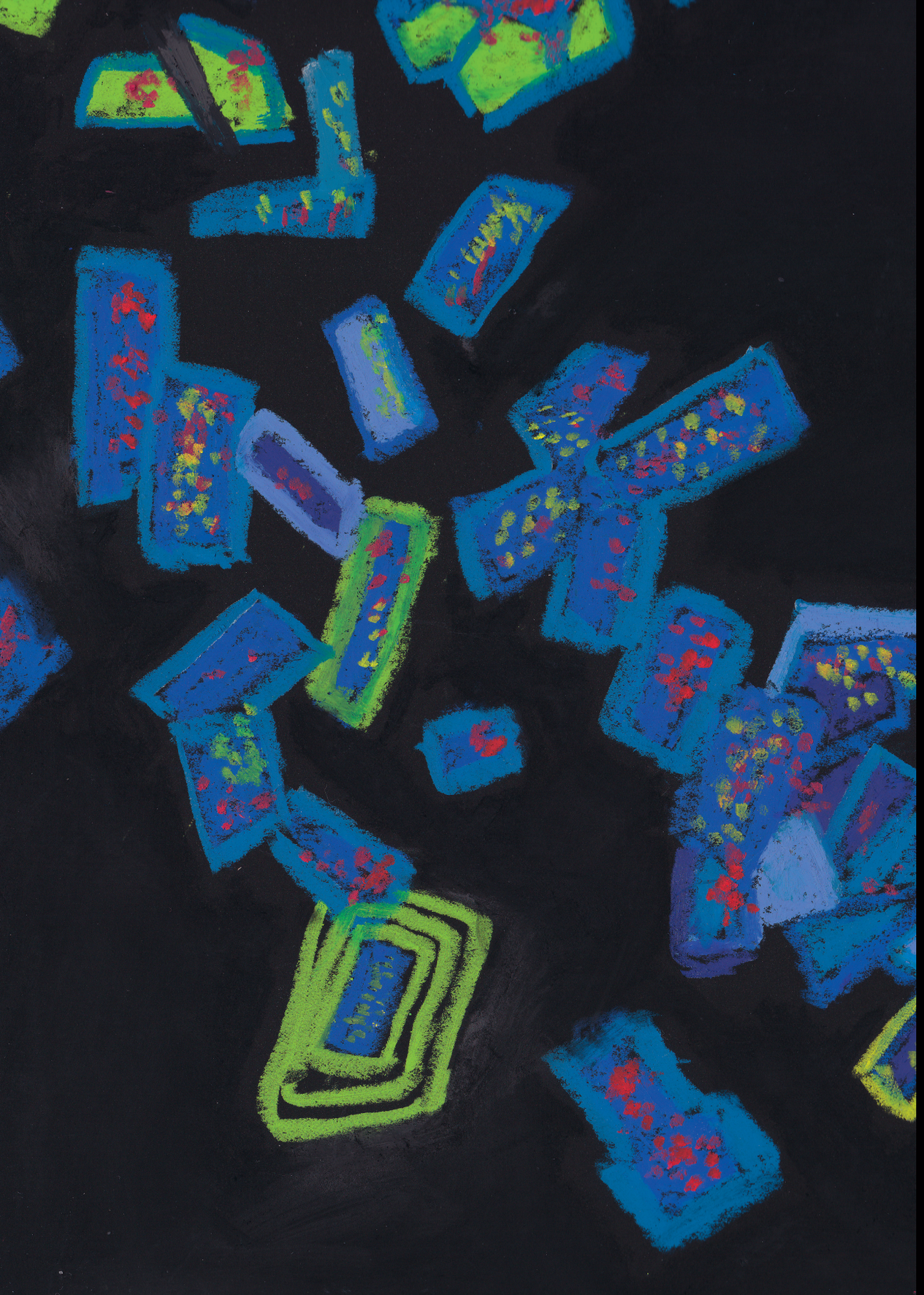
Telomere shortening and fibrotic remodeling in progressive fibrosing interstitial lung disease

Aernoud A. van Batenburg



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# Telomere shortening and fibrotic remodeling in progressive fibrosing interstitial lung disease

**Telomeer verkorting en fibrotische verandering in progressief fibrotische interstitiële longaandoeningen**

(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

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*Je moet roeien met de riemen die je hebt*

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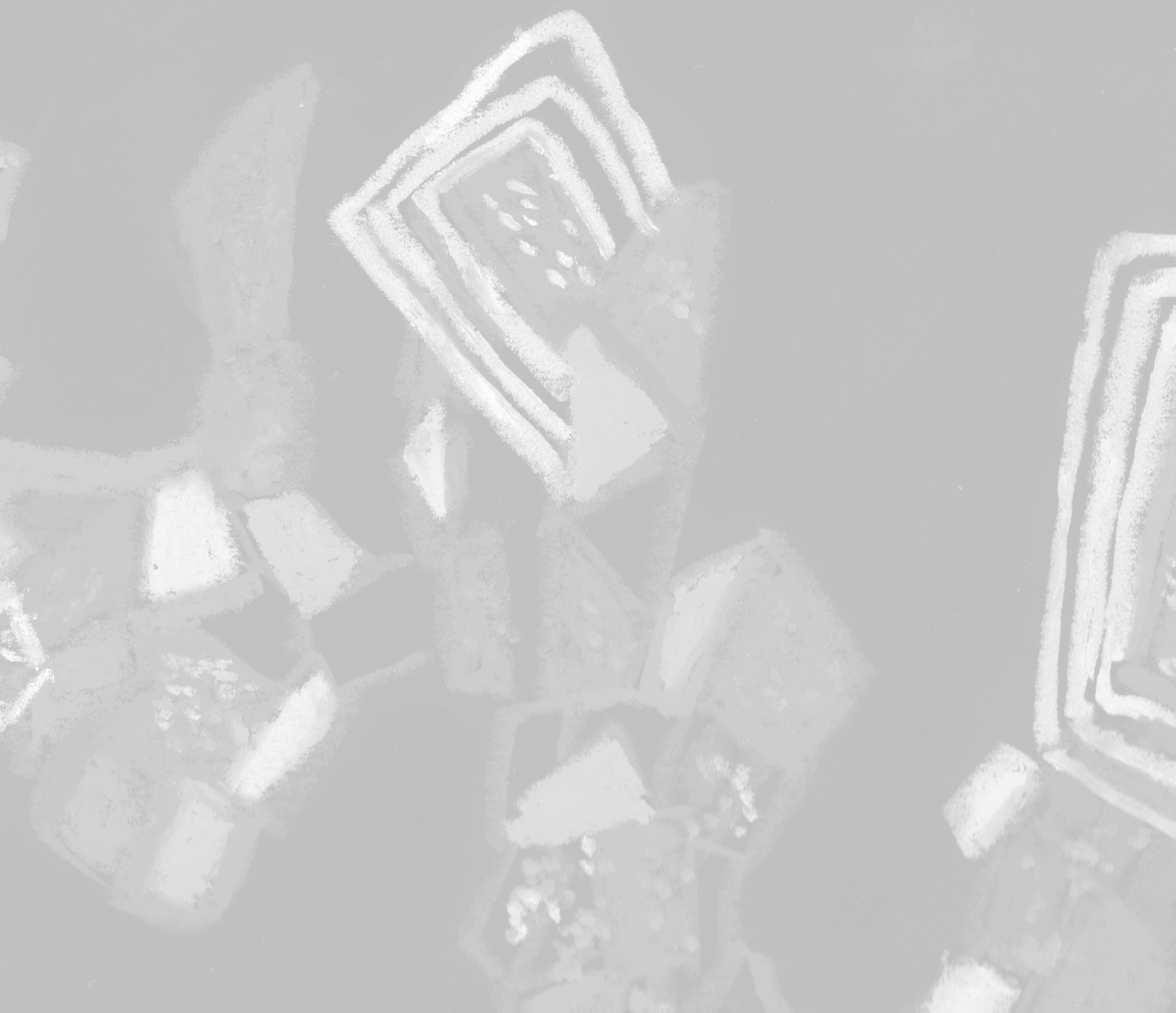
Chapter 1

# General introduction and outline of the thesis



# Introduction

**Progressive fibrosing interstitial lung disease (ILD)**

Interstitial lung diseases (ILDs) are a group of rare diseases that affect the lung parenchyma, including the interstitium. The most life-threatening manifestation is progressive fibrosing ILD, which is characterized by continuous scarring and may occur in sporadic or familial context. The main treatment strategies are removal of causative agents, suppression of inflammation and/or inhibition of fibrosis. In selected cases lung transplantation can be a last resort option. In the majority of ILD patients, the underlying pathological mechanism is still unclear. However, recent advances in the study of the genetics of familial pulmonary fibrosis may help to unravel pathways that are associated with disease progression and/or fibrosing ILD in general. In the research reported on in this thesis, we set out to explore telomere-related pathology in progressive fibrosing ILD.

**Idiopathic pulmonary fibrosis**

Idiopathic Pulmonary Fibrosis (IPF) is regarded as the archetype of progressive fibrosing ILD. IPF is characterized by unclear etiology, heterogeneous fibrosis of the lung with typical constellations of radiological and/or pathological features (i.e. the 'usual interstitial pneumonia (UIP) pattern'), and has a poor prognosis of 2-5 years after diagnosis (2, 3). The incidence varies from 3 to 9 cases per 100.000 people per year in Europe and North America (1). Most patients die from progressive respiratory failure, with 10-20% of cases suffering from acute exacerbations (4). IPF usually presents in smoking men older than 60 years and is associated with aging (5–8). Even though the cause is not known, it has been proposed that IPF is the result of multiple 'hits', with preexisting genetic susceptibility or environmental factors being followed by secondary injuries leading to uncontrolled wound healing response and subsequent fibrosis (9). However, the question remains what factors are involved in IPF development.

Symptoms of IPF consist of progressive shortness of breath, often accompanied by coughing that can be provoked by physical effort (6, 10). Because these symptoms are not very specific, patients with IPF are often initially diagnosed with hearth failure or other lung diseases such as chronic obstructive pulmonary disease (11). It is therefore essential to carefully assess each patient and discuss the diagnosis in a multidisciplinary team of chest physicians, radiologists and pathologists, ideally with expertise in ILD. Such meetings should not only discuss spirometry and other commonly collected clinical data, but also need a high level of alertness to exposures. For example, it is important to consider exposure to molds, damps or certain bacteria, which could indicate disorders that can mimic IPF, such as hypersensitivity pneumonitis. Furthermore, radiological and histological lung images need to be carefully assessed for findings supporting a diagnosis of IPF. If present, these can be classified as a UIP pattern on high-resolution computed tomography (HRCT) and/or histology, and provide a high degree of confidence for a correct diagnosis.

