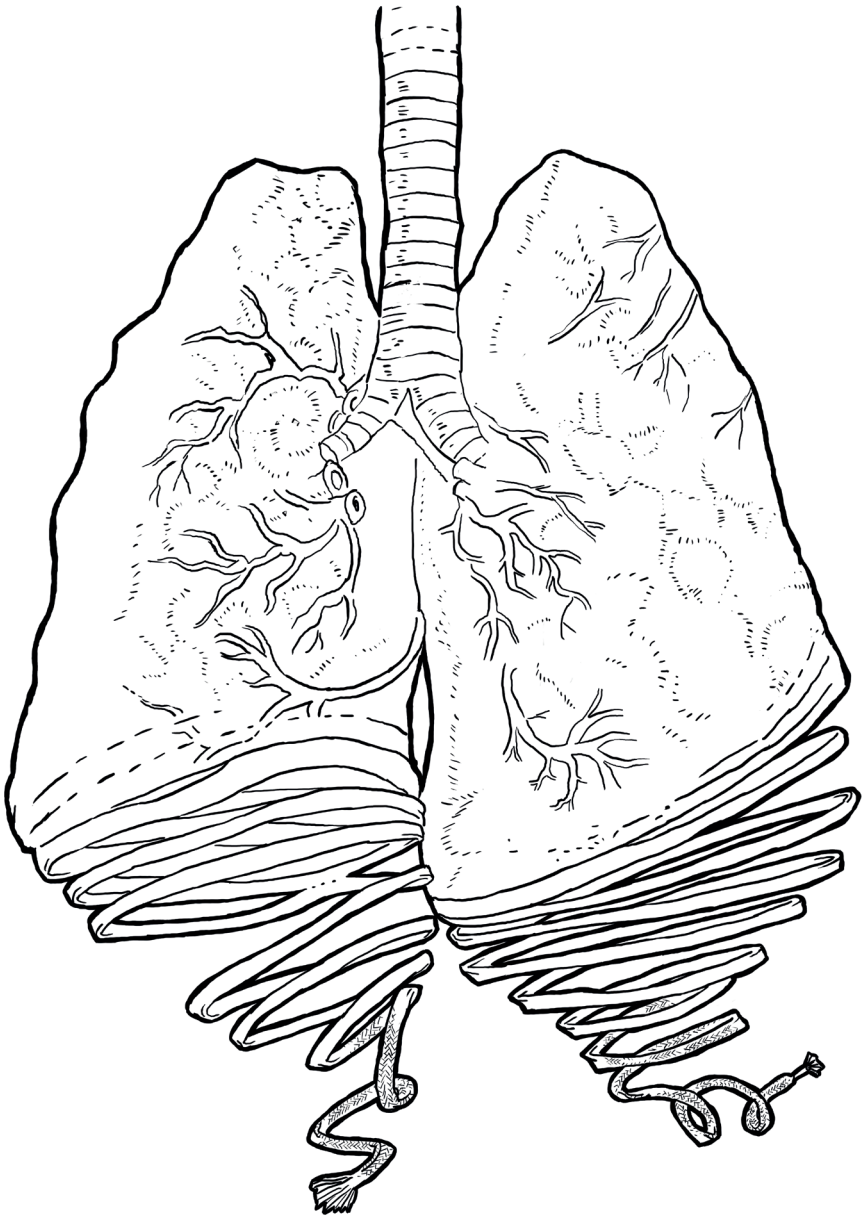
TELOMERE LENGTH AND GENETIC PREDISPOSITION IN IDIOPATHIC PULMONARY FIBROSIS

Reinier Snetselaar



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TELOMERE LENGTH AND GENETIC PREDISPOSITION IN IDIOPATHIC PULMONARY FIBROSIS

Telomeerlengte en genetische predispositie in idiopathische pulmonale fibrose
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 12 juni 2018 des ochtends te 10.30 uur

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ARIS GEERTEN REINIER SNETSELAAR

geboren op 15 juni 1984
te Zeist

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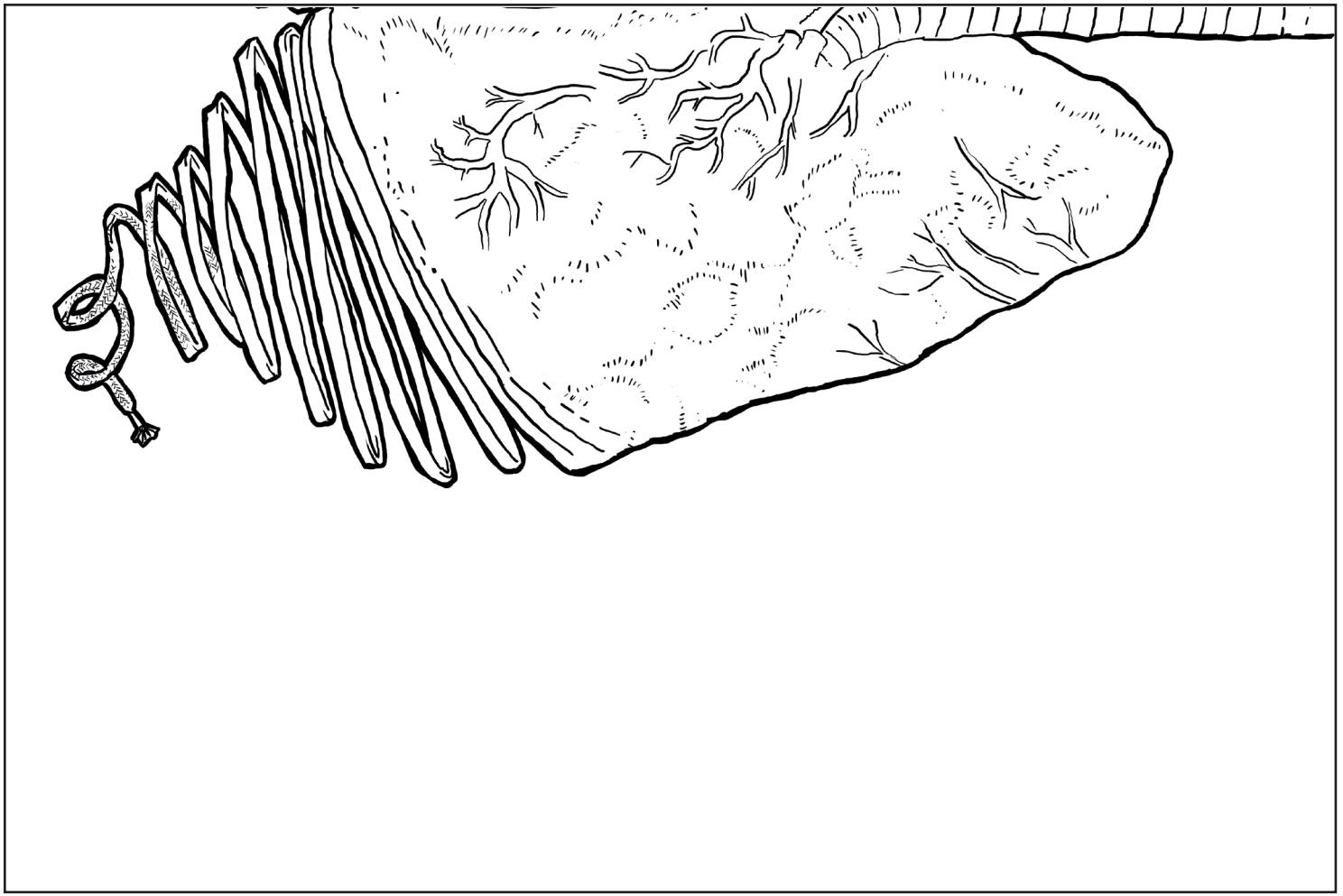
Voor Noam, Rosemijn en Lieve

Contents



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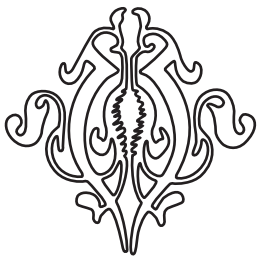
General Introduction



General introduction

Interstitial lung diseases

Interstitial lung diseases (ILD), also referred to as diffuse parenchymal lung diseases, are a class of rare diseases that affect the alveolar interstitium. ILDs are grouped together based on similar clinical, radiological, physiological or pathological features[1]. Within ILD, four subgroups are distinguished: diseases with a known cause, idiopathic interstitial pneumonias (IIP), granulomatous diseases, and a miscellaneous group (figure 1)[2,3]. The most common disease within IIP is idiopathic pulmonary fibrosis (IPF), which has a global estimated prevalence of 2 - 29 per 100.000[4].

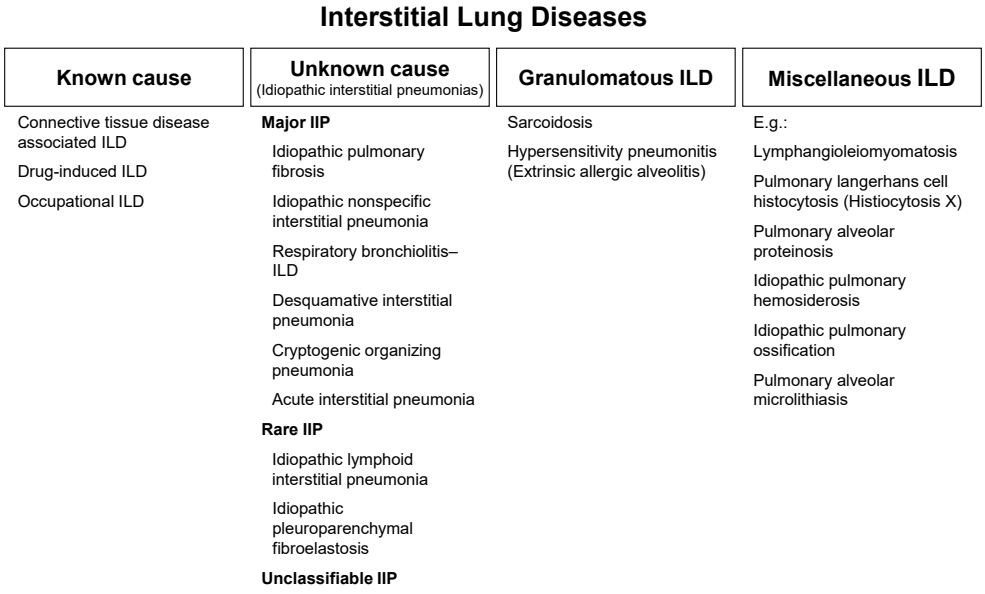


Figure 1. Interstitial lung disease (ILD) classification. Four main subgroups in ILD; diagnoses within each group are given below. Adapted from Raghu et al., 2002 and Travis et al., 2013.

Idiopathic pulmonary fibrosis

IPF is an interstitial lung disease characterized by chronic, progressive formation of scar tissue in the alveolar compartment of the lungs[4,5]. Treatment options for IPF are limited and apart from lung transplantation, none of these are curative[6]. Recently, two anti-fibrotic agents, pirfenidone and nintedanib, have become available. However, although these drugs seem to decrease the decline in lung function of IPF patients, their effect is modest[7]. The development of viable treatment options has been hampered in part by a lack of knowledge on the etiology of IPF.

Common symptoms of IPF include clubbing of the fingers, chronic shortness of breath, often accompanied by cough, provoked by physical effort, and inspiratory crackles at the base of

both lungs[8,9]. IPF is an age related disease with the vast majority of patients aged 60 to 80 and the disease occurs more often in men than in women[8,10,11]. Patients with IPF have a very poor prognosis with median survival ranging from 2 to 5 years after diagnosis[12,13]. This disease occurs mainly sporadic, but in a minority of cases, multiple family members are affected, and these appear to develop IPF at a younger age (55-61)[4,14,15].

Diagnosing IPF

Multi-disciplinary discussion among pulmonologists, radiologists and pathologists is important for a confidant diagnosis of IPF[4,16,17]. Essential for diagnosis is the observation of a specific pattern, called usual interstitial pneumonia (UIP). This pattern should be observed on high resolution computed tomography (HRCT) imagery of the chest, or in histological material when available. Important HRCT features of a UIP pattern include reticular abnormalities, honeycombing, and a distinct localization or accentuation of the abnormalities in the basal and subpleural regions of the lungs[18–20.] Histological features of the UIP pattern include a patchy distribution of affected, fibrotic areas at low magnification[2,9]. Another important feature is the presence of type 2 pneumocyte hyperplasia, and foci of proliferating fibroblasts and myofibroblasts called fibroblast foci[4,21]. Only after exclusion of any known causes of ILD and observation of the UIP pattern on radiological imagery and, when available, in histological material, a patient can be confidently diagnosed with IPF.

IPF pathogenesis

Several key elements are involved in the formation of pulmonary fibrosis in IPF, including destruction of healthy epithelium, aberrant re-epithelialization, and finally progressive formation of scar tissue[22]. In heathy lungs, alveoli are for >95% lined with thin alveolar type 1 epithelial cells that permit effective gas diffusion[23,24]. A second cell-type present at the alveolar epithelium is the alveolar type 2 (AT2) cell which play a major role in surfactant production in healthy lungs[25]. AT2 cells also serve as progenitor cells that, under normal circumstances, have the potential to proliferate and differentiate into AT1 cells in order to replace damaged AT1 cells[26–28]. A distinctive histological feature of IPF is AT2 cell hyperplasia. These AT2 cells line the walls of the alveoli, reepithelializing areas that are denuded as a result of epithelial damage, but subsequently fail to differentiate into the gas-diffusing AT1 cells, leading to AT2 cell hyperplasia[5,29]. The characteristic fibroblast foci are also generally found overlain by hyperplastic AT2 cells[29]. Several hypotheses have been explored to explain these elements, and it is now generally accepted that the interaction between environmental factors and genetic predisposition underlie the onset and progression of IPF in a so-called multi hit model[22].

Environmental factors

Several injurious agents have been reported to constitute the initial source of damage to the epithelium. First of all, both metal and wood dust have been observed to increase IPF risk[30,31]. Secondly, there is evidence that cigarette smoke is associated with IPF[32]. Thirdly, viruses have been implicated in the pathogenesis of IPF, of which Epstein-Barr virus (EBV) is most often identified[33]. This virus can infect the basal regions of the lung as well as replicate uniquely in alveolar epithelial cells located there[34,35]. Furthermore, interaction between EBV and the epithelial cells has been shown to result in the production of fibrosis[22]. Finally, gastro-esophageal reflux has been shown to have a high prevalence in IPF[36]. However, it must be emphasized

that these environmental factors do not lead to IPF in all exposed individuals, which suggests the existence of co-determinant susceptibility factors, including genetic predisposition to develop this disease[37].

Genetic predisposition

Up to 19% of IPF patients have a family history for this disease, which further bolsters the assumption that genetic factors predispose to IPF[38]. Transmission is autosomal dominant, although with reduced penetrance[39]. Familial clustering has been reported in monozygotic twins raised in different environments[40]. First, mutations in the surfactant protein C (*SFTPC*) were found to cause IPF in a subset of familial interstitial pneumonia (FIP) patients. The protein surfactant protein C (SP-C) is exclusively produced in the AT2 cells and is a component of the pulmonary surfactant that protects exterior surfaces of the lungs[41–43]. Further research also revealed mutations in the surfactant protein A1 and A2 gene (*SFTPA1*, *SFTPA2*) to be carried by FIP patients[44–46].

Surfactant genes

IPF causing *SFTPC* mutations are found in two locations. First, is the I73T mutation, which causes abnormal processing of the SP-C protein, leading to accumulation in the endosomal system and subsequent disruption of cell signaling and metabolic processes[47]. The second location is the C-terminal BRICHOS domain. Mutations in this domain also lead to misfolding of the protein after transcription, which prevents correct processing through the endoplasmatic reticulum (ER), which would lead to ER stress and the subsequent unfolded protein response (UPR)[48–51]. This response is invoked to normalize protein levels in the cell. However, prolonged activation due to overload by mutated proteins leads to apoptosis[50,52]. Elevated UPR markers are observed in AT2 cells of patients with surfactant mutations. However, in most cases UPR cannot be explained by mutations in the surfactant genes and is largely attributed to Herpesvirus infection[48,53].

MUC5B

In both familial and sporadic IPF patients, an association with disease susceptibility with odds ratios >8, has been found for a common (risk allele frequency: ~0.10) genetic variant in the mucin 5B (*MUC5B*) gene[54,55]. Mucins have a role in providing a barrier in the lungs and in innate immunity[56]. The polymorphism leads to an increase in *MUC5B* expression in the lung, however its role in IPF pathogenesis remains unclear.

Telomerase genes

Mutations underlying IPF have also been identified in the genes telomerase reverse transcriptase (*TERT*) and Telomerase RNA component (*TERC*)[57,58]. These genes encode the catalytic protein component (*TERT*) and the RNA template (*TERC*) of the ribonucleoprotein telomerase[59]. Telomerase is essential to maintain replicative potential in select cell types, as it is needed to maintain telomere length (TL) (figure 2). Telomeres are non-coding tandem repeats of a TTAGGG sequence, that cap chromosomes[60,61]. Telomeres serve as a buffer against the shortening of chromosomes, thereby preventing the loss of vital genetic information[62]. Telomere shortening occurs due to the inability of DNA polymerase to replicate DNA to the ends of linear chromosomes, leading to the loss of 30 to 50 base pairs per cell division[63–66]. It follows that a finite number of cell divisions can occur before telomeres become too short

to protect the chromosomes. Upon reaching this critically short length, the cell division is halted either by proliferative senescence or apoptosis, to prevent chromosome fusion leading to genomic instability[64,67,68]. Secondly, telomeres are spatially organized by several distinct proteins forming the shelterin complex[69,70]. This complex prevents end-to-end fusion of the chromosomes and protects against recognition by the double-strand breaks repair mechanism of the cell[71]. Transcription of telomerase is highly regulated and occurs only in specific human cell types[72], in particular stem cells and other progenitor cells, of which continuous cell cycles are required[73,74]. Due to the limit that telomeres shortening imposes on cell proliferation, it has been suggested to be a mitotic clock and thereby a measure for aging[62,75].

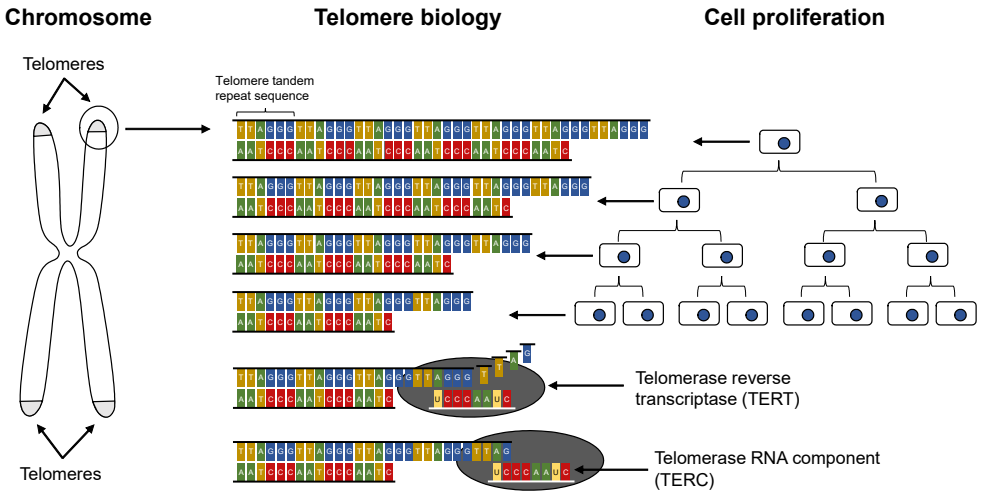


Figure 2. Telomere biology. Telomeres cap linear chromosomes to prevent damage to the genetic information as chromosomes shorten upon each cell cycle. Telomerase is a protein that can elongate shortened telomeres using the telomerase RNA component as a template.

Discovery of a link between pulmonary fibrosis and mutations in telomerase genes was prompted by observing IPF-like patterns of fibrosis in a patient with dyskeratosis congenita (DC)[57,76]. This disease is a rare hereditary disorder which in most cases, leads to aplastic anemia and subsequent bone
marrow failure as the most common cause of death[77]. A minority of DC patients inherits the disease in an autosomal dominant pattern, which is underlain by heterozygous mutations in *TERT* and *TERC*[57,78]. This form of DC usually does not manifest itself before adulthood, and is sometimes characterized by anticipation, where in successive generations, at an earlier age, a more severe form of the disease is observed[76,79]. This suggests that not the mutations in *TERT* and *TERC*, but the resulting short TL, also in germ cells, determines disease severity[57]. The telomerase gene mutations thereafter found in families with IPF, were shown to influence TL and the patients showed very short TL in their blood cells[57]. More recent studies have also discovered mutations in familial IPF patients, located in other telomere biology related genes beside *TERT* and *TERC*, such as *DKC1* in the telomerase complex, *TINF2* in the shelterin complex, and *RTEL1*, which interacts with the shelterin complex. This converging of IPF causing mutations in telomere maintenance related genes suggests telomere dysfunc-

tion in general underlies IPF, instead of the individual function of these gene transcripts. Furthermore, it was shown that non carrying descendants of *TERT* mutation carriers, did have shorter TL compared to controls, showing anticipation of TL in IPF[80].
Evidence that telomere maintenance could also play a role in sporadic IPF patients came when studies showed that TL in these patients was significantly shorter than in healthy control subjects[81,82]. Short TL was therefore deemed a risk factor for IPF[81]. Further evidence comes from the discovery of a common genetic variant in the *TERT* gene that is associated with IPF susceptibility in sporadic patients[83,84]. This single nucleotide polymorphism (SNP), rs2736100, is also associated with TL and the allele associated with shorter TL is also the one associated with IPF susceptibility[85,86]. All this leads to the hypothesis that in most cases IPF is an age related, degenerative disease that occurs when lung tissue has lost its regenerative capacity as a result of telomere dysfunction[87,88].

From failing regeneration to fibrosis

After injury and the inability to regenerate the alveolar epithelium, the lung undergoes a fibrotic remodeling[5,22]. A clear connection between the genetic variation described above and fibrosis has not been shown yet. However there are indications that these connections could be present. Telomere pathology has been linked to epithelial cell senescence[89] and increased senescence of epithelial cells lining fibroblast foci has been observed in IPF[90]. Furthermore, induced telomere dysfunction leads to senescence in AT2 cells[91]. Senescent cells have a distinct secretory phenotype that could induce lung remodeling and subsequent fibrosis[89,92]. However, short TL in itself does not lead to pulmonary fibrosis in mouse models[93]. Rather, addition of a secondary insult leads to manifest lung disease in this model. In the context of the multi hit model, reaching the limit on the regenerative potential would severely impair the regenerative response of the lungs to damage by the described injurious agents. This in turn could lead to an aberrant wound healing response and progressive fibrosis as observed in IPF (figure 3).

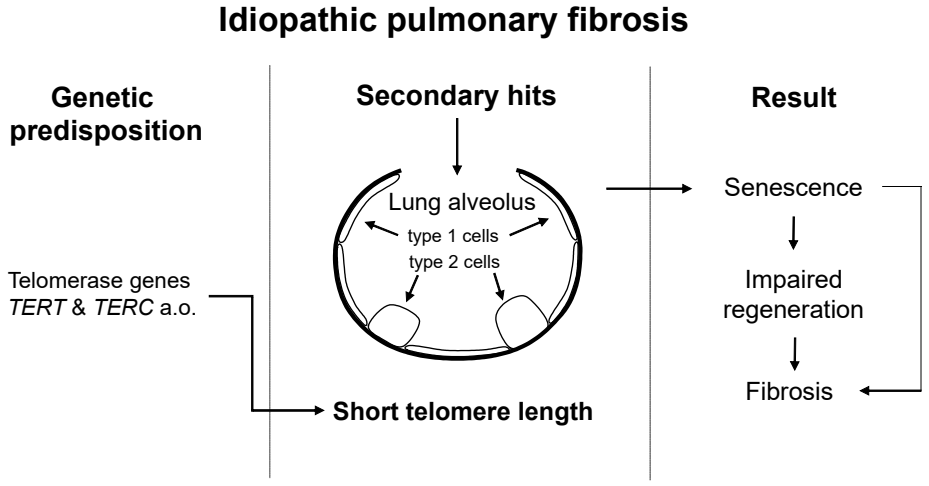


Figure 3. Pathogenesis of IPF. IPF occurs in individuals with a genetic predisposition to disease in the presence of secondary hits in lung alveoli that results in fibrotic remodeling.

Aim of the thesis

The general aim of this thesis is to elucidate the pathophysiological role of telomere shortening in IPF as well as investigate genetic predisposition to this disease. The hypothesis that there is such a role for telomere shortening, derives from observations that telomeres in leukocytes are short in patients with IPF, and that mutations in telomere maintenance genes confer a strong risk for developing IPF. These observations suggest that telomere biology is important in the pathogenesis of IPF. Next to genetic variation in *TERT*, a variant in the *MUC5B* gene has been shown to predispose to IPF.

Therefore we will investigate, the role of (a) telomere length in disease development and prognosis, and (b) genetic risk factors in the *MUC5B* and telomerase genes. Studying predisposing risk alleles and telomere length, not only in IPF, but in a spectrum of interstitial lung diseases allows for comparison between diseases and may determine if the role of telomere length and genetic risk factors is unique for IPF. Furthermore, we aim to determine if there is a relation between telomere shortening and fibrosis in the alveolar compartment. A study focused on telomere length in IPF lung tissue will point to the primary affected cell of telomere shortening. Also, we aim to investigate if carriership of multiple genetic risk factors in *TERT*, *TERC* and *MUC5B* confers additional risk compared with carriership of a single risk factor. This could further our understanding of the multifaceted nature of IPFs pathobiology. Finally, we aim to analyze a genetic risk factor in the *TERT* gene for its role in both cancer and degenerative disease, and discuss the viability of therapies aimed at telomeres in IPF.

Outline of the thesis

There are several indications that dysfunctional telomere maintenance is a significant factor in Idiopathic pulmonary fibrosis pathogenesis. Here we aim to further our knowledge on the role telomere biology in IPF by studying telomere length in in blood and lung cells, as well as genetic risk factors in IPF patients.

In **chapter 2**, TL is measured in patients with a broad selection of ILD to determine differences in TL between healthy controls and ILD patients and between the different ILD themselves. The difference in TL between controls and different groups of patients allows differentiation between TL as a causative factor and a secondary effect.

In **chapter 3**, TL is studied in lung cells of IPF patients. TL is compared between AT2 cells in non-fibrotic and fibrotic areas, but also between AT2 cells and surrounding lung cells. Furthermore, the effect of lung TL on patient's survival is studied.

In **chapter 4**, the association between the *MUC5B* genetic risk variant and IPF, FIP and the ILD non-specific interstitial pneumonia is tested in a Dutch cohort. Furthermore, the effect of this variant on survival is tested for these cohorts.

In **chapter 5**, novel candidate genetic variants in *TERT* and *TERC* are studied alongside known variants, for an association with IPF susceptibility. Furthermore, it is determined if carrying multiple variants, including the variant in *MUC5B* influenced the odds of IPF or survival rate.

In **chapter 6**, a meta-analysis is conducted on the association of the IPF associated *TERT* SNP rs2736100 to other diseases including cancer. A contrary role of telomere biology is described for degenerative diseases like IPF and cancer. It is evaluated whether this is reflected in the association to the genetic variants.

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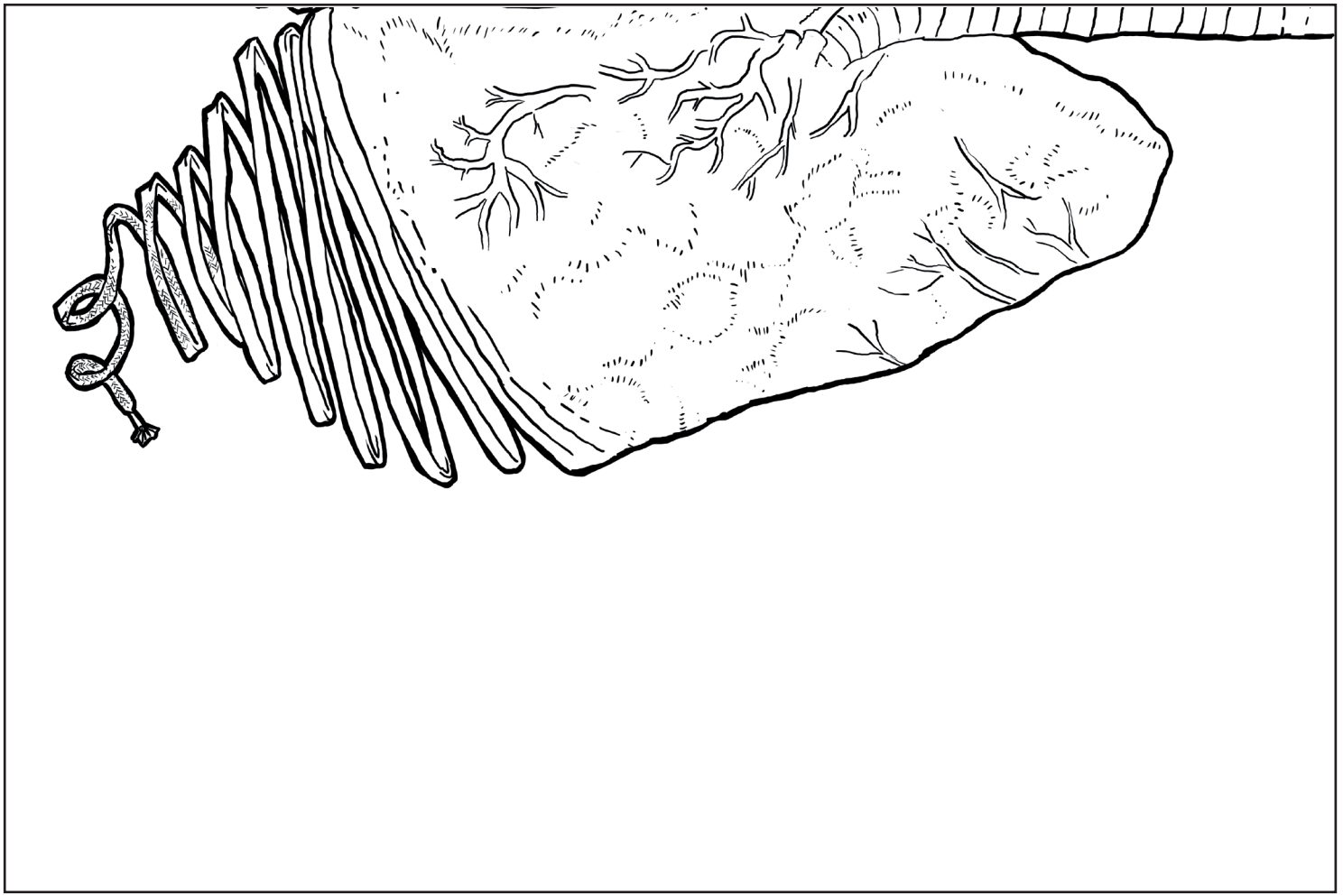

2

Telomere Length in Interstitial Lung Diseases

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Abstract

Interstitial lung disease (ILD) is a heterogeneous group of rare diseases which primarily affect the pulmonary interstitium. Studies have implicated a role for telomere length maintenance in ILD, particularly in idiopathic interstitial pneumonia (IIP). Here we measure telomere length in a wide spectrum of sporadic and familial cohorts of ILD and compare telomere length between patient cohorts and controls.

