-carrier relatives. When comparing telomere lengths amongst age-matched subjects, mutation carriers were below the 10th percentile.⁹

When studying 134 subjects from 21 unrelated families with heterozygous TERT mutations, researchers found that no subjects below the age of 40 presented the

pulmonary fibrosis phenotype.¹⁰ In contrast, researchers found that 60% of male subjects and 50% of the female subjects over the age of 60 presented with clinical IPF.¹⁰ Of the subjects studied, those with TERT and TERC mutations had shorter telomeres than noncarriers (Figure 7). In addition to these findings, it was found that biological family members of subjects affected by pulmonary fibrosis but did not have a TERT mutation had shorter telomeres than healthy unrelated controls and spouses that married into the family.¹⁰ This implies that successive generations inherit telomere length regardless of inheriting TERT mutations. It could not be concluded as to whether or not those affected with short telomeres presented any IPF phenotypes without inheriting the mutations.¹⁰ It is believed that environmental factors played a role in the prevalence of IPF in the subjects with mutations, as 95% of the mutation carriers smoked or had exposure to a fibrotic agent.¹⁰

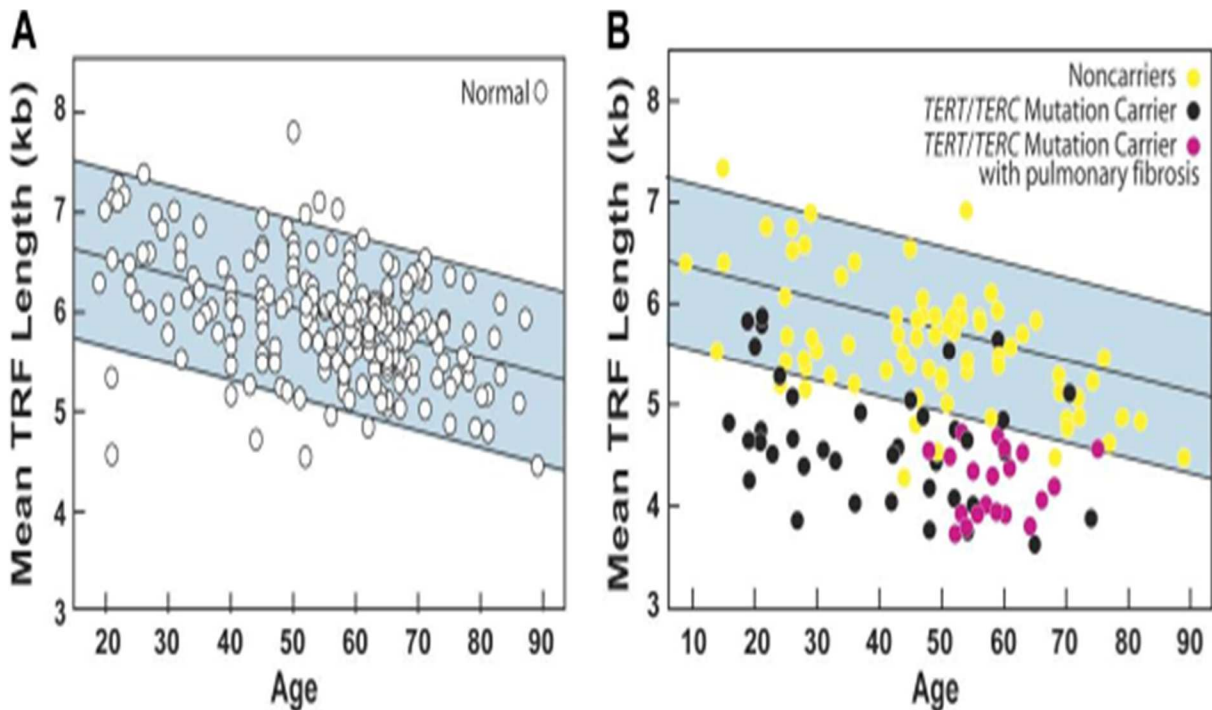


Figure 7—Telomere Length in Normal population vs. TERT/TERC Mutation Carriers:
 A) The graph shows the length of telomeres in the normal population. The data represents the average telomere length in kilobases (kb) across all age groups. B) The graph shows the telomere lengths of noncarriers, mutation carriers, and mutation carriers with the IPF phenotype. Both groups of mutation carriers show a lower mean length than noncarriers. (Adapted from Diaz de Leon, Alberto et al., 2010 (10))

In 2008, a study was done to test if telomere shortening was a common incidence in the manifestation of IPF in patients without the known TERT or TERC mutations. It had been previously established that missense, frameshift, and splice site mutations in TERT was found in both familial and sporadic cases of IPF.¹¹ The study reiterated that telomere lengths in IPF patients with these mutations were found to be significantly shorter and decreasing in circulating leukocytes than those of age-adjusted control subjects.¹¹ The study took patients with familial and sporadic cases of IPF and sequenced the genes that encode the components of the telomerase, TERT and TERC. The telomere

lengths of the circulating leukocytes in both the test and control subjects were analyzed using terminal restriction fragment length (TRFL) analysis of genomic DNA. The TRFL method consists of digesting DNA into sections and measuring the lengths of the fragments. The study inadvertently found six new mutations in TERT.¹¹ In alignment with its goals, the results showed that 25% or more of the subjects with familial or sporadic IPF has short telomere lengths.¹¹ Of the subjects in the study that had familial IPF, a total of 37% showed telomeres below the 10th percentile.¹¹ Of the subject in the study that had sporadic IPF, a total of 25% showed short telomeres.¹¹ These statistics included both subjects with and without mutations in TERT and/or TERC. The results suggested that short telomeres played a role in IPF beyond the mutations. Though, 65% of the subjects with coding mutation in either TERT or TERC met the clinical definition of IPF. The subjects that carried mutations with a diagnosis of IPF were all above 48 years old, indicating that pulmonary fibrosis is correlated with aging. Subjects with mutations were more likely to pass away over the course of the study than those without, as well as subjects with telomere lengths below the tenth percentile versus those with lengths above.¹¹ Smoking has been a known risk factor for IPF, but researchers speculated that there existed an increased risk for those with mutations, as well.¹¹ The male to female ratio of subjects with mutations suggested a greater “penetrance of the pulmonary fibrosis phenotype” in males with the dominant telomerase-mutated genotype.¹¹