Early detection of IPF is important because starting treatment in the initial phase of the disease is likely to improve the patient's prognosis (12, 13). Some patients have symptoms up to 5 years prior to the diagnosis of IPF (11), and pathogenesis may start even many years before that. Although early IPF is generally described as IPF with mild impairment of pulmonary function, small extent of fibrosis on HRCT, minor symptoms and a stage 1 Gender-Age-Physiology index, it is very difficult to detect in clinical practice, and guidelines, e.g. for screening high risk individuals, are currently unavailable (14, 15). Studies of asymptomatic members of families with familial pulmonary fibrosis (FPF) may possibly help to establish greater insight into clinical, radiological, as well as genetic, biological and cellular markers to improve early diagnosis (14).

A confident diagnosis of IPF can be made when the chest HRCT enables pulmonary fibrosis with a UIP pattern to be distinguished from disease without this pattern (16–18). On HRCT, IPF lungs typically show an apicobasal gradient, in which fibrosis is most abundant subpleurally in the basal lung fields, with bilateral reticulation and honeycombing (18). If the imaging of the lung matches these features, and other causes of ILD are ruled out, the diagnosis of IPF can be made (18).

In approximately two-thirds of the cases the chest HRCT is sufficient for a confident diagnosis of IPF (19). However, if clinical and radiological findings are inconsistent and if results are needed to determine therapy, a lung biopsy will usually be needed for diagnosis. Typical histological findings for IPF include healthy air-containing non-fibrotic areas juxtaposed to fibrotic areas with dense collagen, fibroblast foci and honeycomb cysts (classified as histological UIP pattern). Honeycombing is characterized by fibrotic mucus-filled airspaces with an alignment of bronchiolar epithelium. These features are commonly found in the subpleural and paraseptal regions of the lung (18, 20). Inflammatory cell infiltrate is commonly low in IPF (20). However, lymphocytes are predominantly associated with the dense fibrosis in the lung, suggesting it to be a consequence of disease.

**Treatment**

In the early years of the present millennium, and after the publication of initial diagnostic guidelines for IPF, immunosuppressive drugs such as corticosteroids and/or azathioprine were still the mainstay of therapy for this disease. Even though corticosteroids in particular can still be beneficial for children with a genetic surfactant protein C deficiency (21), and a recent study in a mouse model with a heterozygous knockin of the known human Sftpc I73T mutation showed that the disease initially started with a (treatable) inflammatory response followed by fibrogenesis (22), immunosuppressive therapies were later discovered to be harmful in sporadic IPF (sIPF) patients (23, 24). Since then, anti-fibrotic therapy has been proven to beneficially reduce the decline of forced vital capacity (FVC) in sIPF and other progressive fibrotic ILDs (25–28). However, improvement of FVC has rarely been reported, and pirfenidone and nintedanib are associated with reduced tolerability resulting in greater side effects (29). Furthermore, although lung transplantation is the only curative and last resort therapy, it is

only feasible in a select group of patients (30), so the need for a curative therapy in IPF remains.

**Fibrotic hypersensitivity pneumonitis**

While the etiology of IPF is unknown, the situation is different for hypersensitivity pneumonitis (HP). HP is often caused by a sustained allergic response to extrinsic antigens such as bacteria, molds or bird proteins (31), resulting in dyspnea, cough, and mid-inspiratory squeaks (32). In most cases, removal of the trigger resolves the symptoms. However, since these potential antigens are inseparable from human life, the question remains why only certain individuals develop the disease while the majority do not (31). As with IPF, preexisting genetic and environmental factors are implicated in this specificity.

Lungs of patients with HP are traditionally distinguished by air trapping on HRCT and the presence of bronchiolocentrically formed non-necrotizing granulomas on histology (31). In some patients, however, persistent damage results in the development of progressive fibrosis (fibrotic HP or fHP). What provokes the progression toward the fibrotic phase remains unclear (33). In fHP patients, fibrotic remodeling can present in several patterns, including UIP, which may make differentiation of fHP from IPF diagnostically challenging (16, 34–36).

Treatment of HP traditionally includes corticosteroids, but not all patients respond well to first or second-line immunosuppressive drugs, and a proportion develop progressive fibrotic disease. Recently it has been reported that not only IPF but also many progressive fibrosing ILD such as fHP show favorable response to anti-fibrotic treatment. It is therefore important to diagnostically identify fibrotic changes at an early stage.

**Familial pulmonary fibrosis**

In the majority of cases, IPF and fHP present as a sporadic disease, i.e. without other relatives known to suffer from the same disease. However, in up to 0.5 - 20% of cases, one or more first-degree family members also have pulmonary fibrosis (familial pulmonary fibrosis (FPF)) (18, 37–39). Genetic testing has revealed disease-causing mutations in nearly half of the families with FPF, mainly in surfactant protein (*SFTP*)- or telomere-related genes (40). Interestingly, studies of familial disease have demonstrated that the same mutation can cause different types of progressive pulmonary fibrosis within one family.

The international guidelines state that sporadic IPF and IPF with familial background (IPF/FPF) are clinically and histologically indistinguishable (18). Both may be characterized by a UIP pattern in the surgical lung biopsy. However, these statements are based on reports published before the discovery of the involvement of surfactant- and telomere-related mutations, so mutation-specific phenotypes may exist. Besides their role in fibrogenesis, both telomere maintenance and surfactant proteins can alter lymphocytic inflammatory responses. While telomere dysfunction is a systemic disease that can cause immunodeficiency (41, 42), for example in common variable immunodeficiency disease (43, 44), genetic variations in surfactant genes have also been associated with changes in immune cell activation and the severity of infection (45). Moreover, lungs of patients with *SFTP* mutations have been

frequently described to contain lymphocytic infiltrates. Furthermore, a genetic surfactant protein C deficiency was reported to be associated with pulmonary infections in childhood, and immunosuppressive treatment is often beneficial in these patients (21). Studies in *SFTP*-knockout mice and alveolar epithelial cell models showed that surfactant protein plays an important role in inflammatory activation and regulation (46, 47). Interestingly, a recent study in a mouse model with a heterozygous knockin of the known human Sftpc I73T mutation showed that the disease initially started with an inflammatory response followed by fibrogenesis. This suggests that immunosuppressive treatment in early disease stages may be beneficial (22). Furthermore, inflammation was shown to be associated with disease progression (48–50).

**Pathogenesis**

One of the focal areas in the search for the underlying causality of pulmonary fibrosis is that of genetic malformations.

Surfactant genes; ER stress and UPR

Pulmonary surfactant consists of 90% lipids and 10% proteins and is present as a thin layer covering the surface of epithelial cells on the inside of alveoli (51). The four surfactant subtypes SFTPA (A1 and A2), SFTPB, SFTPC and SFTPD in the lung are produced and recycled in alveolar type 2 (AT2) cells. The main function of surfactant is to prevent the collapse of alveoli by the reduction of the alveolar surface tension (52–55). Furthermore, surfactant proteins are involved in innate host defense regulation through complement activation, thereby clearing away apoptotic cells and invading microorganisms (56, 57). Even though all four subtypes of surfactant proteins are produced in the lung, to date only mutations in the *SFTPA1/2* and *SFTPC* genes have been associated with FPF in adults (37, 58–62).

Recently it was reported that mice with induced expression of a *SFTPC* mutant I73T developed lung fibrosis (22). In general, mutations in surfactant-processing genes prevent correct folding or localization of proproteins, which leads mainly to endoplasmic reticulum (ER) stress and subsequent initiation of the unfolded protein response (UPR) (63, 64). Indeed, it has been shown in transgenic mice and A549 cell lines expressing a mutated SFTP protein that ER stress and UPR processes can be experimentally upregulated (65, 66). In turn, this results in altered cellular processes in AT2 cells, such as dysregulated proteostasis, altered surfactant lipid composition and activation of immune cells (65, 67, 68). Finally, when the ER stress is not resolved over time, cellular apoptosis is triggered in AT2 cells (69, 70), which may result in fibrogenesis. Interestingly, activated forms of UPR were also found in the sporadic form of IPF (66, 71), but no initiators have been found. This suggests that undiscovered genetic alterations in surfactant processing genes may underlie the disease development in these patients.

Telomere-related genes; DNA damage, cellular senescence and aging

The largest group of disease-causing mutations in FPF is comprised of genes associated with the telomerase complex, an RNA-protein complex which is important in germline and stem

cell telomere homeostasis (72–74). Diseases caused by these mutations are also known as short telomere syndromes (STSs). To date, mutations in six telomere-related genes have been associated with FPF: telomerase reverse transcriptase (*TERT*) (75), telomerase RNA component (*TERC*) (76), poly (A) specific ribonuclease (*PARN*) (77), regulator of telomere elongation helicase-1 (*RTEL1*) (78), *TRF1* interacting nuclear factor 2 (*TINF2*) (79) and dyskerin pseudouridine synthase 1 (DKC-1) (80). *TERT* encodes the catalytic protein component of telomerase and harbors the majority of mutations discovered so far (38). Together with the RNA template *TERC*, *TERT* forms the core unit of telomerase, while *PARN* (involved in RNA maturation of *TERC*), *RTEL1* (which assists telomere stabilization during replication), *TINF2* (involved in the production of the telomere shelterin protein complex) and *DKC-1* (active role in telomerase stabilization) have important supportive properties (Fig. 1).

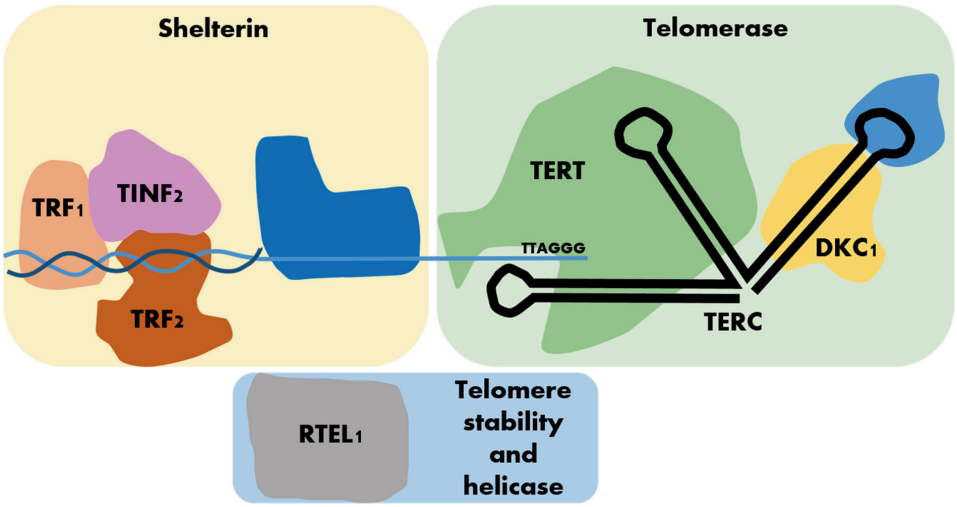



**Figure 1. Telomerase machinery and involved gene products**

Telomeres are short TTAGGG DNA tandem repeats at the ends of chromosomes, acting as a buffer in cell-cycle-dependent shortening of DNA, thereby preventing loss of genomic information (83–85). During healthy aging, leukocyte telomere length declines by approximately 20–30 base pairs per year (86), while in blood leukocytes of patients with pulmonary fibrosis and a TERT mutation telomeres shorten progressively compared to controls and other ILDs (87).