A multiplex quantitative PCR method was used to measure telomere length in 173 healthy subjects and 359 patients with various ILD, including familial interstitial pneumonia (FIP). The FIP cohort was divided in patients carrying telomerase reverse transcriptase (*TERT*) mutations, surfactant protein- A2 or –C (SFTP) mutations and patients without a proven mutation (FIP-no mutation).

Telomere length in all ILDs was significantly shorter compared to controls (p range = 0.038 - 2.28x10-27). Furthermore, telomere length in idiopathic pulmonary fibrosis (IPF) patients was significantly shorter than in other IIP (p=0.002), and than in sarcoidosis patients (p=1.35x10-7). Within the FIP cohort, FIP-TERT patients had the shortest telomeres (p=2.28x10-27), and FIP-no mutation had telomere length comparable to IPF patients (p=0.049). Remarkably, telomere length of FIP-SFTP patients was significantly longer than in IPF patients, but similar to that observed in the other sporadic IIP.

The results show telomere shortening across all ILD diagnoses. The difference in telomere length between FIP-TERT and FIP-SFTP indicates the distinction between acquired and innate telomere shortening. Short telomere length in IPF an FIP-no mutation is indicative of an innate telomere-biology defect, while a stress induced, acquired telomere shortening might be the underlying process for the other ILD diagnoses.

Introduction

Interstitial lung diseases (ILD) are a group of diseases which primarily affects the pulmonary interstitium. Although ILD is a heterogeneous group of diagnoses, they are classified together based on similar clinical, radiological, physiological or pathological features[1]. Four groups are distinguished within ILD: diseases with a known cause, idiopathic interstitial pneumonias (IIP), granulomatous diseases, and a miscellaneous group[2]. The etiology of a number of ILD is unknown, which presents limitations to classification and hence to treatment[3]. Therefore, it is important to investigate which features are common and which are unique in ILD.

Multiple ILDs have been associated with short telomere length[4–6]. Telomeres protect genetic information by acting as a buffer against the chromosomal shortening that is inherent to cell growth. Critical shortening of the telomeres leads to cell cycle arrest. Maintaining telomere length is therefore necessary for ongoing cell proliferation[7]. Loss in telomere length can be restored by the ribonucleoprotein telomerase. The relevance of telomere biology in ILD was first discovered in IIP. Patients with familial disease were found to carry mutations in telomere maintenance genes Telomerase reverse transcriptase (*TERT*) and Telomerase RNA component (*TERC*)[8,9]. These patients also had distinctly short telomeres in their blood cells. Next, a cohort of IIP patients not carrying these mutations, both familial and sporadic, were also shown to have shorter telomeres compared to controls. A significant portion of these non-mutation carrying patients had telomere length below the 10th and even below the 1st percentile of controls[4,6]. Telomere-biology related genetic factors are suggested to underlie telomere shortening in these patients. This suggestion is underlined by recent discoveries of familial disease causing mutations in other telomere biology related genes beside *TERT* and *TERC*, such as *DKC1* in the telomerase complex, *TINF2* in the shelterin complex, and *RTEL1*, which interacts with the shelterin complex[10–12]. Associations with short telomere length have also been described in other diseases, such as asthma, COPD[13,14], cardiovascular disease[15] and cancer[16]. Therefore, it is important to distinguish between, genetically predisposed, innately telomere-related diseases and diseases where short telomeres are acquired due to increased oxidative stress, inflammation or accelerated cell turnover[17]. It has therefore been suggested that the degree of difference in telomere length between healthy controls and patients determines if the short telomeres reflect acquired stress states or innate telomere-driven degenerative changes[18].

We hypothesize that measuring telomere length in a broad selection of ILD diagnoses will allow us to identify ILD diagnoses with an innate telomere-related pathobiology. Therefore, we measured telomere length of peripheral blood cells in healthy controls and in seven different ILD diagnoses. Subsequently we determined the degree of difference between healthy controls and ILD. We also assessed differences in telomere length among ILD, and in particular among the different forms of IIP.

In familial IIP, also called familial interstitial pneumonia (FIP), it has been found that a diag-

nosis of idiopathic pulmonary fibrosis (IPF) is most frequent, but all subtypes of IIP can be present[19]. In this group, two classes of disease causing mutations can be distinguished. Beside the telomerase related mutations there are mutations in surfactant proteins that are known to cause FIP[20,21]. The surfactant mutations cause ER-stress finally leading to pulmonary fibrosis[22], but an effect of these mutations on telomere length has never been investigated. To further explore telomere length in ILD, we subdivided the FIP-cohort in three groups: "surfactant", "telomerase" and "no mutation" carrying FIP patients and compared these to the non-familial ILD data.

Material & Methods

Patients and controls

In this study, 359 patients diagnosed with ILD at the Department of Pulmonology of the St. Antonius Hospital in Nieuwegein were retrospectively included. The patients were diagnosed with IPF, idiopathic non-specific interstitial pneumonia (iNSIP), cryptogenic organizing pneumonia (COP), smoking related interstitial lung disease (SR-ILD), hypersensitivity pneumonitis (HP), sarcoidosis, connective tissue disease-associated ILD (CTD-ILD) or FIP. Diagnosis was made in accordance with international guidelines[2,23–25]. For IIP cases with coexisting patterns, multidisciplinary discussion determined the clinical significance of the individual patterns[25]. FIP was defined as two or more first-degree family members with IIP and was documented in 67 patients consisting of 49 different families, and 18 affected family members. Upon first visit to the out-patient clinic, patients are asked to fill out a questionnaire regarding familial disease status. In case of a positive anamnesis for familial disease, the possibility of FIP and retrieval of further medical information was discussed by the respective physician. Fourteen of the 49 families have been described by van Moorsel and coworkers[21] as "FPF- 1-10, 15 and 17-19". A histopathological pattern of UIP was present in these patients sometimes with co-existence/super-imposition of other patterns as is also known from other FIP reports[8,19]. FIP patients had been screened for mutations on all exons of the genes telomerase reverse transcriptase (*TERT*), telomerase RNA component (*TERC*) and surfactant protein C (*SFTPC*) and on exon 6 of surfactant protein A2 (*SFTPA2*). Mutation carrying familial patients were classified as a separate group and subdivided in surfactant protein- C or -A2 carriers (FIP-SFTP) and telomerase mutation carriers (FIP-TERT); no mutations were found in *TERC*. The remaining FIP cohort therefore consisted only of patients without an identified mutation (FIP-no mutation). The control subjects comprised 173 self-reported healthy employees of the St. Antonius Hospital. The medical ethical committee of the St. Antonius Hospital in Nieuwegein (approval number W14.056 and R05.08A) approved this study and subjects gave formal written informed consent.

qPCR telomere length

Genomic DNA was extracted from peripheral white blood cells, using a magnetic beads based method (chemagic DNA blood 10k kit, Perkin Elmer). Telomere length was measured using the Monochrome Multiplex Quantitative- Polymerase Chain Reaction (MM-Q-PCR) method previously described[26]. Briefly, the relative TL was estimated from the ratio of the telomere (T) repeat copy number to a single gene (S; human β-globin gene) copy number (T/S ratio) for each sample using standard curves from a serial dilution of a genomic DNA-pool. Reactions were performed on a Biorad MyiQ real time PCR detection system and all Q-PCR TL measurements were performed in triplicate. Along each PCR run, 4 independent samples were measured for quality control and an average inter-assay coefficient of variation <10% was found. An average intra-assay variation <5% was found.

Statistical methods

For statistical analysis IBM SPSS Statistics 22. (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) were used. A GLM ANOVA was used to determine differences in telomere length between the tested cohorts. Age and sex were modeled as covariates. Post-hoc analysis was done using Fisher's least significant difference. The relation between relative telomere length and age in controls was used to calculate the 10^{th}, 5^{th}, and 1^{st} percentile of control telomere length. Fisher's exact test was used to compare percentages of cases and controls below the 10^{th}, 5^{th}, and 1^{st} percentile.

Results

Relative telomere length was determined in controls and patients diagnosed with the following ILD: sarcoidosis, HP, CTD-ILD, iNSIP, SR-ILD, COP, IPF, and FIP (table 1).

Telomere length in ILD

First, we determined the effect of an ILD diagnosis on telomere length using a general linear model with age and sex as covariates. This analysis showed that telomere length in the study population was significantly determined by an ILD diagnosis ($p<0.0001$) when adjusted for age and sex. Post-hoc comparison showed a significant difference in telomere length between the control group and all tested ILD separately (figure 1A). Largest differences in average telomere length compared to controls were found for IPF, FIP-no mutation and FIP-TERT ($p<0.001$). Sarcoidosis patients had the smallest difference in telomere length compared to controls ($p=0.004$).

Table 1. ILD cohorts and mean telomere length in peripheral blood

Cohort	N (male/female)		age - median (IQR)	T/S ratio - mean (SD)
control	173	(105/68)	49 (31-57)	0.975 (0.10)
sarcoidosis	67	(38/29)	50 (43-64)	0.927 (0.10)
HP	40	(19/21)	60 (50-67)	0.870 (0.10)
CTD-ILD	29	(11/18)	62 (44-66)	0.865 (0.09)
iNSIP	26	(19/7)	68 (60-76)	0.879 (0.09)
SR-ILD	13	(6/7)	46 (41-55)	0.907 (0.12)
COP	8	(4/4)	67 (60-71)	0.883 (0.06)
IPF	109	(112/21)	68 (59-72)	0.818 (0.11)
FIP-no mutation	28	(19/13)	59 (53-70)	0.822 (0.09)
FIP-TERT	27	(23/6)	63 (57-67)	0.710 (0.09)
FIP-SFTP	12	(7/8)	33 (30-59)	0.895 (0.10)

IPF: idiopathic pulmonary fibrosis, CTD-ILD: connective tissue disease-associated ILD, iNSIP: idiopathic non-specific interstitial pneumonia, COP: cryptogenic organizing pneumonia, SR- ILD: smoking-related interstitial lung disease, HP: hypersensitivity pneumonitis, FIP-no mutation: familial interstitial pneumonia - non mutation carriers, FIP-TERT: telomerase mutation carriers, FIP-SFTP: surfactant mutation carriers, T/S: telomere / single copy gene

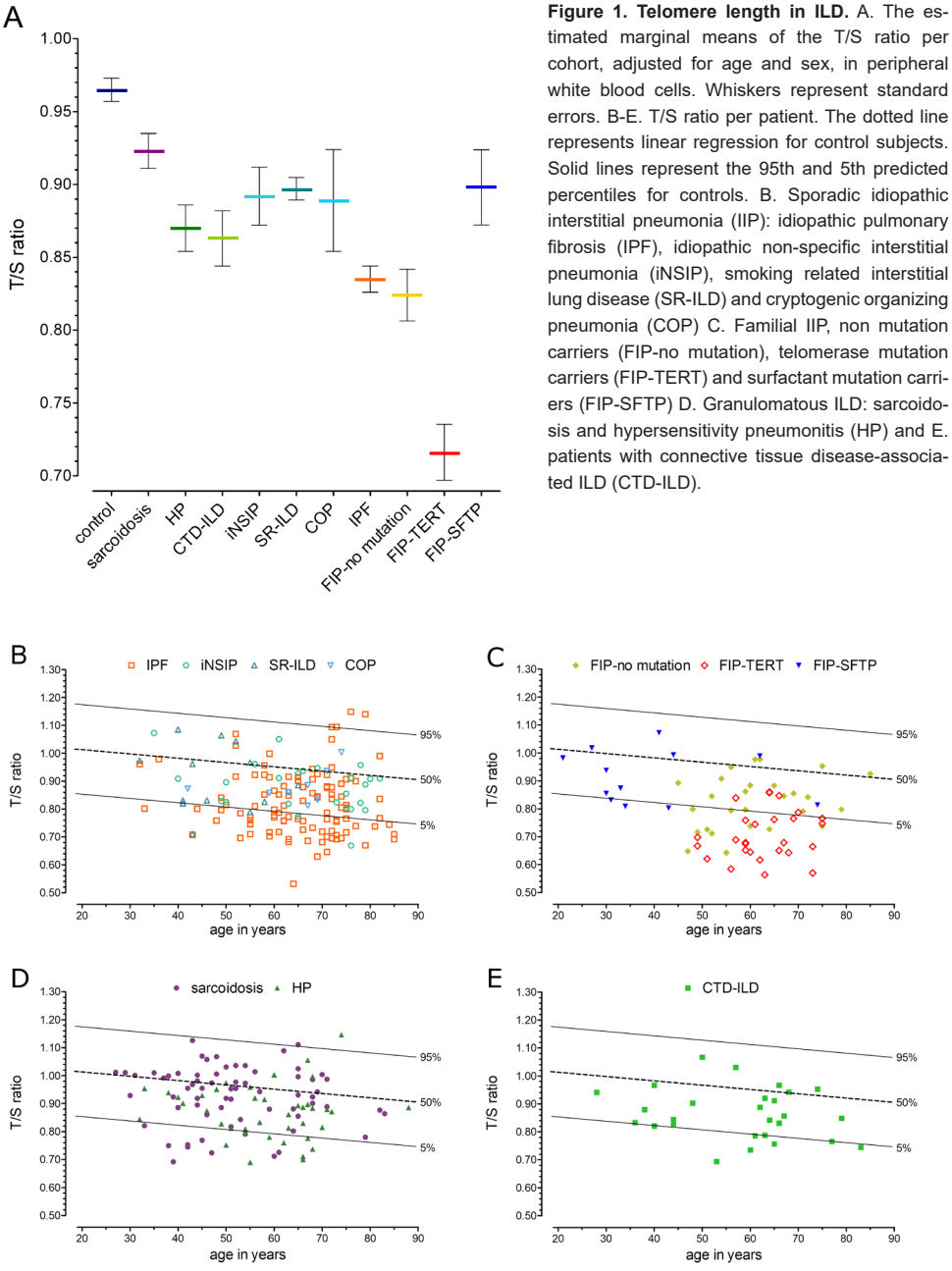

Figure 1. Telomere length in ILD. A. The estimated marginal means of the T/S ratio per cohort, adjusted for age and sex, in peripheral white blood cells. Whiskers represent standard errors. B-E. T/S ratio per patient. The dotted line represents linear regression for control subjects. Solid lines represent the 95th and 5th predicted percentiles for controls. B. Sporadic idiopathic interstitial pneumonia (IIP): idiopathic pulmonary fibrosis (IPF), idiopathic non-specific interstitial pneumonia (iNSIP), smoking related interstitial lung disease (SR-ILD) and cryptogenic organizing pneumonia (COP) C. Familial IIP, non mutation carriers (FIP-no mutation), telomerase mutation carriers (FIP-TERT) and surfactant mutation carriers (FIP-SFTP) D. Granulomatous ILD: sarcoidosis and hypersensitivity pneumonitis (HP) and E. patients with connective tissue disease-associated ILD (CTD-ILD).

Post-hoc comparison also revealed a number of notable differences and similarities in telomere length between separate ILD (figure 1A). First, telomere length in sporadic IPF patients was significantly shorter than in sarcoidosis (p<0.001), and the following IIP diagnoses: iNSIP (p=0.005), SR-ILD (p=0.031), and a trend towards significance was found for COP (p=0.113). Second, a significant difference was found between the two granulomatous ILD, telomere length in sarcoidosis was longer than in HP (p=0.009). Finally, no significant difference was found between CTD-ILD and iNSIP (p=0.276).

Table 2. Percentages of patients per cohort with telomere length below 10th, 5th and 1st percentile

Cohort	10th	5th	1st
Control	8.7	6.9	1.7
Sarcoidosis	19.4*	14.9	9.0*
HP	35.0*	22.5*	7.5
CTD-ILD	41.4*	27.6*	3.4
iNSIP	30.8*	7.7	3.8
SR-ILD	46.2*	23.1*	7.7
COP	12.5	0	0
IPF	55.0*	45.9*	16.5*
FIP- no mutation	50.0*	39.3*	21.4*
FIP-TERT	85.2*	81.5*	59.3*
FIP-SFTP	33.3*	25	0

* p<0.05 compared to controls; IPF: idiopathic pulmonary fibrosis, CTD-ILD: connective tissue disease-associated ILD, iNSIP: idiopathic non-specific interstitial pneumonia, COP: cryptogenic organizing pneumonia, SR-ILD: smoking-related interstitial lung disease, HP: hypersensitivity pneumonitis, FIP-no mutation: familial interstitial pneumonia - non mutation carriers, FIP-TERT: telomerase mutation carriers, FIP-SFTP: surfactant mutation carriers, T/S: telomere / single copy gene

FIP-no mutation patients had telomere length comparable with sporadic IPF patients. Between familial patients, *TERT* mutation carriers had the shortest telomeres and differed significantly from FIP-SFTP patients or FIP-no mutation patients (p<0.001). On the other hand, patients with a SFTP mutation had the longest telomeres, comparable with that found in the IIP: iNSIP, SR-ILD and COP, and significantly longer than telomere length in IPF (p=0.049) and FIP-no mutation (p=0.037).

Patient proportions below 10th percentile

Per ILD, the degree of difference of telomere length compared to controls was examined by determining the proportion of patients that had telomere length below the 10th, 5th and 1st percentile of controls (table 2). In controls, a significant linear relation between relative telomere length and age (p=0.0024) was present and relative telomere length decreased by 0.002 units/year. No such relation between telomere length and age was seen in any of the ILD cohorts. T/S ratio for each ILD per patient is shown in figure 1 B-E.

In all ILD except COP, the proportion of patients with a telomere length below the 10th percentile differed significantly from controls ($p<0.05$, table 2). Below the 5th percentile the proportion of patients with HP, CTD-ILD, SR-ILD, IPF, FIP and FIP-TERT was still significantly higher than in controls ($p<0.05$). Below the 1st percentile, the proportion of sarcoidosis, patients was significantly higher than controls ($p=0.016$). Only in IPF, FIP-no mutation and FIP-TERT a significant proportion of patients had telomere length below the 10th and the 5th and the 1st percentile.
The proportion of FIP patients with a *TERT* mutation below the 10th percentiles was extremely high (85%) and remained high at the 5th and 1st percentile. The FIP-TERT cohort had significantly more patients below the 5th percentile ($p=0.022$) than FIP-SFTP.
Dividing the HP patient cohort in a group with (n=20) and without (n=20) fibrosis did not show a significant difference in relative telomere length (data not shown).

Discussion

This study shows that telomeres of ILD patients are shorter compared to those of healthy control subjects. However, the degree of difference in telomere length differs significantly between the different ILD diagnoses and particularly within the IIP subgroup. Within familial disease, telomere length correlates with the underlying genetic cause. This is a large study on telomere length and ILD, including eight different ILD diagnoses and 532 subjects and the first study to separately analyze different subclasses of IIP and FIP.

Previous studies have shown that a significant proportion of sporadic IIP patients have reduced telomere lengths[4,6]. Two IIP telomere length studies have reported that no significant difference in telomere length between the different IIP diagnoses was found[4,6]. It was suggested that insufficient statistical power was present to detect differences. The studies had been performed in mixed IIP cohorts of 73 patients[6] and 62 patients[4] and consisted of 63% and 81% IPF patients respectively. To analyze difference between IIP cohorts, we included a large number of non-IPF IIP patients in our study and found that telomere length is significantly shorter in IPF compared with iNSIP and SR-ILD, while a trend towards significance was found for COP (p=0.113).

Shortest mean telomere length was found in patients with FIP that carried a *TERT* mutation. In previous studies, FIP patients whether or not carrying a mutation in telomerase maintenance genes *TERT* and *TERC* had been shown to have significantly shorter telomeres compared to controls[6,8,9]. Here we confirm these findings both for FIP and FIP-TERT patients and also determine, for the first time, telomere length in familial patients with a mutation in surfactant genes *SFTPA2* or *SFTPC*. More importantly, FIP-SFTP patients have significantly longer telomeres than FIP patients with a *TERT* mutation. This supports the presence of divergent etiologies for FIP: one based on telomere biology[27–29] which is most profound in FIP-TERT patients and one based on local ER-stress, which is most profound in FIP-SFTP patients[22].

IPF telomere length has previously been compared to controls and HP patients[30]. In that study the proportions of both IPF (5.7%) and HP (5.8%) patients with telomere length below 10th percentile of controls were comparable to the control population[30]. Others have found a proportion of 25% of IPF patients below the 10th percentile[6]. Our results showed a significantly high proportion of 55% and 35% below the 10th percentile for IPF and HP patients respectively.

The difference between these studies could possibly be attributed to a difference in disease severity between the studied cohorts. A limitation of the present study is that all patients were derived from a single center. The ILD out patient clinic of St. Antonius Hospital is a tertiary referral and transplantation center for ILD in the Netherlands, which might lead to a patient population with more severe ILD cases in advanced stages of disease. Telomere length might not only be dependent on the diagnosis but may also change during disease. Serial telomere

length measurements might be performed to further investigate this possibility.

A significant difference in telomere length between controls and sarcoidosis patients has been previously reported[5] and is also found in our study. However, we show that in comparison to other ILD, sarcoidosis has the smallest loss of telomere length . This indicates a minimal role of telomere related pathobiology in sarcoidosis. No difference in telomere length between controls and sarcoidosis has also been reported[31], but was thought to be due to the small sarcoidosis sample size (n=22). A notable observation in our sarcoidosis cohort is the significant proportion of patients with telomere length below the 1st percentile of controls. Of these 6 patients, 5 were aged 38-47 and male, corresponding with previously reported age and sex related acceleration of loss of telomere length in sarcoidosis[5].

Multiple factors influence telomere length. FIP-SFTP patients do not suffer from genetically determined telomeropathy[22,32] but do have severe interstitial lung fibrosis with clinical and pathological findings similar to those seen in IPF[21]. Although not driven by telomeropathy, we found that telomere length was significantly shorter in FIP-SFTP patients than in controls. This degree of telomere shortening in FIP-SFTP patients most likely represents the acquired decrease of telomere length due to disease[18,33]. A similar degree of loss of telomere length is found in iNSIP, SR-ILD and COP, suggesting that these diseases are also not driven by telomere related pathology, but instead have acquired shorter telomeres in peripheral blood cells. Telomere length in HP and CTD-ILD is slightly shorter than that found in FIP-SFTP and other non-IPF IIP. A significant proportion of HP and CTD-ILD patients has telomere length below the 5th percentile and a significant difference in telomere length between these ILD and controls has been shown before[31]. Decreased telomere length can be acquired by exogenous factors like increased oxidative stress due to smoking, but also due to increased proliferation of immune cells[34,35]. Both antigen presentation and chronic viral infection are associated with T cell proliferation and subsequent telomere attrition[36,37]. Multiple ILD, including sarcoidosis, COP, HP and CTD–ILD are associated with a significant systemic up regulation of the immune system. Their short telomere length might therefore be due to increased proliferation of immune cells which has been shown to be associated with decreased telomere length in peripheral blood cells[38]. Interestingly, no difference in telomere length was found between (sub)acute HP and chronic HP. This suggests a limit to the extent of telomere shortening as a result of prolonged immune responses.

The FIP-SFTP patients have a significantly higher mean telomere length than FIP-no mutation, and IPF patients. Within our FIP-no mutation and IPF cohort, some individual patients have a near normal telomere length suggestive of non-telomere or surfactant related disease etiology. On the other hand, some IPF and FIP patients have very short telomeres comparable with those seen in FIP-TERT, suggesting an innate telomere related pathobiology[18]. Telomere length is a heritable trait, and compromised telomerase function leads, over generations, to increasingly shorter telomeres. In IPF, a genetic polymorphisms in *TERT* has been found to confer IPF susceptibility[39]. This supports the possibility of a telomere driven pathology in sporadic IPF patients. Telomere length in these patients would already be compromised upon encountering exogenous risk factors and subsequent cell-proliferative demands. Telomere pathology has been linked to epithelial cell senescence[32] and increased senescence of epithelial cells lining fibroblast foci has been shown in IPF by Minegawa et al[40].

Telomere-induced senescent cells were shown, to produce a senescence-associated secretory phenotype (SASP) in vitro[41]. The SASP could subsequently lead to lung remodeling[32]. Also, additional hits to a senescent epithelium, like cigarette smoke, viral infections or gastro-esophageal reflux, would contribute to the onset of IPF[42,43].

Identification of telomere driven disease in at least a subgroup of IPF could lead to a new classification based on presence or absence of telomere aberrations. However, further research is needed as to the association of such a group with clinical and molecular differentiating markers. Furthermore, it is possible that therapeutic interventions have different outcome for telomere pathology driven IPF compared to IPF based on other etiologies. Because the present study supports an important role for telomere shortening in the pathogenesis of IPF, telomere length might become an important parameter in the design of future clinical trials.

In conclusion, telomere length in ILD is shorter compared to controls, and ILD telomere length differs significantly between different diagnoses. This indicates a varying role of telomere biology in ILD. A remarkable difference is also shown between FIP patients carrying a mutation in *TERT*, who have the shortest telomeres, and FIP patients with a *SFTPC* or *SFTPA2* mutation, carrying the longest telomeres in IIP. The decrease in telomere length in most ILD is comparable to that seen in FIP-SFTP and is likely acquired through increased oxidative stress or inflammatory responses. However, the telomeres in IPF and other FIP patients are much shorter and suggest an important role for an innate, genetically predisposed, telomere driven disease in many of these patients.

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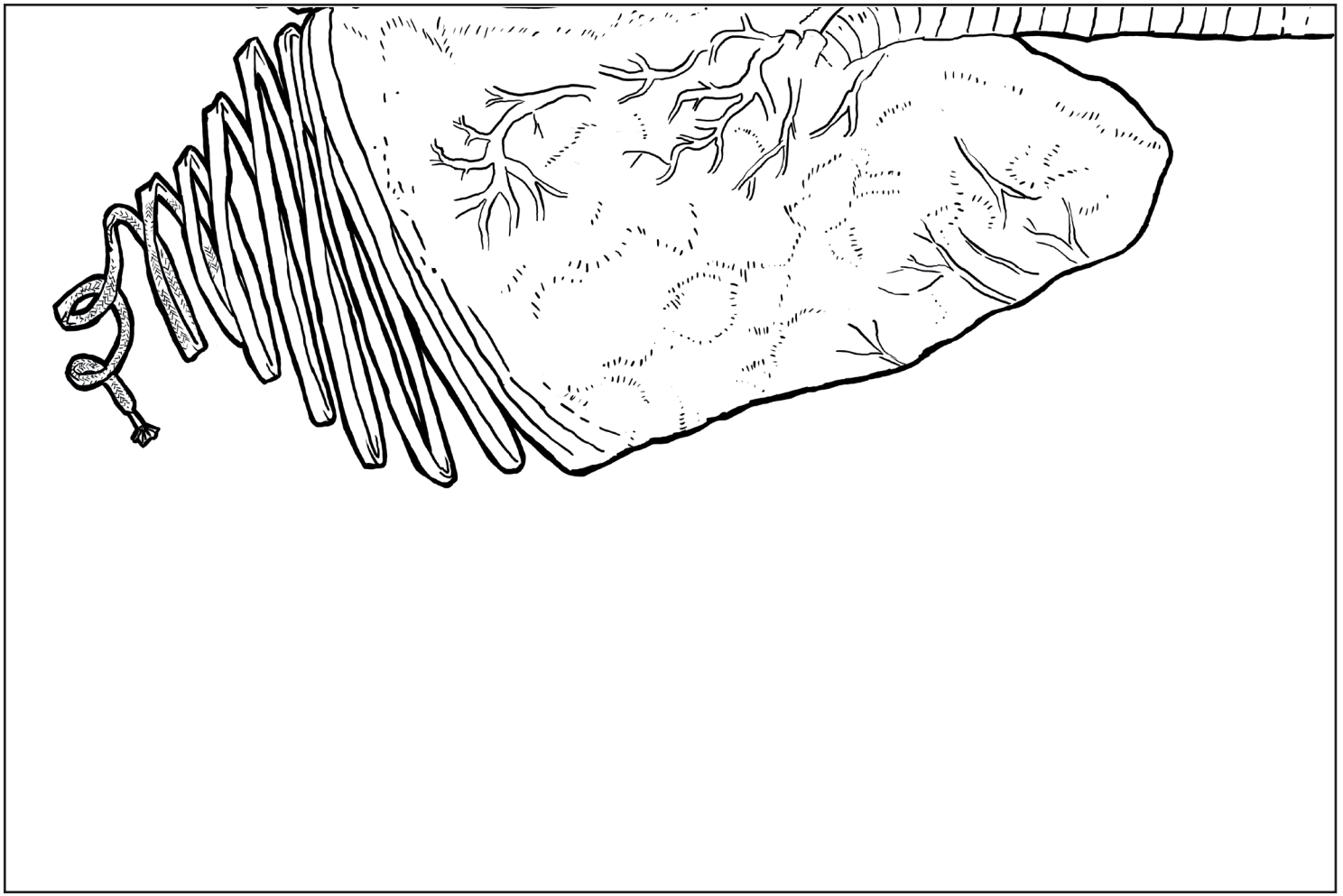

3

Short Telomere Length in IPF Lung Associates with Fibrotic Lesions and Predicts Survival

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Abstract

Telomere maintenance dysfunction has been implicated in the pathogenesis of Idiopathic Pulmonary Fibrosis (IPF). However, the mechanism of how telomere length is related to fibrosis in the lungs is unknown. Surgical lung biopsies of IPF patients typically show a heterogeneous pattern of non-fibrotic and fibrotic areas. Therefore, telomere length (TL) in both lung areas of patients with IPF and familial interstitial pneumonia was compared, specifically in alveolar type 2 (AT2) cells.

Fluorescent in situ hybridization was used to determine TL in non-fibrotic and fibrotic areas of 35 subjects. Monochrome multiplex quantitative polymerase chain reaction (MMqPCR) was used for 51 whole lung biopsies and blood TL measurements.

For sporadic IPF subjects, AT2 cell TL in non-fibrotic areas was 56% longer than in fibrotic areas. No such difference was observed in the surrounding lung cells. In subjects carrying a telomerase reverse transcriptase (*TERT*) mutation, AT2 cell TL was significantly shorter than in sporadic subjects. However, no difference in surrounding cell TL was observed between these subject groups. Finally, using biopsy MMqPCR TL measurements, it was determined that IPF subjects with shortest lung TL had a significantly worse survival than patients with long TL.