RTEL1

Mutations in TERT and TERC cannot completely account for the cause of telomere dysfunction seen in IPF. Regulator of telomere elongation helicase 1 (RTEL1) is a gene that “encodes for a DNA helicase that is responsible for the stability, protection, and elongation of telomeres and interacts with proteins in the shelterin complex known to protect telomeres during DNA replication.”¹⁶ After ruling out TERT and TERC mutations as the cause of FPF, a study in France took the DNA of 47 individuals diagnosed with familial pulmonary fibrosis (FPF) and performed whole human exome sequencing to search for mutations that may play a role in the development of their condition. The study discovered a prevalence of RTEL1 mutations in 4 of the subjects coming from 4 independent families.¹⁶ Whole-blood cell analysis concluded that those individuals had shorter telomeres than age-matched controls. Statistics show that up to 25% of IPF patients and 40% of FPF patients exhibit short telomeres with half of these FPF patients carrying TERT or TERC mutations.¹⁶ The study proposes that RTEL1 mutations account for half of the unexplained FPF patients with shortened telomeres.¹⁶ Similarly to patients with TERT mutations, researchers found a history of smoking in a majority of the patients with RTEL1 mutations afflicted with IPF.¹⁶

TINF2

The gene TRF1-Interacting Nuclear Factor 2 (TINF2) codes for one of the proteins that make up the shelterin complex that protects telomeres. A family with IPF was found to have a heterozygous mutation in TINF2, though there was no evidence of shortened telomeres in peripheral blood cells.¹⁷ This opens up a new role of telomere

dysfunction in IPF that does not result in short telomere lengths. In mice, it has been previously shown that deletion of Trf1 or Trf2 in type II alveolar epithelial cells (AECs) results in telomere damage response, senescence, and a pulmonary fibrosis phenotype with showing short telomeres.¹⁷

PARN

Exome sequencing of patients with IPF linked mutations in PARN with shorter telomeres.¹⁸ When the genetic sequencing of seven relatives with IPF was taken, they were all found to have the PARN mutation.¹⁸ Though this implicates a role of PARN mutations in the pathogenesis of IPF, the pathway through which PARN mutations cause telomere dysfunction is still unknown.¹⁸

All studies agree that shorter telomeres are associated with worse survival rates in patients with IPF. These results are independent of age, sex, and mutations in telomerase.¹⁹

Mechanism of Telomeropathies in IPF

While the exact mechanism behind short telomeres in IPF is unknown, a pathway has been proposed. The short telomeres, being dysfunctional, activate a DNA damage pathway that ultimately results in cell death or cell cycle arrest. This is seen in the clinical setting as tissue death that results in organ failure in tissues that have a high turnover rate of cells, such as in the lungs.⁹ The epithelium of the pulmonary tract is constantly

regenerating and replenishing itself, thus IPF manifests with telomere deficiency. This mechanism would mean that the fibrosis seen is an indirect result of insufficient alveolar epithelium and not a direct consequence of a fibrogenic defect. The proposed pathogenesis would lead to lung remodeling. This would explain the lack of success associated with anti-inflammatory treatments of IPF.⁹ This etiology would also help explain the effect that smoking has on the risk of developing IPF, as smoking induces increased turnover of cells. Paired with the natural shortening of telomeres with increased age, smoking could explain the development of pulmonary fibrosis in those with wild-type telomerase.⁹

A mechanism for a specific cell type has been proposed type II alveolar epithelial cells as the key player in the pathogenesis. A study found that shortened telomeres in type II alveolar epithelial cells causes lung remodeling and fibrosis (Figure 8). Researchers found an abundance of senescent cells accumulated in areas of heavy scarring in the lungs.²⁰ The lung remodeling occurred with age and did not present with previous lung injury or a loss of type II AECs²⁰, reaffirming the preceding theories. In addition, there was evidence of DNA-damage response²⁰, providing further support for the suggested mechanism.

In analyzing this mechanism, a more detailed explanation has been suggested. It is known that short telomeres induce the epithelial damage response via apoptotic pathways.²² When the epithelial tissue of the lung is damaged, it requires regeneration of the dead tissue, as mentioned earlier. When the body interprets the cell death issue as tissue damage, it will initiate proliferation of cells that produce and deposit collagen

fibers.²² The epithelium will release an increased amount of fibrogenic mediators—growth factors—in response. These mediators activate mesenchymal cells. Mesenchymal cells take the form of fibroblasts and myofibroblasts, which cause the build-up of scar tissue that is seen in IPF.²²