Interestingly, like *SFTP* genes, telomere-related gene variants were also found to be associated with the sporadic form of IPF. A variant in *TERT, RTEL1* or *PARN* has been found in 11.3% of these patients (81). Furthermore, it has recently been reported that telomere-related genetic variants were also found in patients with fHP (82).

If telomeres become critically short, the blunt ends are recognized as double-strand DNA breaks. This results in the phosphorylation of H2A histone family member X (γH2AX) initiating the DNA damage response. In healthy circumstances, double-strand DNA breaks take

approximately 72 hours to resolve (88). However, if they are not fixed properly in time, the DNA damage response becomes persistent and eventually leads to cellular senescence (89–92) and/or apoptosis (84), processes associated with fibrogenesis in IPF lungs and fibrotic mouse models (93, 94). Cellular senescence is a stable state of growth arrest which protects tissue from the proliferation of damaged cells (95, 96). Previous studies using mice models with a telomere-repeat factor-1 (Trf1) knockout in alveolar or bronchiolar epithelial cells demonstrated telomere shortening and increased DNA damage foci in both cell types (97, 98). Furthermore, factors involved in senescence in human IPF lungs, were upregulated in alveolar epithelial cells and fibroblasts compared with controls (93, 99). However, to what extent these cells are affected by telomere shortening and DNA damage is not yet known.

Since germline mutations in telomere-related genes of STSs affect telomere length throughout the body, various organs can be involved in these patients (100). Little is known, however, about telomere length in different human organs such as the lung, or the pathology involved in this. Even though several studies discovered differences in telomere length between human organs (101–104), STS patients were never included. One primate study showed that in healthy macaques, telomeres were excessively shortened in a subset of organs, including the lungs (105). Furthermore, the mechanism by which telomere length is related to fibrosis in non-fibrotic versus fibrotic areas and the apicobasal gradient of IPF lungs is unknown.

**Cell types involved in tissue remodeling underlying fibrogenesis**

Even though the processes behind the fibrotic tissue remodeling in IPF still have to be largely unraveled, a contemporary view of the pathogenesis focuses on several cell types.

AT2 cells

AT2 cells are a heterogeneous group of cell types that play a critical role in secretory and regenerative functions of the alveolus. Lineage tracing experiments in mice show that AT2 cells are capable of long-term self-renewal and multipotent differentiation to yield alveolar type I (AT1) cells, the gas-exchanging cells in alveoli (106). Interestingly, AT2 cells are considered the key cells in the distal airways that are affected by chronic (repetitive) injury in the initial fibrogenesis of IPF (107, 108). Both intrinsic processes, such as genetics and aging, and environmental processes, such as smoking and viral infections (109, 110), or a combination of the two, can underlie chronic injury to these cells, eventually leading to senescence or apoptosis. Senescence is an important process in AT2 cells of patients with pulmonary fibrosis upon radiation injury in vivo (111). Furthermore, the proportion of apoptotic AT2 cells in human IPF lungs is significantly higher than in lungs with no fibrosis (112, 113), which in turn, leads to diminished capacity for differentiation into AT1 cells in the IPF lungs, and a further elevated burden of renewal (107). I has also been demonstrated that mice with induced depletion of AT2 cells developed lung fibrosis (114).

AT2 cells were the first to be associated with disease, due to genetic alterations. In fact, studies demonstrated that genetic variations provide the most convincing evidence for the

pathogenic role of AT2 cells in IPF. Mutations in SFTP genes are associated with pulmonary fibrosis and SFTPA and SFTPC are exclusively expressed and secreted by AT2 cells in the lung (53, 54, 59). AT2 cell involvement was backed up by the discovery of the association between telomere-related genes and aging in pulmonary fibrosis, in humans (115) as well as in mouse models (97, 116), and by the excessively shortened telomeres in these cells of human IPF lungs (117, 118). Emerging evidence demonstrates that induction of AT2 senescence in itself is sufficient to promote lung fibrosis (119). Furthermore, a direct functional link was found between progressive lung fibrosis and elevated mechanical tension caused by impaired alveolar regeneration (120).

Environmental factors have also been proved to contribute to repetitive injury to the already genetically burdened epithelial cells. It was demonstrated that, cigarette smoke was the main environmental risk factor for developing pulmonary fibrosis (121).

It is not only depletion, but also dysfunction of AT2 cells which promotes fibrogenesis. Dysfunctional AT2 cells, mainly present in a hyperplastic form next to fibroblast foci, showed reduced renewal capacity (122) and the production of pro-fibrotic factors (108). It has even been demonstrated that epithelial cell plasticity could be the source of emerging fibroblasts (123). Insufficient AT2 cells, and the presence of dysfunctional AT2 cells, both lead to impaired secretory and regenerative functions and alveolar focused disease.

Club cells

Even though increasing evidence suggests that defective AT2 cells play a central role in fibrogenesis, interest has been increasing in a role of club cells in pulmonary fibrosis. Similar to AT2 cells in alveoli, club cells are progenitor cells responsible for the maintenance and renewal of bronchiolar epithelium, but have also been proposed to contribute to alveolar epithelial renewal and replacement of AT2 cells. It was demonstrated in mouse terminal bronchioles that progenitors of club cells, which were positively stained for both SFTPC and CC10, contributed to repair subsequent to chemical injury, and expanded after the induction of activating mutations of the K-ras oncogene (124). Recent studies showed that SFTPC/CC10 dual lineage-labeled cells have the potential to contribute to repair following injury to either airways or alveoli (124, 125). The localization of these progenitor cells seems to play a leading part in this. An important location is the bronchioalveolar-duct junction (BADJ), which is an intermediate zone connecting the bronchioles and alveoli, and also harbors the variant club cell (v-CC). v-CC cells contribute to the bronchiolar epithelium homeostasis by generating club cells and ciliated cells, as well as reconstituting alveolar or bronchiolar epithelia during injury (126). Furthermore, a group of α6β4+ progenitor cells was discovered in bronchioles and alveoli of adult mice which showed accelerated division capacity and expression of SFTPC and CC10 upon bleomycin injury (127). Moreover, in pulmonary fibrosis, club cells drive bronchiolization, a process in which bronchiolar epithelial cells migrate from the BADJ to the alveoli and repopulate them (128, 129). Interestingly, the majority of stem cells apparently only participate actively in major sustained injuries (130, 131).

Even though telomere maintenance is important in stem cells, and telomeres in AT2 cells of patients with IPF are critically short, cell-specific telomere shortening in club cells has never been assessed specifically in IPF or other types of pulmonary fibrosis such as fHP.

Myofibroblasts

A third cell type, the myofibroblast, regulates the extracellular matrix during wound healing and, when damage becomes persistent, is the main source of collagen deposition, driving fibrogenesis. During chronic injury, healthy mechanisms become disrupted and resistant to apoptosis, remaining persistently activated (108, 132–134). Although clusters of myofibroblasts forming fibroblast foci (FF) are considered reactive to fibrosis, they area histological hallmark of IPF (135). Furthermore, FF is a marker of the level of fibrosis in IPF lungs (136), and may also relate to the activity of the fibrotic process, since the presence of FF has been associated with the prognosis of IPF (48, 49, 137, 138), although not consistently so (139).



## Aim and outline of this thesis

IPF is a clinically heterogeneous and progressive disease of the lung (140). Even though the histological pattern of IPF is well characterized, the underlying cause is still uncertain in the majority of cases of this disease. The modest effect of the only available anti-fibrotic treatment on the mean survival of 2-5 years has made it clear that there is still much to learn about factors driving fibrogenesis in these patients. Over the past decade, several reports have shown that telomeres in leukocytes of patients with IPF were significantly shortened. In order to further explore this lead, the general aim of the research underlying this thesis was to elucidate the pathophysiological role of telomere shortening in progressive fibrosing ILD. This involved assessing the distribution of telomere length in AT2 in histological samples of IPF lung, both in space, by comparing different lung localizations, and in time, by assessing lung tissue from different affection stages in IPF and monogenetic telomere-related pulmonary fibrosis. Furthermore, we aimed to develop a method to simultaneously assess telomere length and DNA damage in different cell types which are key to the pathogenesis of pulmonary fibrosis. We also investigated the distribution of inflammatory cell infiltrate and fibrosis in telomere and non-telomere-related fibrosing ILD.

The studies described in this thesis analyzed the association between telomere shortening and progressive fibrosing ILD on multiple levels. In **chapter 2**, we compared telomere length between alveolar AT2 cells and their surrounding lung cells located in non-fibrotic and fibrotic regions in the lungs of patients with IPF, FPF with a telomere-related mutation (TERT-PF) and FPF without an known cause. We also studied the effect of telomere length on survival. In **chapter 3,** we used multi-organ analyses in controls and IPF cases to find out whether telomere shortening is specific to the lung. Furthermore, telomere length was analyzed over time in diagnostic biopsies and samples from explanted lungs, as well as in whole lungs to

address the telomere length in upper and lower lobes. Finally, we used whole exome sequencing to find a genetic explanation for the very short lung telomeres, similar to those in TERT-PF, in a subgroup of IPF patients. **Chapter 4** describes a procedure to measure cell-specific telomere length and DNA damage in multiple cell types located in different areas of the lung. This was achieved by a complex combination of FISH and immunofluorescent staining techniques. We also studied the best way to adjust for the amount of DNA captured in a microscopic image. This procedure is described in **chapter 5,** where, in order to establish the possible causative relationship between telomere shortening and DNA damage, these factors were measured in AT2 cells, club cells and myofibroblasts of IPF, TERT-PF and fHP lungs. We also assessed telomere length and DNA damage in surrogate cell lines of AT2 cells, club cells and myofibroblasts. The study reported in **chapter 6** measured inflammatory cell infiltrate and fibrosis to study the histological differences between IPF and FPF. Furthermore, we analyzed the association between these factors and survival. Finally, **chapter** 7 summarizes the results of this thesis in a comprehensive discussion.

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**Chapter 2**

# 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, S1 Table) 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

**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 (Fig 1A and B). All identifications were done by pathologists (MvO and SR), who are highly experienced in the field of interstitial lung diseases.

**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 $H_2O_2$ 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) (Fig 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.