This study shows that shortening of telomeres critically affects AT2 cells in fibrotic areas, implying TL as a cause of fibrogenesis. Furthermore, short lung telomere length is associated with decreased survival.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a rare lung disease characterized by progressive fibrosis of lung parenchyma[1]. Patients with the disease have a median post-diagnostic survival of 2 - 5 years[2]. IPF can be both a sporadic and a familial disease. The familial form can be caused by mutations in surfactant related genes, or genes that influence telomere maintenance[3–10]. Analysis of familial IPF patients with mutations in telomerase reverse transcriptase (*TERT*) or telomerase RNA component (*TERC*) showed a diminished telomerase activity and prematurely shortened telomere length (TL) in blood leukocytes. Similar results were found in sporadic patients not carrying telomerase mutations, when compared to healthy controls[11–13]. It was also shown that TL of the lung alveolar type 2 (AT2) cells of IPF patients was shorter compared to controls[11]. Together, these findings indicate that telomere related pathology plays a role in both familial and sporadic IPF. However, it remains unknown whether the short TL in AT2 cells is related to fibrosis.

A contemporary view on the pathogenesis of IPF focuses on the role of AT2 cell during disease development[9,14–16]. Evidence for this can be found in patients diagnosed with a surfactant-related familial interstitial pneumonia (FIP). Since AT2 cells are the exclusive producers of surfactant protein-C, these cells are considered to be the precursor cells leading to pulmonary fibrosis[17]. Conversely, a link between mutations in telomerase related genes and the AT2 cell is not clear. In healthy lung tissue, the AT2 cell provides the regenerative capacity of the lung alveoli[18]. Faulty telomere maintenance could underlie an impaired proliferative capacity of the AT2 cells[19]. Recently it has been demonstrated that mice with telomere repeat binding factor 1 (TRF1)-deleted AT2 cells develop lung fibrosis and showed short telomeres in AT2 cells[20,21]. This might explain the human AT2 cell TL shortening in IPF, which could result in a similar response characterized by progressive fibrosis[22]. If telomere shortening plays a role in IPF disease development, it would be expected to occur primarily in AT2 cells.

IPF lungs show a patchy distribution of affected fibrotic and relatively preserved, non-fibrotic tissue[23,24]. This heterogeneous distribution allows for a comparison of TL between non-fibrotic and fibrotic tissue in a single surgical biopsy. In this study we investigated how the distribution of telomere shortening in lung tissue biopsies of patients is related to fibrotic remodeling of the tissue. We show that in sporadic IPF, AT2 TL was significantly longer in non-fibrotic areas than in fibrotic regions, thereby implicating telomere shortening as a cause of fibrotic remodeling of lung tissue in IPF. In addition, familial patients with a *TERT* mutation show significant shorter telomeres than in sporadic IPF. Furthermore, short whole biopsy telomere length in sporadic IPF patients is associated with worse survival.

Material and Methods

Human subjects

In this study, 63 patients diagnosed with IPF at the St. Antonius ILD Center of Excellence Nieuwegein were included retrospectively (table 1). In these patients, TL was measured in AT2 cells, whole lung biopsies and white blood cells. Patients were classified as either sporadic IPF (n=39) or familial interstitial pneumonia (FIP) (n=24). Diagnoses were based on the ATS/ERS/JRS/ALAT guidelines after multidisciplinary discussion[1,25]. The disease was designated as familial if two or more first-degree family members suffered from idiopathic interstitial pneumonia. FIP patients were screened on mutations in *TERT*, *TERC*, surfactant protein C (*SFTPC*), surfactant protein A2 (*SFTPA2*) exon 6 and TRF1-Interacting Nuclear Factor 2 (*TINF2*) exon 6. Based on these results, the FIP group was subdivided in two subgroups: patients that carried a mutation in *TERT*: FIP-TERT (n=10, table 2) and patients that did not carry a known mutation in telomere related genes: FIP-nonTERT (n=14). The latter subgroup included 3 patients with a *SFTPC* or *SFTPA2* mutation. Of the remaining 11 patients, no known pathogenic mutations were found. To assess lung function parameters, diffusing capacity of the lungs for carbon monoxide (DLCO) and forced vital capacity (FVC) data were collected within a 3-month window before or after diagnosis (n=39). To cross reference results, a control group was formed using normal lung tissue obtained during post-mortem examination of five subjects not suffering from lung related pathology. Patient characteristics were retrieved from medical reports. The study was approved by the Medical research Ethics Committees United (MEC-U) of the St Antonius Hospital (approval number W14.056 and R05.08A). All patient data were anonymized.

Table 1. Patient group characteristics

	IPF	FIP-TERT	FIP-nonTERT
Total n (% male)	39 (90%)	10 (80%)	14 (57%)
Mean (SD)			
Age at diagnosis in years	61 (10)	64 (7)	54 (12)
DLCO % predicted	47 (18)	47 (10)	43 (15)
FVC % predicted	69 (22)	85 (8)	63 (22)

IPF: idiopathic pulmonary fibrosis, FIP: familial interstitial pneumonia, SD: standard deviation, FVC: forced vital capacity, DLCO: diffusing capacity of the lungs for carbon monoxide

Table 2. Specific mutations carried by the FIP-TERT subjects in this study

cDNA Position	Variant name	SIFT*	Poly-Phen-2#	Allele frequency (Exac)	Allele frequency (1000G)	Reference no.
c.455T>A	L152Q	D	D	not found	not found	NA
c.1584T>G	C528W	T	P	not found	not found	NA
c.1698_1700delCAC	T567del	NA	NA	not found	not found	NA
c.2005C>T	R669W	D	B	0.00006376	not found	rs372140951
c.2011C>T	R671W	D	P	0.00006342^	not found	NA
c.2303A>T	D768V	D	D	not found	not found	NA
c.2406C>G	S802R	D	D	not found	not found	NA
c.2146G>A	A716T	D	D	not found	not found	rs387907249
c.2701C>T	R901W	T	P	not found	not found	rs199422304+
c.2701C>T	R901W	T	P	not found	not found	rs199422304+

* SIFT prediction: Damaging (D) <=0,05; Tolerated (T) >0,05
# PolyPhen-2 HumVar analysis (% = False Positive Rate): Damaging (D) <=10%; Possibly damaging (P) >10% and <=20%; Benign (B) >20%.
^ Variant is associated with pulmonary fibrosis[26].
+ Variant is described in a patient with Hoyeraal-Hreidarsson syndrome, which is characterized by short telomeres[27].

Lung tissue

Residual lung tissue was obtained from biopsies carried out for diagnostic purposes and was fixed in formalin and embedded in paraffin (FFPE). Serial sections of 4 µm were cut. Non-fibrotic and fibrotic areas were identified on hematoxylin & eosin (H&E) stained sections (figure 1A and B). All identifications were done by pathologists (MvO and SR), who are highly experienced in the field of interstitial lung diseases.

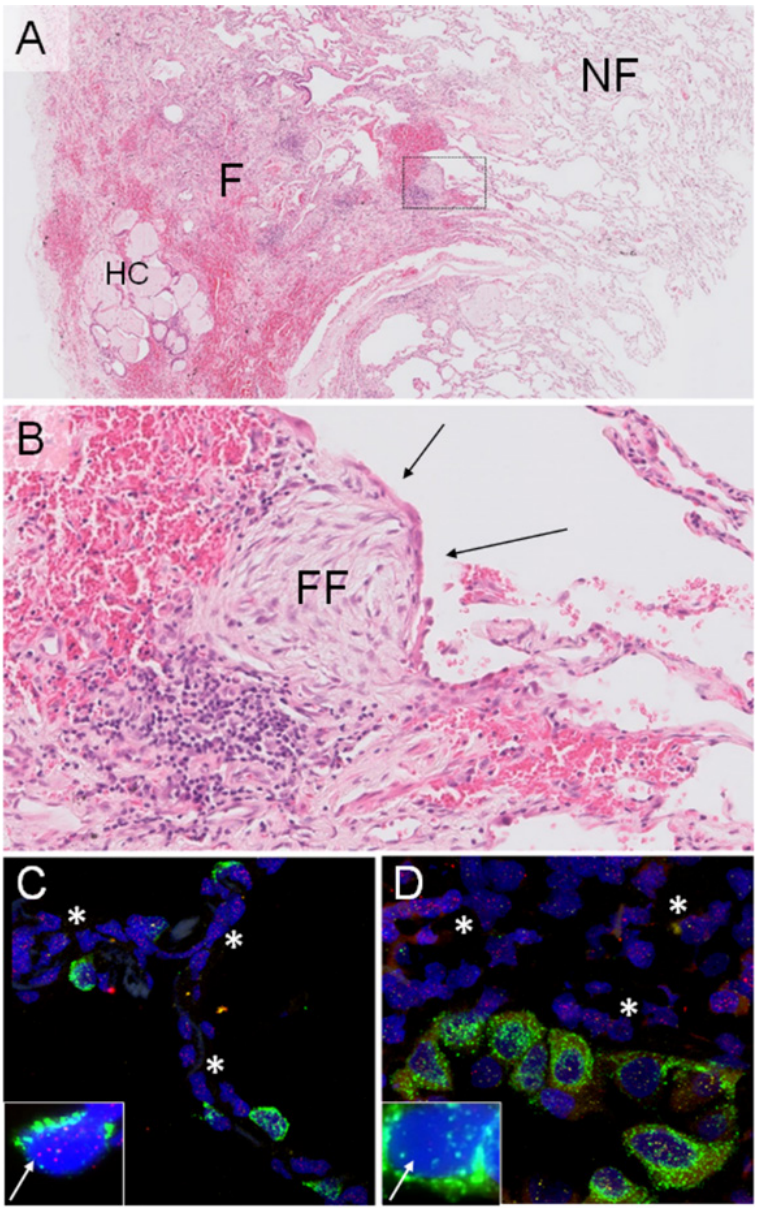

Figure 1. Images showing representative regions for non-fibrotic and fibrotic areas within one lung biopsy. (**A**) Low magnification image of H&E stained IPF lung tissue showing non-fibrotic (NF) and fibrotic (F) areas, and characteristic honeycombing (HC). (**B**) Enlarged boxed area of image **A** representing a fibrotic area in IPF lung biopsy. Characteristic fibroblast focus (FF) and alveolar type 2 (AT2) cell hyperplasia (black arrows) are highlighted. (**C**,**D**) Combined fluorescent images of AT2 and surrounding cells in non-fibrotic areas (**C**) and fibrotic areas (**D**) in IPF lung tissue. DNA in nucleus is displayed in blue (DAPI), pro-SPC in green and telomeres in red. Note the lower signal of AT2 cell (white arrows) telomere signal (red dots) in fibrotic areas (**D**) vs non-fibrotic (**C**) tissue, reflecting shorter telomeres in AT2 cells in fibrotic areas. Examples of pro-SPC negative surrounding cells are marked with an asterisk (*).

Fluorescent in situ hybridization

After identification of fibrotic and non-fibrotic areas, the sequential section of the biopsy was used for a fluorescence in situ hybridization (FISH). Tissue slides were deparaffinized using a xylene series. Next, they were placed in H2O2 block buffer (1.5%), washed in Phosphate-buffered saline (PBS) and treated with Borax (1 mg/mL). For antigen retrieval, specimens were boiled in a citrate solution for 20 min (2.94 g/L, pH 6). Telomeres were labeled with a telomere-Cy3 PNA probe (Panagene, Daejeon, South-Korea) and pro-SPC (AB3786, 1/500,

Merck Millipore, Darmstadt, Germany) was fluorescently labeled to identify AT2 cells (secondary antibody; A-11008, 1/300, Thermo Fisher Scientific, Waltham, MA, USA) (Figure 1C and D). Pro-SPC negative surrounding cells were used as reference. Surrounding cells were located within 2 cells of AT2 cells. Finally, DNA of samples was stained with 4',6-diamidino-2-phenylindole (DAPI, 25 μg/mL) and finished with Vectashield antifade mounting medium (Vector laboratories, Burlingame, CA, USA). Slides were stored at 4°C until analysis.

Imaging and signal quantification

FISH-TL was measured using a method adopted from Meeker et al.[11,28,29]. Images were captured using a Fluorescence microscope (Leica DM 5500 B) at high magnification (100x). Per biopsy, up to 15 images were made per area. Z-stacking of 9 focal planes with 0.5μm intervals was used to maximize the coverage of cell nuclei. Total telomere (cy3) fluorescent signal was quantified per nucleus using the Telometer image analysis plugin (available at http://demarzolab.pathology.jhmi.edu/telometer/index.html) of ImageJ (http://rsb.info.nih.gov/ij/). To account for sub-optimal capturing of the nuclei caused by the cutting planes, total telomere signal was divided by the total DNA (DAPI) signal. All images were taken at fixed time points between 1 to 3 days after staining to circumvent data variability by DAPI fluorescence fading.

MMqPCR for telomere length in FFPE tissue

DNA was isolated from FFPE tissue sections using an AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Slides were cut from sequential sections used for FISH. The paraffin was removed using paraffin dissolver (Macherey-Nagel, Düren, Germany). DNA was quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) using an absorbance ratio of 260 and 280nm. Samples within a ratio of 1.8-2.0 were included. To measure whole lung biopsy and white blood TL, monochrome multiplex qPCR (MMqPCR) was performed as described earlier[13,30]. Because amplification of telomere and β-globin in FFPE DNA is delayed compared to blood derived DNA we adjusted cycle counts for all FFPE samples with -5 and -7 respectively. The relative TL for each sample was estimated from the ratio telomere repeat copy number (T) to a single human β-globin gene copy number (S) (T/S ratio), using standard curves from a serial dilution of a genomic DNA-pool[30]. Quadruplicate reactions were performed on a MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). MMqPCR is proven to be a sensitive method to discriminate between patients with high and low TL[13].

Statistics

Ratios were calculated for non-fibrotic / fibrotic and AT2 cell / surrounding cell comparisons. Values below 1 indicate shorter FISH-TL in non-fibrotic areas and AT2 cell respectively.
All analyses were performed using non-parametric statistical tests. Mann-Whitney and Wilcoxon signed ranked tests were used to compare TL. P-values for two-sided t-tests are shown. Correlations were determined using Spearman's rank coefficient test. Survival analysis was done using Kaplan-Meier estimation. For statistical analysis IBM SPSS Statistics 22. (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 and 6 (GraphPad Software, San Diego, CA, USA) were used.

Results

DAPI is a valid measure to correct for total DNA per cell

For the FISH-TL measurements, DAPI was used to account for the total amount of DNA per cell. To verify whether DAPI staining was valid measure, we compared DAPI with a centromere FISH[31]. Similar results were found between both assays (n=4, data not shown). Therefore we conclude that using DAPI as a counterstain is a valid method, as was found by Meeker and coworkers and Kropski and coworkers[29,32].

Telomeres in non-fibrotic areas of sporadic IPF subjects are longer than in fibrotic areas

In order to investigate whether AT2 telomere shortening is related to fibrosis, we performed a FISH staining on FFPE material in a group of 16 sporadic IPF subjects. Median AT2 cell TL was significantly longer ($p<0.001$) in non-fibrotic areas compared to fibrotic areas (figure 2), resulting in 2.24 times difference (FISH-TL ratio in table 3). To get an idea of the general TL in non-fibrotic and fibrotic areas, we measured FISH-TL in pro-SPC negative surrounding cells. Here, no significant difference ($p=0.30$, data not shown) was found between non-fibrotic and fibrotic areas (FISH-TL ratio: 1.15, table 3).

Figure 2 shows that FISH-TL is variable between subjects. To assess how FISH-TL diversity within subjects is distributed, the correlation between non-fibrotic and fibrotic FISH-TL was analyzed (data not shown). This resulted in a significant correlation for both AT2 ($r=0.855$, $p=2{\cdot}10^{-5}$) and surrounding cells ($r=0.689$, $p=0.003$), indicating that FISH-TL variability among subjects is high, but correlates positively between non-fibrotic and fibrotic areas within a subject.

Table 3. Median telomere length for non-fibrotic versus fibrotic areas per cohort

		AT2 FISH-TL			Surrounding cell FISH-TL		
Subgroup	**n**	Non-Fibrotic (nf)	Fibrotic (f)	Ratio (nf/f)	Non-Fibrotic nf)	Fibrotic (f)	Ratio (nf/f)
Controls	5	23.5	N/A	N/A	26	N/A	N/A
Spor IPF	16	3.22	1.44*	2.24	12	10.70	1.15
FIP-nonTERT	10	3.74	2.15*	1.74	13	7.46	1.70
FIP-TERT	9	1#	1	1	9.2	10.20	0.90

FISH: fluorescence in situ hybridization, TL: telomere length, AT2: alveolar type 2 cell, IPF: idiopathic pulmonary fibrosis, FIP: familial interstitial pneumonia. Numbers indicate median telomere signal, of which ratios were calculated. Ratio (nf/f) = Non-fibrotic / Fibrotic, i.e. if ratio=2 telomeres in non-fibrotic areas are two times longer than in fibrotic areas.

* In non-fibrotic areas, AT2 FISH-TL is significantly longer than in fibrotic regions (spor IPF: $p = 0.0006$, FIP-nonTERT: $p = 0.02$).

AT2 FISH-TL in FIP-TERT is significantly shorter than in sporadic IPF ($p= 0.02$).

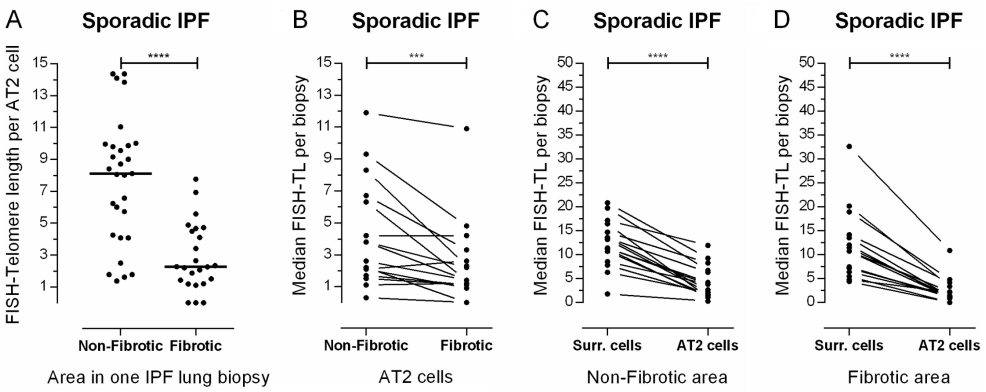

Figure 2. Telomere length in sporadic IPF lungs. (A) Representative example of telomere length per alveolar type 2 (AT2) cell for one sporadic IPF subject. Each dot represents one cell. Non-fibrotic AT2 FISH-TL is significantly higher compared to FISH-TL in fibrotic areas (p<0.0001). Bars represent medians and p-value is calculated using a Mann-Whitney test. (B, C, D) Median surrounding and AT2 cell FISH-TL in non-fibrotic and fibrotic areas for 16 sporadic IPF subjects. Each line connects, per subject, the FISH-TL of (B) AT2 cells in the non-fibrotic and fibrotic area or median FISH-TL differences between surrounding and AT2 cells in (C) non-fibrotic areas or (D) fibrotic areas. AT2 FISH-TL differences between non-fibrotic and fibrotic areas and between surrounding and AT2 cells were significant (2-tailed p<0.0006 and p<0.0001 respectively), which were indicated by asterisks (*** = p<0.001, **** = p<0.0001).

Non-fibrotic and fibrotic AT2 cell telomere length in sporadic IPF subjects is shorter than in surrounding cells.

Next, to elucidate further on telomere shortening in AT2 cells specifically, we compared telomere length of AT2 cells with surrounding cells. In non-fibrotic areas, the telomeres in AT2 cells were 4 times shorter than in surrounding cells (p<0.0001, figure 2C and FISH-TL ratio: 0.26, Table 4). The difference was even larger in fibrotic areas: telomeres in AT2 cells were 8 times shorter than in surrounding cells (p<0.0001, figure 2D and FISH-TL ratio: 0.13, table 4). To place this in perspective, we determined the FISH-TL ratio between AT2 cells and surrounding cells in control subjects (n=5). In controls no significant difference was found between AT2 and surrounding cells, indicating that under non-pathological conditions, AT2 cells do not have shortened telomeres (FISH-TL ratio: 0.90, table 4).

Lung telomere length in Familial Interstitial Pneumonias: TERT

To investigate TL differences between sporadic IPF subjects and subjects with an established telomere syndrome, FISH-TL was determined in *TERT* mutation carriers (FIP-TERT). FIP-TERT showed no difference in AT2 FISH-TL between non-fibrotic and fibrotic areas (p=0.36, figure 3A). However, AT2 FISH-TL was substantially shorter in non-fibrotic areas compared to sporadic IPF (median 1.00 vs 3.22, p=0.02, table 3). In surrounding cells FISH-TL was concordant between FIP-TERT and sporadic IPF in both areas. FISH-TL in AT2 cells was significantly shorter than in surrounding cells in both non-fibrotic and fibrotic areas (p<0.01, figure 3B and C). These data show that AT2 TL distribution between non-fibrotic and fibrotic tissue in FIP-TERT differs from sporadic IPF subjects, underlining the effect of a defective telomerase enzyme.

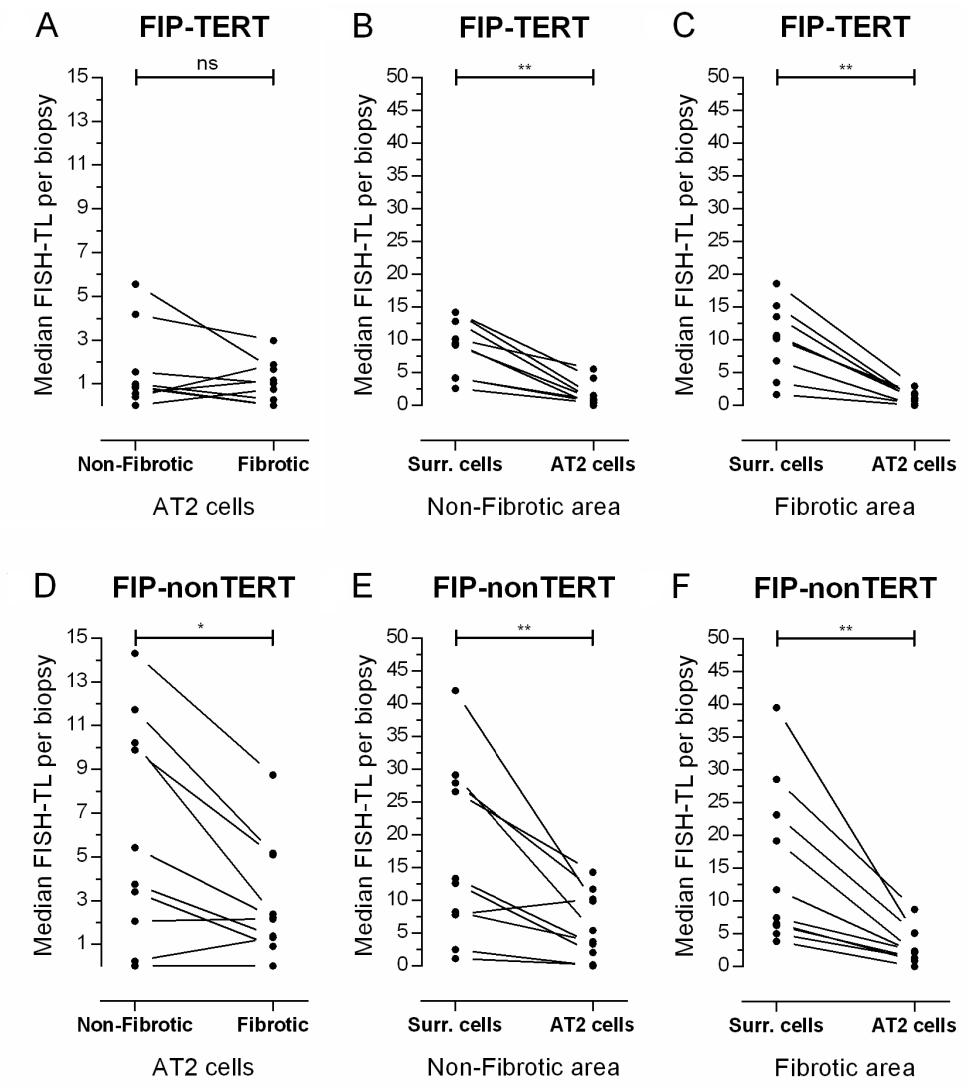

Figure 3. FISH Telomere length in lungs of FIP-TERT and FIP-nonTERT subjects. (**A**) Median FISH-TL in alveolar type 2 (AT2) cells in non-fibrotic and fibrotic areas of 9 FIP-TERT subjects. Telomeres are generally short and no significant difference between areas was observed (2-tailed, p=0.36). (**B, C**) Median FISH-TL of same subjects as in figure **A**, showing differences between surrounding and AT2 cells in (**B**) non-fibrotic and (**C**) fibrotic areas. (**D**) Median FISH-TL in alveolar type 2 (AT2) cells in non-fibrotic and fibrotic areas of 10 FIP-nonTERT subjects. Non-fibrotic AT2 FISH-TL is significantly higher compared to FISH-TL in fibrotic areas (2-tailed, p=0.02). (**E, F**) Median FISH-TL of same subjects as in figure **D**, showing differences between surrounding and AT2 cells in (**E**) non-fibrotic and (**F**) fibrotic areas. Asterisks indicate significant differences calculated by Wilcoxon matched-pairs signed rank analysis (* = p<0.05, ** = p<0.01).

Lung telomere length in Familial Interstitial Pneumonias: nonTERT

Next, we analyzed the familial subjects, who did not carry a known telomere related mutation (FIP-nonTERT). For these subjects, FISH-TL patterns were the same as in the sporadic IPF group; non-fibrotic AT2 cell FISH-TL was 1.74 times longer than fibrotic AT2 cells (p = 0.02, figure 3D, table 3). Furthermore, in both non-fibrotic and fibrotic areas the AT2

Table 4. Median telomere length for alveolar type 2 (AT2) cells versus surrounding cells per cohort

Subgroup	n	Non-fibrotic area FISH-TL			Fibrotic area FISH-TL		
		AT2	Surr.	Ratio (AT2/ Surr.)	AT2	Surr.	Ratio (AT2/ Surr.)
Controls	5	23.47	26.01	0.9	N/A	N/A	N/A
IPF	16	3.22	12.30*	0.26	1.44	10.73*	0.13
FIP-nonTERT	10	3.74	12.60*	0.29	2.15	7.46*	0.29
FIP-TERT	9	1.00#	9.21*	0.11	1.00	10.20*	0.10

FISH: fluorescence in situ hybridization, TL: telomere length, AT2: alveolar type 2 cell, IPF: idiopathic pulmonary fibrosis, FIP: familial interstitial pneumonia, surr.: pro-SPC negative surrounding cells. Numbers indicate median telomere signal, of which ratios were calculated. Ratio (AT2/Surr.) = *AT2 cell / surrounding cell*, i.e. if ratio=2 telomeres in AT2 cells are two times longer than in surrounding cells.

AT2 FISH-TL in FIP-TERT is significantly shorter than in sporadic IPF (p= 0.02).

* In both non-fibrotic and fibrotic areas, AT2 FISH-TL is signifincty shorter than in surrounding cells (spor IPF: p < 0.0001, FIP-nonTERT and FIP-TERT: p < 0.01).

cell FISH-TL was 3.5 times shorter than surrounding cells (p<0.01, figure 3E and F, FISH-TL ratio: 0.29, table 4). Compared to FIP-TERT, FIP-nonTERT subjects had similar AT2 FISH-TL in non-fibrotic areas (p=0.081).

In order to get an overview, we compared the sporadic IPF, FIP-TERT and FIP-nonTERT subject groups. All data is summarized in tables 3 and 4. No statistical age differences were found between groups. Looking specifically at fibrotic areas, AT2 FISH-TL was not significantly different (p=0.16) between subject groups. In non-fibrotic areas a significant difference in AT2 cell FISH-TL was observed between FIP-TERT and sporadic IPF subjects (p=0.02) and a trend between FIP-TERT and FIP-nonTERT subjects (p=0.081). These data indicate that shortest AT2 telomeres in non-fibrotic areas were found in FIP-TERT subjects. In addition, no significant difference in surrounding cells FISH-TL was found between the three subgroups in either area (p=0.67), suggesting that in all subject groups TL shortening is most evident in AT2 cells.

Telomere length by MMqPCR: lung

To test whether whole lung biopsy TL as measured by MMqPCR (biopsy T/S) correlates with FISH-TL of AT2 cells, we extracted DNA from biopsy sections and performed MMqPCR as described by Cawthon *et al.* (IPF n=15, FIP-TERT n=9, FIP-nonTERT n=10)[30]. In sporadic IPF subjects, a significant positive correlation was found between biopsy T/S and AT2 FISH-TL in both non-fibrotic (r^2=0.53, p=0.002) and fibrotic (r^2=0.73, p<0.0001) areas (figure 4). No correlations were found in the FIP-TERT and FIP-nonTERT (data not shown).

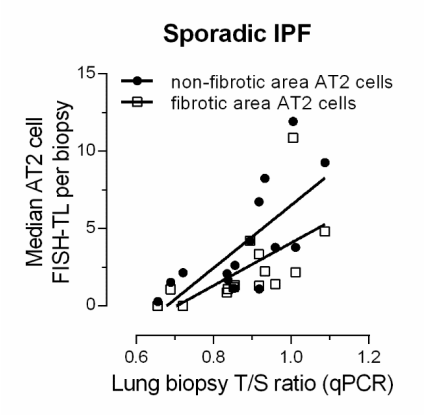

Figure 4. Correlation between FISH-TL and biopsy T/S in IPF subjects. Correlation (Spearman) between FISH-TL measured in alveolar type 2 (AT2) cells and biopsy T/S measured by MM-qPCR (n=15) in non-fibrotic areas (black dots) and fibrotic areas (open squares). Significant correlations were established between biopsy T/S and AT2 cell FISH-TL in non-fibrotic (r2=0.53, p=0.002) and fibrotic (r2=0.73, p<0.0001) areas.

Telomere length by MMqPCR: blood

We tested for a correlation between peripheral white blood cell TL measured with MM-qPCR (blood T/S) and FISH-TL of lung tissue. We found no significant correlation between AT2 FISH-TL in fibrotic areas and blood T/S, except in FIP-TERT subjects. (r^2=0.67, p=0.007) (figure 5). Also no significant correlation was found between MMqPCR measurements biopsy T/S and blood T/S (sporadic IPF; n=26, FIP-nonTERT; n=12 and FIP-TERT; n=10, figure 6).

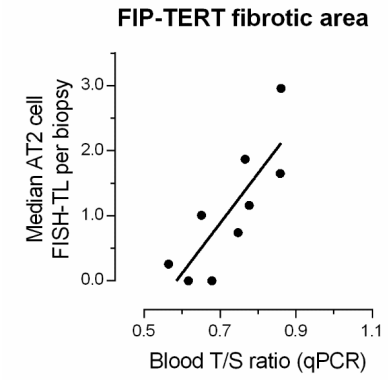

Figure 5. Correlation between FISH-TL and MMqPCR leukocyte TL (blood T/S) in FIP-TERT fibrotic areas. Positive FIP-TERT correlation (Spearman) between alveolar type 2 (AT2) cell FISH-TL in fibrotic areas and MMqPCR blood T/S (n=9, r2=0.67, p=0.007).