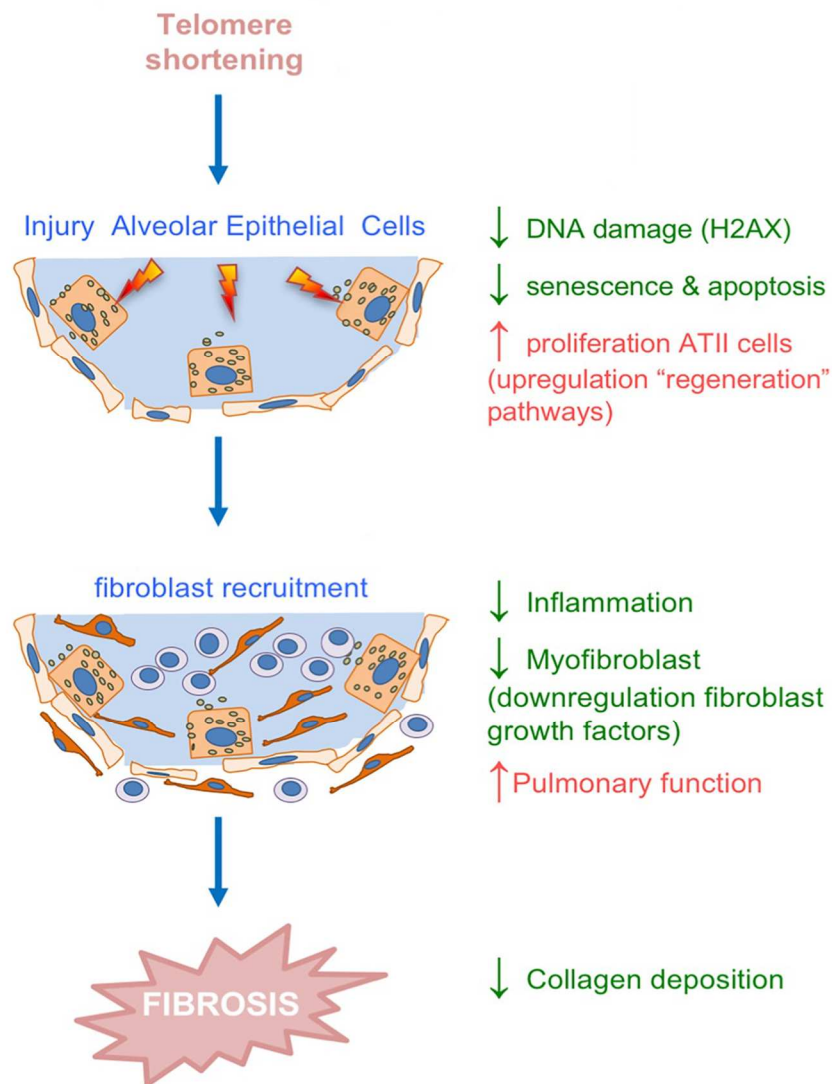


Figure 8: Proposed Pathway From Telomeropathy to IPF: On the right, the steps of the telomere-related mechanism of IPF pathology are shown. Short telomeres and AEC injury (and regeneration) leads to DNA damage which causes the activation of apoptosis pathways. This leads to the recruitment of fibroblasts, which produce and deposit collagen. On the left, the figure shows the desired modifications that would reverse the issues that arise at each step of the pathway. (Adapted from Povedano, Juan Manuel, et al., 2018 (21))

The Role of Growth Factors in IPF

The proliferation of these mesenchymal cells is a downstream consequence of tyrosine kinase receptors being activated on the extracellular surface. The molecules that activate these receptors are known as growth factors. The families of growth factors that play a role in IPF are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). PDGF is released in response to damaged tissue and acts to promote wound repair and the development of scar tissue.²² In IPF, PDGF has been shown to stimulate the proliferation of fibroblasts, which deposit the collagen in the lungs²². VEGF is important for survival and differentiation of endothelial cells in the lung and is, in part, responsible for the maintenance and repair of the lung.²² In IPF, VEGF has been shown to increase permeability of blood vessels in the lungs for factors that increase remodeling and wound healing. Thus, if VEGF is activated in response to a perceived tissue injury in the lungs, it would increase the deposition of collagen and formation of scar tissue in the lungs. FGF is a family of growth factors that play a role in the regulation of most processes related to tissue injury response. FGF acts to aid regulation of “[cell proliferation, differentiation, angiogenesis, wound healing, fibroblast proliferation, and production of collagen].”²² In IPF, the family of FGFs has been shown to induce proliferation of fibroblast in the lung, which causes synthesis of connective tissue.²² The role of these growth factors provide a target for IPF therapy.

Telomeropathies Manifestations

In addition to causing IPF, short telomeres have manifested a number of other conditions. At Brigham and Women's Hospital (BWH) in Boston, Massachusetts, a small pilot study looking to establish screening practices for subjects with clinically diagnosed IPF who had the potential to have underlying short telomeres or telomerase mutations was established in 2011.²³ This study emerged following a case in which a patient with IPF underwent lung transplant, but subsequently developed severe bone marrow and liver failure. The association with these three organ systems was suggested to be related to telomeropathy given the known organ dysfunction associated with functional mutations in genes responsible for telomere homeostasis.²³

In September 2011, the lung transplant program at BWH published a protocol and clinical guidelines to choose potential study subjects. Inclusion criteria were patients that had two or more visits to physicians within the lung transplant program and exclusion criteria were those patients that had connective tissue disease (CTD) or sarcoidosis-associated IPF. The protocol involved screening patients for the following:

- Abnormally low complete blood count (CBC) values including white blood cells, platelets, and hematocrit
- High mean corpuscular volume (MCV)—or large average size of red blood cells
- Family history of IPF, aplastic anemia, or liver disease
- Abnormal liver enzymes and function
- Abnormal liver findings on imaging such as ultrasound or CT scans

Those subjects that met one of the above criteria as well as the inclusion criteria had their telomere length checked by flow fluorescence in situ hybridization. Those with telomere lengths shorter than the 10th percentile of the population were qualified as having short telomeres. These patients were sent for further bone marrow and liver testing as part of their lung transplant work up.²³ Additionally, those who were characterized as having short telomeres were consented for further genetic testing in regards to functional mutations in the TERC and TERT genes.²³

During the enrollment period from September 2011 to December 2013, 127 subjects were eligible for inclusion based on the criteria of having IPF not associated with sarcoidosis or CTD and having two or more visits to the lung transplant program outpatient clinic in that time. Of the 127 subjects, 30 of them were suspected of having short telomeres because they met at least one of the screening criteria as defined above. Of the 30, 22 were consented for telomere length testing. The other eight did not on the basis of either being too well to be considered for lung transplantation in the near future, being rejected for transplant for other reasons, or having passed away in the enrollment period.²³ Fifteen of the 22 people that had their telomere length checked were found to have short telomeres.²³ Of the subjects with short telomeres, seven agreed to genetic testing. Of those seven subjects, two patients were found to have mutations in TERT believed likely to be pathogenic because they caused coding abnormalities, three patients were found to have a variant of TERT that was previously found to be a benign mutation given no changes in coding sequence of the protein, and two patients had no mutations in either gene.²³

Interestingly, it was found that macrocytosis (high MCV of red blood cells) had a positive predictive value of 86.6% in recognizing patients with short telomeres as 13 of 15 patients with short telomeres had an MCV above the limit of normal.²³ In the patients with short telomeres, 13 of them underwent bone marrow biopsies to better characterize marrow disease, even when their peripheral blood showed few abnormalities (such as mild anemia and macrocytosis). Significant abnormalities were found in the marrows of eight of the 13 patients that underwent these biopsies.²³

In addition to the exploration of bone marrow abnormalities, investigators inquired as to subclinical liver dysfunction. Of the 15 patients with short telomeres, one had abnormal liver function tests on blood work and seven of them underwent liver biopsies.²³ On biopsy, six of seven samples had abnormalities ranging from mild regenerative changes to mild portal chronic inflammation.²³ Notably, none of the patients had cirrhosis. The results of this study were consistent with the hypothesis that short telomeres can cause a spectrum of diseases that lead to multiorgan failure include lung, bone marrow, and liver.²³

Most Notable Treatment Options to Date

N-Acetylcysteine, Azathioprine, and Prednisone & Pirfenidone

In 2011, clinical trials of treatments for IPF included N-acetylcysteine used in combination with prednisone and azathioprine. N-acetylcysteine is an antioxidant and combined with prednisone, a corticosteroid, and azathioprine, an immunosuppressive drug, did produce a slowing of the “rate of decline in forced vital capacity and diffusing capacity for carbon monoxide” after a year of treatment.⁷ These observations, however, were not of definitive clinical significance. Clinical trials of treatment also included pirfenidone. Pirfenidone, a compound that works by down-regulating growth factors such as transforming growth factor-beta (TGF β) also decreased the rate of decline in vital capacity, as well as increase the survival time over the course of a year.⁷ A clinical trial in which prednisone and an anticoagulant were administered was suspended by the National Heart, Lung, and Blood Institute (NHLBI), as they felt there would be no added effect in treatment of IPF.^{6,7} Pirfenidone is the only choice of these treatments for IPF with positive clinically significant results, but is not a cure and cannot resolve telomere dysfunction.