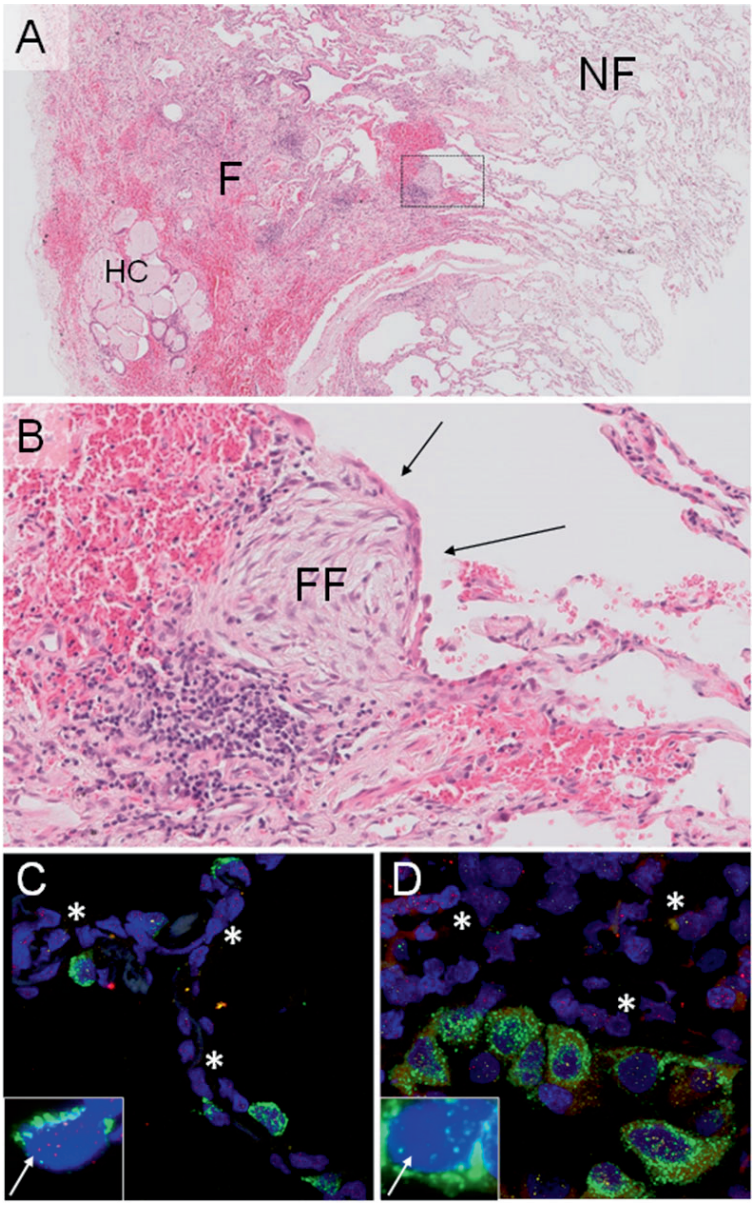


**Fig 1. Images showing representative regions for non-fibrotic and fibrotic areas within one lung biopsy.** (**A**) Low magnification image of H&E stained IPF lung tissue showing non-fibrotic (NF) and fibrotic (F) areas, and characteristic honeycombing (HC). (**B**) Enlarged boxed area of image **A** 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 (*).

**Imaging and signal quantification**

FISH-TL was measured using a method adopted from Meeker *et al*. [11,26,27]. 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 [13,28]. 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 [28]. Quadruplicate reactions were performed on a MyiQ™ Single-Color Real-Time PCRDetection 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 [29]. 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 [27,30].

**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 (Fig 2), resulting in 2.24 times difference (FISH-TL ratio in Table 2). 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 2).

Fig 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 ($r=0.855$, $p=2\cdot10^{-5}$) 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 2. 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 | 23.47 | N/A | N/A | 26.01 | N/A | N/A |
| Spor IPF | 16 | 3.22 | 1.44* | 2.24 | 12.30 | 10.73 | 1.15 |
| FIP-nonTERT | 10 | 3.74 | 2.15* | 1.74 | 12.60 | 7.46 | 1.68 |
| FIP-TERT | 9 | 1.00# | 1.00 | 1.00 | 9.21 | 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).

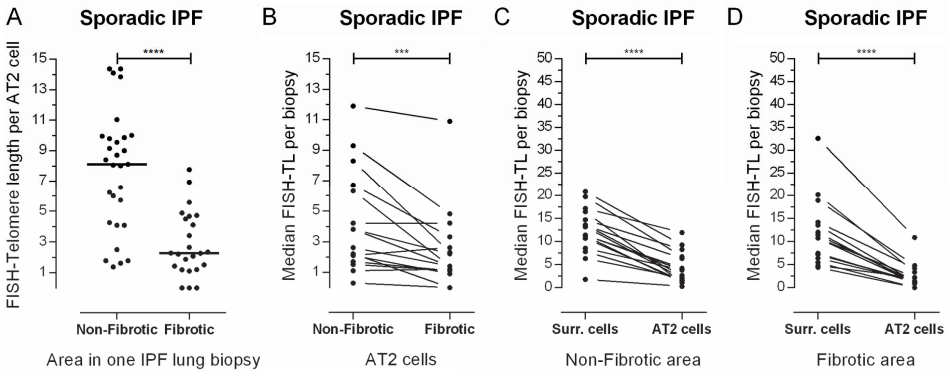


**Fig 2. Telomere length in sporadic IPF lungs.** (**A**) Representative example of telomere length per alveolar type 2 (AT2) cell for one sporadic IPF subject. Each dot represents one cell. Non-fibrotic AT2 FISH-TL is significantly higher compared to FISH-TL in fibrotic areas ($p<0.0001$). Bars represent 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 (*** = $p<0.001$, **** = $p<0.0001$).

**Table 3. Median telomere length for alveolar type 2 (AT2) cells versus surrounding cells per cohort.**

| | | **Non-fibrotic area FISH-TL** | | | **Fibrotic area FISH-TL** | | |
|---|---|---|---|---|---|---|---|
| **Subgroup** | **n** | AT2 | Surr. | Ratio (AT2/Surr.) | AT2 | Surr. | Ratio (AT2/Surr.) |
| Controls | 5 | 23.47 | 26.01 | 0.90 | 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 significantly shorter than in surrounding cells (spor IPF: $p < 0.0001$, FIP-nonTERT and FIP-TERT: $p < 0.01$).

### 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, Fig 2C and FISH-TL ratio: 0.26, Table 3). The difference was even larger in fibrotic areas: telomeres in AT2 cells were 8 times shorter than in surrounding cells (p<0.0001, Fig 2D and FISH-TL ratio: 0.13, Table 3). 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 3).

**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, Fig 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 2). 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, Fig 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.

**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, Fig 3D, Table 2). Furthermore, in both non-fibrotic and fibrotic areas the AT2 cell FISH-TL was 3.5 times shorter than surrounding cells (p<0.01, Fig 3E and F, FISH-TL ratio: 0.29, Table 3). 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 2 and 3. 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.

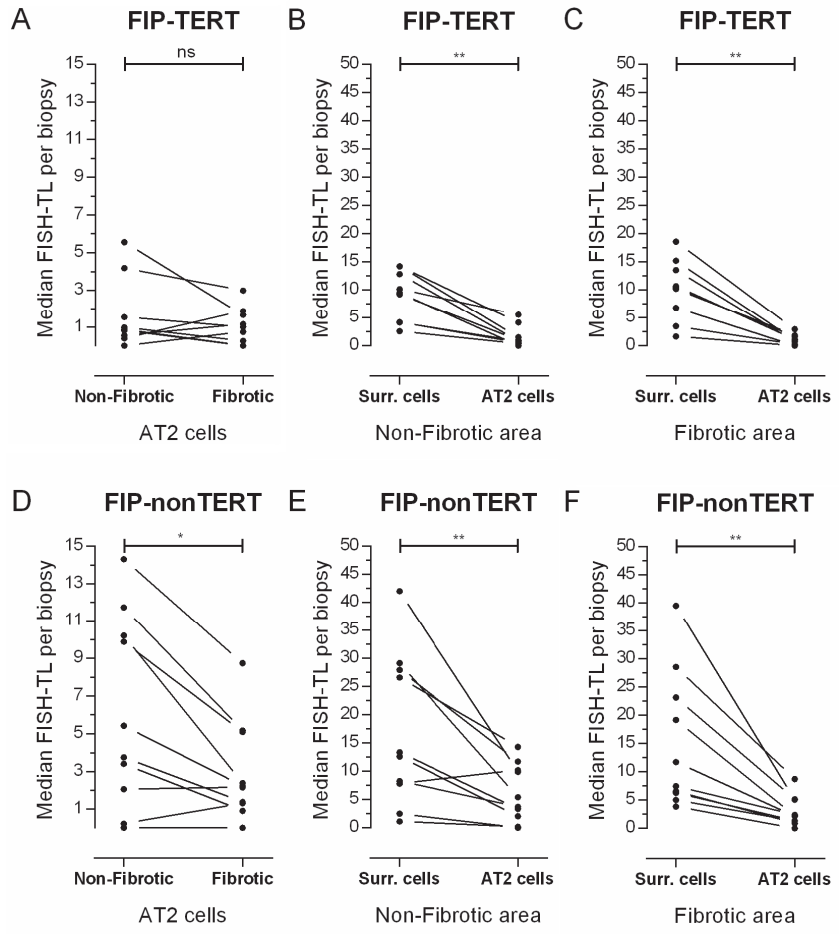


**Fig 3. FISH Telomere length in lungs of FIP-TERT and FIP-nonTERT subjects.** (**A**) Median FISH-TL in alveolar type 2 (AT2) cells in non-fibrotic and fibrotic areas of 9 FIP-TERT subjects. Telomeres are generally short and no significant difference between areas was observed (2-tailed, p=0.36). (**B, 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).

### 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) [28]. 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 (Fig 4). No correlations were found in the FIP-TERT and FIP-nonTERT (data not shown).

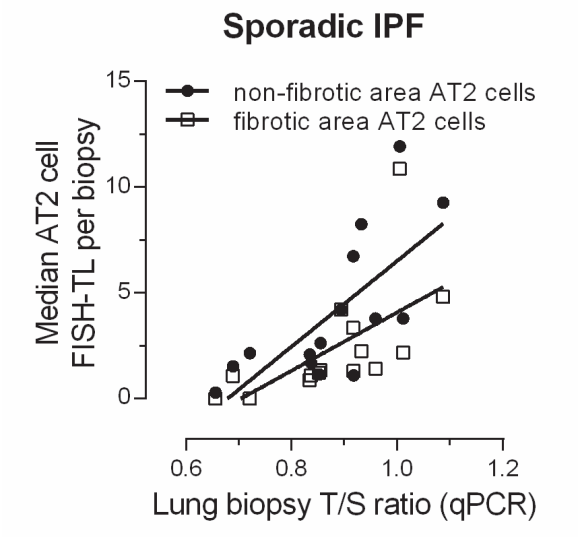


**Fig 4. Correlation between FISH-TL and biopsy T/S in IPF subjects.** Correlation (Spearman) between FISH-TL measured in alveolar type 2 (AT2) cells and biopsy T/S measured by MMqPCR (n=15) in non-fibrotic areas (black dots) and fibrotic areas (open squares). Significant correlations were established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

**Telomere length by MMqPCR: blood**

We tested for a correlation between peripheral white blood cell TL measured with MMqPCR (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) (Fig 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, Fig 6).