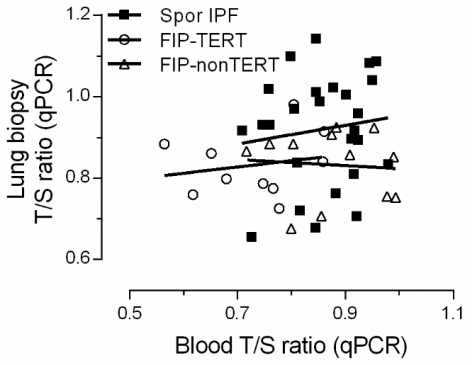

Figure 6. Correlations between MMqPCR measurements biopsy T/S and blood T/S. No correlation between biopsy T/S and blood T/S was observed in sporadic IPF (n=26), FIP-nonTERT (n=12) and FIP-TERT (n=10) subjects.

Short telomeres are associated with worse survival

In the literature, short peripheral leukocyte telomere length has been associated with worse survival time in IPF[33,34]. Here we investigated whether a shorter survival time is similarly associated with FISH-TL. We showed that in non-fibrotic regions AT2 FISH-TL variability between IPF subjects was substantial (figures 2 and 3). To test whether this variability is associated with survival we divided this group (n=15) at the median AT2 cell TL. Survival was calculated from date of biopsy until death (n=9) or censoring of the patient (lung transplantation n=3, still alive n=3). Kaplan Meier survival analysis showed that patients with shortest AT2 cell TL had a lower median survival rate than patients with longest AT2 cell TL (26 months vs 60 months, p=0.353, figure 7A). Because lack of significance could be caused by underpowered analysis and because a significant positive correlation between biopsy T/S and AT2 FISH-TL was established above, we also performed a survival analysis using biopsy T/S (n=34). Dividing the patient group at the median T/S, a significant difference in survival rate (p=0.003) was found. Patients with a low T/S had decreased median survival of 22 months and lived 41 months shorter than patients with high T/S (figure 7B). There were no significant differences in mean age at date of biopsy between the group with TL above median and the group with TL below median in either AT2 FISH-TL and biopsy T/S analyses.

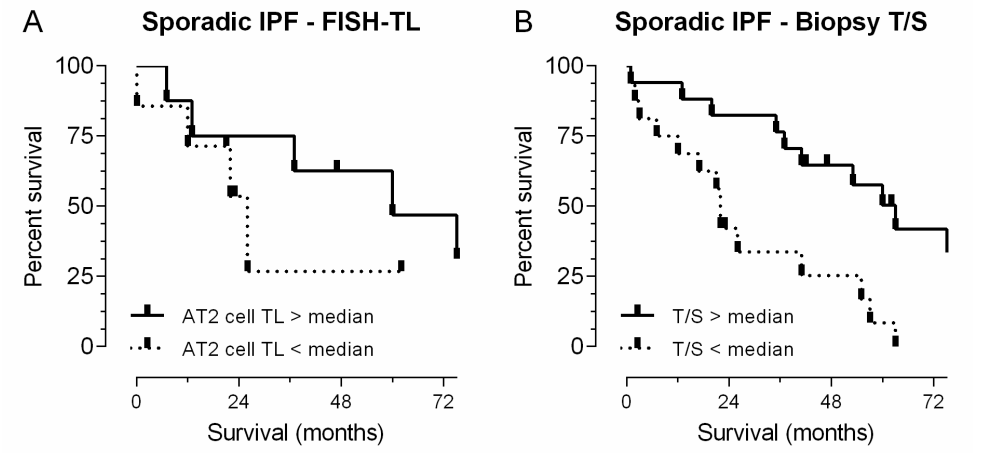

Figure 7. Survival of IPF patients. (A) FISH-TL on lung biopsy; Kaplan-Meier curve of 15 IPF patients showing a median survival of 60 months for patients with AT2 cell FISH-TL in non-fibrotic areas above median TL (solid line) and a median survival of 26 months for patients with AT2 cell TL below median TL (dotted line). The difference in median survival is 34 months (p=0.353, Mantel-Cox). (B) MMqPCR on lung biopsy; Kaplan-Meier curve of 34 IPF patients showing a median survival of 63 months for IPF patients with a lung biopsy MMqPCR T/S ratio above the median (solid line) and a median survival of 22 months for patients with a lung biopsy MMqPCR T/S ratio below the median T/S (dotted line). The significant difference in median survival is 41 months (p=0.003, Mantel-Cox). Deceased n=27, lung transplantation n=4, still alive n=3.

Discussion

In this study, we found that telomere shortening is predominantly observed in AT2 cells and associates with fibrotic lesions in IPF lung biopsies (figures 2 and 3). Furthermore, patients with short lung telomeres had significantly worse survival than patients with longer telomeres (figure 7). Telomere shortening in AT2 cells is in accordance with two experimental observations where mice with telomere repeat binding factor 1 (*TRF1*)-deleted AT2 cells develop lung fibrosis and present short telomeres in AT2 cells[20,21].

To date, no study investigated the association between AT2 TL and the characteristic non-fibrotic and fibrotic regions in human IPF lungs with (FIP-TERT) or without a mutation in the telomerase gene. In fibrotic areas of sporadic IPF and FIP-TERT subjects we found that AT2 cells contain short telomeres, confirming results of Alder and coworkers[11]. However, in contrast to sporadic IPF, FIP-TERT AT2 telomere length was equally short in non-fibrotic areas and fibrotic areas (p=0.36, figure 3A). This suggests that patients with a telomere mutation are born with "aged-short telomere containing" lungs or that their lungs age at an increased rate. The latter is most probable because we also found that telomere length of surrounding cells was similar in FIP-TERT lungs, sporadic IPF and FIP-nonTERT lungs.

The pivotal role of AT2 cells in IPF pathogenesis is highly supported by the discovery of disease causing mutations in *SFTPC*. AT2 cells exclusively produce Surfactant protein C[35–37]. Additionally, besides producing and regulating surfactant fluid in the alveoli, AT2 cells are the progenitor cells that can differentiate into the gas diffusing AT1 cells[38]. This regenerative function of AT2 cells requires active telomere maintenance[39]. Indeed, telomerase has been shown to be active and upregulated in subpopulations of rat AT2 cells after hypoxic injury[39–41]. Additionally, literature postulated that shortened telomere length in blood is a risk factor for sporadic IPF and FIP-TERT subjects[6,7,11–13,33].

To clinically target the potential pathogenic AT2 cells in fibrosis, it might be feasible in the near future to introduce AT2 cell transplantation. It has been shown in the literature that AT2 cell transplantation is safe and well tolerated in IPF patients[42]. However, the therapeutic effect on fibrogenesis still has to be elucidated.

In contrast to AT2 cells, no difference was observed in TL of surrounding cells between non-fibrotic and fibrotic areas. Additionally, in healthy lung tissue no difference was found between AT2 and surrounding cells (table 3). This also suggests that in pulmonary fibrosis, anomalous telomere shortening primarily affects AT2 cells. In fibrotic areas, no differences were found in AT2 TL (p=0.16) between subject groups. This could suggest that a critically short TL threshold must be reached for the development of fibrosis.

In general, critically short telomeres eventually lead to cell senescence or apoptosis, limiting the regenerative capacity of tissue[43,44]. Furthermore, mice with AT2 cell dysfunctional telomeres showed impaired response to induced injury[9]. In IPF an increase of apoptosis and

senescence signaling has been reported in fibrotic areas. This might be the causal link between telomere shortening and IPF onset[45–48]. Moreover, mice with critically AT2 short telomeres are linked to elevated levels of pro-fibrotic TGF- β1 release[20], which also may lead to the development of lung fibrosis[49].

Next, we showed that measuring TL using MMqPCR on DNA extracted from whole lung biopsy sections of sporadic IPF can replace TL measured by FISH (figure 4). This MMqPCR technique allows a time and labor efficient method of estimating lung telomere length and allowed us to efficiently double the sample size for survival analysis. Using whole lung biopsies, a significant negative survival was found in sporadic IPF patients with short TL (figure 7B). The association is in accordance with previous studies reporting short leukocyte telomere length (measured by MMqPCR) to negatively influence survival[33,34].

There are conflicting reports concerning the correlation between blood TL and lung FISH-TL[11,28]. In our study, TL measured in blood cells did not correlate with FISH-TL in the lung (data not shown). This is in concordance with a report by Kropski et al[28]. However, in FIP-TERT patients we did find a correlation between TL in blood and in fibrotic area AT2 cells (figure 5). This suggest that only in the presence of a *TERT* mutation telomeres in peripheral leukocytes and AT2 cells in fibrotic areas have comparable rates of shortening. Germ line *TERT* mutations affect all cells in the body and therefore TL in lung and all other cells are linked. Because the association between blood and lung telomere length is absent in sporadic patients we argue that telomere shortening in sporadic IPF patients is partly determined by the patient's genetic constitution (explaining the shortened blood telomeres) and partly by lung specific factors (explaining the absence of a correlation between blood and lung telomere length). This is in accordance with the second hit theory in IPF, which implies that besides telomere shortening a local second hit, like a virus or smoking, might be responsible for elevated cell stress and development of fibrosis[16,50].

Given the influence of biopsy T/S on survival it might be useful to incorporate lung TL as a prognostic molecular biomarker in the interpretation and stratification of ongoing and future clinical trials. However, since the risk of complications of surgical lung biopsies is high[51,52] and diagnosis in IPF is often based on typical radiographic pattern of usual interstitial pneumonia, future studies will need to focus on less invasive methods to assess TL.

Strength of this study comprises the inclusion of three types of IPF cohorts; sporadic IPF, FIP-TERT and FIP-nonTERT. Comparison of TL between these groups is novel in this field of research. Furthermore, the comparison of fibrotic and non-fibrotic areas in one biopsy make the result independent of inter-assay differences. However, there are also some limitations in this study. The FIP-nonTERT group was chosen from familial patients with no telomere-related gene mutations. Screening was performed for *TERT*, *TERC*, *SFTPC*, *SFTPA2* exon 6 and *TINF2* exon 6. Other previously described IPF-related mutations, e.g. in dyskeratosis congenital 1 (*DKC1*), regulator of telomere elongation helicase 1 (*RTEL1*) and Poly(A)-specific ribonuclease (*PARN*) were not tested[8,32]. Therefore, we cannot exclude the presence of telomere-related genes in the FIP-nonTERT cohort.

In conclusion, this study shows that shortest telomeres are found in AT2 cells in fibrotic areas

of IPF lung. Furthermore we show that short telomeres associate with shorter survival time. This provides new evidence for a critical role of short AT2 cell TL in the pathogenesis of IPF and maintenance of telomere length as a target for therapy.

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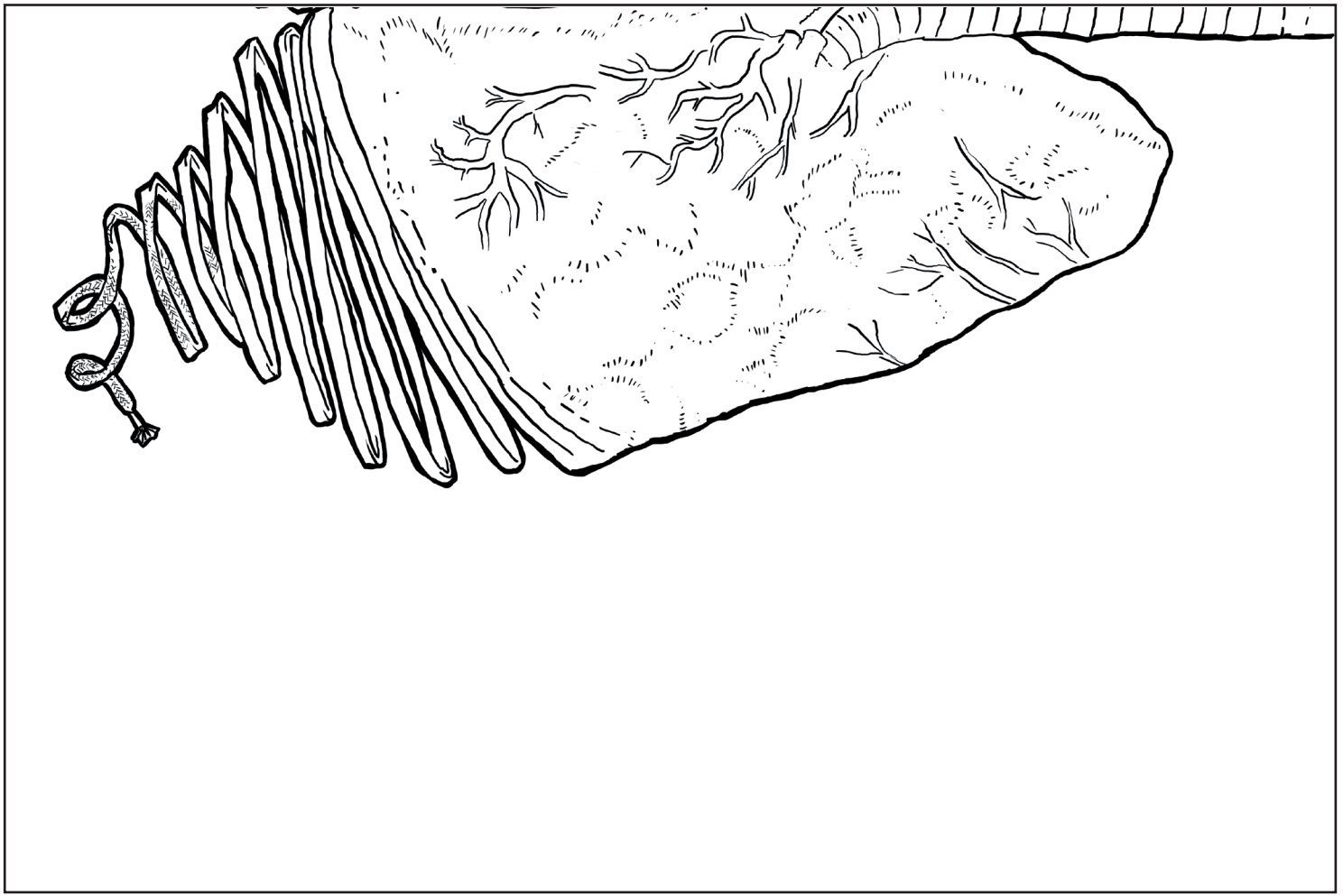

4

Effect of MUC5B Promoter Polymorphism on Disease Predisposition and Survival in Idiopathic Interstitial Pneumonias

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Abstract

Background and objective:

A common polymorphism in the *MUC5B* gene (rs35705950) is associated with susceptibility to idiopathic pulmonary fibrosis (IPF) and familial interstitial pneumonia (FIP). We investigated predisposition of the *MUC5B* polymorphism to fibrotic interstitial pneumonias in Dutch Caucasian patient cohorts. Furthermore, we investigated the correlation between *MUC5B* genotype and survival in these cohorts.

Methods:

Sporadic IPF (spIPF, n=115), FIP (n=55), idiopathic nonspecific interstitial pneumonia (iNSIP, n=43), connective tissue disease associated interstitial pneumonia (CTD_IP, n=35) and a control cohort (n=249) were genotyped for rs35705950.

Results:

Rs35705950 minor allele frequency (MAF) in controls was 0.09. Case-control analysis showed significant allelic association with spIPF (MAF=0.27;$p=5.0x10^{-10}$), FIP (MAF=0.30;$p=2.7x10^{-9}$) and iNSIP (MAF=0.22;$p=3.4x10^{-4}$). No association was observed in CTD_IP (MAF=0.07). FIP subgroup analysis revealed an association between *MUC5B* and telomerase mutated FIP (p=0.003), and between *MUC5B* and FIP with unknown genetic cause ($p=1.2x10^{-8}$). In spIPF carriership of *MUC5B* minor allele did not influence survival. In FIP *MUC5B* minor allele carriers had better survival (non-carriers 37 vs. carriers 53 months, p=0.01). In iNSIP survival analysis showed an opposite effect. Worse survival was found in iNSIP patients that carried the *MUC5B* minor allele (non-carriers 118 vs. carriers 46 months, p=0.027)

Conclusions:

This study showed that *MUC5B* minor allele predisposes to spIPF, FIP and iNSIP. In spIPF, survival is not influenced by *MUC5B* alleles. In FIP, *MUC5B* minor allele predicts better survival, pointing towards a subgroup of FIP patients with a milder, MUC5B-driven form of pulmonary fibrosis.

Introduction

The idiopathic interstitial pneumonias (IIP) form a heterogeneous group of diffuse parenchymal lung diseases (DPLD) characterized by varying patterns of inflammation and fibrosis of the lung parenchyma. Idiopathic pulmonary fibrosis (IPF) is the most common IIP and forms together with idiopathic nonspecific interstitial pneumonia (iNSIP) the subcategory of chronic interstitial pneumonias[1]. IPF is a progressive disease with a median survival of approximately 3 years[2]. The prognosis is variable and hard to predict in individual cases. In contrast to IPF, the prognosis of iNSIP is relatively good, with 74% survival at 5 years, because most patients respond to treatment with corticosteroids or cytotoxic agents[3]. Interstitial pneumonia (IP) occurs not only as an idiopathic form, but can also evolve secondary to other diseases such as connective tissue disease (CTD).

A significant percentage of IIP patients report to have familial disease. The genetic basis of FIP has been partially uncovered and has elucidated biological mechanisms that are involved in the pathogenesis of pulmonary fibrosis. Specific mutations leading to IIP, have been discovered in the genes surfactant protein C and A2 (*SFTPC* and *SFTPA2*), telomerase reverse transcriptase gene (*TERT*) and telomerase RNA component gene (*TERC*)[4–7]. Recently, linkage and fine mapping analysis identified a common variant (rs35705950) in the putative promoter of the gene encoding mucin 5B (*MUC5B*) that is related to disease development of IPF, familial interstitial pneumonia (FIP) and iNSIP[8–10]. Previous studies did not find an association between *MUC5B* promoter polymorphism and development of pulmonary fibrosis in patients with systemic sclerosis[11–13]. Moreover, the risk allele of *MUC5B* was significantly associated with improved survival in IPF[14].

In this study we investigated whether the *MUC5B* promoter polymorphism is associated with development of different forms of IP. We therefore included sporadic IPF (spIPF) patients, FIP patients with a *TERT* mutation and those without a known mutation, iNSIP patients and IP-CTD patients. Furthermore, we investigated if the *MUC5B* promoter polymorphism is associated with survival outcome in the different IIP cohorts.

Material and Methods

Subjects

A total of 248 unrelated Caucasian patients with IP were included for *MUC5B* rs35705950 genotyping: 115 spIPF, 55 FIP, 43 iNSIP and 35 CTD_IP patients. FIP was defined as two or more first-degree family members with IIP. For each family one designated proband was included in the analysis. FIP patients were screened for mutations in *SFTPC*, *SFTPA2*, *TERT* or *TERC*. Based on these results FIP patients were distributed over two subgroups: FIP_unknown-cause with patients that had no mutation in one of these genes, and FIP_TERT with patients who carried a *TERT* mutation.

For rs35705950 dependent survival analysis 115 IPF patients, 55 FIP patients and 43 iNSIP were included. All patients visited the Centre for Interstitial Lung Diseases at the St. Antonius Hospital Nieuwegein. IP diagnoses were established by an experienced multidisciplinary team, in accordance with American Thoracic Society/European Respiratory Society criteria. Diagnoses made before 2002 were reviewed by an experienced clinician and included when the diagnoses met the current guidelines[1,2,15]. The control subjects comprised 249 healthy, unrelated Dutch Caucasians. Local ethics review board of the St Antonius Hospital gave medical-ethical approval for this study (R05-08A) and all subjects gave written informed consent.

Clinical parameters

From spIPF, FIP and iNSIP patients we collected age at diagnosis, survival data, DLCO% predicted and FVC% predicted at diagnosis and smoking status. The interval between pulmonary function testing and time at diagnosis was less than three months. Age at diagnosis and survival data were available of all patients. DLCO% predicted data were available of 82 IPF, 41 FIP and 26 iNSIP patients, FVC% predicted data were available of 81 IPF, 39 FIP and 28 iNSIP patients. Further, smoking status was available of 109 IPF, 53 FIP and 39 iNSIP patients.

DNA analysis

Genomic DNA was extracted from peripheral blood of each individual using standard methods. A pre-designed taqman SNP genotyping assay and an ABI 7500Fast analyser (Applied Biosystems, Foster City, CA) were used to genotype rs35705950.

Statistical analysis

SPSS 22 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 5 (Graphpad software INC., San Diego, CA, USA) was used for statistical analysis. The Mann-Whitney U test was used to analyse differences between carriers and non-carriers of the rs35705950 minor allele. The Kaplan-Meier method with log-rank test was used to analyse if rs35705950 carriership was associated with survival time. Patients who underwent lung transplantation were censored. Differences between the allele frequencies were calculated with the Pearson's goodness-of-fit Chi-square test, together with the Odd Ratio (OR) and 95%CI. Fisher's exact test were used

to test for deviation from Hardy-Weinberg equilibrium. Computations were performed online at: http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Differences with a p-value<0.05 were considered statistically significant.

The power of this study was >96% for all cohorts, based on the FIP allele frequency of 33.8% published by Seibold et al[8].

Results

Clinical and demographic characteristics of IIP patients are summarised in table 1.

Table 1. Clinical and demographic characteristic of the IIP cohorts

	spIPF	FIP	iNSIP
n	115	55	43
Characteristics			
survival time*), median (SE), mo	41 (5.1)	47 (3.1)	98 (23.9)
Deaths, n (%)	81 (70)	28 (51)	23 (53)
transplant event, n (%)	14 (12)	10 (18)	1 (2)
male, n (%)	97 (84)	38 (69)	32 (74)
age at diagnosis, mean (SD), y	63.5 (11.0)	57.6 (11.6)	61.5 (11.8)
ever smokers, n (%)	77 (71)	34 (64)	23 (59)
FVC % predicted, mean (SD)	71.4 (23.4)	79.6 (27.1)	76.15 (18.7)
DLCO % predicted, mean (SD)	44.0 (15.7)	47.2 (27.1)	43,8 (15.7)
MUC5B MAF rs35705950	0.27	0.30	0.22

*) Kaplan-Meier survival analysis

Abbreviations: spIPF, sporadic idiopathic pulmonary fibrosis; FIP, familial interstitial pneumonia; iNSIP, idiopathic non-specific interstitial pneumonia; SE, standard error; SD, standard deviation; y, year; mo, months; FVC, forced vital capacity; DLCO, diffusing capacity of the lungs for carbon monoxide; MAF, minor allele frequency

Genotype frequencies for rs35705950 were consistent with Hardy-Weinberg equilibrium in all subgroups. Case-control analysis showed significant allelic association of rs35705950 with spIPF, FIP and iNSIP. The minor allele frequencies (MAF) in spIPF, FIP and iNSIP were 27%, 30% and 22% respectively compared to a MAF of 9% in controls (table 2). CTD_IP was not significantly associated with the rs35705950 minor allele. The MAF of iNSIP was also significantly higher than that of the CTD_IP cohort (22% vs 7%, p=0.01). Subgroup analysis of the FIP cohort revealed a significant association of rs35705950 minor allele with FIP-TERT (p=0.003) and FIP-unknown cause ($p=1.2x10^{-8}$) (table 2). Odds Ratio (OR) for disease among heterozygous subjects and homozygous subjects for the minor allele are presented in table 3.

In spIPF, comparison of survival between carriers and non-carriers of the rs35705950 minor allele showed no significant difference in survival (p=0.80, figure 1a). There were also no significant associations between *MUC5B* minor allele carriership and age at diagnosis, DLCO% predicted at diagnosis, FVC% predicted at diagnosis, gender and smoking status (ever vs never).

Table 2. *MUC5*B rs35705950 genotype distribution and minor allele frequency in spIPF, FIP, iNSIP and CTD_IP compared to controls

	n	GG (%)	GT (%)	TT (%)	MAF	p value
Control	249	205 (82)	43 (17)	1 (0)	0.09	
spIPF	115	59 (51)	51 (45)	5 (4)	0.27	$5.0x10^{-10}$
FIP	55	24 (44)	29 (53)	2 (4)	0.30	$2.7x10^{-9}$
FIP_unknown-cause	27	10 (37)	15 (56)	2 (7)	0.35	$1.2x10^{-8}$
FIP_TERT	18	8 (44)	10 (56)	0	0.28	0.003*)
iNSIP	43	26 (60)	15 (35)	2 (5)	0.22	$3.4x10^{-4}$
CTD_IP	35	30 (86)	5 (14)	0	0.07	0.60

*) Fisher exact test

Abbreviations: MAF, minor allele frequency; OR, Odds Ratio; spIPF, sporadic type of idiopathic pulmonary fibrosis; FIP, familial interstitial pneumonia; FIP_TERT, FIP caused by mutation in *TERT* gene; FIP_SFTP, FIP caused by mutation in *SFTPC* or *SFTPA2* gene; iNSIP, idiopathic non specific interstitial pneumonia; CTD_IP, connective tissue disease associated interstitial pneumonia.

Table 3. Odds ratio for disease among subjects heterozygous and homozygous for the *MUC5B* rs35705950 minor allele

		heterozygous			homozygous		
	n	OR	95% CI	p value	OR	95% CI	p value
Control	249						
spIPF	115	4.1	2.5 - 6.8	$8.5x10^{-9}$	17.4	2.0 - 152	$5.1x10^{-4}$
FIP	55	5.8	3.1 - 10.8	$7.0x10^{-9}$	17.1	1.5 - 195	0.002
FIP_unknown-cause	27	7.2	3.0 – 17.0	$6.7x10^{-7}$	41	3.4 - 491	$2.9x10^{-6}$
FIP_TERT	18	6	2.2 – 16.0	$9.0x10^{-5}$	NA		
iNSIP	43	2.8	1.3 - 5.6	0.004	15.8	1.4 - 180	0.003
CTD_IP	35	0.8	0.3 - 2.2	0.7	NA		

Abbreviations: MAF, minor allele frequency; OR, Odds Ratio; CI, confidence interval; spIPF, sporadic idiopathic pulmonary fibrosis; FIP, familial interstitial pneumonia; FIP_TERT, FIP caused by mutation in *TERT* gene; FIP_SFTP, FIP caused by mutation in *SFTPC* or *SFTPA2* gene; iNSIP, idiopathic non specific interstitial pneumonia; CTD_IP, connective tissue disease associated interstitial pneumonia; NA, not applicable.

In FIP, comparison of survival between carriers and non-carriers of the rs35705950 minor allele showed a significantly longer survival for patients carrying the minor allele (p=0.011; figure 1b). There were no differences in age at diagnosis, DLCO% predicted at diagnosis, FVC% predicted at diagnosis, gender and smoking status between carriers and non-carriers of the rs35705950 minor allele. Subgroup analysis of the FIP cohort confirmed the association between better survival and minor allele carriership in the FIP_unknown-cause cohort (p=0.002; figure 1c).

In iNSIP, comparison of survival between carriers and non-carriers of the rs35705950 minor allele showed a significantly worse survival for patients carrying the minor allele (p=0.027: figure 1d). There were no differences in DLCO% predicted at diagnosis, FVC% predicted at diagnosis, gender and smoking status between carriers and non-carriers of the rs35705950 minor allele.

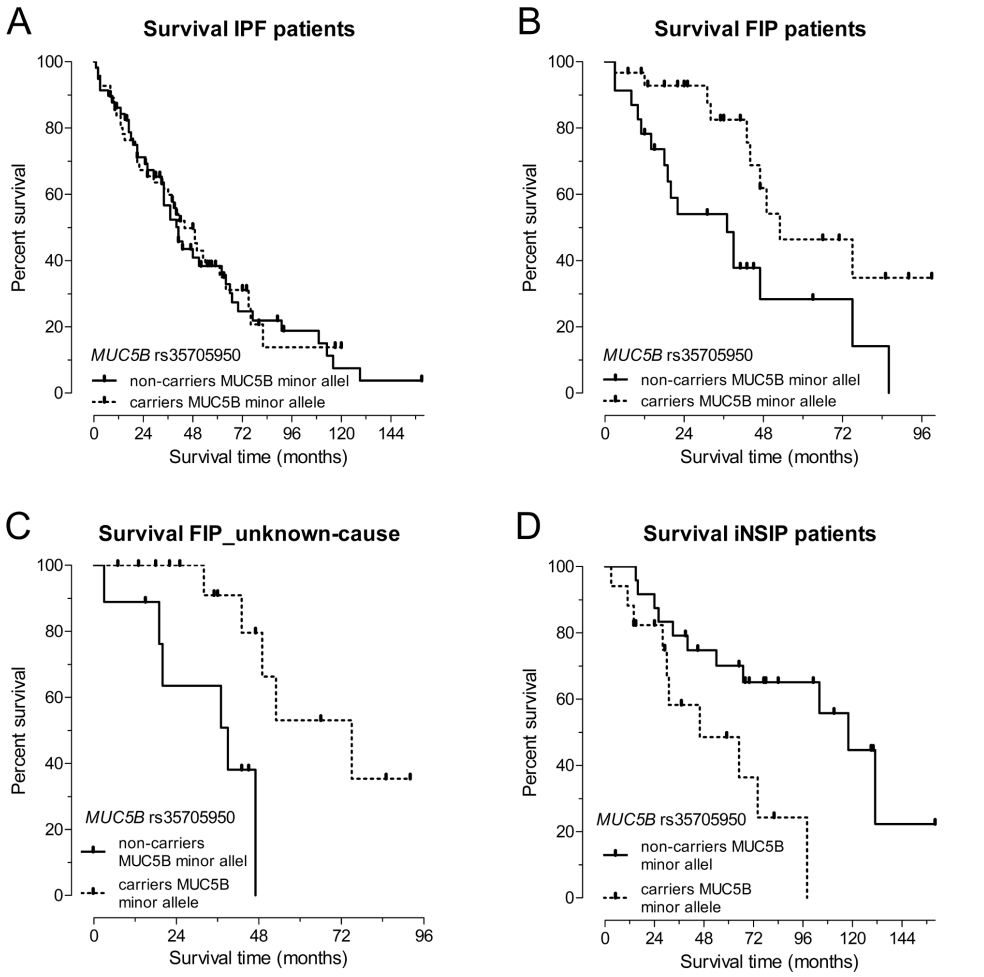

Figure 1. Survival of carriers versus non-carriers of MUC5B minor allele per IIP cohort. Patients who underwent a lung transplantation or lost to follow up were censored. **A**: Kaplan-Meier curve of IPF patients showing a median survival of 40 months in non-carriers of the *MUC5B* minor allele (n=59; dashed line) and a median survival of 44 months in carriers of the *MUC5B* minor allele (n=56; solid line). There is no significant difference between the groups (p=0.80). **B**: Kaplan-Meier curve of FIP patients showing a median survival of 37 months in non- carriers of the *MUC5B* minor allele (n=24; dashed line) and a median survival of 53 months in carriers of the *MUC5B* minor allele (n=31; solid line). The difference between the two curves is statistically significant, p=0.01. **C**: Kaplan-Meier curve of FIP_unknown-cause patients showing a median survival of 37 months in non-carriers of the *MUC5B* minor allele (n=10; dashed line) and a median survival of 75 months in carriers of the *MUC5B* minor allele (n=17; solid line). The difference between the two curves is statistically significant, p=0.002. **D**: Kaplan-Meier curve of iNSIP patients showing a median survival of 118 months in non-carriers of the *MUC5B* minor allele (n=26; dashed line) and a median survival of 46 months in carriers of the *MUC5B* minor allele (n=17; solid line). The difference between the two curves is statistically significant, p=0.027.