Danazol

A study done in 1999 showed that estrogen, a steroid hormone, activates telomerase by acting on the TERT promoter.²⁴ Estrogen activates transcription of the TERT gene, which promotes activation of the telomerase enzyme to elongate telomeres.²⁴

This study laid the foundation for the experimental hormonal activation of telomerase. While the initial intent of the study was to observe the effect of estrogen on telomerase in the endometrium, it later inspired further research into how steroid hormones could be used as treatments for telomeropathies.²⁴

Androgens have been used to treat bone marrow deficiency.²⁵ Androgens were found to normalize telomerase activity in the subjects with loss-of-function mutated TERT alleles.²⁵ The study claimed that these findings could suggest possible treatment options for “patients with aplastic anemia carrying telomerase complex mutations and in dyskeratosis congenita, ”-- two illnesses with a telomerase-deficient etiology.²⁵ The results of the study established the mechanism for activation. TERT is expressed when aromatized sex hormones (estrogen metabolites) bind to the estrogen receptors, which then locates and binds to the DNA binding sequence (ERE) located in the TERT promoter region (Figure 9).²⁵ Knowing that estrogen metabolites are formed from aromatized androgens, this mechanism suggests that androgens are converted before binding to ERs in leukocytes.²⁵

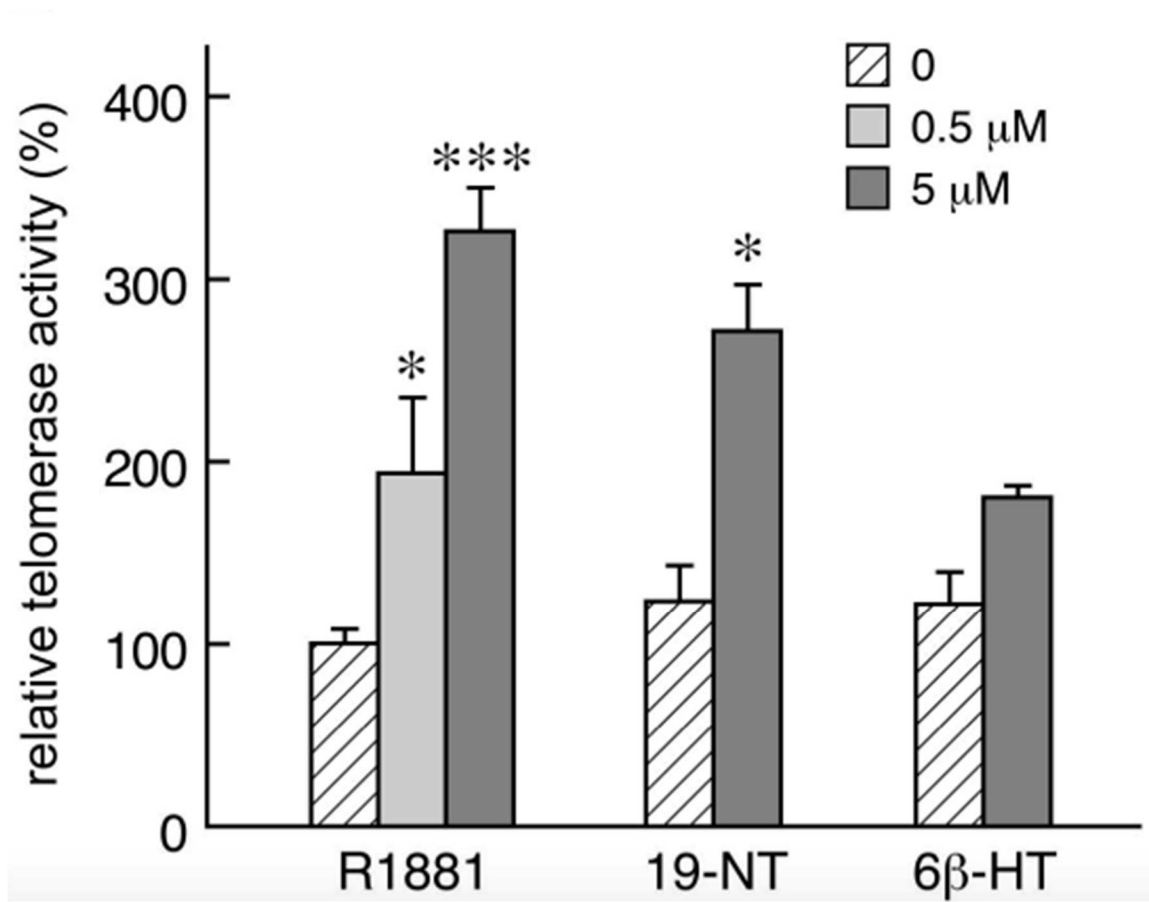


Figure 9—Sex Hormones Induce Telomerase: As the concentration of the 3 synthetic sex hormones increase, upregulation of telomerase activity increases. (Adapted from Calado, Rodrigo T., et al., 2009 (25))

As mentioned previously, short telomere syndromes result in other clinical manifestations in addition to lung fibrosis. Bone marrow deficiency and liver cirrhosis have been well characterized in patients with short telomeres as a result of loss-of-function mutations in telomerase genes (TERT and TERC in particular).²⁶ Male steroid hormones have been used for decades to increase the production of red blood cells by defective bone marrow and it is that observation that lead Townsley et al. (2016) to study the effects of danazol on the length of telomeres in patients with short telomere

syndrome. In this two-year prospective study, patients with telomere lengths in the 10th percentile or less were given oral doses of danazol daily and their telomere lengths (as well as blood counts, liver function tests, and pulmonary function tests) were evaluated every six months. A majority of patients experienced a significant lengthening of telomeres shortly after beginning danazol treatment (Figure 10).²⁶ Additionally, those patients receiving danazol had increased red blood cell count and no progression of their lung disease as compared to their counterparts in the study that were not receiving danazol, suggesting that the elongation of telomeres has a therapeutic effect on the clinical progression of the disease.²⁶