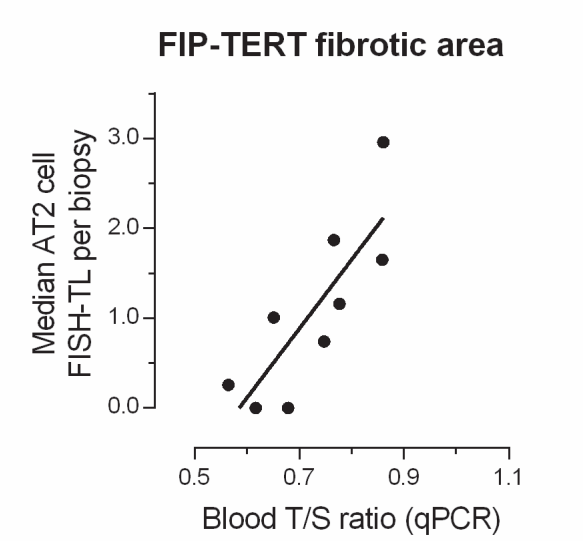


**Fig 5. Correlation between FISH-TL and MMqPCR leukocyte TL (blood T/S) in FIP-TERT fibrotic areas.** Positive FIP-TERT correlation (Spearman) between alveolar type 2 (AT2) cell FISH-TL in fibrotic areas and MMqPCR blood T/S (n=9, $r^2$=0.67, p=0.007).

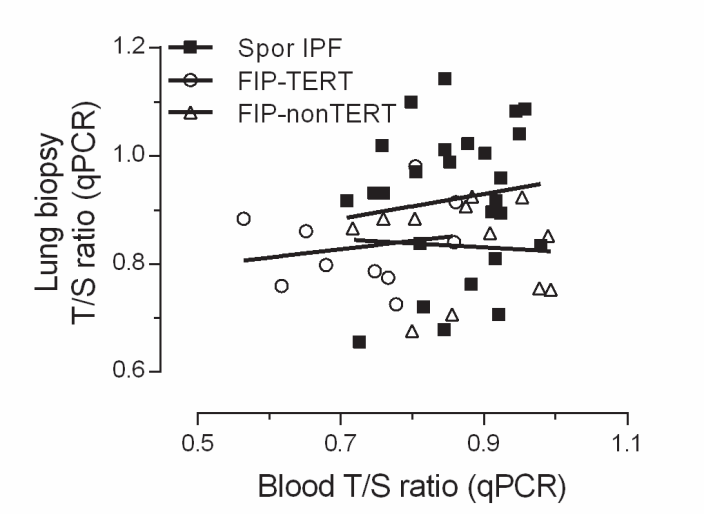


**Fig 6. Correlations between MMqPCR measurements biopsy T/S and blood T/S in IPF.** No correlation between biopsy T/S and blood T/S was observed in sporadic IPF (n=26), FIP-nonTERT (n=12) and FIP-TERT (n=10) subjects.

**Short telomeres are associated with worse survival**

In the literature, short peripheral leukocyte telomere length has been associated with worse survival time in IPF [31,32]. 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 (Figs 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, Fig 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 (Fig 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.



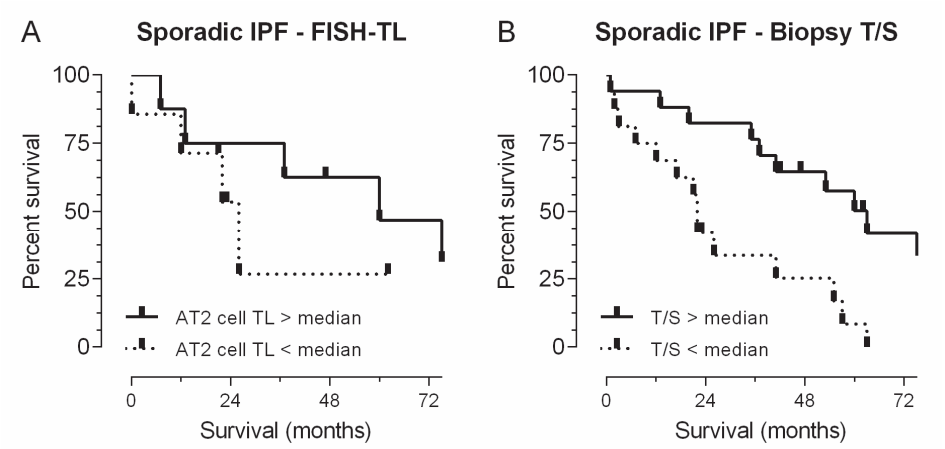


**Fig 7. Survival of IPF patients.** (**A**) FISH-TL on lung biopsy; Kaplan-Meier curve of 15 IPF patients showing a median survival of 60 months for patients with AT2 cell FISH-TL in non-fibrotic areas above median TL (solid line) and a median survival of 26 months for patients with AT2 cell TL below 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 (Figs 2 and 3). Furthermore, patients with

short lung telomeres had significantly worse survival than patients with longer telomeres (Fig 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, Fig 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 [33-35]. 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 [36]. This regenerative function of AT2 cells requires active telomere maintenance [37]. Indeed, telomerase has been shown to be active and upregulated in subpopulations of rat AT2 cells after hypoxic injury [37-39]. Additionally, literature postulated that shortened telomere length in blood is a risk factor for sporadic IPF and FIP-TERT subjects [6,7,11-13,31].

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 [40]. 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 2). 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 [41,42]. 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 [43-46]. 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 [47].

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 (Fig 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 (Fig 7B). The association is in accordance with previous studies reporting short leukocyte telomere length (measured by MMqPCR) to negatively influence survival [31,32].

There are conflicting reports concerning the correlation between blood TL and lung FISH-TL [11,26]. 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 [26]. However, in FIP-TERT patients we did find a correlation between TL in blood and in fibrotic area AT2 cells (Fig 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 [16,48].

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 [49,50] 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 [8,30]. 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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## Supporting information

**S1 Table. Specific mutations carried by the FIP-TERT subjects in this study.**

| cDNA Position | Variant name | SIFT* | PolyPhen-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 [1]

[+]Variant is described in a patient with Hoyeraal-Hreidarsson syndrome, which is characterized by short telomeres [2].

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Chapter 3

# From Organ to Cell: Multi-level Telomere Length Assessment in Patients with Idiopathic Pulmonary Fibrosis

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## Abstract

*Rationale*: A subset of patients with idiopathic pulmonary fibrosis (IPF) contains short leukocyte telomeres or telomere related mutations. We previously showed that alveolar type 2 cells have short telomeres in fibrotic lesions. Our objectives were to better understand how telomere shortening associates with fibrosis in IPF lung and identify a subset of patients with telomere-related disease.
*Methods*: Average telomere length was determined in multiple organs, basal and apical lung, and diagnostic and end-stage fibrotic lung biopsies. Alveolar type 2 cells telomere length was determined in different areas of IPF lungs.
*Results*: In IPF but not in controls, telomere length in lung was shorter than in other organs, providing rationale to focus on telomere length in lung. Telomere length did not correlate with age and no difference in telomere length was found between diagnostic and explant lung or between basal and apical lung, irrespective of the presence of a radiological apicobasal gradient or fibrosis. Fifteen out of 28 IPF patients had average lung telomere length in the range of patients with a telomerase (*TERT*) mutation, and formed the $IPF_{short}$ group. Only in this $IPF_{short}$ and TERT group telomeres of alveolar type 2 cells were extremely short in fibrotic areas. Additionally, whole exome sequencing of IPF patients revealed two genetic variations in *RTEL1* and one in *PARN* in the $IPF_{short}$ group.
*Conclusions*: Average lung tissue telomere shortening does not associated with fibrotic patterns in IPF, however, approximately half of IPF patients show excessive lung telomere shortening that is associated with pulmonary fibrosis driven by telomere attrition.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a life-threatening disease of the lung, caused by progressive decay of alveolar epithelium and parenchymal scarring. Median survival of IPF patients after diagnosis is approximately 4 years [1,2].
IPF is a heterogeneous disease that can occur sporadically or in the context of familial disease. A major genetic cause underlying familial disease comprise of mutations in telomerase related genes involved in the maintenance of telomere length [3-6]. Mutations are most commonly found in the gene encoding telomerase reverse transcriptase (*TERT*). Defects in telomerase result in extremely short telomeres and lead to abnormal DNA repair, or DNA degradation, and eventually to cellular senescence, a phenomenon known to be involved in acute wound healing as well as in lung fibrosis [7-12]. In families carrying *TERT* mutations, pulmonary fibrosis was shown to be the most common disease manifestation [13]. Moreover, it was shown that not the presence of the mutation itself, but the resulting short telomeres were associated with development of fibrosis [14]. IPF cases and patients with telomere related gene mutations have short leukocyte telomere length, but present with high inter-individual

variation. It is therefore thought that in a subgroup of patients with IPF telomere maintenance is a key to disease pathogenesis whereas in others it is not [15-18].
During healthy ageing, leukocyte telomere length declines approximately 20 – 30 base pairs per year due to shortening per cell division [19]. In other organs, cell turnover rates are different and therefore telomere length may vary between organs [20]. Several studies discovered differences in telomere length between human organs [20-23]. However, the lung was never included. One primate study showed that in healthy macaques telomeres were excessively shorter in a subset of organs, including the lungs [24].
Not only variation in telomere length between organs may exist, also variation within organs may be present. IPF lungs are typically characterized by a usual interstitial pneumonia (UIP) pattern on histology and high-resolution computed tomography (HRCT). The histological pattern of UIP consists of healthy air-containing non-fibrotic areas juxtaposed to dense fibrotic areas with fibroblast foci and honeycombing features. Previously we showed that telomere shortening in alveolar type 2 cells associates with fibrotic lesions [25]. On HRCT, IPF lungs typically show an apicobasal gradient, in which fibrosis is most abundant subpleurally in the basal lung fields [1]. In control lungs, in which such a gradient is absent, it was recently found that telomere length in the basal fields was significantly shorter than in the apical fields of the same lung [26]. It is unknown whether telomere shortening in the IPF lung associates with the apicobasal gradient.
In this study, we showed that average telomere length does not associate with lung localization in apical and basal regions or disease duration. Furthermore, measurements of telomere length enabled us to identify a subgroup of sporadic IPF patients with lung telomere length similar to that found in patients carrying a telomerase mutation.