Discussion

This study showed that the *MUC5B* rs35705950 minor allele predisposes to iNSIP and not to CTD_IP, and confirmed the association of *MUC5B* promoter polymorphism with IPF and FIP in Dutch patients. This is the first study showing that the *MUC5B* promoter polymorphism is a significant predictor of disease progression in FIP and iNSIP.

In previous studies *MUC5B* rs35705950 minor allele was found to predispose to IPF[8–12,14,16]. Only one study found a significant improved survival for carriers of the GT and TT *MUC5B* risk genotypes which was replicated in a second cohort[14]. A survival benefit of *MUC5B* minor allele carriage could not be found in our spIPF cohort. However in FIP, survival analysis revealed significant better survival for carriers of the *MUC5B* minor allele. To the best of our knowledge, in previously used IPF cohorts for survival analysis, familial patients were not strictly excluded. Familial anamneses in our outpatient clinic are extensively checked by physicians and additionally all patients are asked to fill out a questionnaire regarding disease status in their family. It has been found that our centre has a high number of familial patients, many with mutations in causal genes[17,18]. As a consequence, and in contrast to previous studies, the IPF cohort used in this study is completely made up of patients with sporadic disease. From this study we conclude that in true sporadic patients *MUC5B* minor allele carriership does not influence survival. In patients with familial disease however, we found that carriership of the *MUC5B* minor allele does associate with better survival.

Previously, the *MUC5B* minor allele had been associated with less severe pathological changes in FIP[19]. This observation corresponds to the improved survival that we found for FIP patients who carry the *MUC5B* minor allele. Interestingly, the results are most prominent in the FIP_unknown–cause subgroup. No causal mutation in *TERT*, *TERC*, *SFTPC* or *SFTPA2* was identified in the FIP_unknown–cause subgroup. These results suggest that penetrance of disease in *MUC5B* risk allele carriers in these families is several magnitudes higher than in the general population. In families where *MUC5B* plays such a prominent role it appears to positively influence survival. Overall, the results suggest that in FIP a subgroup of patients with a milder, MUC5B-driven form of pulmonary fibrosis can be distinguished.

We found that the *MUC5B* rs35705950 minor allele predisposes to iNSIP. This finding confirms the study of Horismashu and co-workers, which showed a comparable association in a German iNSIP cohort[10]. Combining results from the published German (n=31, MAF=27%) and current Dutch (n=43, MAF=22%) iNSIP cohorts reveals a significant association (n=74, MAF=24%, $p=9.0x10^{-08}$) with an OR for disease of 3.5 (95%CI: 2.2-5.6).

Remarkably, survival analysis in our iNSIP revealed significantly worse survival for carriers of the *MUC5B* minor allele compared to non-carriers (46 vs. 118 months). While median survival of the total iNSIP cohort was 98 months. Survival of iNSIP patients carrying *MUC5B* minor allele is concordant with survival time of that observed in spIPF patients (46 vs. 42 months). This data suggest that iNSIP patients carrying the *MUC5B* risk allele have disease

progression similar to that found in spIPF patients. In the future, *MUC5B* genotyping could be of added value in disease management of iNSIP, but further research is needed to confirm the findings and to investigate if resistance to corticosteroid treatment in iNSIP is also associated with *MUC5B* promoter polymorphism.

We showed that the *MUC5B* rs35705950 minor allele did not predispose to CTD_IP. This finding confirms previous negative observations in studies comparing allele frequencies between systemic sclerosis and controls[11–13]. Furthermore, the association between *MUC5B* genotype and development of spIPF, FIP and iNSIP suggests similarities in pathogenesis of these diseases. While the significant difference in allele frequency between iNSIP and CTD_IP indicates that *MUC5B* is not involved in immunological and inflammatory pathways leading to pulmonary fibrosis in patients with connective tissue disease.

The association of *MUC5B* promoter polymorphism and development of pulmonary fibrosis was also present in patients with a *TERT* mutation, 10 out of 18 patients with a *TERT* mutation were carrier of the rs35705950 risk allele. Mutations in *TERT* or *TERC* are associated with several telomere related diseases such as dyskeratosis congenita, aplastic anaemia, liver cirrhosis and IPF[6,7,20–22]. One specific mutation in *TERT* can lead to different phenotypes not always involving the lungs[23]. Because of this, it is appealing to hypothesize that patients with a *TERT* mutation and at least one *MUC5B* rs35705950 minor allele are more prone to lung involvement than patients with a *TERT* mutation who do not carry this risk allele. As such, genotyping of *MUC5B* rs35705950 does not only provide information on disease predisposition and prognosis in IIP, but might also be used to predict organ involvement in families with telomeropathies. Further research is needed to understand the role of rs35705950 minor allele carriership in lung involvement of families with a mutation in telomere maintenance genes.

A limitation of this study is the relatively small sample size of the iNSIP and FIP-subgroups to investigate predisposition to disease. Also, regarding the effect of *MUC5B* genotype on survival time in both iNSIP and FIP, further studies using larger cohorts are necessary to replicate the findings of this study.

In conclusion, this study showed that *MUC5B* promoter polymorphism predisposes to spIPF, FIP and iNSIP. In spIPF, survival is not influenced by *MUC5B* alleles. In FIP, *MUC5B* minor allele predicts better survival, pointing towards a subgroup of FIP patients with a milder, MUC5B-driven form of pulmonary fibrosis. Remarkably *MUC5B* minor allele associates with worse survival in iNSIP.

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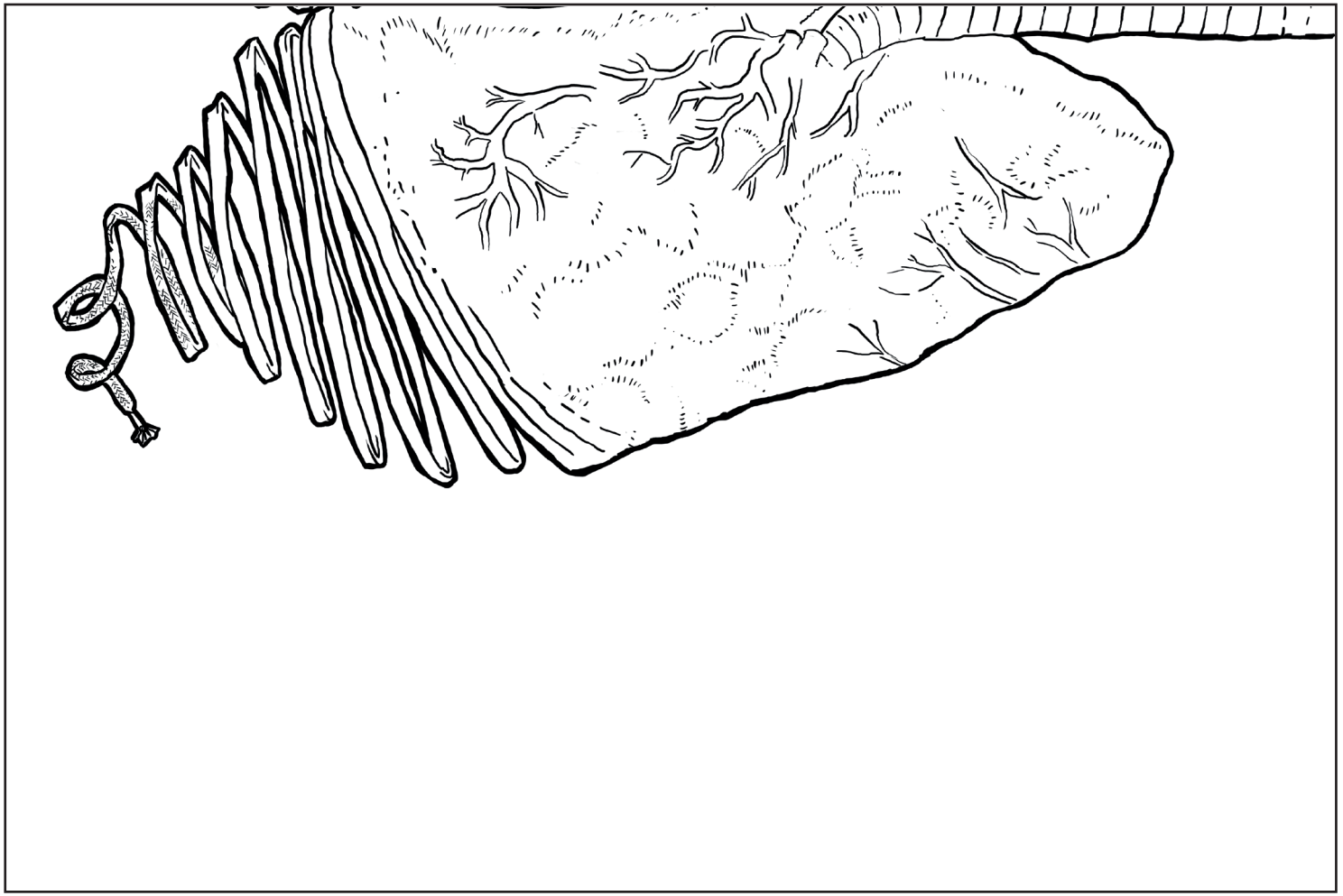

5

Common Variants in TERT and TERC Predispose to Idiopathic Pulmonary Fibrosis and Decreased Survival

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a rare lung disease characterized by progressive scarring of the lung and a poor life expectancy. Short leukocyte telomere length is a common feature in IPF. In healthy individuals, telomere length is associated with common genetic variants in telomerase genes. Therefore, we aimed to test these common variants as risk alleles for disease and survival in sporadic and familial IPF patients.

Four genetic variants in the Telomerase RNA complex (*TERC*) and three variants in Telomerase reverse transcriptase (*TERT*), which have been shown to associate with telomere maintenance were tested. Analysis was performed on two separate cohorts. The cohort consisted of 106 IPF patients, 24 patients with familial interstitial pneumonia (FIP), not carrying a disease causing mutation in telomerase or surfactant genes, and 148 healthy controls. All genotyped variants, except *TERT* variant MNS16A, were found to be associated with susceptibility to disease ($p<0.05$). Using haplotype analysis, an increased frequency of both a *TERC* and a *TERT* risk allele haplotype was found in both patients groups, compared with controls. Carriership of both risk haplotypes leads to a significantly increased risk for disease (OR=2.40 [CI95%=1.35-4.28], p=0.01) and a significantly lower survival rate in patients (p=0.001). Median survival in carriers was 20 months compared to 50 months in non-carriers. Combining risk haplotype data with mucin 5B (*MUC5B*) rs35705950 risk allele data showed that carriers of all risk alleles had an increased susceptibility to IPF in the sporadic and familial cohort (OR=11.20 [CI95%=3.22-39.0], $p=9.0 \cdot 10^{-6}$; OR=19.90 [CI95%=4.70-84.24], $p<0.001$ resp.).

Here we demonstrate the importance of variation in telomerase biology genes in IPF patients. Carriership of the common, telomere related risk alleles have a significant effect on disease predisposition and survival. Concomitant carriership of the *MUC5B* risk allele further increases IPF susceptibility. This suggests that multiple risk loci act together in the development of IPF and FIP.

Introduction

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease, characterized by progressive scarring of the lung and subsequent shortness of breath[1]. The median survival is 2-5 years and due to the unknown aetiology of this disease, no effective curative therapy is available[1,2]. Up to 18% of IPF cases have been reported to be familial[3–5]. A presumed genetic background for the familial form was confirmed by the discovery of disease causing mutations in the surfactant protein genes A2 and C and telomere maintenance genes telomerase reverse transcriptase (*TERT*) and telomerase RNA component (*TERC*)[3,5–9]. Together these mutations explain disease in up to 25% of familial cases but only 1-3% of sporadic cases[4].

TERT and *TERC* encode the two components of the ribonucleoprotein telomerase[10]. Telomerase maintains the length of telomeres, the non-coding ends of chromosomes, thereby protecting the coding DNA from chromosome shortening that is inherent to the cell-division cycle. Critical loss of telomere length induces cell senescence to prevent chromosomal instability. Telomerase is therefore an essential component in maintaining the proliferative potential of cells[11].

Mutations in the telomerase genes are found in up to 18 percent of familial patients[4]. Patients carrying these mutations were shown to have diminished telomerase function and abnormally short leukocyte telomere length[3,5]. In sporadic IPF, mutations in *TERT* and *TERC* are very rare (~3%), however short telomeres are a common feature (~50%)[12–14]. This has led to the hypothesis that not only the mutations in *TERT* and *TERC*, but short telomeres itself are a risk factor for IPF[13]. The cause of the short telomeres in non-mutation carriers however is unclear.

Potential factors contributing to shortness of telomeres are common genetic variants in the telomerase genes. The variant rs2736100 in the *TERT* gene, is associated with inter-individual variation in mean telomere length[15]. Interestingly, this variant is also associated with IPF susceptibility[16]. Besides rs2736100, several common variants (Minor allele frequency (MAF)>0.20) in the *TERT* and *TERC* region, have been associated with telomere maintenance in literature. One other genetic variants that is a known risk factor for IPF, is rs35705950, a SNP in the promotor region of Mucin 5B (*MUC5B*)[17,18]. Mucins have a barrier function in the lung and have a role in innate immunity[19], however the role of rs35705950 in the pathogenesis of IPF remains unclear.

The aim of this study is first to further explore the association between genetic variants in the *TERT* and *TERC* gene region and IPF susceptibility, and to further explore the association of these variants in combination with the disease predisposing *MUC5B* SNP. Therefore we analyzed our findings in *TERT* and *TERC*, together with genotype data in our cohort on variant *MUC5B* variant rs35705950[20].

Material and Methods

Human Subjects

For this study, 106 patients diagnosed at the Department of Pulmonology, St. Antonius hospital Nieuwegein with sporadic idiopathic pulmonary fibrosis and 24 patients with familial interstitial pneumonia (FIP) were analysed. FIP patients did not carry any mutation in telomerase or surfactant genes. Diagnosis was in accordance with current ATS/ERS criteria for interstitial pneumonia[1,21]. Patients were considered to have familial disease when idiopathic IP was present in a first or second degree family member. A total of 148 self-reported healthy employees of the St. Antonius Hospital were included as the control group.

The medical ethical committee of the St. Antonius Hospital in Nieuwegein approved this study and subjects gave formal written informed consent.

Variant selection

Candidate genetic variants were selected from literature. Genetic variants in *TERT* or *TERC*, reported to be associated with telomere length or telomerase function were selected for analysis. Four single nucleotide polymorphisms (SNP) in *TERC*: rs2293607[22,23], rs16847897[24,25], rs12696304[25–27], rs3772190[26,28] and three variants in *TERT*, namely the SNPs rs2736100[16,17] and rs2735940[29] and the Variable number tandem repeat (VNTR) MNS16A30. The VNTR MNS16A variants of different lengths are classified as either Long or Short[30,31].

Genotyping

Genomic DNA was extracted from peripheral blood using standard methods. SNP genotyping of the *TERC* variants and VNTR MNS16a was conducted using the Restriction Fragment Length Polymorphism (RLFP) technique. Primers were designed to amplify the regions around rs16847897, rs12696304, rs3772190 and rs2293607. Restriction enzyme sites distinguished the two alleles of the SNPs (table 1). Restriction digests were separated on 2% agarose gel by electrophoresis. MNS16a primers were used as previously reported[30].

Rs2736100, rs2735940 and rs35705950 were analyzed by a predesigned TaqMan genotyping assays using the ABI 7500Fast analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1. RLFP details per common genetic variant

Variant	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3	[MgCl2] (mM)	restriction enzyme	incubation time restriction (h)
rs16847897	GCCCTCCTTTTC GGCCTGAG	GGAGCTCACA GGCACCCCTT	3	CAC8I	2
rs12696304	AGACATGCACTT GTCTGTAGTTCA	CCTCTAGAGTC CACTGTTGCT	3	Hpy166II	16
rs3772190	CCCAAACCCACT CCCCAACCC	TGCTCAAGCCA GCACACTGACC	3	BsmAI	2
rs2293607	CCCACTGCCACC GCGAAGAG	GAACGGCTCC AGGCAACCCC	3	BsrDI	2
MNS16A	AGGATTCTGATC TCTGAAGGGTG	TCTGCCTGAGG AAGGACGTATG	1.5	N/A	N/A

Statistics

Statistical analysis on genotype and allele frequency associations between cases and controls was done using the publicly available web application provided by the Institute of Human Genetics, Munich, Germany (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). In this application Pearson's goodness-of–fit Chi-square test was used to test for deviation from the Hardy-Weinberg equilibrium and for genotype and allele frequency associations. Cochran–Armitage test for trend was used to test for additive effect (weights: 0, 1, 2). The software package Phase 2.1.1[32,33] was used to determine Haplotype frequencies. The minimum haplotype reconstruction probability score for the *TERC* and *TERT* risk haplotype was >0.970, except in one control where the score was 0.798. Haploview 4.2 (Daly Lab, Broad Institute, Cambridge, MA, USA) was used for Haplotype analysis. Survival analysis was done using Kaplan-Meier estimation and Cox regression in IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA).

Results

Common variants associated with IPF susceptibility

Genotype and allele frequencies for the *TERC* and *TERT* variants did not deviate from Hardy-Weinberg equilibrium, except for the genotype distribution of variant MNS16A in the sporadic IPF cohort and rs2736100 in the FIP cohort ($p<0.05$, genotype data not shown).

A significant difference in allele frequency was observed between IPF cases and controls for all tested SNPs in *TERC* and for variants rs2736100 and rs2735940 in *TERT* (table 2). No difference in allele frequency was found for MNS16A. Risk allele carriership of all *TERC* variants and *TERT* variant rs2736100 was significantly associated with susceptibility to IPF in this cohort. For these specific variants we also found a significant ($p\leq0.01$) additive effect of carriership of the risk allele. For variant MNS16A we found an association only between homozygous carriership of the short variant and IPF susceptibility (OR=2.96 [1.30-6.77], p=0.008).

Similar risk allele association for all variants in *TERC* and *TERT*, except MNS16A, was found in the FIP cohort. Furthermore, in the FIP cohort, similar carriership associations were found for all variants in *TERC* and for *TERT* variant rs2736100, but also for variant rs2735940 ($p<0.05$) (table 2). For all *TERT* and *TERC* variants we also found a significant additive effect ($p<0.05$). In the FIP cohort, no association was found for variant MNS16A.

Table 2. Allele frequency in controls, IPF and FIP cases

gene	variant	Controls (n=148)	Sporadic IPF cohort (n=106)			FIP cohort (n=24)		
		allele fequency	allele fequency		risk allele carriership	allele fequency		risk allele carriership
				OR [95%CI]	OR [95%CI]		OR [95%CI]	OR [95%CI]
TERC	rs12696304	C 0.78 G[1] 0.22	C 0.67 G 0.33	OR=1.72* [1.15-2.55]	OR=1.79* [1.08-2.96]	C 0.60 G 0.40	OR=2.33* [1.23-4.42]	OR=2.66* [1.10-6.48]
TERC	rs2293607	A 0.80 G[1] 0.20	A 0.69 G 0.31	OR=1.74* [1.16-2.61]	OR=1.99* [1.20-3.31]	A 0.60 G 0.40	OR=2.58* [1.35-4.91]	OR=3.08* [1.26-7.51]
TERC	rs3772190	C 0.79 T[1] 0.21	C 0.69 T 0.31	OR=1.73* [1.15-2.59]	OR=1.97* [1.19-3.28]	C 0.60 T 0.40	OR=2.52* [1.33-4.80]	OR=2.99* [1.22-7.29]
TERC	rs16847897	G 0.76 C[1] 0.24	G 0.65 C 0.35	OR=1.72* [1.18-2.56]	OR=2.05* [1.28-3.53]	G 0.60 C 0.40	OR=2.08* [1.10-3.93]	OR=2.51* [1.03-6.12]
TERT	MNS16A	L 0.69 S 0.31	L 0.64 S 0.36	OR=1.29 [0.89-1.87]	OR=0.86 [0.52-1.42]	L 0.74 S 0.26	OR=0.89 [0.45-1.77]	OR=0.76 [0.32-1.83]
TERT	rs2736100	C 0.57 A[1] 0.43	C 0.39 A 0.61	OR=2.08* [1.45-2.98]	OR=3.03* [1.54-5.97]	C 0.31 A 0.69	OR=2.89* [1.50-5.54]	OR=20.87* [1.24-351]
TERT	rs2735940	A 0.54 G[1] 0.46	A 0.44 G 0.56	OR=1.47* [1.04-2.11]	OR=1.48 [0.83-2.65]	A 0.33 G 0.67	OR=2.35* [1.24-4.47]	OR=4.51* [1.02-20.00]

Allele frequencies of variants rs2293607, rs16847897, rs12696304 and rs3772190 in *TERC* and rs2736100, rs2735940 and mns16A in *TERT* for controls, idiopathic pulmonary fibrosis (IPF) patients and familial interstitial pneumonia (FIP) patients. Odds ratios (OR) are given for differences in allele frequency and in risk allele carriership in controls versus IPF and versus FIP. The OR 95% confidence interval is shown between brackets. Significant differences are marked with an asterisk (*). Risk alleles are marked with [1]. L = Long variant and S = short variant of MNS16A.

Haplotype analysis

Figure 1 shows the observed linkage disequilibrium (LD) between the genotyped alleles per gene in our study population. We observed linkage disequilibrium, with r^2 values ranging from 0.41 to 0.98, between the four variants in *TERC* and between rs2736100 and rs2735940 in *TERT*. Haplotypes in each gene were derived from SNPs with LD higher than 0.4 (table 3). In *TERC* all 4 four SNPs were used and five haplotypes were found with a frequency above 0.01 in the IPF cohort. Haplotype analysis showed a significantly higher carrier frequency of *TERC* haplotype 2, consisting of all four risk alleles, in IPF cases compared to controls (p=0.005) (table 3). This was confirmed in the FIP group (p=0.002) where the carrier frequency of this haplotype was even higher compared to IPF. In *TERT*, risk allele haplotype 2 also had a significantly higher carrier frequency in cases compared to controls (p=0.012). This was also confirmed in the FIP group (p=0.004) where 22 of the 24 patients carried this haplotype.

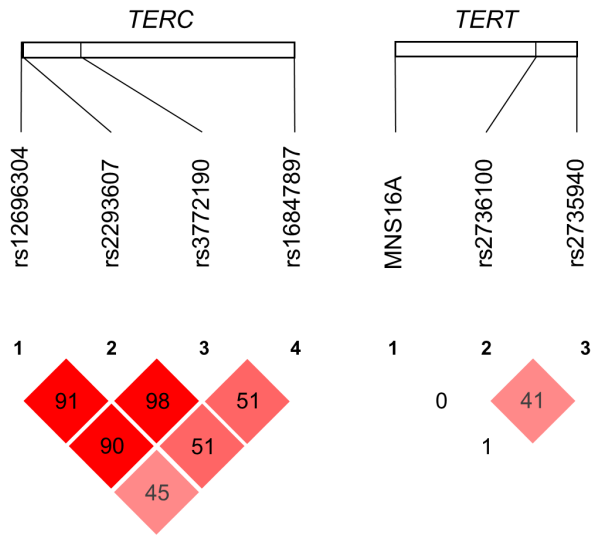

Figure 1. Linkage disequilibrium (LD) (r^2) in TERC and TERT based on genotyped cases and controls. An LD of at least 0.4 to the other genotyped variants was found for all variants, except VNTR MNS16A.

Table 3. Haplotype and carrier frequencies for SNPs in *TERC* and *TERT*

TERC	**Variant**				**Controls** (n=148)	**IPF** (n=106)		**FIP** (n=24)	
Haplotype	rs12696304	rs2293607	rs3772190	rs16847897	**Carrier (haplotype)** frequency	**Carrier (haplotype)** frequency	**Carriership** OR [CI 95%]	**Carrier (haplotype)** frequency	**Carriership** OR [CI 95%]
1	C	A	C	G	0.91 (0.71)	0.82 (0.59)	0.48 [0.23-1.00]*	0.79 (0.58)	ns
2**	G¹	G¹	T¹	C¹	0.30 (0.17)	0.47 (0.27)	2.11 [1.26-3.55]*	0.63 (0.38)	3.94 [1.60-9.67]*
3	C	A	C	C¹	0.09 (0.06)	0.15 (0.08)	ns	0.04 (0.02)	ns
4	G¹	G¹	T¹	G	0.05 (0.03)	0.05 (0.04)	ns	0.04 (0.02)	ns
5	G¹	A	C	G	0.03 (0.02)	0.03 (0.02)	ns	0.03 (0.00)	ns

TERT	**Variant**		**Controls** (n=148)	**IPF** (n=106)		**FIP** (n=24)	
Haplotype	rs2736100	rs2735940	**Carrier (haplotype)** frequency	**Carrier (haplotype)** frequency	**Carriership** OR [CI 95%]	**Carrier (haplotype)** frequency	**Carriership** OR [CI 95%]
1	C	A	0.76 (0.48)	0.54 (0.32)	0.36 [0.21-0.62]*	0.50 (0.25)	0.31 [0.13-0.75]*
2**	A¹	G¹	0.61 (0.38)	0.76 (0.49)	2.03 [1.16-3.54]*	0.92 (0.60)	6.89 [1.56-30.42]*
3	A¹	A	0.09 (0.06)	0.15 (0.12)	ns	0.13 (0.08)	ns
4	C	G¹	0.10 (0.08)	0.12 (0.07)	ns	0.13 (0.06)	ns

Risk alleles are marked with ¹. Risk haplotypes are marked with ** and consists of only risk alleles in a gene. Significant differences are marked with an asterisk (*). The OR 95% confidence interval is shown between brackets. IPF = Idiopathic pulmonary fibrosis, FIP = Familial interstitial pneumonia, ns = not significant

Table 4. Presence of *TERC* and *TERT* risk haplotypes in controls and patients. Presence and absence of the risk haplotypes is displayed as proportions of the cohort. OR and p values display a comparison between cases and controls.

risk haplotypes in *TERC* and *TERT*	**controls** (n=148)	**IPF** (n=106)		**FIP** (n=24)	
	proportion	proportion	OR [CI 95%]	proportion	OR [CI 95%]
carrying none	0.37	0.11	0.34 [0.17-0.70]*	0.04	0.12 [0.02-0.90]*
carrying one	0.55	0.54	ns	0.38	ns
carrying both	0.18	0.35	2.40 [1.35-4.28]*	0.58	6.27 [2.52-15.63]*

Significant differences are marked with an asterisk (*). The OR 95% confidence interval is shown between brackets. IPF = Idiopathic pulmonary fibrosis, FIP = Familial interstitial pneumonia, ns = not significant

Risk haplotypes associated with susceptibility

In order to analyze the effect of carrying both a risk alleles in *TERT* and *TERC*, cases and controls were divided in three groups based on carriership of the risk haplotypes. In the first group, subjects did not carry either haplotype. Subjects in the second group only carried the risk haplotype in either *TERT* or *TERC*. The third group was comprised of subjects carrying the risk haplotype in both *TERT* and *TERC*. Analysis showed a significant difference in frequency between cases and controls for both the first group, who did not carry any risk haplotype and for the group carrying both risk alleles (table 4). No significant association with IPF susceptibility was found when only one risk haplotype was present. Similar results were found in the FIP cohort.

Risk haplotype carriership impacts survival

To investigate the consequence of carriership of both *TERC* and *TERT* risk haplotypes, we compared the survival rate of carriers of both risk haplotypes to non-carriers. Subjects were considered non-carriers when carrying none or one risk haplotype. A significant effect of carrying both telomere risk haplotypes on survival was observed (p=0.001 Log Rank (Mantel-Cox)). In non-carriers a median survival of 50 months was observed, while this was 20 months in carriers. Median risk haplotypes carriers survival is therefore 2.5 years shorter compared to non-carriers (see figure 2).

For the FIP cohort, no significant difference in survival was found (p=0.723).

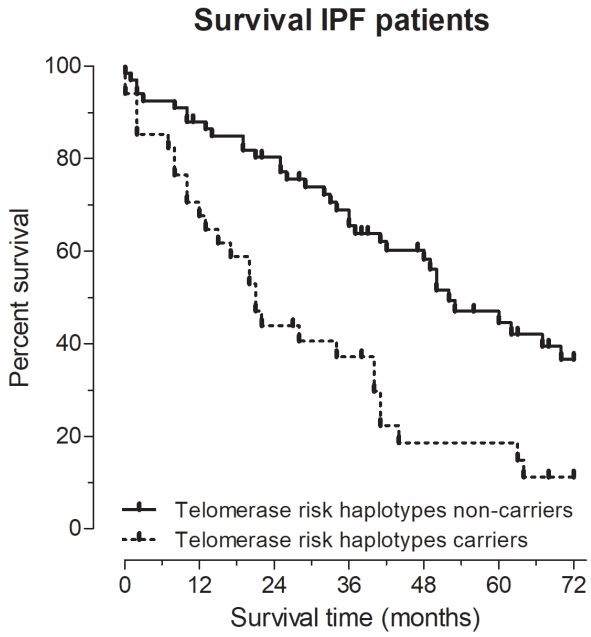

Figure 2. **Kaplan-Meier analysis of survival in the sporadic IPF cohort.** There was a significant difference in survival rate (p=0.001 Log Rank (Mantel-Cox)) for non-carriers and carriers of both risk haplotypes in *TERC* and *TERT*.

Table 5. Allele frequency in controls, IPF and FIP cases

		Controls (n=148)	**Sporadic IPF cohort** (n=106)			**FIP cohort** (n=24)		
gene	**variant**	**allele fequency**	**allele fequency**		**risk allele carri-ership**	**allele fequency**		**risk allele carriership**
				OR [95%CI]	OR [95%CI]		OR [95%CI]	OR [95%CI]
MUC5B	rs35705950	G 0.90 T[1] 0.10	G 0.72 T 0.28	OR=3.30* [2.04-5.32]	OR=3.77* [2.18-6.54]	C 0.71 G 0.29	OR=3.55* [1.71-7.27]	OR=4.46* [1.82-10.92]

Allele frequencies of variant rs35705950 in *MUC5B* controls. idiopathic pulmonary fibrosis (IPF) and familial interstitial pneumonia (FIP) patients. Odds ratios (OR) are given for differences in allele frequency and in risk allele carriership in controls versus IPF. The OR 95% confidence interval is shown between brackets. Significant differences are marked with an asterisk (*). Risk alleles are marked with [1]. L = Long variant and S = short variant of MNS16A.