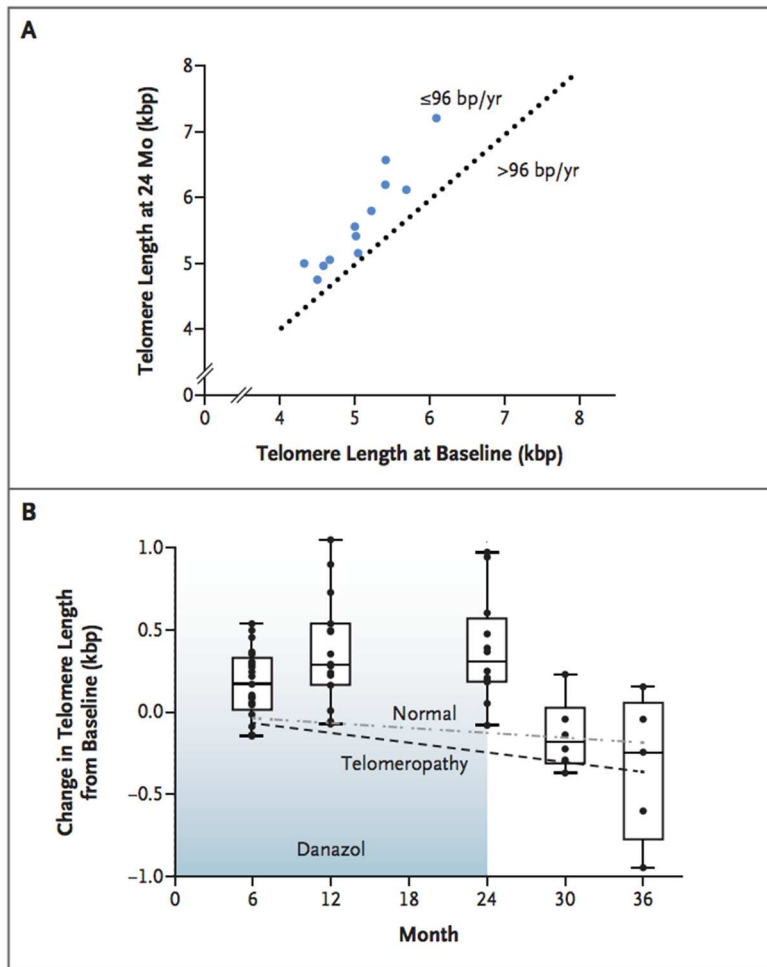


Figure 10—Telomere Length Increases After Danazol Administration: A) The length of telomeres (in kilobase pairs) of the 12 patients enrolled in the study receiving daily doses of 800mg of danazol at the end of 2 years. The dotted line represents the previously decided upon threshold for significant increase in length as compared to baseline. All 12 patients met that criterion. B) The change in length of telomeres in patients taking danazol as compared to their baseline at 6, 12, 24, 30, and 36 months. Fine dotted line represents the average decay of telomeres in healthy individuals (~60 bp/year) and bold dotted line represents the rate of decay in patients with short telomere syndrome (~120 bp/year). These results show danazol increased length of telomeres in patients with short telomere syndrome, but the effects were lost after the discontinuation of danazol at 24 months. (Adapted from Townsley, Danielle M., et al., 2016 (26))

The Use of Nintedanib

Nintedanib, a receptor tyrosine kinase (RTK) inhibitor, was studied *in vitro* as a potential treatment for IPF. Lung fibroblasts were extracted from patients with IPF and control, non-fibrotic subjects and nintedanib was applied. The goal was to observe whether or not there was a decrease in the deposition of extracellular matrix (ECM) and a proliferation of the cells that produce this ECM. It was found that nintedanib prevented fibroblast proliferation caused by growth factors (Figure 11) and collagen production was significantly reduced.²⁷ The drug acted to block the receptors of the growth factors PDGF, FGF, and VEGF (Figure 12), which have been found to play a role in IPF.²⁷

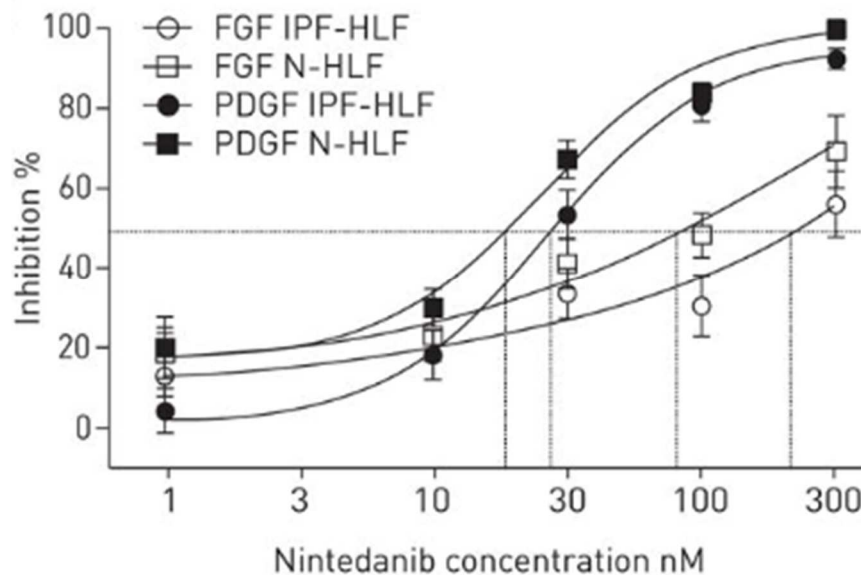


Figure 11—Nintedanib Concentration vs. Percent Growth Factor Inhibition: Nintedanib concentration positively correlates with inhibition of FGF and PDGF in cells of mice with IPF and mice without IPF. This shows that nintedanib is a generalized inhibitor regardless of growth factor concentration.