## Materials and Methods

**Patient and tissue selection**

In total 82 subjects were included in the study, consisting of 49 patients with sporadic IPF, 18 pulmonary fibrosis patients with a *TERT* mutation, and 15 controls. The study was approved by Medical research Ethics Committees United (MEC-U) of the St Antonius Hospital (approval number W14.056 and R05-08A). All patients were recruited when visiting the St Antonius Hospital for ILD care and provided written informed consent. Research was conducted at the department of pulmonology of the St Antonius Hospital Nieuwegein and at the department of pathology of the University Medical Center Utrecht. Diagnoses were made according to ATS/ERS/JRS/ALAT guidelines [1,2] by a multidisciplinary team including an experienced pulmonologist (JCG), radiologist (HWvE) and pathologist (MFMvO) at St. Antonius ILD centre of excellence Nieuwegein, The Netherlands.
Biopsy specimens originated from three different sources: group 1 consisted of autopsy specimens, group 2 of diagnostic biopsies, and group 3 of material from explant lung (S1 Fig). Formalin-fixed paraffin-embedded (FFPE) lung slides were cut from residual biopsies. Surgical

biopsy specimens from IPF and TERT subjects, obtained between 1994 and 2015, were randomly included if histological usual interstitial pneumonia (UIP) characteristics were present. Biopsies were taken subpleurally and showed a patchy fibrotic/non-fibrotic pattern, fibroblast foci and honeycombing. Furthermore, in the IPF group, familial subjects were excluded and IPF patients were screened negative for mutations in TERT, TERC, surfactant protein C (SFTPC), surfactant protein A2 (SFTPA2) exon 6 and TRF1-Interacting Nuclear Factor 2 (TINF2) exon 6. Relevant demographic details of patients with a diagnostic biopsy are presented in Table 1. Additional information on patient selection and clinical data collection are provided in an online data supplement (S1 File and S1 Table).

**Table 1. Characteristics of patients with a diagnostic biopsy**

| | $IPF_{normal}$ | $IPF_{short}$ | *TERT* |
|---|---|---|---|
| *N* | 17 | 15 | 17 |
| *Male/Female* | 16/1 | 13/2 | 14/3 |
| *Mean Age at diagnosis (SD)* | 61.2 (9.1) | 58.8 (10) | 58.7 (9.8) |
| *Mean FVC%pred (SD)* | 67.9 (23.7) | 73 (19.8) | 81 (15.6) |
| *Mean DLCO%pred (SD)* | 47.9 (20.3) | 44.9 (11.1) | 43.6 (6.2) |
| *Smoking status (CS:FS:NS:U)* | 0:11:5:1 | 2:10:1:2 | 0:14:3:0 |
| *Pack years (SD)* | 16.4 (15.6) | 23.9 (22.5) | 18 (15.5) |

IPF = Idiopathic Pulmonary Fibrosis; FVC = Forced Vital Capacity; DLCO = Diffusing Capacity of the Lungs for Carbon Monoxide. Pred = Predicted; $IPF_{short}$ = lung telomere length in the same range as TERT patients: < 0.857; $IPF_{normal}$ = lung telomere length above the range of TERT patients: > 0.857; CS = Current Smoker; FS = Former Smoker; NS = Never Smoker; U = Unknown.
No significant differences were present between patient groups (Kruskal-Wallis multiple comparison tests).

**Tissue preparation and fluorescence in situ hybridization**

FFPE biopsies were prepared, stained and analysed for telomere signals in alveolar type 2 (AT2) cells, using fluorescence *in situ* hybridization (FISH) as described previously [27]. Immunofluorescent staining of proSP-C, a protein exclusively produced by AT2 cells, was used for identification of AT2 cells. A summary is provided in an online data supplement (S1 File).

**Telomere length measurements by MMqPCR in FFPE tissue and peripheral blood leukocytes**

DNA was isolated from FFPE samples and T/S ratios were measured as described previously [28]. The T/S ratio is a measure for average telomere length in biopsy or peripheral blood leukocytes obtained by MMqPCR and is proven to be a sensitive method to discriminate between patients with high and low telomere length signals [15,29]. The overall mean coefficient of variation was 2.5 and only samples with a coefficient of variation below 10%

were included. A summary of the MMqPCR protocol is provided in an online data supplement (S1 File).

**Whole exome sequencing**

DNA extracted from peripheral blood leukocytes of all subjects with sporadic IPF was obtained for whole exome sequencing (WES) at Novogene (Hong Kong, China) using the Agilent SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, CA, USA) on an Illumina PE150 sequencing platform (Illumina, San Diego, California, USA) according to standard protocol. Additional detail on analysis of the sequence data is provided in an online data supplement (S1 File).

**Statistical analysis**

Statistical significances were computed using non-parametric tests in GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA). Telomere length differences were determined by Mann-Whitney tests and a combined Kruskall-Wallis and Dunn's multiple comparisons tests. Paired data were computed using a Wilcoxon matched-pairs signed rank analysis. Spearman's rank coefficient was used to calculate correlations.



## Results

**In three IPF patients, organ telomere length is shortest in lung**

To compare telomere length between fibrotic lung and other organs, we measured average telomere length in lung, kidney, thyroid, liver and bladder of two age and sex matched controls, two sporadic IPF patients and one IPF patient carrying a *TERT* mutation using MMqPCR. In the three patients with pulmonary fibrosis shortest telomere length was present in lung tissue (Fig 1).

**IPF lung telomere length does not correlate with age**

Lung telomere length of diagnostic biopsy specimens from sporadic IPF patients was measured in a cohort with an age ranging between 35 and 75 years old (n = 32; median age = 61 years old) using MMqPCR. In sporadic IPF no correlation was present between lung telomere length and age ($r = 0.015$, $p = 0.935$; Fig 2a). In control lung, correlation analysis showed a trend towards significance between increasing age and shortening of lung telomere length (n = 18, $r = -0.4$, $p = 0.115$; S2 Fig).

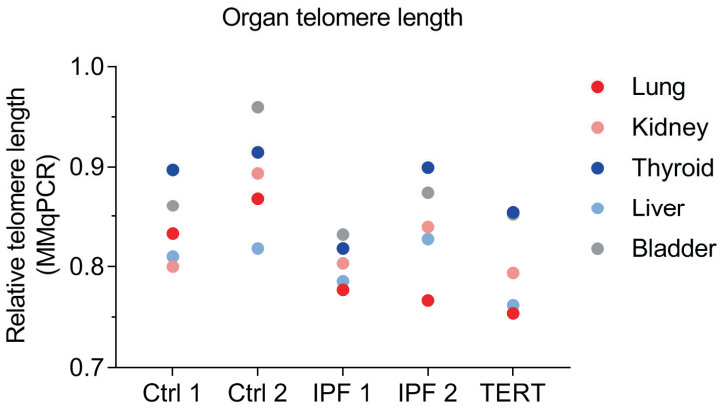


**Fig 1. Organ telomere length per subject.** Biopsy telomere length measurements by MMqPCR in lung, kidney, thyroid, liver and bladder tissue in 2 controls (Ctrls), 2 spradic IPF patients and one pulmonary fibrosis case with a *TERT* mutation. In patients with pulmonary fibrosis the shortest telomere length was found in lung tissue.

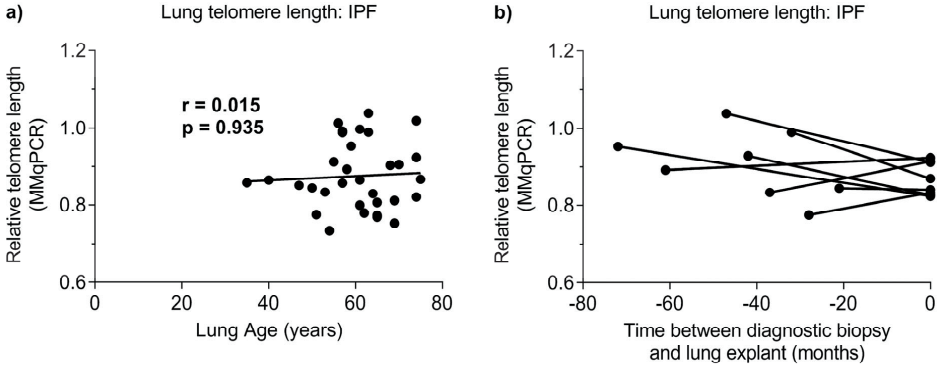


**Fig 2. Lung telomere length in IPF.** (**a**) Telomere length measurements in lungs of 32 subjects with IPF by MMqPCR. No correlation was found (Spearman correlation; r = 0.015, p = 0.935). (**b**) Comparison of telomere length between 8 diagnostic biopsy and 8 explant lung specimens. Samples belonging to the same patient are connected. Wilcoxon matched-pairs signed rank tests showed no differences in telomere length between samples (p = 0.25).

### Average lung telomere length remains the same during disease

To assess if telomere length changes during IPF disease evolution, we compared MMqPCR lung telomere length of eight diagnostic biopsy specimens with that of explant lung specimens with end-stage fibrosis, taken from the same lobe of the same patients. A median of 45 months passed between the diagnostic biopsy and the transplantation procedure. No difference was found between diagnostic biopsy and explant lung telomere length (p = 0.251; Fig 2b).

**Telomere shortening is not associated with the apicobasal UIP gradient**

Average lung telomere length was measured in apical and basal specimens from IPF explant lung with (n = 8) and without (n = 7) an apicobasal gradient on HRCT (Figs 3a and 3b). The percentage of fibrosis, measured on a macroscopic tissue level, in lungs with an apicobasal gradient was 51% in apical and 96% in basal tissue. In lungs without an apicobasal gradient these numbers were 78% for apical and 88% for basal tissue. Using MMqPCR, no significant difference in telomere length was present between basal and apical tissue (Figs 3c and 3d) and no significant differences were found between lungs with and without an apicobasal gradient ($p > 0.75$;). Similarly, using FISH for measurement of cell specific AT2 cell telomere length, no difference in AT2 cell telomere length between apical and basal specimens was found ($p = 0.193$; Fig 4d and S3 Fig).

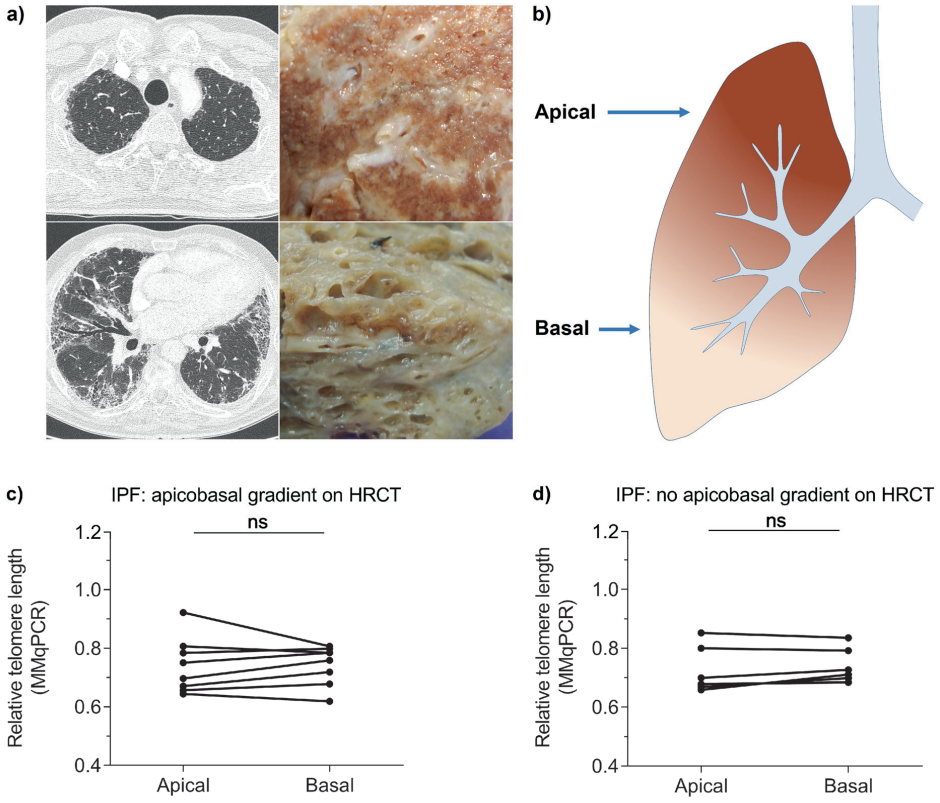


**Fig 3. Telomere shortening is not associated with apical or basal localisation in whole explant lung.** (**a**) HRCT on the left and formalin fixed explant images of IPF lung with an apicobasal fibrotic gradient on the right. The top and bottom figures represent apical and basal locations respectively, corresponding to (**b**) the schematic picture. (**c, d**) Apical and basal lung telomere length comparison of (**c**) 8 lungs with an apicobasal gradient and (**d**) 7 lungs without apicobasal gradient measured by MMqPCR. Samples belonging to the same person are connected. Wilcoxon matched-pairs signed rank tests showed no differences in telomere length between lung sections.