Table 6. Presence of the *TERC* and *TERT* risk haplotypes and the *MUC5*B risk allele in controls and patients. Presence and absence of the risk haplotypes and allele is displayed as proportions of the cohort. OR and p values display a comparison between cases and controls.

Risk locus		**controls** (n=148)	**IPF** (n=106)		**FIP** (n=24)	
telomere risk haplotypes	*Muc5B*	proportion	proportion	OR [CI 95%]	proportion	OR [CI 95%]
0=absent, 1=present						
0	0	0.63	0.33	0.29 [0.17-0.49]*	0.17	0.12 [0.04-0.36]*
0	1	0.19	0.32	2.02 [1.13-3.61]*	0.25	ns
1	0	0.16	0.17	ns	0.29	ns
1	1	0.02	0.18	11.20 [3.22-39.0]*	0.29	19.90 [4.70-84.24]*

Significant differences are marked with an asterisk (*). The OR 95% confidence interval is shown between brackets.
IPF = Idiopathic pulmonary fibrosis, FIP = Familial interstitial pneumonia, ns = not significant

IPF susceptibility depends on multiple risk loci

IPF susceptibility has also been associated with *MUC5B* promoter polymorphism rs35705950. A significant difference in allele frequency was observed between IPF cases and controls for this polymorphism in *MUC5B* (table 5).

We combined genotype data of this *MUC5B* SNP with the above presented telomere risk haplotype data[20]. Patients were divided in four groups. The first group neither carried the *MUC5B* risk allele, nor the telomere risk haplotypes. The second and third group carried either the *MUC5B* risk allele, or the telomere risk haplotypes. The fourth group carried both the telomere risk haplotypes, as well as the risk allele in *MUC5B*. In both the IPF and FIP cohort, carriership of both the *MUC5B* risk variant and telomere risk haplotypes is significantly associated with IPF susceptibility (IPF $p=9.0 \cdot 10^{-6}$, FIP $p=1.4 \cdot 10^{-7}$) (table 6).

Furthermore we investigated the effect on survival of carriership of the risk allele in *MUC5B* and the telomere risk haplotypes in one model. *MUC5B* and telomerase risk haplotypes data were used as covariates in a Cox regression survival analysis. When analysed together, the *MUC5B* did not have a significant effect on survival (p=0.790), but the telomere risk haplotypes together did ($p=7.5 \cdot 10^{-4}$, HR=2.28 [1.41-3.67]) in IPF patients.

Discussion

In this study we analyzed common variants in *TERT* and *TERC* that influence telomere length and found an association between these genetic polymorphisms and susceptibility to IPF. The associations were confirmed in a cohort of FIP patients. Furthermore we show that carriership of risk haplotypes in both *TERT* and *TERC* has a significant effect on survival in sporadic IPF. Finally, by combining telomere risk haplotypes data with *MUC5B* rs35705950 we show that multiple risk loci underlie IPF susceptibility.

Mutations in the telomerase genes *TERT* and *TERC* have previously been shown to underlie IPF in familial disease[3–5]. Subsequent studies have observed significantly shorter telomere length in sporadic IIP and IPF patients, compared to controls[12–14]. To investigate if genetic predisposition for short telomere length is more prevalent in sporadic IPF patients, we genotyped seven alleles in *TERC* or *TERT*, associated with short telomere length or diminished telomerase activity.

In *TERC*, the four SNPs are associated with telomere length in the general population. Both minor alleles of rs12696304 (G) and rs16847897 (C) were found to be association with telomere length in blood in a combined study population of 12,409 individuals, using an additive model[27]. This was replicated in a separate study of 3,554 individuals by Prescott and colleagues, who also found this association for the minor allele of rs3772190 (T)[24]. Finally, the minor allele of rs2293607 (G) was also found to be associated with telomere length in a study population of 2,953 individuals[23].
In *TERT* SNP rs2736100 (A) was shown to be associated with telomere length in a combined study population of 48,423 individuals. SNP rs2735940 (G) was associated with short telomeres in 133 individuals and variable tandem repeat MNS16A has been shown to influence telomerase activity[29,30].

In the present study, we found an association of all the single nucleotide polymorphisms to IPF susceptibility in both sporadic and familial patients. The alleles of these variants that are associated with shorter telomere length are also the alleles associated with IPF. The association of *TERT* variant rs2736100 to IPF susceptibility has been shown before by a genome wide association study in a Japanese cohort[16]. Mushiroda et al. found a significant association with carriership of the risk allele A with an OR of 2.15 [1.35-3.43], quite similar to the one found in the present study (OR=3.03 [1.54-5.97]) even though there was a difference in risk allele frequency in controls: 0.43 in the Dutch cohort, compared to 0.59 in the Japanese cohort.

This *TERT* rs2736100 A allele is also identified as a susceptibility locus in an idiopathic interstitial pneumonia (IIP) cohort (carriership risk allele A, OR=1.44 [1.28-1.61]) of which the majority (77%) were diagnosed with IPF. Although confidence intervals are still overlapping, it is tempting to attribute the difference in OR between this study and our first cohort to the inclusion of non-IPF IIP's. However, this is contradicted by a study by Wei et al[34]. This study shows an association of this allele with "other ILD (non-IPF)" (OR=1.43 [1.11-1.85]),

but not with IPF(OR=1.08 [0.78-1.49])[34]. Further research is therefore needed to understand the role of *TERT* SNPs in non-IPF ILD.
The rs2736100 *TERT* variant has also been identified as a risk factor in several forms of cancer[35], including lung cancer[36,37]. Telomere biology is an important factor in the development of cancer and an elevated risk of lung cancer for IPF patients has been described[38]. However, instead of the A allele that is associated with IPF, the C allele of this locus is associated with lung cancer risk. This excludes a role for rs2736100 as a possible link between IPF and lung cancer. Nonetheless, this supports the functional role of this *TERT* SNP in telomere maintenance and its effect on telomere length variance.

TERC variant rs12696304 has been implicated as a susceptibility locus in IIP, after imputation of genotypes found in the case-control genome wide association study (GWAS) with IIP patients performed Fingerlin and co-workers[17]. Allele frequencies in cases and controls and odds ratios were similar with the data presented here. Interestingly however, in our study higher susceptibility odds ratios were found for the *TERC* variants other than rs12696304.

In our cohort, most *TERT* and *TERC* SNP are individually associated with IPF susceptibility. However, when all SNPs were analysed as haplotypes, we only found significant frequency differences between IPF patients and controls for carriers of both risk haplotypes and individuals carrying neither (table 4). This would initially suggest that IPF susceptibility is only associated with joint carriership of variants in *TERT* and *TERC*. Furthermore, adding the risk allele of *MUC5B* variant rs35705950 to this genetic profile, shows that jointly carrying risk alleles in all three genes confers an even greater risk. We found this joint carriership of telomerase-*MUC5B* risk alleles in 18% of IPF patients and 29% of FIP patients but only in 2% of controls . This suggest that a combination of genetic factors underlies diseases in IPF and non-mutation carrying FIP patients. Inclusion of more genetic variants is needed to verify this hypothesis.

In IPF, carriers of both risk haplotypes have a significant lower survival rate compared to non-carriers. In FIP however, there was no significant difference in survival rate. This is probably due to lack of statistical power. Moreover, the FIP cohort comprised only 1 patient who did not carry any risk haplotype in *TERC* or *TERT*. The composition of the FIP non-carriers group did therefore not mirror the IPF non-carrier group. The relevance of telomere biology in IPF survival has previously been shown in a study that determined that telomere length in white blood cells is associated with survival in IPF[39]. Here we show that a telomere length related genetic background is also associated with a lower survival rate. This is compelling evidence for a fundamental role for telomere biology in IPF pathogenesis.

In conclusion, we demonstrate the importance of genetic variance in telomerase genes in patients with sporadic IPF and FIP. Carriership of risk haplotypes in both *TERC* and *TERC* predisposes for IPF, but is also associated with a significantly lower survival rate in IPF. This underlines the importance of addressing telomere biology in IPF in future studies and of investigating treatment options that influence telomere maintenance. Furthermore we show the importance of multiple risk loci in IPF disease development that goes beyond variation in single genes. A combined analysis of IPF risk loci will be necessary to understand IPF genetics.

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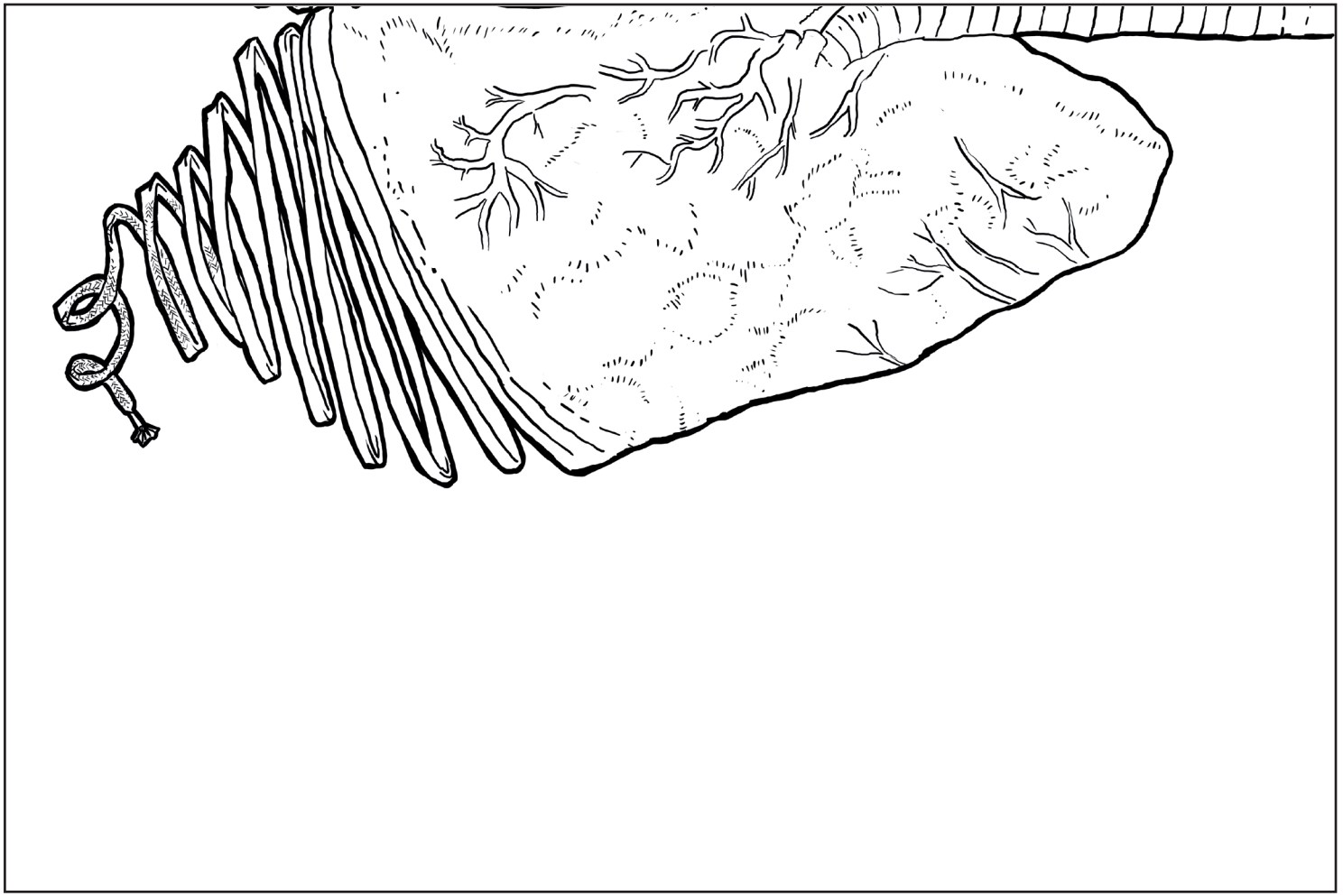

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Telomerase Reverse Transcriptase Polymorphism rs2736100: A Balancing Act between Cancer and Non-Cancer Disease, a Meta-Analysis

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Abstract

The enzyme telomerase reverse transcriptase (*TERT*) is essential for telomere maintenance. In replicating cells, maintenance of telomere length is important for the preservation of vital genetic information and prevention of genomic instability. A common genetic variant in *TERT*, rs2736100 C/A, is associated with both telomere length and multiple diseases. Carriage of the C-allele is associated with longer telomere length, while carriage of the A- allele is associated with shorter telomere length. Furthermore, some diseases have a positive association with the C and some with the A allele. In this study, meta-analyses were performed for two groups of diseases, cancerous diseases, e.g. lung cancer and non-cancerous diseases, e.g. pulmonary fibrosis, using data from genome wide association studies and case-control studies. In the meta-analysis it was found that cancer positively associated with the C allele (pooled OR 1.16 [95%CI 1.09-1.23]) and non-cancerous diseases negatively associate with the C allele (pooled OR 0.81 [95%CI 0.65-0.99]). This observation illustrates that the ambiguous role of telomere maintenance in disease hinges, at least in part, on a single locus in telomerase genes. The dual role of this SNP also emphasizes that therapeutic agents aimed at influencing telomere maintenance should be used with caution.

Introduction

Telomere biology is emerging as a significant factor in an increasing number of diseases [1–4]. Studies have found disease associations with both abnormal telomere length and with genetic variants related to telomere biology[5–7]. Telomeres are non-coding tandem repeats (TTAGGG) spatially organized by specialized proteins, that maintain stability of the chromosome ends[8–10]. Furthermore, telomeres serve as a buffer against the shortening of chromosomes, thereby preventing the loss of vital genetic information[11]. To maintain replicative potential, telomeres can be elongated by the ribonucleoprotein telomerase[12,13]. Telomerase consists of a catalytic protein component, encoded by the gene Telomerase reverse transcriptase (*TERT*), and a RNA template, encoded by Telomerase RNA component (*TERC).*

Over the course of the human lifespan, the average length of telomeres can be disproportionally influenced by a number of factors, leading to a broad spectrum of diseases. Genetic variation in telomere maintenance genes has been shown to either accelerate or prohibit telomere shortening. Genomic mutations in the coding regions of telomerase genes are primarily found in degenerative diseases like dyskeratosis congenita, aplastic anemia and idiopathic pulmonary fibrosis (IPF)[14–16]. These mutations generally lead to a decrease in telomerase activity[17] and shorter telomeres in mutation carriers who develop fatal disease due to organ failure. Mutations in the coding regions of telomerase genes are very rare in cancer, however somatic mutations in the promotor region of the *TERT* gene has been reported in the context of several cancer types[18–20]. These mutations generally lead to an increase in telomerase activity, which corroborates the observation of high levels of telomerase activity in cancer cells[21]. Telomerase activation and the subsequent telomere elongation leads to the immortalization of cells and prevents fatal instability of the chromosomes, opening up the possibility of unrestricted cell proliferation[21,22].

However, Telomere biology is an ambiguous factor in cancer pathology[23–25]. In healthy individuals it is thought that the restricted transcription of telomerase and the resulting limited number of cell divisions present a barrier to unlimited replication of somatic cells, thus preventing cancer[26,27]. However, other research suggests that telomere shortening can lead to chromosomal instability in the form of chromosome fusion, genomic copy addition, deletion and mutation, which in turn can lead to tumor initiation[22,28–31]. This duplicity is apparent in humans, where both long and short telomere length of white blood cells has been associated with different cancers[32–36]. As short telomeres can lead to damaged chromosomes, it is proposed that long telomeres postpones senescence thereby increasing the risk for cells to acquire genetic abnormalities that facilitate tumorigenesis[36–38].

Besides rare mutations in telomerase genes, common genetic variation in these genes has also been associated with disease. A well-studied example is the single nucleotide polymorphism (SNP) rs2736100 in the *TERT* gene (5p15.33). Interestingly, the first report on a disease association to this SNP, was to the non-cancerous disease IPF[39]. An IPF susceptibility odds ratio of 1.82 [95%CI:1.47-2.22] was found for the A allele of this SNP. The second report

on this SNP showed an association between lung cancer and the C allele of this SNP[40]. Later studies have shown an association between the A allele and shorter blood cell telomere length while it follows that the C allele is associated with longer telomeres[41,42]. This duality in disease association of the rs2736100 alleles might reflect a fundamentally different role of telomere biology in cancerous diseases as opposed to non-cancerous diseases. Such a dichotomy would underline, that therapeutic agents influencing telomere length or telomerase activity should be used with caution, as both (too) long and short telomeres could lead to disease.

The aim of this study is to conduct a systematic review and meta-analysis of disease association studies with *TERT* SNP rs2736100, and to gain insight in the balancing act between telomere maintenance and disease predisposition.

Material and Methods

Study selection

The electronic databases PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and Embase (https://www.embase.com/#quickSearch/default) were queried for studies on *TERT* SNP rs2736100 by using "rs2736100" as search input (figure 1). Initially 92 studies were found. After selecting for papers pertaining to the subject of this review 57 studies remained. Of these, 49 studies described an associations between *TERT* SNP rs2736100 and cancer, and 8 studies described associations between this SNP and non-cancer disease. Another 77 studies were added through references found in the original studies. Excluded were reviews and meta-analyses, and studies from which no odds ratio data was available or could be calculated. Furthermore studies were excluded that did not provide definite data on which allele was associated with risk for the investigated disease. Finally, 85 studies were included of which 77 described association with cancer and 8 with non-cancer and the *TERT* SNP, respectively.

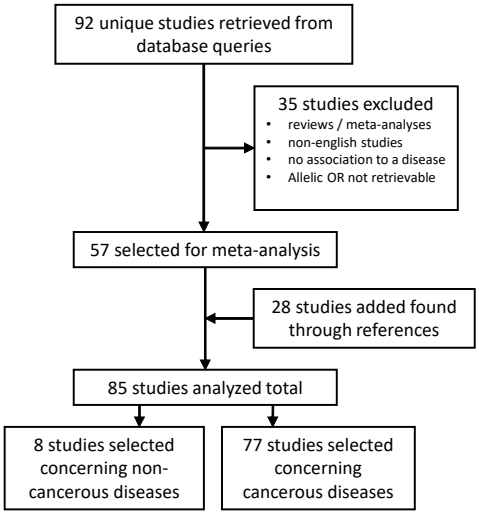

Figure 1. Flowchart of studies included in the meta-analysis.

Eligibility criteria

Included in this meta-analysis were case-control and genome-wide association studies assessing the association between *TERT* SNP rs2736100 and disease. These studies were furthermore included when the associated allele and the used inheritance model were clearly derivable from the study. Results of meta-analyses were excluded, however these studies were searched for eligible studies to be included in the present study. This lead to further inclusion of studies that did not find a significant association between rs2736100 and disease, thereby preventing major publication bias. Finally, systematic reviews, abstracts, non-english studies and studies investigating rs2736100 not in the context of disease were excluded from the meta-analysis.

Data extraction

The following data was derived from each study: first author, year of publication, number of cases and controls, associated disease (cancer and non-cancer), and OR and 95% confidence interval. Furthermore, the used inheritance model was checked as well as which allele specifically was associated with the studied disease. When this information was not provided it was derived from the provided genotype data when possible.

Bias evaluation

(Publication) bias was evaluated by visual inspection of Doi plots, as well as calculate the Luis Furuya-Kanamori (LFK) index, which provides a statistic for the amount of bias in a Doi plot. (No asymmetry: LFK index within ±1, minor asymmetry: LFK index exceeds ±1 but within ±2, major asymmetry: LFK index exceeds ±2)

Statistical analysis

Rs2736100 associations and (publication) bias were both analyzed using meta-analysis software MetaXL 4.0 (EpiGear International, Sunrise Beach, Australia). Due to the large number of different diseases that were included in this study, a high level of variation in study outcome, heterogeneity, was expected. The meta-analysis was therefore performed using the Inverse variance heterogeneity method, to determine the pooled result and heterogeneity[43]. Many studies do not provide genotype data. Therefore we performed meta-analysis on the odds ratio (OR) and 95% confidence interval (95%CI) for allelic association.

Results

Non-cancerous diseases

A meta-analysis was performed to analyze the association of *TERT* SNP rs2736100 with a group of cancer diseases and with a group of non-cancerous diseases. Table 1 shows all included studies. For the non-cancer group, 8 studies were found with diagnoses of pulmonary fibrosis and coronary heart disease among others. Each of these 8 studies showed either a negative association between the C-allele and disease or a non-significant result. Figure 2 shows a forest plot of a meta-analysis of these studies using the Inverse Variance Heterogeneity model[43]. The odds ratios (OR) in this figure are an effect measure for the association with the C allele of rs2736100 in a co-dominant model. The pooled negative association with disease for the C-allele was significant with an OR ratio of 0.81 [95%CI: 0.65-0.99]. Presence of the C-allele is protective for non-cancerous diseases.

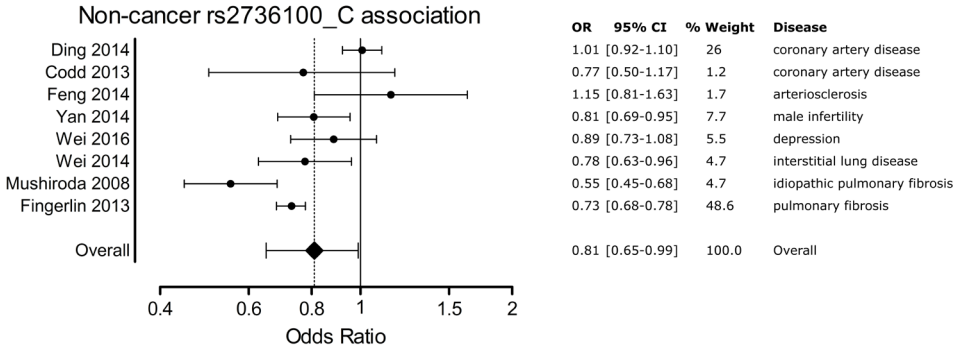

Figure 2. Meta-analysis of non-cancer association to rs2736100 allele C. OR = Odds ratio, 95% CI = 95% confidence interval.

Association with cancer

Meta-analysis for associations with cancer included 77 studies. These studies included a variety of cancers of which the majority (n=46) involved studies on lung cancer (n=33) and glioma (n=13). The majority of studies reported a positive association with the C-allele of rs2736100. However, four of the included studies reported a negative association with the C-allele, these included two studies on testicular cancer one on colorectal cancer and one on pancreatic cancer[44–46] In the meta-analysis of cancer studies, the pooled effect size was significant with a pooled OR of 1.16 [95%CI:1.09-1.23] (figure 3) and shows that the C-allele is a risk allele for cancer.

(Continued on page 100)

Table 1. Studies included in the meta-analyses

			Association rs2736100_C		
Study	**Cases**	**Controls**	**OR**	**95% CI**	**Disease**
47	245	489	1.12	0.90-1.39	Pediatric brain tumor
48	445	497	1.29	1.07-1.55	Lung cancer
49	1154	1137	1.24	1.10-1.39	Lung cancer
50	1896	1939	1.13	1.03-1.24	Pancreatic cancer
51	1094	1100	1.14	1.01-1.30	Lung cancer
52	4441	5194	1.22	1.15-1.29	Lung cancer
46	5550	7585	0.91	0.99-0.84	Pancreatic cancer
53	976	1057	1.26	1.11-1.43	Glioma
54	196	229	1.65	1.17-2.32	Lung cancer
55	13265	40245	1.08	1.12-1.04	Endometrial cancer
56	243	246	1.2	0.92-1.55	Gastric cancer
57	386	587	0.94	0.78-1.14	Liver cancer
41	22233	64762	0.77	0.50-1.17	Coronary artery disease
58	663	420	0.99	0.79-1.25	Renal cell carcinoma
59	845	1190	1.27	1.12-1.44	Glioma
60	1514	2470	1.01	0.92-1.10	Coronary artery disease
61	1136	1012	1.02	0.90-1.15	Gastric cancer
62	639	649	1.37	1.18-1.61	Glioma
63	3131	3702	1.02	0.94-1.11	Skin cancer
64	84	257	1.15	0.81-1.63	Arteriosclerosis
65	2477	6550	0.73	0.68-0.78	Pulmonary fibrosis
66	970	525	1.17	1.04-1.33	Bladder cancer
67	510	913	1.17	1.00-1.37	Skin cancer
68	3264	1793	0.98	0.77-1.25	Colorectal cancer
69	2308	2321	1.48	1.36-1.62	Lung cancer
70	8559	9378	1.25	1.20-1.31	Lung cancer
71	1145	1142	0.96	0.78-1.17	Breast cancer
72	716	716	1.17	1.00-1.38	Lung cancer
73	717	202	1.57	1.25-1.96	Myeloproliferative neoplasms
74	104	135	1.01	0.70-1.46	Colorectal cancer
75	855	844	1.16	1.02-1.33	Lung cancer
76	1212	1339	1.14	1.02-2.27	Lung cancer

(Continued)

Table 1. Continued

			Association rs2736100_C		
Study	**Cases**	**Controls**	**OR**	**95% CI**	**Disease**
77	349	914	0.81	0.68-0.97	Testicular cancer
45	16039	16430	0.93	0.91-0.96	Colorectal cancer
78	370	1173	1.38	1.23-1.56	Lung cancer
79	584	400	1.77	1.47-2.12	Myeloproliferative Neoplasms
80	518	1201	1.64	1.42-1.91	Glioma
81	855	1160	1.16	1.01-1.33	Glioma
82	4543	5505	1.38	1.31-1.47	Lung cancer
83	193	197	1.29	1.00-1.67	Lung cancer
84	5739	5848	1.09	1.03-1.15	Lung cancer
85	370	1263	1.1	0.93-1.29	Colorectal cancer
86	2283	2785	1.18	1.09-1.27	Lung cancer
87	304	319	1.33	1.06-1.67	Lung cancer
88	690	1538	1.19	1.03-1.38	Bladder cancer
89	5457	4493	1.38	1.30-1.47	Lung cancer
40	5870	9319	1.14	1.08-1.20	Lung cancer
90	2086	11034	1.27	1.19-1.37	Lung cancer
91	226	806	1.22	0.99-1.51	Acute myeloid leukemia
92	226	806	1.44	1.10-1.88	Glioma
39	242	1496	0.55	0.45-0.68	Idiopathic pulmonary fibrosis
93	352	447	1.18	0.97-1.45	Lung cancer
35	277	831	1.19	1.04-1.37	Skin cancer
94	1681	1635	1.16	1.04-1.30	Lung cancer
95	3534	4098	1.08	1.02-1.16	Breast cancer
96	1955	1995	1.11	1.00-1.23	Pancreatic cancer
97	596	1480	1.08	0.94-1.23	Endometrial cancer
98	1854	4949	1.3	1.19-1.41	Glioma
99	810	3080	1.23	1.10-1.37	Glioma
100	1029	1668	1.31	1.17-1.47	Glioma
101	660	523	0.92	0.78-1.09	Breast cancer
101	372	363	0.95	0.77-1.16	Prostate cancer
102	569	656	1.23	1.05-1.45	Acute lymphoblastic leukemia
103	1878	3670	1.27	1.19-1.37	Glioma

(Continued)

Table 1. Continued

			Association rs2736100_C		
Study	**Cases**	**Controls**	**OR**	**95% CI**	**Disease**
104	5992	13531	1.34	1.28-1.41	Lung cancer
105	807	708	1.24	1.05-1.48	Lung cancer
106	810	4479	1.31	1.16-1.48	Myeloproliferative neoplasms
107	719	6030	1.08	0.97-1.21	Lung cancer
108	10812	13913	1.15	1.10-1.20	Lung cancer
109	661	1347	1.32	1.14-1.52	Lung cancer
44	1045	8403	0.75	0.67-0.85	Testicular cancer
110	1660	1299	1.39	1.28-1.50	Glioma
111	1618	7736	1.39	1.28-1.50	Glioma
112	239	553	1.3	1.04-1.61	Lung cancer
113	1033	1053	1.16	1.03-1.32	Cervical cancer
114	1552	1605	1.2	1.09-1.33	Lung cancer
115	370	686	0.78	0.63-0.96	Interstitial lung disease
116	1404	5040	1.24	1.10-1.39	Lung cancer
117	239	1197	0.89	0.73-1.08	Depression
118	692	3992	1.51	1.35-1.71	Glioma
119	580	580	0.81	0.69-0.95	Male infertility
120	1735	1036	1.11	0.99-1.24	Lung cancer
121	524	524	1.34	1.13-1.60	Lung cancer
122	1425	3011	1.26	1.11-1.43	Lung cancer
123	784	782	1.28	1.11-1.47	Lung cancer
124	2096	2147	1.38	1.27-1.51	Esophageal cancer

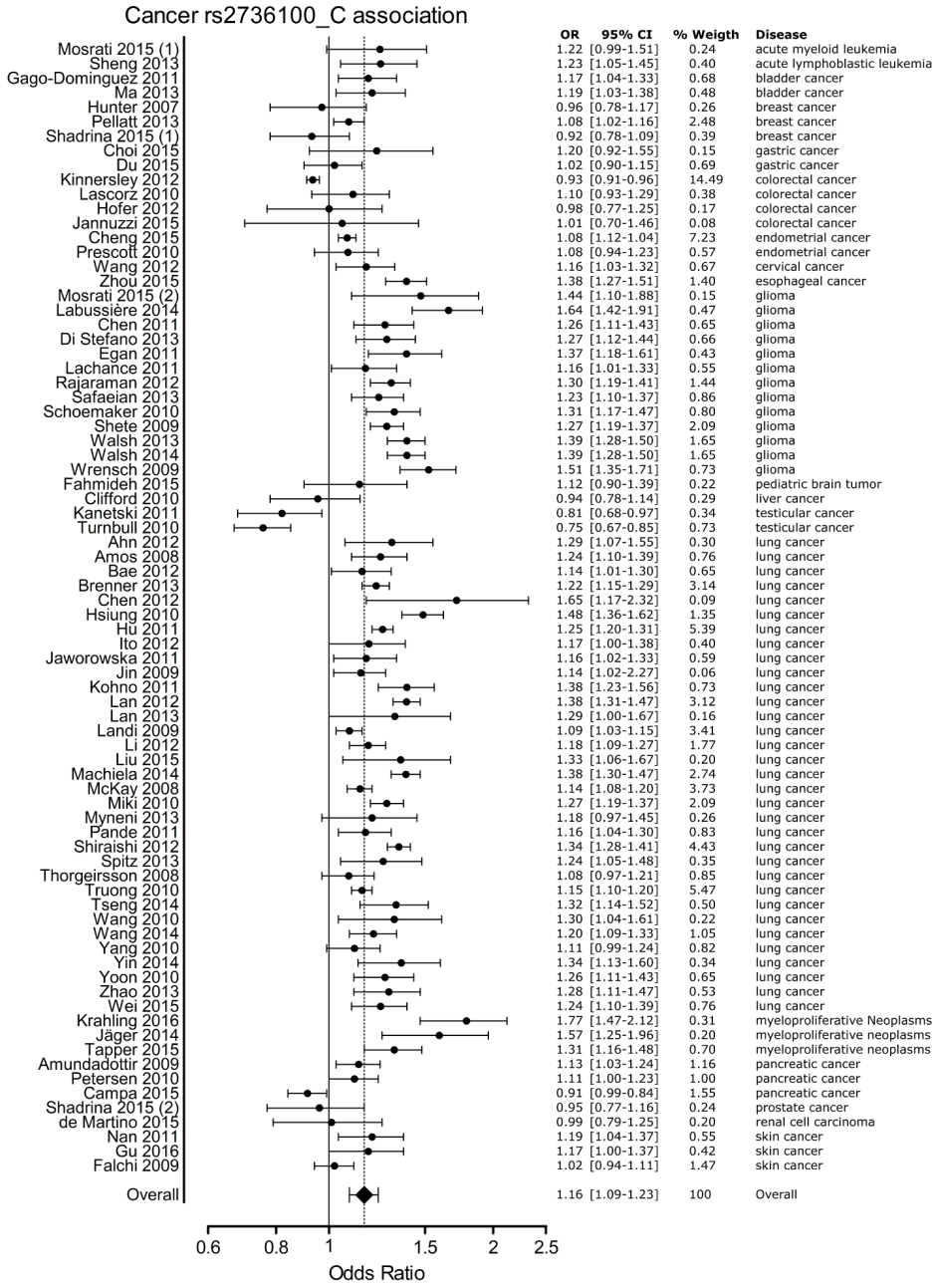

Figure 3. Meta-analysis of cancer association to rs2736100 allele C. OR = Odds ratio, 95% CI = 95% confidence interval.