(Adapted from Hostettler, Katrin E., et al., 2014 (27))

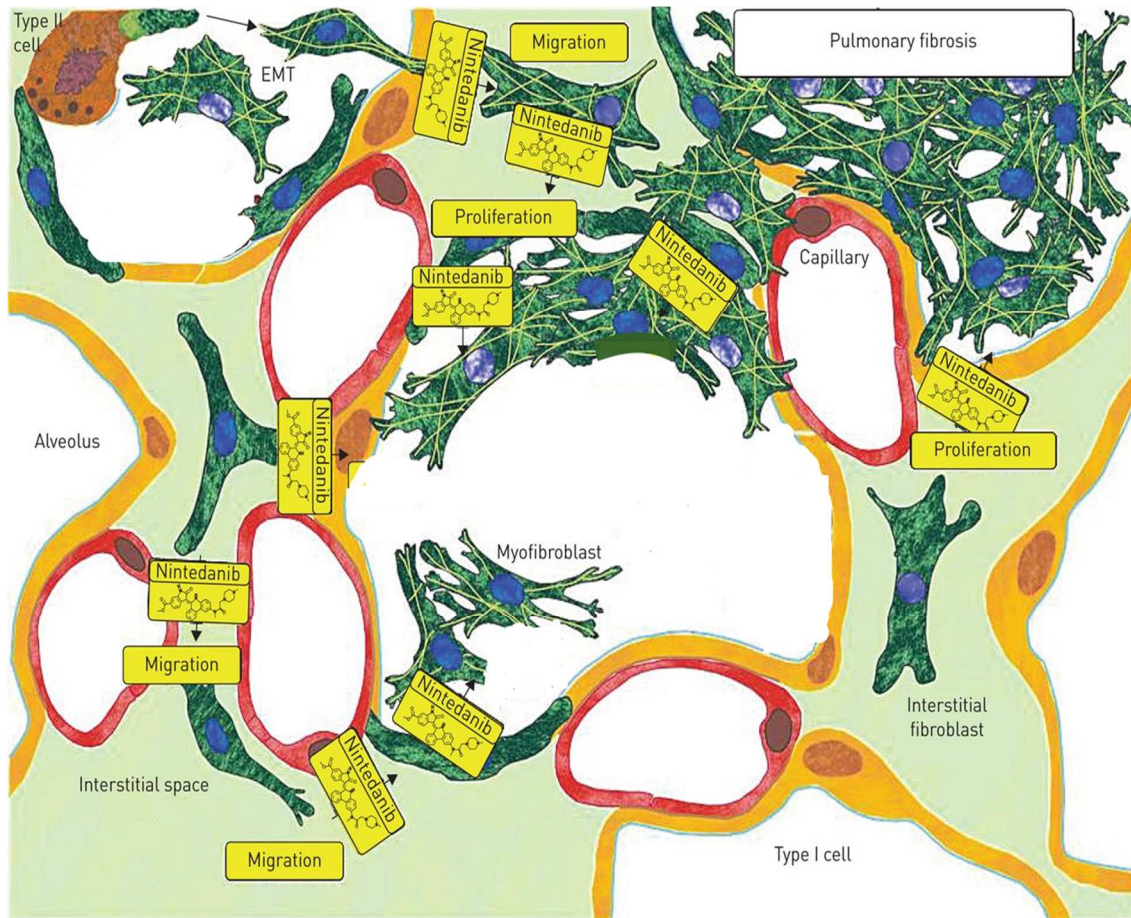


Figure 12—Nintedanib Mode of Action: The figure shows the molecules of nintedanib directly attacking the areas of fibrosis, preventing proliferation of myofibroblasts (green cells) and migration of growth mediators. The abnormal count of myofibroblasts and irregular migration of interstitial fibroblasts are an indication of the IPF phenotype. The transition of type II AEC to myofibroblast (or epithelial-mesenchymal transition, denoted as EMT) seen in the top left corner of the figure is also a sign of IPF. (Adapted from Wollin, Lutz, et al., 2015 (28))

The effectiveness of adding statins to the nintedanib treatment course was observed in a clinical trial completed in 2017.²⁹ Subjects were split into a total of four groups: 192 patients receiving both statins and nintedanib, 120 receiving only statins and a placebo, 446 patients receiving only nintedanib, and 303 in a placebo group. When

comparing the two placebo groups, researchers found there was no statistically significant difference in progression of IPF.²⁹ This implies that there was no harm done in administering the statins, though there also was no benefit. The study also determined that there was no statistically significant difference between the subjects taking nintedanib and statins and the subjects only taking nintedanib.²⁹ Though the study notes a numerical difference in both comparisons, without a statistical significance, no correlation can be made. The addition of statins to the nintedanib did produce some increase in adverse affects, such as an increase in diarrhea occurrence and an increase in major adverse cardiac events.²⁹ The researchers do not mention the statistics of these findings, however, it is claimed to have been “expected.”²⁹

Low Dose Inhaled Carbon Monoxide

Carbon monoxide (CO) is endogenously produced in the body with a variety of functions in normal biological processes such as “protection against oxidative injury and cell injury, inhibition of cell proliferation, suppression of matrix production and inflammation, and increased fibrinolysis.”³¹ These are all important factors in IPF and therefore, presented CO as a potential treatment option. A clinical trial of low-dose inhaled CO was executed in a randomized, placebo-controlled study with 29 subjects in each group. The placebo group received oxygen (21%) and the experimental group received 100 or 200 ppm CO gas via facemask. The result of the trial showed no statistically significant difference in either group, meaning the CO was not shown to be

effective as a treatment option.³¹ Though the researchers reported a number non-serious and serious adverse effects, it has been shown that inhaled CO is safe to administer.³¹

Stem-Cell Therapy

Amongst the stem cell research done, the study completed with somatic cell nuclear transplant (SCNT) of telomere haplo-insufficient cells from mice was successful in producing naïve pluripotent stem cells, as well as elongated telomeres. Somatic cell nuclear transplant takes the DNA from one somatic cell and places it in another. In this particular study, the somatic cells used are adipose stem cells (ADSCs). This provided an opportunity for a potential patient-specific treatment for IPF patients. The researchers took cells from mice with heterozygous TERC mutations and replaced the mutated DNA with wild-type TERC cells' DNA. In using adipose cells, this particular treatment was intended to address a theory that the pathology of IPF originates in an activated immune pathway—a theory that rivals the injury-based etiology described earlier. Researchers used these mesenchymal stem cells (MSCs) because, as immune-privileged cells (cells that do not produce immune responses), they possess anti-inflammatory effects—effects that dampen the response to infection. In agreement with the studies that indicate the mechanism is injury-based,^{9,20,22} the trial executed with IPF patients showed no clinically significant benefit of the treatment.^{32,33}

GRN510

In 2013, a study of the small molecule activator, GRN510, had a promising conclusion. The study found that the small molecule activated telomerase in lung and bone marrow tissue in mice with induced pulmonary fibrosis (Figure 13).³⁴ This resulted in a conclusion that GRN510 increased the telomere length and ameliorated changes lung structure and function.³⁴ While the mechanism of action of the molecule could not be concluded, it worked on lung epithelial cells, not lung fibroblasts.³⁴ The combination of positive results seen with GRN510 use and the conclusion that the molecule acts on lung epithelial cells³⁴ correlates with the proposed mechanism of type II AECs in IPF pathology.²⁰ The authors of the study suggested that the activator up-regulated TERT.³⁴ Though this analysis had positive results, the experiment was done with mice and, therefore, requires further studies to establish if it is effective as a treatment in IPF patients.³⁴