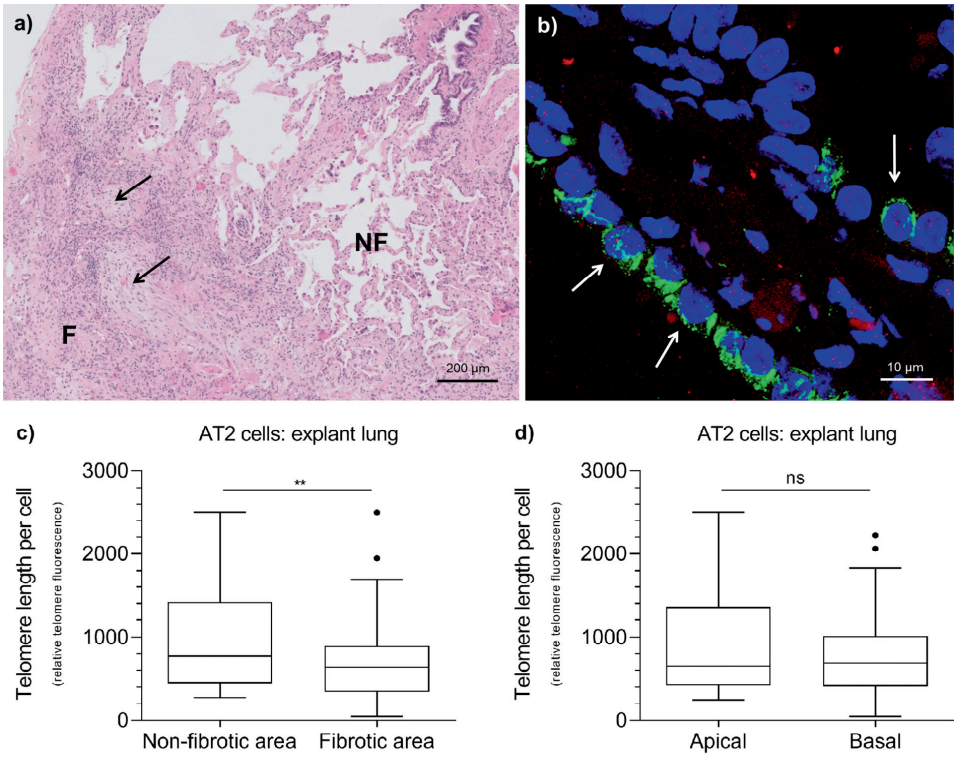


**Fig 4. AT2 cell telomere length in IPF explant lung tissue measured by FISH.** (**a**) Hematoxylin and Eosin (H&E) staining representing non-fibrotic (NF) and fibrotic (F) areas of a typical IPF lung biopsy. Black arrows indicate fibroblast foci. (**b**) Example of a combined fluorescent image of AT2 cells (white arrows) in a fibrotic area of IPF lung tissue. DNA in nucleus is displayed in blue (DAPI), proSP-C in green and telomeres in red (dots). (**c**) Within a tissue specimen, telomere length in AT2 cells was significantly longer in non-fibrotic areas than in fibrotic areas. (n = 3; Mann-Whitney test: ** < 0.01). (**d**) No difference in AT2 cell telomere length was observed between apical and basal lung tissue. Boxes represent data between 1st and 3rd quartile and whiskers extend to the highest and lowest values of expression that are not considered outliers.

### AT2 telomere length is short in fibrotic lesions in IPF explant lungs

Previously we showed that AT2 cell telomere length associated with fibrotic lesions in sporadic IPF diagnostic lung biopsies [25]. To verify whether this is also true for explant lungs, we performed a FISH analysis (Fig 4b) of juxtaposed microscopic non-fibrotic and fibrotic areas within three tissue specimens (Fig 4a). In non-fibrotic areas, AT2 telomere length was significantly longer than in fibrotic areas of the same biopsy ($p = 0.009$; Fig 4c and S3 Fig).

### A subgroup of IPF has extremely short lung telomeres

To identify a subgroup of IPF cases with possible telomere related pathology we determined blood and lung telomere length using MMqPCR in 32 sporadic IPF and 17 pulmonary fibrosis subjects with a *TERT* mutation. In IPF, blood and biopsy telomere length significantly

correlated (MMqPCR, r = 0.531, p = 0.002; Fig 5a), while in TERT cases no such correlation was found (r = -0.157, p = 0.545; Fig 5b). The highest biopsy value in TERT patients served as a threshold to form an $IPF_{short}$ group with telomere length < 0.857 (n = 15) and an $IPF_{normal}$ group with telomere length > 0.857 (n = 17; Fig 5c). Median biopsy telomere length in $IPF_{short}$ was, as expected, in the range of the TERT group (p > 0.999; Fig 5c). Surprisingly, median biopsy telomere length in $IPF_{normal}$ was in the same range as that of age-matched controls. No significant differences in clinical status were observed between the patient groups (Table 1).

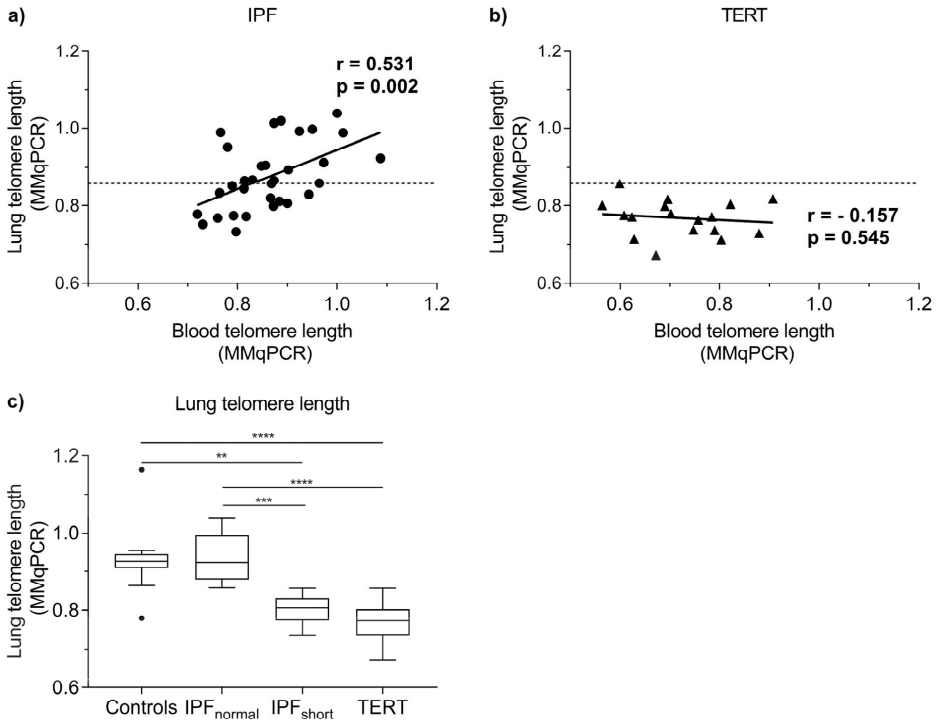


**Fig 5. Blood and biopsy telomere length in IPF and TERT subjects measured by MMqPCR.** (**a, b**) Spearman correlation of blood and biopsy telomere length in (**a**) 32 IPF and (**b**) 17 TERT cases. A significant positive correlation was established in IPF cases (r = 0.531, p = 0.002). In TERT cases, no significant correlation was found (r = -0.157, p = 0.545). Dashed lines represents threshold for lung telomere length associated with telomere related pathology. $IPF_{normal}$ = lung telomere length above threshold, $IPF_{short}$ = lung telomere length below threshold. (**c**) Tukey boxplots of lung telomere length measured by MMqPCR in control (n = 13), $IPF_{normal}$ (n = 17), $IPF_{short}$ (n = 15) and TERT (n = 17) lungs. Lung telomere length in the $IPF_{short}$ group was significantly shorter than telomere length in age-matched controls (p = 0.0014) and $IPF_{normal}$ (p = 0.0001). Telomere length in the TERT group was also significantly shorter than telomere length in controls (p < 0.0001) and in $IPF_{normal}$ (p < 0.0001). Telomere length in the $IPF_{normal}$ group and controls were comparable. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests (** = p < 0.01, *** = p < 0.001, **** = p < 0.0001). Boxes represent data between 1st and 3rd quartile and whiskers extend to the highest and lowest values of expression that are not considered outliers.