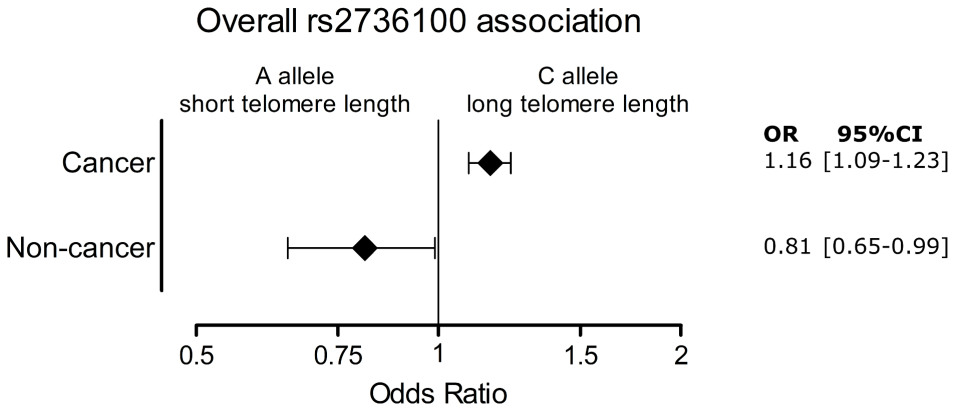

Figure 4. Overall association of the cancer and age-related group of diseases with TERT rs2736100 allele C. OR = Odds ratio, 95% CI = 95% confidence interval.

Figure 4 shows the significant pooled ORs for the meta-analysis of cancer and age-related diseases.

Potential bias from publication selection and other sources was evaluated using Doi plots and quantified by the LFK index (figure 5). Figure 5A shows the Doi plot for the cancer meta-analysis. A LFK index of 1,09 was found indication minor influence from publication bias or bias from other sources. Figure 5B shows the Doi plot for non-cancer diseases.

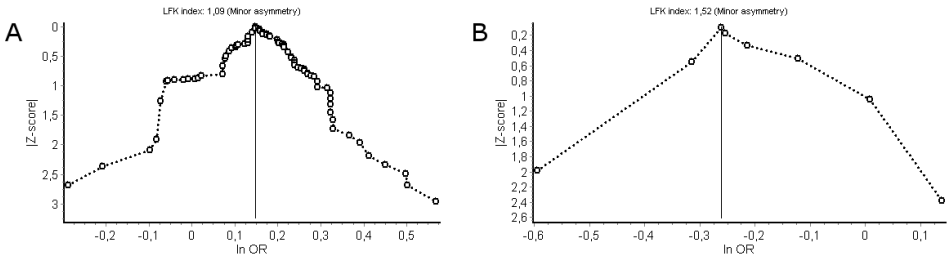

Figure 5. Evaluation of (publication) bias for the cancer (A) and non-cancer (B) meta-analysis. LFK index = Luis Furuya-Kanamori index, OR = Odds ratio

Discussion

The results of the meta-analyses illustrate the duality of telomere biology in disease predisposition. Pooled analysis showed that non-cancerous age-related diseases, such as pulmonary fibrosis and coronary artery disease, associate positively with the telomerase A-allele that is linked to shorter telomeres. Pooled analysis of cancer studies however showed an association with the opposite allele, the C-allele that is linked to longer telomere length. This is supported by a recent study, that showed an association of genetically increased telomere length and cancer, while the opposite protected against non-cancerous diseases[125]. This two-sided association suggests opposite roles of telomere length in cancerous and age-related diseases.

The *TERT* SNP rs2736100 has robustly been associated with telomere length in healthy controls[41,42]. Studies showed that presence of the A-allele is associated with shorter telomere length. This is in congruence with observations made in IPF, cardiovascular disease and male infertility, where patients have been shown to have relatively short leukocyte telomeres[126–128]. This SNP could therefore be part of the genetic background that increases susceptibility to these diseases in combination with other cellular and environmental factors that might cause increased cellular turnover[129]. On the other hand, the C-allele, which is associated with most cancer types, is associated with longer telomere length in health[42,102]. How the rs2736100 SNP influences telomere length is presently not understood. The SNP is located in intron 2 in *TERT*, and was suggested to influences telomerase activity or to be in strong linkage with a functional variant in *TERT*[102,130].

The dual associations of the SNP in this study, would support the hypothesis that telomere maintenance is at an intersection between cancer and -premature- aging[131,132]. Cancer and aging share many molecular pathways, including telomere maintenance pathways. And while aging is associated with a progressive decrease in telomere length, cancer is characterized by immortalization of the cell often through telomerase activation[21,133]. Short telomeres accelerate aging through cell senescence, but long telomeres postpone senescence, which in turn, facilitates survival of cells with acquired oncogenic DNA-alterations and thereby promotes tumorigenesis[36–38]. The meta-analysis of cancer studies showed a pooled positive correlation with the *TERT* allele that is known to cause longer telomeres. This suggest that the majority of patients develop cancer due to a.o. the presence of long telomeres in tumor initiating cells. On the other hand, it is also well understood that critically short telomeres lead to chromosomal instability, which can cause tumorigenesis[32–34]. Although the results of the meta-analysis suggest this if not the cause in the majority of cases, four cancer studies were included that originally reported an association with the *TERT* allele for short telomeres. For colorectal cancer there is general agreement that short telomere length increases tumor initiation by causing chromosomal instability[134]. This would account for the association of colorectal cancer with the A allele of rs2736100 that is also associated with short telomere length[45]. Testicular cancer is also associated with the A allele[44]. Telomerase activity is restricted in most tissues, one exception being germ cells[21]. It is assumed that in highly proliferative tissues, a genetic factor decreasing telomerase activity may cause chromosomal instability leading to cancer.

Most non-cancer diseases showed an association with the A allele of rs2736100 SNP. Regarding telomere biology, disease susceptibility for pulmonary fibrosis is opposite to that of lung cancer, while both disease are highly associated with smoking behavior. In case of short telomeres, smoking may cause increased senescence with subsequent pulmonary fibrosis, while in case of long telomeres, smoking may cause DNA-damage in cells with sustained proliferative capacity. For coronary artery disease no significant allelic association has been found for rs2736100. But reports have shown a significant effect of this variant when analyzed in combination with other risk loci or when analyzed in a dominant model[41,60,64]. However, conflicting data were found for the direction of the association, which could have been due to the ethnic background of the population. Codd et al. report an association between coronary artery disease and the A-allele in a Caucasian cohort. Both Feng et al. and Ding et al. study Asian populations, and found no association or an association with the C-allele[60,64].

A limitation is that although an exhaustive literature search was performed, studies could have been overlooked. Some studies were excluded because of missing genotype data and some describe an association without reporting the associated allele. Furthermore, it should be emphasized that the pathogenesis for most of the diseases mentioned in this study is not fully known and is suggested to be complex, involving both genetic and environmental factors. Another limitation is the discrepancy in the number of studies found for cancer vs non-cancer. This results in a strong influence of interstitial lung disease on the pooled OR. Finally, (publication) bias was evaluated for both the cancer and non-cancer study (figure 5). For both meta-analyses asymmetry was minor and the corresponding bias introduced by study selection and other sources is therefore also considered to be of minor influence on the pooled result.

The ambiguous effects of telomere maintenance do pose a great challenge for the development of therapeutic agents, for instance putative anti-aging therapeutics. Therapeutic agents aimed at increasing telomere length should be used with caution[23,135] Telomerase activating agents used in the context of degenerative or aging-related diseases could facilitate tumorigenesis or lead to proliferation of untargeted tissues[132,136]. On the other hand, in relation to cancer, telomerase inhibiting agents are attractive candidates but could lead to accelerated tissue degeneration or negatively affect stem cell function and immune response as this requires increased telomerase activity to sustain a high level of cell proliferation[136,137]. To develop disease specific treatment, future research should be aimed at further understanding of optimal telomere length per specific tissues during life, and at the ability to target specific cell-types or tissues very accurately.

Conclusion

Meta-analyses showed that the *TERT* SNP rs2736100 C-allele is positively associated with multiple cancerous diseases while the A-allele is positively associated with predisposition to non-cancerous (age-related) diseases. Because the SNP is known for its influence on telomere length, this result illustrates that optimal telomere maintenance balances between increasing the risk for cancer or for non-cancerous diseases. This underlines the caution that should be taken, when developing therapies that influence telomere length.

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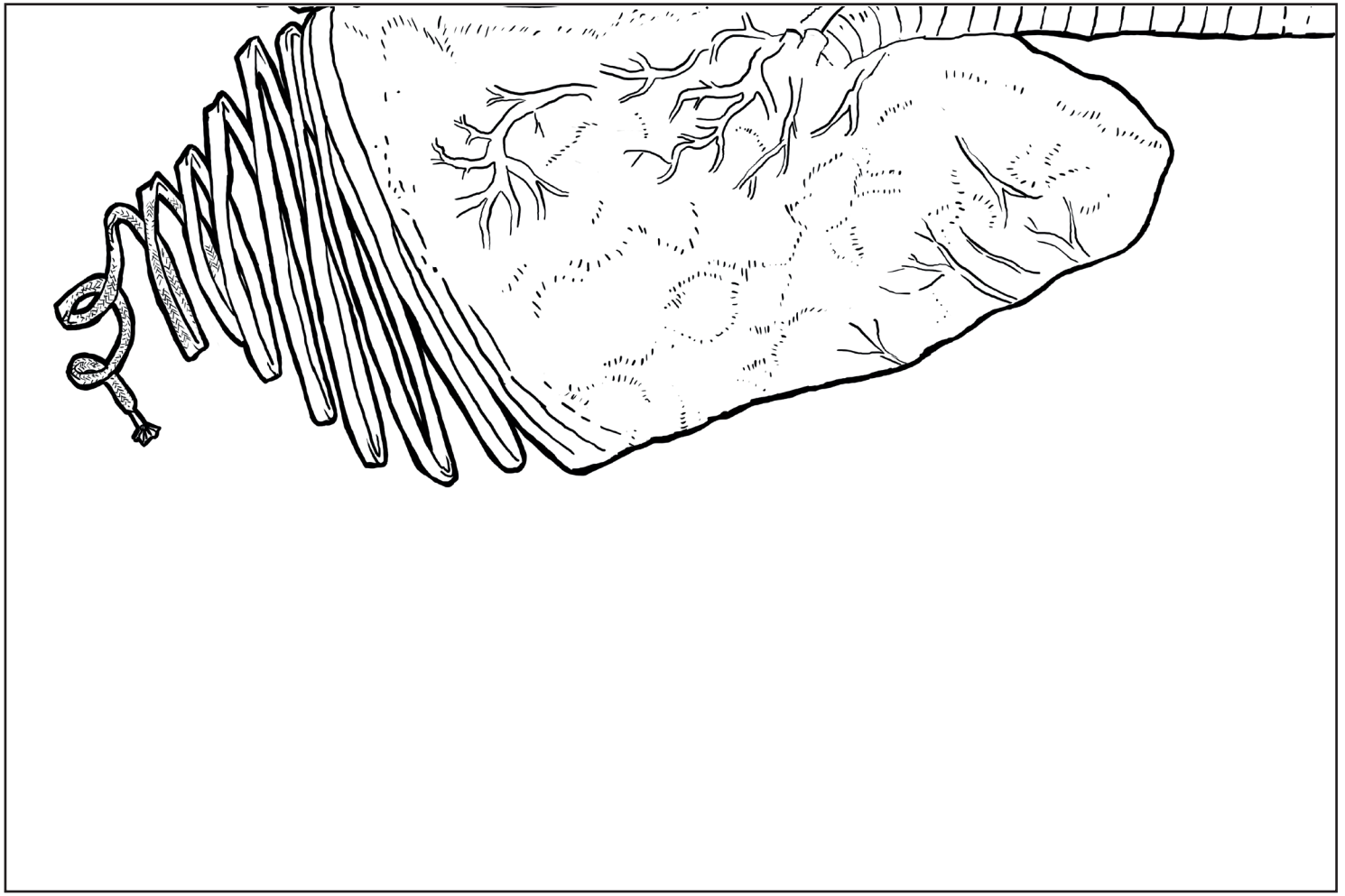
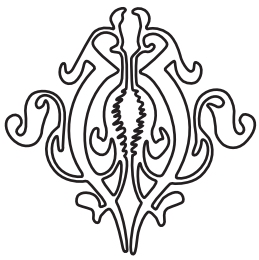


7

Summary and General Discussion



Summary

The aim of this thesis is to elucidate the pathophysiological role of telomere shortening in IPF as well as investigate genetic predisposition to this disease. The hypothesis that there is such a role for telomere shortening derives from observations that telomeres in leukocytes appear to be short in many IPF patients, and that mutations in telomere maintenance genes confer a risk for developing IPF. These observations suggest that telomere biology is important in the pathogenesis in IPF. Next to genetic variation in the telomere maintenance genes, a variant in the *MUC5B* gene has been shown to predispose to IPF.

In **chapter 2** Telomere length (TL) was measured in patients with a broad selection of interstitial lung diseases (ILD) (n= 359: idiopathic pulmonary fibrosis (IPF; n=109), familial interstitial pneumonia (FIP; n=57), idiopathic non-specific interstitial pneumonia (iNSIP; n=26), hypersensitivity pneumonitis (HP; n=40), sarcoidosis (n= 67), connective tissue disease-associated (CTD)-ILD (n=29), smoking related (SR)-ILD (n=13) and cryptogenic organizing pneumonia (COP; n=8)). TL was measured in white blood cells using a multiplex quantitative PCR method. In all ILD patients, TL was significantly shorter compared to healthy controls (n=173) (p range=0.038 - $2.28x10^{-27}$). Furthermore, we show that TL in IPF patients was significantly shorter than in patients with other forms of lung fibrosis (p=0.002), and in sarcoidosis patients (p=$1.35x10^{-7}$). Within the FIP cohort, patients carrying a telomerase reverse transcriptase (*TERT*) mutation (FIP-TERT) showed the shortest telomeres (p=$2.28x10^{-27}$), and FIP-no mutation patients had TL comparable to IPF patients (p=0.049). Remarkably, TL of FIP patients carrying a surfactant mutation (FIP-SFTP) was significantly longer than in IPF patients, but similar to that observed in the other sporadic idiopathic interstitial pneumonias. These results show telomere shortening across all ILD diagnoses. The difference in telomere length between FIP-TERT and FIP-SFTP indicates the distinction between acquired and innate telomere shortening. Short telomere length in IPF an FIP-no mutation is indicative of an innate telomere-biology defect, while a stress induced, acquired telomere shortening might be the underlying process for the other ILD diagnoses.

In **chapter 3** we measured TL in histological lung slides from patients with IPF in fibrotic and non-fibrotic areas. TL was measured using in-situ hybridization combined with an immunohistochemical marker to identify alveolar type 2 (AT-2) cells. We showed that in IPF, TL in AT2 cells in fibrotic areas was 56% shorter than in non-fibrotic areas (p<0.001), thereby showing a relation between short TL and fibrosis. No such difference was observed in the surrounding cells (p=0.30). Furthermore, AT2 cell TL is significantly shorter compared to the TL of their surrounding cells (p<0.001). In patients carrying a mutation in the telomerase gene *TERT* (FIP-TERT), AT2 cell TL was significantly shorter than IPF patients (p=0.02). However, no significant difference was observed between the surrounding cells of IPF and FIP-TERT patients. These findings may suggest that AT2 cells are the culprit cells in the pathogenesis of IPF, despite their genetic predisposition.

Since we have learned that telomere biology plays an important role in the development and

prognosis of IPF we sought an alternative, less laborious method than in situ hybridization to test histological samples (lung tissue biopsies). We measured TL using the multiplex quantitative PCR method, where DNA was extracted both from FFPE tissue blocks and circulating white blood cells. Using this technical approach, we found a correlation between the average TL of lung biopsies and TL in AT2 cell (non-fibrotic AT2 cells r^2=0.53, p=0.002). However no correlation was found between blood cell TL and lung TL. Finally, using qPCR TL measurements, it was determined that IPF patients with shortest lung TL had a significantly worse survival of just 26 months (p=0.003), demonstrating the potential prognostic value of this lung TL.

In **chapter 4** a significant association between the minor allele of mucin 5B (*MUC5B)* single nucleotide polymorphism (SNP) rs35705950 and IPF ($p=5.0x10^{-10}$), FIP ($p=2.7x10^{-9}$) and iNSIP ($p=3.4x10^{-4}$) was found. No association was observed in CTD-ILD. A FIP subgroup analysis revealed an association between *MUC5B* and telomerase mutated FIP (p=0.003), and between *MUC5B* and FIP with unknown genetic cause ($p=1.2x10^{-8}$). Furthermore, for IPF it was shown that carriership of *MUC5B* minor allele did not influence survival. In FIP *MUC5B* minor allele carriers had better survival (non-carriers 37 vs. carriers 53 months, p=0.01). In iNSIP survival analysis showed an opposite effect. Worse survival was found in iNSIP patients that carried the *MUC5B* minor allele (non-carriers 118 vs. carriers 46 months, p=0.027). This study showed that *MUC5B* minor allele predisposes to IPF, FIP and iNSIP. In IPF, survival is not influenced by *MUC5B* alleles. In FIP, *MUC5B* minor allele predicts better survival, pointing towards a subgroup of FIP patients with a milder, MUC5B-driven form of pulmonary fibrosis.

In **chapter 5**, we investigated the association between candidate alleles in telomerase genes *TERT* and *TERC*, and disease susceptibility in IPF and FIP patients. We showed that all genotyped variants in *TERT* and telomerase RNA component (*TERC*), except tandem repeat minisatellite of human telomerase gene 16A (MNS16A), were associated with disease susceptibility (p<0.05) in both sporadic and familial patients. Furthermore, an increased frequency of both a *TERC* and *TERT* risk allele haplotype was found in both patients groups, compared to controls. Carriership of both risk haplotypes leads to a significantly increased risk for disease (OR=2.40 [CI95%=1.35-4.28], p=0.01) and a significantly lower survival rate in IPF patients (p=0.001). Median survival in carriers was 20 months compared to 50 months in non-carriers. Combining risk haplotype data with *MUC5B* rs35705950 risk allele data showed that carriers of both risk alleles had an increased susceptibility to IPF (OR=11.20 [CI95%=3.22-39.0], $p=9.0{\cdot}10^{-6}$). This was also found in the FIP cohort (OR=11.22 [CI95%2.43-51.86], p<0.001). These findings suggest that genetic predisposition of IPF in sporadic patients depends on variation in multiple risk loci.

In **chapter 6**, we analyzed the role of the *TERT* variant rs2736100 in degenerative and cancerous disease. This genetic variant rs2736100 has been shown to be associated to multiple diseases including IPF. However not all diseases are associated to the same allele. We performed meta-analyses to determine if cancerous diseases are associated to one allele, while non-cancerous diseases are associated to the opposite allele. We found that cancer associates to the C allele (OR 1.16 [95%CI 1.09-1.23]) that is also associated to longer TL. Meanwhile, the odds ratio for the association of non-cancer diseases shows that these diseases associate

with the opposite allele (OR 0.81 [95%CI 0.65-0.99]), the allele that is associated with short TL. This suggests that this variant, and possibly *TERT*, acts as a balancing point between cancer and non-cancer disease, based on the consequences of either too long or too short telomeres.

Thus to conclude, (short) telomere length is an important factor in the pathogenesis and prognosis of IPF as demonstrated by TL measurements in blood and lung cells. The importance of telomere biology in IPF is further given weight by the association between IPF susceptibility and genetic variation in telomerase genes. The role of *MUC5B* in IPF is confirmed and is shown to increase the risk of IPF especially in combination with carriership of unfavourable variants in telomerase genes. Finally, one of these telomerase risk factors, rs2736100, is also associated with other diseases and appears to be a balancing point between cancer and non-cancer disease.

General discussion

In this thesis, we set out to further our understanding on the role of (a) genetic risk factors and (b) telomere length in disease development and prognosis, and identify telomere shortening effector cells in the alveolar compartment. We have shown that among ILD, IPF patients had distinguishably short telomeres. In IPF lungs, telomeres were shortest in AT2 cells located in fibrotic areas. TL in AT2 cells was also shorter than in surrounding cells. Patients with familial interstitial pneumonia carrying a *TERT* mutation had a significantly shorter TL in AT2 cells compared to sporadic IPF patients. However, there was no significant difference in TL of surrounding cells between IPF and FIP-TERT patients. In IPF patients, TL in AT2 cells correlated with average lung TL of biopsy slides as measured by qPCR. Patients with shortest average biopsy slide TL, also had the worst survival rate. Next, we showed that carrying risk alleles in *TERT*, *TERC* or *MUC5B* underlies IPF susceptibility. Moreover, we found that carrying both the *MUC5B* risk allele and the telomerase risk haplotypes increases the odds ratio for disease risk, compared to only carrying either. Carriership of the telomerase risk haplotypes also results in a significantly reduced survival time. Lastly, meta-analyses performed on the association of *TERT* SNP rs2736100 revealed an ambiguous role of this variant in disease as one allele is associated with cancer, while the opposite allele is associated with degenerative disease.

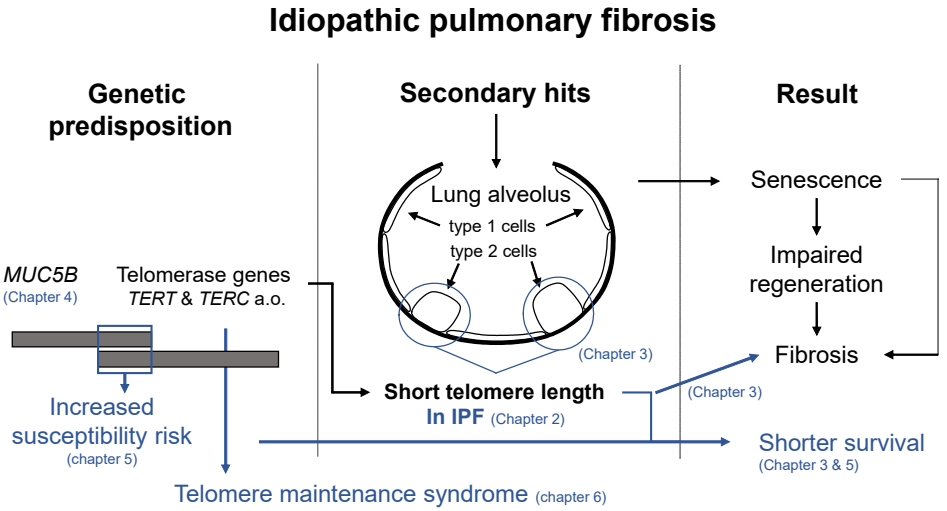


Figure 1. Pathogenesis of IPF. This diagram shows an IPF disease model complemented with the results of this thesis in blue. Chapter 2: TL in ILD, chapter 3: TL in IPF lungs, chapter 4: *MUC5B* risk allele IIP, chapter 5: *TERT* , *TERC* and *MUC5B* risk alleles in IPF, chapter 6: *TERT* risk allele in disease.

To illustrate how this thesis contributes to the knowledge on IPF pathogenesis, its findings have been added to the model that was introduced in the general introduction (chapter 1, figure 3). The updated model shows the findings of this thesis in blue, along with a referral to the corresponding chapters (figure 1). The general discussion will further address these

findings in a broader context with the main focus on if and how dysfunctional telomere maintenance would cause IPF. First, we discuss TL in blood and lung cells and how this underlines short TL as a causative factor. Second, we elaborate on how the findings presented here fit the IPF multiple hit hypothesis. Next, we hypothesize how not only a gene-environment interaction, but also a combination of genetic risk factors could also constitute the multiple hits causing IPF. Finally we discuss the prognostic value of TL on survival, the potential value of this thesis' results in the clinic, and our recommendations for future research.

Telomere length in ILD

Compromised telomere homeostasis has been implicated in a wide range of diseases[1–4]. Findings indicating the importance of telomere biology in IPF include the discovery of germ-line mutations in the telomerase genes *TERT* and *TERC* in families with IPF, and abnormally short blood cell TL in both sporadic and familial IPF patients[5–8]. TL however, is influenced by multiple external factors, including air pollution, tobacco exposure, stress and chronic inflammatory processes[9–12]. It is therefore important to determine if TL distinguishes IPF from other diseases. A significantly short TL in IPF compared to other ILD would suggest faulty telomere maintenance being a part of IPF's pathogenesis and not a secondary effect of the disease state. Therefore, we measured TL across the full spectrum of ILD (**chapter 2**). Tested non-IPF ILD patients had a mean TL, significantly shorter than control TL, and roughly comparable to the TL of surfactant mutations carrying familial interstitial pneumonia patients. Increased proliferation of immune cells is associated with shortened TL in peripheral blood cells[13] . Short TL in non-IPF ILD is therefore likely caused by an increased cell turnover in blood cells and represents an acquired state of short TL[14]. Systemic up-regulation of the immune system is associated with the ILDs sarcoidosis, COP, HP and CTD–ILD and could therefore cause the short TL in these patients. Of further importance was the finding of significantly shorter TL in sporadic IPF patients compared to the other ILD, which approached TL of familial patients with *TERT* mutations. Taken together these finding strongly suggest a causative role of impaired telomere maintenance in IPF.

Telomere length in lung cells

Multiple studies on TL so far, were all performed on white blood cell samples. However, as IPF by definition exclusively affects the lungs, it is important to determine the state of telomeres in lung cells themselves. Therefore, we also examined TL in the lung cells of IPF patients in both fibrotic an non-fibrotic areas. We showed that TL is shortest in AT2 cells of fibrotic lung areas as compared to non-fibrotic lung areas (**chapter 3**). Whereas TL in cells surrounding AT2 cells did not differ between fibrotic and non-fibrotic lung areas. This resulted in an important addition to the model in figure 1, namely the finding that short lung TL in AT2 cells is linked with fibrosis, and supports a contemporary view on the AT2 cell as a key factor in IPF pathogenesis[15–17]. The significance of this cell type in IPF has been established by the discovery of IPF causing mutations in the surfactant protein C (*SFTPC*) gene, of which the transcript is exclusively produced in the AT2 cells[18,19]. AT2 cells are the progenitor cells of the alveolar epithelium[20]. In this capacity, AT2 ensure the regenerative capacity of the epithelium upon injury[21]. Upregulated telomerase activity has been measured in AT2 cells upon hypoxic injury, establishing the importance of sustained telomere homeostasis in these cells and its vulnerability to telomere maintenance disruption under injurious circumstances[22]. Our results affirm this vulnerability to telomere shortening by showing that

AT2 cells have significantly shorter telomeres compared to surrounding cells in both fibrotic and non-fibrotic areas (**chapter 3**).

In IPF, repetitive injury to AT2 cells is thought to ultimately exceed the regenerative capacity of the alveolar epithelium[23,24]. Impairment of the AT2 cells' renewal capacity from idiopathic pulmonary fibrosis tissue leads to severe fibrosis[73]. Fibrosis itself is thought to arise through the induction of resident fibroblasts to differentiate into myofibroblasts[25]. Both celltypes induce extracellular matrix deposition and are found in the characteristic fibroblast foci in IPF lungs[15]. A study by Minagawa et al has shown that epithelial cells lining fibroblast foci showed increased levels of senescence[26]. Fibroblast foci sites typically contain AT2 cell hyperplasia[27]. In mice, it has been shown that telomere dysfunction leads to age-dependent lung remodeling and fibrosis[28]. A functional link between telomere maintenance and impaired AT2 cell regeneration is provided by the finding of senescence induced in AT2 cells through telomere dysfunction[29,30]. Senescence is a cell state that is classically linked to critically short telomere length[31]. As TL is shortened beyond a specific length the cell stops proliferating to prevent chromosomal instability[32]. However, this does not fully prevent further adverse consequences to the tissue. Gene expression profiles of senescent cells change, and in vivo, senescent cells have been shown to excrete a distinct profile of cytokines, chemokines and proteases called the senescence-associated secretory phenotype (SASP)[33,34]. These secretory products are in turn thought to induce fibrosis, providing a causative link between short TL and fibrosis mediated by the AT2 cell[30,35]. On the other hand, there are multiple indications that suggest that short TL alone is not sufficient to induce pulmonary fibrosis, and point to the necessity of a secondary hit to the alveolar epithelium.

Multiple hit hypothesis

IPF is currently thought to result from a convergence of specific genetic variation and secondary (environmental) hits to the alveolar epithelium, coined the multiple hit hypothesis[1,15,36]. In our opinion this hypothesis greatly accommodates a role for telomere biology in IPF, as will be argued here. In theory, a connection between telomere dysfunction and IPF is contradictory. That is, TL homeostasis ensures chromosomal stability and is thereby a universal part of cell maintenance. However, diagnosing a patient with IPF requires the exclusion of a fundamental, i.e. systemic cause[37]. This would imply an incompatibility between systemic telomere dysfunction and localized pulmonary fibrosis. However, there are several studies that, taken together provide support for this paradoxical relation specifically through the multi hit hypothesis.

In general, critically short telomeres limits the regenerative capacity of tissues[38,39]. But, short TL alone has been shown to be insufficient to cause pulmonary fibrosis in mouse models[40,41]. A secondary hit in these models was necessary to induce pulmonary fibrosis or emphysema. A similar phenomenon is observed in mice with short telomeres, where liver damage was only found after treatment with a toxin[42]. This would explain, why individuals who are genetically predisposed to short TL either through genetic variant carriership or inherited short TL, *and* encounter specific organ targeting triggers like cigarette smoke, develop disease symptoms only in the lung and not systemically.