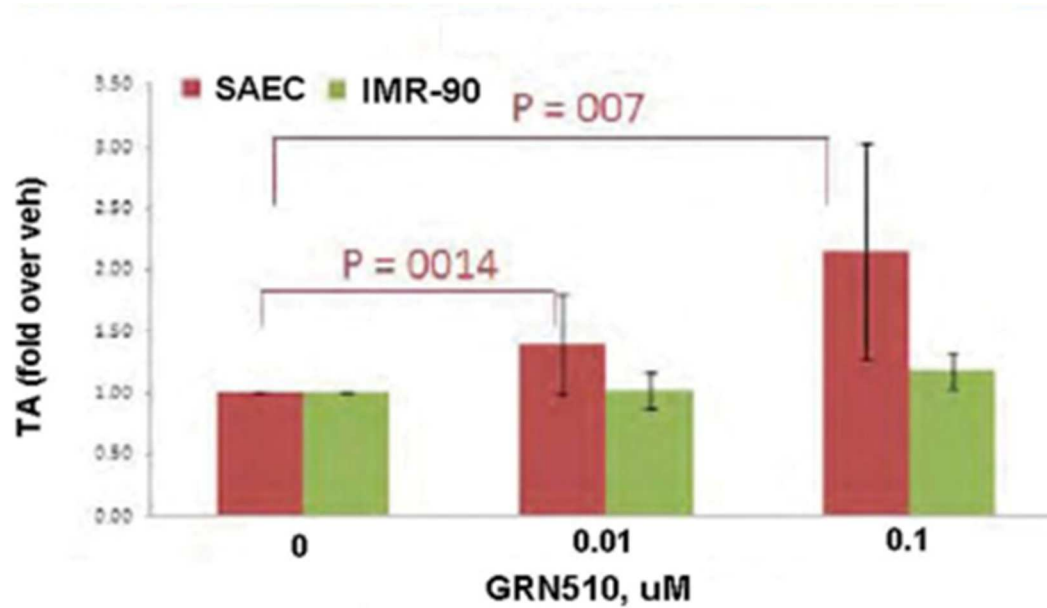


Figure 13—GRN510’s Effect On Telomerase Activity in Lung Epithelial Cells and Fibroblasts: The graph shows the upregulation of telomerase activity (TA) in small airway (bronchial) epithelial (red) with increasing concentration of GRN510. The graph also shows a minimal increase in TA in lung fibroblasts (green) at maximum concentration of GRN510, 1uM. This correlates with the mechanism of the role of epithelial cells in IPF. (Adapted from Le Saux, Claude Jourdan, et al., 2013 (34))

DISCUSSION

The literature available on the role of telomeropathies in idiopathic pulmonary fibrosis all seems to agree that, regardless of the cause of the etiology, telomere dysfunction commonly causes lung fibrosis. Short telomeres have been found in pulmonary leukocytes, type II AECs, and myofibroblasts in patients with IPF. The current mechanism supported is one in which telomere dysfunction leads to the activation of an epithelial cell injury pathway. This pathophysiology of IPF is supported both by literature specifically published on the matter and in studies that analyze the effectiveness of treatments. The treatment options that target this mechanism have been shown to be more effective. The studies available show that telomere dysfunction can result from a variety of known telomerase and shelterin complex mutations, but that there is also still unknown causes. TERT and TERC mutations account for half of telomere-related FPF cases and RTEL1 mutations may account for up to half of the rest of the telomere-related FPF cases.

The studies presented did, however, have limitations. The study performed by Diaz de Leon, Alberto et al (2010) was limited due to the familial nature of the patients. The rate of incidence was likely to be higher than with subjects of a random population study.¹⁰ In addition, due to the observational nature of the study, not all the subjects received the same work up to establish an IPF diagnosis.¹⁰ The rate of incidence within any family could have been lower or higher than reported depending on the variable diagnostic guidelines. There was also a prevalence of bias as a result of collecting information on patients with lung disease. The subjects diagnosed with IPF were more

likely to report environmental conditions that support their diagnosis.¹⁰ Despite these limitations, the study confirmed the relationship between age and the manifestation of IPF.

The study done by Armanios, M Y, et al. (2007) was limited by the size of the sample of the subjects and conclusions made should be verified by a larger scale study. Understanding this limitation, telomere lengths were found to be below the 10th percentile and this difference is significant enough to reasonably accept the relationship between mutation and telomere length proposed. The research done with respect to the prevalence of short telomeres regardless of mutations by Cronkhite, Jennifer T. et al. (2008) was completed before whole-exome sequencing discovered the telomere –related mutations unrelated to TERT or TERC.¹¹ It was unknown if there were other mutations present causing the manifestation of IPF. It was also unknown that the degree of telomere shortening in circulating leukocytes was representative of cells in the lung environment.¹¹ Researchers cannot be certain that the telomere lengths of the peripheral cells measured in the study would be the same as cells in the lungs of the subjects. The finding that more patients with mutations or with telomeres below the tenth percentile died over the course of the study than subjects without mutations or with telomere lengths above the tenth percentile was not found to be statistically relevant.¹¹ The TRFL assay is not a direct measurement of telomere lengths, as its undigested lengths of DNA contain both telomeres and subtelomeric segments. While the researchers attempted to minimize the subtelomeric segments with multiple restriction enzymes, this could be a source of error in measure of telomere length.¹¹ Despite this possible source of error,¹¹ results of telomere

lengths recorded at below the 10th percentile (significantly shorter than the average) are likely able to withstand scrutiny.

The study that compiled the extrapulmonary effects of short telomeres²³ had many limitations, most notably low power and the issue of validity due to the narrow scope of the subjects (i.e. all chosen from one center). Additionally, the inclusion criteria featured patients who were being evaluated for lung transplant, signifying substantially advanced disease, which is likely to be correlated with multiorgan dysfunction, regardless of the relationship the researchers found with telomere length. However, the results of this small pilot study were promising and offered a new screening tool for patients with IPF and allowed for further genetic testing in family members at risk of developing disease.

While IPF is a relatively rare disease, with telomere-deficient IPF affecting an even smaller subgroup, the rate of incidence increases annually. There have been, and continue to be, a variety of studies done in efforts to provide a substantial treatment plan. Some of the treatment plans discussed specifically target the telomere dysfunction in telomere-related IPF, while others address the direct causes of symptoms of IPF. The longest-running treatment option to date is lung transplantation.⁷ It is equivalent to a “band-aid solution” and only approximately 44% of patients have shown a survival time of 5 years post-transplant.⁷ This means a transplant cannot be considered a cure for the condition. N-Acetylcysteine, administered either alone or in combination with prednisone and azathioprine, was at one point the standard choice of treatment, but has not been clinically significant as an effective treatment.⁶ Though the treatment showed a decrease in the decline of FVC, the results were not considered statistically significant.⁶ This

treatment plan should not be recommended to patients by their physicians. Pirfenidone, though shown to be clinically significant, is not a cure for IPF and does not resolve the issue of telomere dysfunction.

While low-dose carbon monoxide was found to be effective in experimentally induced pulmonary fibrosis in a study done with mice, it was found to have no effect as a treatment in a clinical trial. The researchers reported no specific limitations of their study, though they reported that the CO was safe when they also reported a number of both serious and non-serious adverse effects. Low-dose CO should not be considered as a viable treatment option.

The clinical trial that tested the effectiveness of danazol showed great promise. Of the 12 patients that stayed with the trial for the entire 24 months, 11 showed an average of 30% telomere attrition. In addition to this observed attrition, the telomeres of these patients elongated by 386 base pairs, on average. This elongation seemed to occur in the patients with TERT mutations more so than the other patients, though it could not be concluded that danazol works by up-regulating TERT. The study also claims hematologic improvements accompanied treatment. The study evaluated its own limitations, among which were being unable to identify mutations in all patients, not measuring telomere attrition for a longer period, using the highest dosage of danazol approved for humans, and not having a randomized study with a control group. In being unable to identify mutations in all patients, the genetic heterogeneity could have resulted in a biased measurement of the estimated telomere attrition rate. This applies for the shorter observation time, as well. In using the highest dosage of danazol, side effects

were possibly worsened and the study suggests that a follow up study be done to determine appropriate dosing. A lack of randomization and a control group in a clinical trial increases bias. In addition to this, it should be observed that the study was done with a very small group of subjects, and increasingly became smaller as patients dropped out through the 36 months of the trial. This makes it difficult to conclude the efficacy of the trial. With an ongoing clinical trial attempting to establish appropriate dosing,³⁵ danazol is a worthy option for treating telomere-related IPF.

The 2013 study done with the small molecule activator, GRN510, had a promising conclusion. Though this analysis had positive results, the experiment was done with mice and, therefore, requires further studies to establish if it is effective as a treatment in IPF patients. In addition, the study was performed on mice with induced TERT mutations, which brings into questions the validity of using GRN510 to treat IPF related to other mutations. While GRN510 was shown to increase telomerase activity, this may not be the result in cells with telomere dysfunction due to other mutations. Without a clinical trial to draw conclusions from, GRN510 is still far from being a reasonable therapy choice for IPF.

The experiment done with SCNT of telomere haplo-insufficient cells from mice was successful in producing naïve pluripotent stem cells, as well as elongated telomeres. This was not the case in a longitudinal clinical study. The study claims no relationship can be defined between the stem cell therapy and disease progression, as its effectiveness was not being observed. However, the results of the study indicate no change in the disease progression than that in patients receiving only pirfenidone. Stem cell therapy,

though a patient-specific therapy, cannot currently be considered an option for IPF treatment.

The small-molecule tyrosine kinase inhibitor, nintedanib, showed itself to have a potent inhibitory effect on the proliferation and migration of human lung fibroblast in the clinical trials completed in 2015. The two-phase study was double-blind and placebo-controlled and in both phases, nintedanib showed a slowed annual rate of decline in FVC versus the placebo group. The study showed many adverse effects, which brings into questions the safety of long-term use. In addition, a case study described below indicates that physicians should monitor discontinuation of its use.

In two cases of IPF, 3 weeks after cessation of nintedanib, the patients' conditions deteriorated rapidly. One patient was brought into the hospital 3 weeks after discontinuing nintedanib with a worsened dyspnea on exertion, tachypnea, respiratory failure, and bilateral crackles could be heard. No other change of medicine or environment occurred and no signs of infection could be detected. Three months later the patient died as a result of a spontaneous pneumothorax.²⁹ The second case consisted of a patient who had been administered nintedanib 3 weeks prior to admission and had to discontinue that line of treatment while under hospital care due to a nonstop loss of appetite. The patient's condition deteriorated soon after, requiring oxygen to be administered even at rest. There was no sign of infection and antibiotics showed no improvement.²⁹ In both cases, other possibilities for exacerbation had been ruled out. Infection drug toxicity, inhaled antigens, and congestive heart failure were denoted unlikely as causes.²⁹ The physicians who admitted these patients strongly believed that

the rapid deterioration of their condition was due to the cessation of nintedanib and urge physicians to monitor any plans to discontinue the medication as a course of treatment closely. It has previously been discovered that the discontinuation of tyrosine kinase inhibitors in other conditions have correlated with a disease flare, though the underlying mechanism is not understood.²⁹

Though its discontinuation should be monitored more closely and a clinical trial should be administered to establish appropriate dosing, nintedanib is a strong option for effectively decreasing the fibrosis caused by any etiology of IPF.

In a follow-up study, statins were tested as treatment, both alone and in combination with nintedanib.³⁰ The study was not randomized and, therefore, no causality can be established. The study concludes that future prospective studies of the effects of statins in the treatment of IPF are needed.³⁰ There is no evidence in the results of the present study that indicate that this would be beneficial. The results show no statistically significant benefit of the use of statins and report an increase in adverse events.

CONCLUSION

In conclusion, it is shown that telomere dysfunction is highly correlated with IPF diagnoses. The mechanism with the most supporting evidence is that shortened telomeres activate an epithelial injury pathway that results in proliferation of cells that produce and deposit collagen in the lungs. Danazol is the most supported telomere-directed treatment, though a clinical trial of lower dosing should be completed. This drug could also be a potential preventative measure in those with telomere-related FPF in their pedigree. Nintedanib is the supported choice for all cases of IPF, as it targets fibrosis in a direct fashion. After appropriate dosing of each drug has been established (and proven to be nontoxic), a combination of the two drugs should be tested for patients with telomere-mediated IPF. Targeting telomere attrition and lengthening could prevent further manifestations of telomeropathy, while an RTK inhibitor could reverse the prevalent condition.

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VITA