Secondly, it is assumed that the regulatory environments of TL differ between compartments

of the body. But also the sensitivity between compartments to short telomeres differs and could explain the localization of telomere driven disease[36]. This difference could be facilitated by the *telomere position effect*, which is the effect of TL on the expression of genes located close to chromosome ends[43]. A different vulnerability to telomere shortening between blood cells and lung tissue could be the result. This is corroborated by our findings, as we show that there is no correlation between lung and blood cell TL suggesting asynchronous telomere shortening between these compartments (**chapter 3**). Another finding even narrows the vulnerability to telomere dysfunction down to one cell type. FIP-TERT patients have a significantly shorter AT2 cell TL compared to IPF patients. However, no difference was found in surrounding cell TL between these patient groups (**chapter 3**). This suggests that the AT2 cell is most vulnerable to telomere dysfunction in lung tissue, but would still require a second hit to develop disease.

Finally there are observations in *TERT* mutation carriers supporting the multiple hit hypothesis involving telomere dysfunction. A singular *TERT* mutation can lead to different phenotypes in different patients, possibly as a result of encountering different environmental hits[6]. Furthermore, the observed incomplete penetrance observed in familial patients carrying mutations in *TERT* further suggest a role for the effect of different environmental factors[5]. This all would explain why telomere malfunction can lead to disease exclusively in the Lung.

The multiple hit model further entails that, at least partially, IPF is a manifestation of a broader spectrum of diseases caused by compromised TL[36,44]. Telomerase gene mutations were first found due to the presence of pulmonary fibrosis in patients with dyskeratosis congenita, a disease caused by compromised telomere homeostasis[5]. Manifestations of telomere driven diseases can be found in multiple organs. Examples are liver cirrhosis and aplastic anaemia in adulthood[3,45]. For sporadic IPF to be part of such a short telomere syndrome, a genetic background related to short telomere length is to be expected in these patients.

Genetic predisposition

In search of the genetic predisposition of IPF, several genetic variants associated with susceptibility, have been discovered[46]. We confirmed the association of *MUC5B* SNP rs35705950 and *TERT* SNP rs2736100 with IPF susceptibility and present variants in *TERC* as significant factors in IPF (**chapter 4 and 5,**[47–50]). More interestingly from a pathogenic perspective, we showed that 10 out of 18 FIP patients carrying a *TERT* mutation also carried the *MUC5B* risk allele (**chapter 4**). Also in sporadic patients we show higher IPF odds ratios for patients carrying both the *MUC5B* and telomerase risk alleles compared to carriership of either of these alleles alone (**chapter 5**). With these findings we can now expand the IPF disease model as given in figure 1 and add the involvement of *TERC* SNPs. Moreover, we can add the greater IPF risk conveyed by the joint carriership of genetic variants in both telomerase genes and *MUC5B*, compared to carrying either. In the context of the multiple hit hypothesis, it is conceivable that a second hit could also be of a genetic nature instead of an environmental one. Specifically our results suggest that a telomerase hit combined with a *MUC5B* hit could lead to IPF. A hypothesis on how the *MUC5B* SNP could cause IPF as a secondary hit, has been presented by Van Moorsel et al.[46]. The IPF risk allele of this SNP is also associated with interstitial lung abnormalities in the general population[51]. Furthermore, areas of the lung that are exposed to most mechanical stress during respiration are also the most fibrotic regions in

IPF lungs[52]. The demand on the regenerative capacity of the lung and AT2 cells specifically will be highest in these high stress areas.

Compromised telomere maintenance as a primary hit in these areas wouldlead to accelerated depletion of the regenerative capacity[35]. The protein Mucin-5B reduces the surface tension of the surfactant fluid which prevents the collapse of alveoli during the respirational movement of the lung. Carrying the risk allele induces higher expression levels of *MUC5B*, which could disturb surface tension levels. Telomere dysfunction alone (*first hit*) does not lead directly to IPF. However in combination with the increased stress as a result of the altered surfactant composition(*second hit*) it could result in pulmonary fibrosis, even without an extra physical hit like cigarette smoking.

Patients with HP are at risk of developing pulmonary fibrosis. In a cohort of 217 HP patients, an association was found between the extent of radiographic fibrosis, carriership of the *MUC5B* risk allele and short telomere length[53]. For the patients we genotyped up to 18% of the IPF patients carried both the *MUC5B* risk allele and the telomerase risk haplotypes, compared to 2% of the controls (OR: 11.20 [95%CI: 3.22 - 39.0] p = $9.0{\cdot}10^{-6}$) (**chapter 5**). It is therefore tempting to speculate that the underlying cause of IPF in these patients is telomere dysfunction, but that fibrosis only develops in combination with the detrimental effect of overexpressed *MUC5B* on the alveolar surface tension. This would also explain the observation why 10 out of 18 *TERT* mutation carriers also carried the *MUC5B* risk allele (**chapter 4**). The discovery of an IPF associated variant in the desmoplakin (*DSP*) gene, would further support this hypothesis. This gene encodes a protein that is important for maintaining epithelial integrity through the formation of structural connections between cells that are essential for tissues under mechanical stress[50,54]. Dysfunction of this protein could also exacerbate the damage caused by mechanical stress in the lung. In combination with impaired regenerative capacity due to telomere dysfunction, this could be a second route to pulmonary fibrosis. It is important to note here that analysis of possible secondary hits is complicated by the presence of genetic anticipation in families harboring telomere maintenance mutations[55]. Telomere length itself influences disease penetrance and expressivity[56]. This poses a challenge as patients without a genetic background implicating telomere dysfunction can still suffer from an impaired regenerative capacity.

Survival

A final addition to the knowledge on IPF is our findings concerning the survival prognosis. The *MUC5B* SNP risk allele has been associated with an improved survival rate in IPF[57]. In our cohort, no such association was found in IPF (**chapter 4**). Moreover, in a Cox regression survival analysis which included both the *MUC5B* and telomerase risk haplotypes as covariates, we found that only carriership of the telomerase risk haplotypes together was a significant determinant in IPF survival, not *MUC5B* carriership (**chapter 5**). Although the telomerase SNPs are associated with TL in the general population[58–62] we did not detect this effect in our cohort. However, we did find an association between short TL of lung biopsy slides, and diminished survival. This association has also been found between blood cell TL and survival in IPF[63,64]. Although we corroborated the previous results presented by Kropski et al., that showed no correlation between the TL in white blood cells and in lung tissue, survival is predicted by TL in both compartments[65]. It is therefore possible that survival based

on blood cell TL is not related to IPF and reflects the effect of telomere length on survival in general.
A detrimental effect of carrying the *MUC5B* risk allele on survival was found for iNSIP patients. This could be a result of the detrimental effect this allele has on lung integrity found in the general population as discussed above[51]. However it could also be a reflection of the observed clinical heterogeneity between IPF cohorts[66]. This is further underlined by the finding that *TERT* SNP rs2736100 is not associated with IPF, but is with a cohort of non-IPF ILD[67]. It might therefore be necessary to classify IPF and indeed idiopathic interstitial pneumonias (IIP) based on genetic background or at least stratify IIP patient groups, to generate more homogenous cohorts for experimentation and more importantly for clinical trials.

Translation
A number of results, presented in this thesis, might have potential use in clinical practice or should be taken into consideration during development of new therapeutics, and evaluation of clinical trials. First, although it is not possible to distinguish between ILD based on TL, **chapter 2** does provide a measure for the shortness of TL in individual patients compared to controls. This measure is an indication for the extent to which a patient is subject to telomere driven disease and could be used as a prognostic marker for survival[63,64]. This is also true for the average TL of lung biopsy slides (**chapter 3**). Although surgical biopsies are not by default taken in IPF patients, average TL from these biopsies could be used as a prognostic marker for survival. Next, findings in **chapter 3**, further cement the AT2 cell as a central point of interest in IPF pathogenesis. Prospective therapeutics for the treatment of IPF should therefore be aimed at the reconstruction and preservation of this cell type. Furthermore, genetic predisposition studies (**chapter 4 & 5**) in this thesis show the potential use of risk allele carriership in *MUC5B* and telomerase genes as prognostic markers for survival in iNSIP and FIP, and IPF respectively. Finally, analysis of the *TERT* SNP rs2736100 showed both sides of this genetic variant: it is a pivot between non-cancerous diseases of which multiple have a degenerative character, and cancerous disease (**chapter 6**). This genetic variant will be a part of multi-facetted genetic predisposition that drives the role of telomere homeostasis in the different diseases. However, it is a first tangible indication for the balancing act of telomere biology in disease. More importantly, this finding proves that great care should be taken concerning therapeutic agents that influence TL. For example, preemptive use of putative telomerase activating agents could facilitate tumorigenesis in a degenerative disease like IPF[68,69]. In order to develop reliable telomere therapeutics it is important to determine factors that cause the scale of telomere homeostasis to tip to one side (tumorigenesis) or the other (degenerative disorder).

Future research
One recurring observation in particular requires further study, namely the possibility that telomere dysfunction marks a separate group in IPF. In the IPF cohort, a number of patients showed TL, similar the TL of *TERT*-mutation carriers (**chapter 2**). While other IPF patients had TL similar to controls and surfactant-mutation carriers. This observation suggests a different pathogenesis between these groups of IPF patients based on telomere length. This in turn could resemble the apparent difference in disease etiology between familial patients carrying telomerase or surfactant mutations. Considerable variation in TL was also observed between lung biopsies, and telomerase risk alleles only underlie a distinct portion of IPF

patients (**chapter 3 and 5**). Both IPF patients with telomere and non-telomere driven disease will have pulmonary fibrosis comparable in appearance. However, the different etiologies could influence the effectiveness of prospective therapies. Furture studies should aim at further defining the genetic background of the telomere dysfunction group or determine if this group can be distinguished by other markers than TL of white blood cells. Also, we show that other ILD beside IPF have significantly shorter TL compared to controls (**chapter 2**). Therefore, research should be conducted to verify this cause of telomere shortening in the non-IPF ILD.

Chapter 3 shows that short TL is associated with fibrotic areas in the IPF lung. A next logical step would be to investigate if these cell show the result of short TL, like end-to-end fusion of chromosomes or increased DNA damage repair responses. Senescent AT2 cells in alveolar organoid have been shown to trigger upregulated cytokine signaling pathways[29]. Further investigation should demonstrate this in IPF lungs and ascertain if they contain higher levels of SASP and putative subsequent fibrosis.

Finally, our findings suggests that IPF predisposition in sporadic patients could be based on carriership of multiple genetic risk alleles (**chapter 5**). Multiple risk alleles in several distinct genes have been found to associate with IPF risk. Beside alleles in telomerase genes and *MUC5B*, risk alleles have been found in immunity related genes like Toll-like receptors 3 (*TLR3*) and interleukin 1 receptor antagonist (*IL1RN),* cell-cell adhesion related genes dipeptidyl peptidase 9 *(DPP9)* and desmoplakin *(DSP),* the Rho guanine nucleotide exchange factor gene *AKAP13* that is involved in profibrotic signaling pathways, and family with sequence similarity 13 member A (*FAM13A*) which is associated with susceptibility to chronic lung diseases [46,70–72]. Future research should investigate if combining more risk alleles would yield more genetic risk profiles for IPF. Risk enhancing combinations could further lead to new insights in how different processes in the lung lead to IPF.

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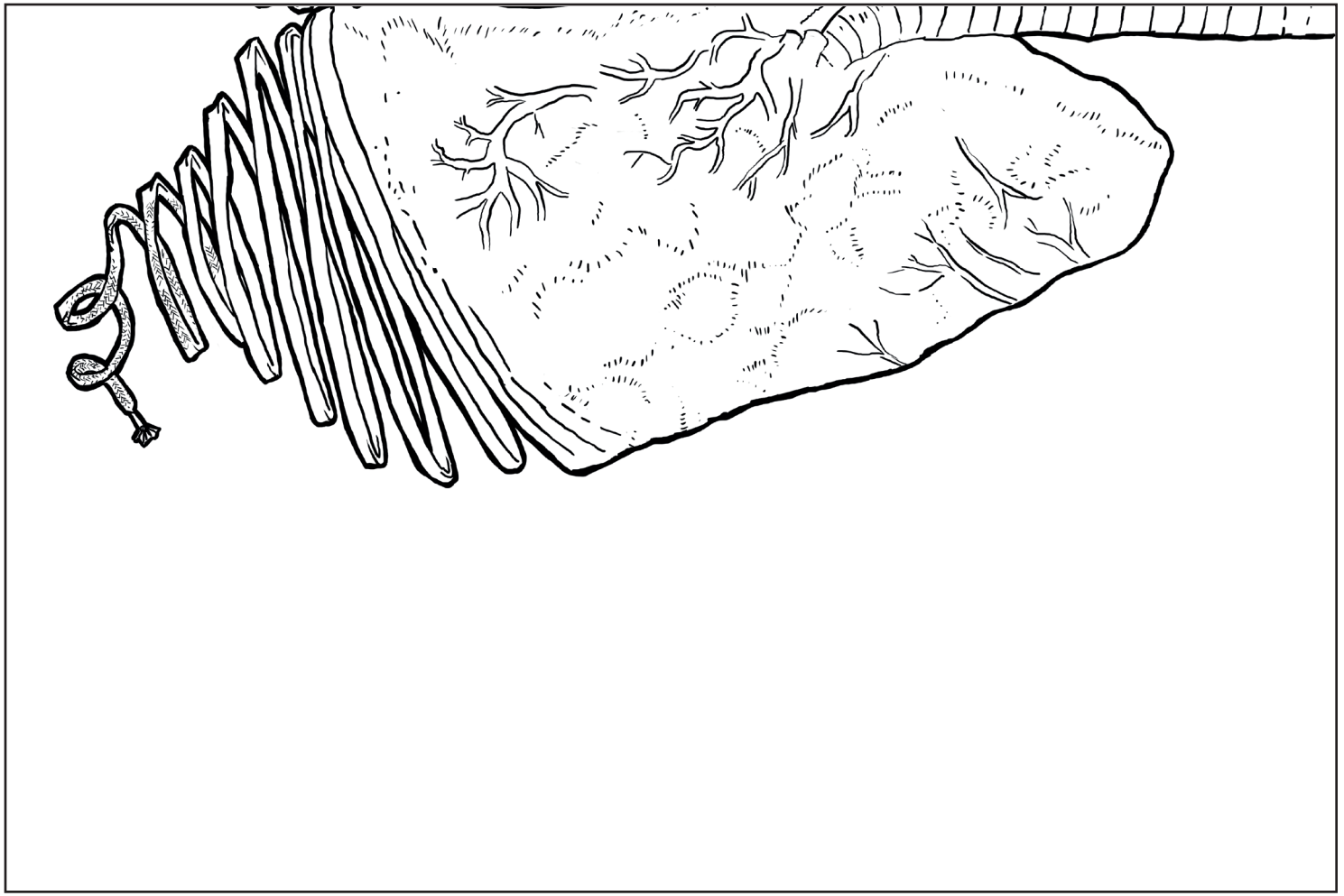

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Hoofdstuk 1. – Inleiding

Idiopathische pulmonale fibrose (IPF) is een longziekte die wordt gekenmerkt door een progressieve verlittekening van het alveolaire interstitium, het weefsel achter het oppervlak van de longblaasjes. Deze verlittekening bemoeilijkt de gasuitwisseling met als gevolg een afnemende longfunctie. Er zijn slechts een beperkte hoeveelheid behandelopties beschikbaar en deze zijn niet curatief. Het ontwikkelen van een doeltreffende therapie wordt gehinderd door het feit dat de oorzaak van IPF onbekend is. IPF is een leeftijd gerelateerde aandoening die vooral voorkomt bij mensen ouder dan 60 jaar. In een aantal gevallen (tot 20%) hebben meerdere familieleden pulmonale fibrose. Dit wordt dan familiaire interstitiële pneumonitis (FIP) genoemd. Bij IPF patiënten wordt de longfunctie steeds slechter en uiteindelijk overlijden patiënten meestal 2 tot 5 jaar na diagnose. De huidige medicatie zorgt er voor dat de afname van de longfunctie iets langzamer verloopt. De enige optie die een IPF-patiënt nu heeft om te overleven is een longtransplantatie. Donorlongen zijn echter zeer beperkt beschikbaar, de kosten van een longtransplantatie zijn hoog en de gemiddelde overleving (ca. 10 jaar in Nederland) na transplantatie is beperkt.

Onderzoek bij patiënten met de familiaire vorm van IPF heeft uitgewezen dat het gen *TERT* (Telomerase reverse transcriptase) gemuteerd is in een aantal van deze patiënten. *TERT* codeert voor de eiwit component van het eiwit-RNA complex *telomerase*. (De RNA component wordt gecodeerd door het gen telomerase RNA component (*TERC)*). De belangrijkste functie van telomerase is het verlengen van de uiteinden van chromosomen, de telomeren. Wanneer cellen delen wordt het DNA in deze cellen gekopieerd en verdeeld over twee nieuw gevormde cellen. Inherent aan het kopiëren van DNA is dat de uiteinden van de chromosomen verkorten. Om te voorkomen dat met dit verkorten genetische informatie verloren gaat, bevinden zich aan het uiteinde van de chromosomen de telomeren. De telomeren bestaan uit een repeterende DNA sequentie van zes nucleotiden: TTAGGG. De telomeren bevatten zelf geen genetische informatie en zorgen met de ruimtelijke structuur die ze vormen met andere eiwitten voor de stabiliteit van de chromosomen.

Wanneer na een beperkt aantal celdelingen de telomeren uitgeput zijn, zal een cel stoppen met delen. In een beperkt aantal celtypen, zoals stamcellen, wordt in beperkte mate door het telomerase eiwit de telomeren weer verlengd, zodat celdeling door kan gaan. Per saldo echter worden telomeren in menselijke cellen korter naarmate een hogere leeftijd wordt bereikt. De lengte van telomeren wordt daarom gezien als een maat voor veroudering. In de FIP patienten met de mutaties in het *TERT* gen worden kortere telomeren gemeten dan in gezonde leeftijdsgenoten. Dit zou betekenen dat deze patiënten vervroegd verouderen en dat dit zich openbaart in de longen. In het bloed van sporadische IPF patiënten (IPF patiënten zonder familiegeschiedenis) worden ook kortere telomeren gemeten, hoewel deze patiënten geen mutaties hebben in telomerase genen.

Doel van het onderzoek
Het doel van het onderzoek, beschreven in deze thesis is tweeledig. Ten eerste willen we nader onderzoek doen naar de rol van telomeerlengte in IPF door deze te meten in het bloed en de longen van IPF patiënten. Ten tweede willen we onderzoeken of veelvoorkomende genetische varianten in de telomerase genen predisposeren voor IPF, al dan niet in combinatie met een genetische variant in het gen *MUC5B*.

Hoofdstuk 2. – Telomeerlengte in Interstitiële Longziekten
IPF maakt deel uit van een groep longziekten die interstitiële longziekten (interstitial lung diseases (ILD) in het Engels) worden genoemd. Het is bekend dat telomeerlengte korter kan worden door verscheidene psychologische en fysiologische stressoren. Om te onderzoeken of de korte telomeren in IPF niet een bijverschijnsel zijn van het ziek zijn zelf is de telomeerlengte van bloedcellen van IPF patiënten vergeleken met die van gezonde controles, maar ook met de telomeerlengte van patiënten met andere interstitiële longziekten. Uit het onderzoek bleek dat de telomeerlengte van alle interstitiële longziekten significant korter was dan die van de controles. Ten opzichte van de meeste andere interstitiële longziekten waren de telomeren van IPF patiënten echter significant nog korter. Telomeerlengte is ook gemeten in FIP patiënten. FIP patiënten werden onderverdeeld in een groep patiënten met een mutatie in het telomerase gen *TERT*, een groep patiënten waarbij geen bekende longfibrose veroorzakende mutatie is gevonden, en een groep patiënten met een mutatie in het gen voor het longsurfactant eiwit C of A2 (*SFTPC*, *SFTPA2*). Mutaties in het longsurfactant gen veroorzaken ook longfibrose, maar hebben geen rechtstreeks verband met telomeren biologie. De telomeerlengte van FIP patiënten met een *TERT* mutatie waren het kortst, telomeren van patiënten zonder een bekende mutatie waren vergelijkbaar in lengte met de telomeerlengte van IPF patiënten, en de telomeerlengte van FIP patiënten met een surfactant mutatie is vergelijkbaar met de lengte van andere ILD. Het verschil in telomeerlengte tussen FIP patienten met een *TERT* mutatie en patiënten met een surfactant mutatie wijst erop dat er twee categorieën van telomeerverkorting te onderscheiden zijn in ILD. In de meeste ILD behalve IPF, lijkt verkorting van de telomeerlengte op te treden als secundair gevolg van het ziek zijn, zoals bij FIP patiënten met een surfactant mutatie. Dit in tegenstelling tot IPF, waar telomeren significant korter zijn, wat duidt op een aangeboren telomeren biologie defect.

Hoofdstuk 3. – Telomeerlengte in de long
Naast telomeerlengte in bloed is de lengte van telomeren gemeten in longcellen van IPF patiënten. Op histologische longcoupes zijn met behulp van een in-situ hybridisatie (FISH) methode de telomeren gemarkeerd met een fluorescente probe. Door de mate van fluorescentie intensiteit te bepalen is het mogelijk om de gemiddelde relatieve telomeerlengte per cel te bepalen. Dit is specifiek gebeurd voor de alveolaire type 2 (AT2) cel door deze te onderscheiden van de overige longcellen met een immunohistochemische marker. De AT2 cel is een belangrijke cel in de longblaasjes. De AT2 cel produceert het longsurfactant dat de long beschermd tegen ziektekiemen en ademhaling faciliteert. Daarnaast kan de AT2 cel delen en differentiëren tot AT1 cellen, de cellen die de longblaasjes bekleden en door welke de gasdiffusie plaatsvindt. Uit metingen blijkt dat in IPF patiënten telomeren 56% korter zijn in AT2 cellen in niet-fibrotische gebieden, vergeleken met AT2 cellen in fibrotische gebieden van een longcoupe ($p<0{,}001$). Dit duidt op een relatie tussen telomeerlengte en fibrose in

IPF longen. Wanneer de telomeerlengte wordt gemeten in niet-AT2 cellen, cellen rondom de AT2 cellen, blijkt er geen verschil te zijn in telomeerlengte tussen fibrotische en niet-fibrotische gebieden. In beide gebieden is de telomeerlengte van de AT2 cellen korter dan van de omgevende cellen. In patiënten met een *TERT* mutatie was de telomeerlengte in AT2 cellen korter dan die in IPF patiënten zonder mutatie. Echter, er is geen verschil in telomeerlengte van omgevende cellen tussen patiënten met en zonder *TERT* mutatie. Dit suggereert dat de AT2 cel een belangrijke cel is in de pathogenese van IPF, ongeacht de genetische predispositie van de patiënt.
Telomeerlengte in de longen is ook bestudeerd met behulp van een multiplex kwantitatieve PCR (qPCR) methode. Tevens is met deze methode de telomeerlengte in het bloed van dezelfde patiënten gemeten. Er is geen correlatie gevonden tussen de lengte van telomeren in het bloed en in longweefsel. Wel is een correlatie gevonden tussen telomeerlengte gemeten met behulp van de qPCR en FISH methode voor AT2 cellen in non-fibrotische gebieden (r^2=0.53, p=0.002).
Ten slotte is vastgesteld dat IPF patiënten met kortere telomeren (zoals vastgesteld met de qPCR methode) een kortere overleving hebben (van slechts 26 maanden na afname van het longbiopt,) ten opzichte van patiënten met langere telomeren. Dit laat zien dat telomeerlengte in de long mogelijk gebruikt zou kunnen worden als prognostische marker in IPF.

Hoofdstuk 4. – Genetische variatie in MUC5B

Er is een significante associatie tussen een enkel-nucleotide polymorfie (single nucleotide polymorphism (SNP) in het Engels) (rs35705950) en zowel IPF ($p=5.0x10^{-10}$), FIP ($p=2.7x10^{-9}$) als idiopathische niet-specifieke interstitiële pneumonitis (iNSIP, $p=3.4x10^{-4}$). Een dergelijke associatie is niet gevonden voor interstitiële longaandoening als gevolg van bindweefselziekten. Wanneer de FIP patiënten opgedeeld worden in een groep met een telomerase mutatie en een groep waarvoor geen ziekte veroorzakende mutatie is gevonden, blijken beide groepen significant geassocieerd met het polymorfisme in *MUC5B* (p=0.003 en $p=1.2x10^{-8}$ resp.).
In het IPF cohort is geen verband gevonden tussen dragerschap van het polymorfisme en overleving na diagnose. Een dergelijk verband is wel aangetoond in het FIP cohort. Dragers van het minor allel (allel met de laagste frequentie in de algemene populatie) in het FIP cohort hebben een langere overlevingstijd (gemiddeld 52 maanden) ten opzichte van niet-dragers (37 maanden). Dit duidt erop dat er binnen het FIP cohort een deel van de patiënten een mildere, door *MUC5B* gedreven vorm van pulmonale fibrose heeft. In het iNSIP cohort werd ook een significant verband aangetoond, echter hier was het effect tegenovergesteld. Dragers van het minor allel hadden gemiddeld een kortere overlevingstijd (46 maanden) ten opzichte van niet-dragers (118 maanden).

Hoofdstuk 5. – Genetische variatie in telomerase genen

Een groot deel van de IPF patiënten heeft significant korte telomeren. Om te achterhalen of genetische variatie in de telomerase genen hier een rol in speelt zijn een aantal varianten in de telomerase genen *TERT* en *TERC* die in de algemene populatie geassocieerd zijn met telomeerlengte, onderzocht op associatie met IPF en FIP. Het betrof 4 SNPs in het gen *TERC*, 2 SNPs in het gen *TERT* en een variabele repeterende sequentie in *TERT*. Voor alle SNPs is een significante associatie gevonden tussen het minor allel en zowel IPF als FIP. Daarnaast wordt in de IPF en FIP een groep significant vaker een haplotype van alle risico

allelen aangetroffen dan in controles. Dragerschap van de beide risico haplotypes in zowel *TERT* als *TERC* is ook geassocieerd met verhoogd risico op ziekte. (OR=2.40 [CI95%=1.35-4.28], p=0.01) Daarnaast is de overleving binnen het IPF cohort significant lager van dragers van beide risico haplotypes vergeleken met niet dragers (20 en 50 maanden respectievelijk p=0.001). Wanneer deze data wordt gecombineerd met de data van het onderzoek naar variatie in het *MUC5B* gen, blijkt dat wanneer de risico allelen in zowel de telomerase genen als *MUC5B* worden gedragen de odds ratio voor de ziekte hoger wordt (IPF: OR=11.20 [CI95%=3.22-39.0], $p=9.0 \cdot 10^{-6}$; FIP: OR=11.22 [CI95%2.43-51.86], p<0.001). Deze bevinding suggereert dat genetische predispositie voor pulmonale fibrose afhankelijk is van meerdere risico loci.

Hoofdstuk 6. – Meta analyse *TERT* variant rs2736100

TERT SNP rs2736100 is naast IPF ook geassocieerd met meerdere andere ziekten. Uit een meta-analyse van 85 studies blijkt dat het C allel, dat geassocieerd is met langere telomeerlengte, ook geassocieerd is met verschillende kankers, terwijl het A allel, geassocieerd met kortere telomeren ook geassocieerd is met niet-kanker ziektes (waaronder IPF). Dit suggereert dat deze SNP, en mogelijk het *TERT* gen, een balanceerpunt is tussen kankers en niet-kankers waarbij de eerste groep te lange en de tweede groep te korte telomeerlengte heeft.

Hoofdstuk 7. – Discussie en conclusie

Telomeerlengte is een belangrijke factor in de pathogenese en prognose van IPF. Dit is gebleken uit telomeerlengte metingen in zowel het bloed als de longen van IPF patiënten. Daarnaast is het belang van telomeren biologie in IPF aangetoond door de associatie tussen genetische variatie in de telomerase genen *TERT* en *TERC* en vatbaarheid voor IPF. De rol van *MUC5B* in IPF is bevestigd en daarnaast is aangetoond dat het risico op IPF wordt verhoogd door dragerschap van risk allelen in zowel *MUC5B* als de telomerase genen. IPF is een aandoening die verondersteld wordt, veroorzaakt te worden door meerdere factoren, omschreven als een "multi-hit model". Mogelijk zijn de afzonderlijke varianten in *MUC5B* enerzijds en de telomerase genen anderzijds, verschillende factoren die, wanneer ze in een individu samenkomen, leiden tot IPF. Ten slotte is een van de SNPs in *TERT*, rs2736100, geassocieerd met meerdere ziekten, waarbij het allel C, geassocieerd met langere telomeerlengte, geassocieerd blijkt met kanker, terwijl het allel A geassocieerd met kortere telomeerlengte, geassocieerd blijkt met niet-kanker ziektes, waaronder IPF.

De rol van telomeren in de vatbaarheid voor IPF en het beloop van deze ziekte, duidt op een vroegtijdige veroudering van de long. Met de huidige medicatie is deze veroudering moeilijk te stoppen. Toekomstig onderzoek zou daarom gericht moeten worden op het ontwikkelen van medicatie waarmee vroegtijdige veroudering van de long gestopt kan worden.

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List of abbreviations

ILD	interstitial lung disease
DPLD	diffuse parenchymal lung diseases
IPF	idiopathic pulmonary fibrosis
TL	telomere length
IIP	idiopathic interstitial pneumonia
IP	interstitial pneumonias
FIP	familial interstitial pneumonia
SFTP	surfactant protein
TERT	telomerase reverse transcriptase
TERC	telomerase RNA-component
iNSIP	idiopathic non-specific interstitial pneumonia
COP	cryptogenic organizing pneumonia
SR-ILD	smoking-related interstitial lung disease
HP	hypersensitivity pneumonitis
CTD-ILD	connective tissue disease-associated interstitial lung disease
T/S ratio	telomere repeat copy number to a single gene copy number ratio
AT2	alveolar type 2
MMqPCR	monochrome multiplex quantitative polymerase chain reaction
DLCO	diffusing capacity of the lungs for carbon monoxide
FVC	and forced vital capacity
FFPE	Formalin fixed paraffin embedded
MAF	minor allele frequency
SNP	single nucleotide polymorphism
OR	odds ratio
CI	confidence interval

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Curriculum vitae

Reinier Snetselaar was born on June 15th 1984 in Zeist, the Netherlands. After graduating from Gymnasium at Christelijk College Zeist in 2002, he finished his bachelor in Biology at Utrecht University in 2006 and in 2008 obtained a master's degree in Plant Biology. He worked as a microbiological technician at Silliker, Ede and subsequently as a junior researcher at the ILD-research group at the St. Antonius hospital in Nieuwegein. He investigated the role of telomere length and genetic predisposition in the development and prognosis of idiopathic pulmonary fibrosis and obtained his Ph.D. degree on June 12th 2018. Currently he works as project leader at the Product Development department of MRC-Holland, Amsterdam.