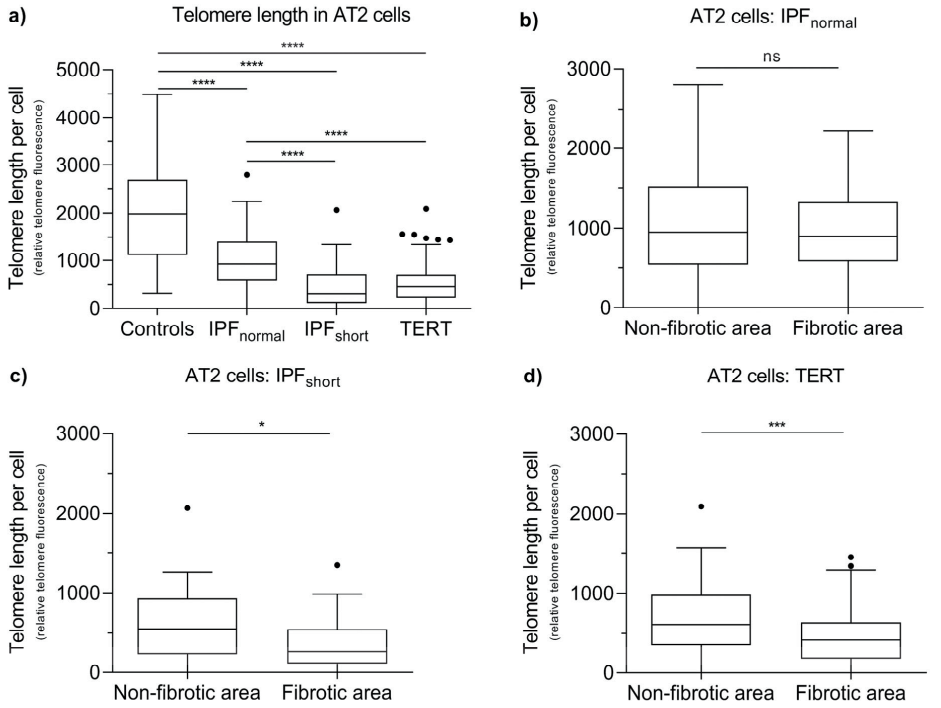


**Fig 6. AT2 cell telomere length measured by fluorescence *in situ* hybridization (FISH) in 5 control, 5 $IPF_{normal}$, 5 $IPF_{short}$ and 6 TERT lungs.** (**a**) Tukey box-plots of telomere length measured in AT2 cells by fluorescence in situ hybridization (FISH). All patient groups showed significant shorter AT2 cell telomere length than in controls ($p < 0.0001$).Telomere length in the $IPF_{short}$ group was significantly shorter than in controls ($p < 0.0001$) and in $IPF_{normal}$ ($p < 0.0001$). Telomere length in TERT was also significantly shorter than in controls ($p < 0.0001$) and in $IPF_{normal}$ ($p < 0.0001$). No difference in AT2 telomere length was present between $IPF_{short}$ and TERT. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests. (**b, c, d**) AT2 cell telomere length in non-fibrotic and fibrotic lung areas in (**b**) $IPF_{normal}$, (**c**) $IPF_{short}$ and (**d**) TERT lungs. In $IPF_{normal}$ no difference was found between areas. AT2 cell telomere length in non-fibrotic areas was significantly longer than fibrotic areas in $IPF_{short}$ ($p = 0.0237$) and TERT ($p = 0.0001$) lungs. Asterisks indicate significant differences calculated by Mann-Whitney analyses (ns = not significant, * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$). Boxes represent data between 1st and 3rd quartile and whiskers extend to the highest and lowest values of expression that are not considered outliers.

### AT2 cell telomere length in $IPF_{short}$ equals that of TERT samples

Next, we determined the distribution of telomere length of AT2 cells in the $IPF_{normal}$, $IPF_{short}$ and TERT groups with FISH staining. AT2 cell telomere length in $IPF_{normal}$ and $IPF_{short}$ patient groups were respectively 2.1 and 6.5 times shorter than in controls ($p < 0.0001$; Fig 6a and S3 Fig). Comparison between the patient groups showed that AT2 cell telomere length in $IPF_{normal}$ was 2 times longer than in the TERT group, while no difference between the $IPF_{short}$ and the TERT group was found.

In a more detailed analysis, comparison of AT2 cell telomere length in fibrotic versus non-fibrotic areas of the same biopsy (Fig 4a) showed that only in $IPF_{short}$ and TERT biopsies the telomere length of AT2 cells in fibrotic areas was shorter than in non-fibrotic areas ($p = 0.024$ and $p < 0.001$ respectively; Figs 6c and 6d). In contrast, in $IPF_{normal}$ no significant difference was observed ($p = 0.722$; Fig 6b and S3 Fig).

**Table 2. Telomere-related genetic variants discovered in IPF cases by WES**

| *Lung telomere length (MMqPCR)* | 0.857 ($IPF_{short}$) | 0.798 ($IPF_{short}$) | 0.829 ($IPF_{short}$) |
|---|---|---|---|
| *Gene* | *RTEL1* | *RTEL1* | *PARN* |
| *Chromosome* | 20 | 20 | 16 |
| *Position GRCh37/hg19* | g.62324600 | g.62321484 | g.14649519 |
| *Annotation* | c.3028C>T (NM_032957.4) p.(Arg1010Ter) (NP_116575.3) | c.2258G>A (NM_032957.4) p.(Arg753His) (NP_116575.3) | c.1310G>A (NM_002582.4) p.(Gly437Glu) (NP_002573) |
| *Reference* | rs373740199 | rs777153220 | N/A |
| *Effect* | Stop gain | Missense | Missense |
| *ACMG classification* [30] | Likely pathogenic | Uncertain significance | Uncertain significance |
| *CADD score (minimum value for pathogenicity)* | 35 (19.35) | 27.2 (19.35) | 33 (34) |
| *SIFT prediction* | N/A | Damaging | Damaging |
| *PolyPhen-2 prediction* | N/A | Probably damaging | Probably damaging |
| *ExAC %* | 0.008 | 0.001 | 0 |
| *Literature* | [31,32] | | |

IPF = Idiopathic Pulmonary Fibrosis; CADD = Combined Annotation Dependent Depletion score; ExAC = Exome Aggregation Consortium;

PolyPhen-2 = Polymorphism Phenotyping version 2; SIFT = Sorting Intolerant From Tolerant; N/A = Not Applicable.

Refsnp (rs) single-nucleotide polymorphism identifiers are provided where available. Pathogenicity of CADD scores were interpreted using the Gene-Aware Variant INterpretation (GAVIN) method (minimum value needed for pathogenicity is noted between brackets).

**Whole exome sequencing in IPF**

To identify mutations in telomere related genes associated with pulmonary fibrosis, we decided to perform whole exome sequencing in all 32 sporadic IPF patients. Sequencing data revealed 3 telomere-related variants, all in the $IPF_{short}$ group, including a stop gain (c.3028C>T)

and a missense (c.2258G>A) variant in *RTEL1* and a missense (c.1310G>A) variant in *PARN* (Table 2). High CADD scores for both *RTEL1* variants indicated that these were pathogenic, while the CADD score of 22 for the *PARN* mutation implied a trend towards pathogenicity as reported by the GAVIN method.

## Discussion

Aberrant maintenance of telomere length is accepted as a possible cause of pulmonary fibrosis in familial and sporadic IPF. Patients with short telomeres showed short median survival of 22 months, which was even shorter than that generally found in IPF [25]. Nonetheless, little is known about organ telomere length in IPF. Previous studies showed that telomeres were relatively short in skin, thyroid and lung tissues of healthy macaques [24]. In human controls, skin and liver samples had relatively short telomeres, while lung and thyroid were not evaluated [20]. In this study, we show that the lung of three IPF subjects had the shortest telomere length among 5 organs samples, while this was not found in the two control lungs. This may be indicative of accelerated aging of fibrotic IPF lungs and the reason why IPF patients only suffer from a diseased lung. The finding provides rationale for further investigation of the association of lung telomere length and fibrosis in IPF lung.

Telomere shortening is a hallmark of aging and just recently it was shown that, similar to other organs, telomeres in control lungs shorten with age [26]. However, lung telomere length in our IPF samples was shorter than in age-matched controls and did not correlate with age. Similar findings are known from patients suffering from bone-marrow failure due to telomere related gene mutations in which leukocyte telomere length no longer correlates with age [33]. In addition, we found that liver telomeres were short in both IPF and controls. Even though liver cirrhosis is also described to be a consequence of telomere-related defects [34,35], no signs of chronic liver disease were found in our group.

Next, by comparing diagnostic biopsy samples with samples from explant lungs, we were able to follow the evolution of telomere length during the fibrosis. To our surprise, no difference in average telomere length was observed over the course of the disease. This implies that genetic or environmental factors must have added significantly to telomere shortening prior to disease diagnosis. In addition, it is also still possible that long-term asymptomatic disease is responsible for the observed shortening. At diagnosis, IPF patients already have severely impaired diffusing capacity for carbon monoxide (DLCO), averaging around 50% of predicted [36] and it is unknown at what point in time the causative pathogenic processes initiated.

In paired samples from control lung it was recently demonstrated that telomeres in basal regions are shorter than in apical regions of the lung [26]. IPF lung characteristically present a UIP pattern on HRCT with an apicobasal fibrotic gradient. When we assessed telomere length in IPF explant lungs with and without this gradient, no difference between apical and basal lung telomere length was found, irrespective of presence of the apicobasal gradient. Thus in IPF, the effects of natural aging on lung telomere length are completely masked. Because

differences in average lung telomere length in time and location are absent in IPF, it is likely that the duration of the presence of short telomeres associates with the degree of fibrosis. Previously it was reported that short telomeres induce cellular senescence, thereby signalling the pro-fibrotic senescence-associated secretory phenotype (SASP) [8-10]. Therefore, as cellular senescence is not resolved, fibrotic remodelling may accumulate over time.

The current concept of IPF pathogenesis describes that AT2 cells, alveolar progenitor cells with high division capacity, play a fundamental role in the onset of fibrogenesis [37,38]. This is supported by studies in which mice with selectively knocked out telomere repeat binding factor-1 (TRF-1) in AT2 cells were prone to develop pulmonary fibrosis [39].

In our IPF cohort, average lung telomere length proved to be highly variable. Grouping the sporadic IPF patients with short – TERT-like – lung telomere length together, showed that they had very short AT2 cell telomere length, particularly in fibrotic lesions. Because the TERT group also demonstrated AT2 cell telomere shortening predominantly in fibrotic lesions, this so-called $IPF_{short}$ subgroup might be underlying telomere driven disease. A previous study investigating the cell proliferation marker Ki-67 showed that elevated cell proliferation is likely not the cause of telomere shortening in IPF [40]. Furthermore, 3 patients with telomere related gene mutations were found in this group, supporting the likelihood of telomere related disease pathogenesis. The *RTEL1* c.3028C>T sequence variant was previously associated with IPF, dyskeratosis congenita and Hoyeraal-Hreidarsson Syndrome [31,32]. Recently an *RTEL1* c.2257C>T variant resulting in an amino acid substitution at the same codon as our c.2258G>A variant was described in a patient with pulmonary fibrosis [32]. Moreover, *in silico* prediction models such as high CADD scores, SIFT and Polyphen-2 predictions and low ExAc frequencies support pathogenicity. The relatively high number of variants in *PARN* and *RTEL1* in our cohort was justified by previous data in which 11.3% of a sporadic IPF cohort harboured qualifying genetic variant in *TERT*, *RTEL1* or *PARN* [32].

Most interestingly, the remaining group with lung telomere length above the range of TERT cases, had normal average lung telomere length comparable with those in controls. And although AT2 telomere length in this so-called $IPF_{normal}$ group was shorter than in controls, it was significantly longer than in the TERT and $IPF_{short}$ group. This is highly suggestive of a disease pathogenesis other than telomere driven disease, which may take a toll on AT2 proliferative capacity and is driven by other pro-fibrotic processes. We found a higher number of pack years in the $IPF_{short}$ group, which would be suggestive of environmental driven disease, however, the difference was not significant ($p = 0.364$). Therefore, other processes involving alveolar homeostasis may be involved.

Some limitations of the study are worth noting. The data presented here are based on associations; no causative links could be concluded from telomere length or WES analysis. Next, control tissue was obtained from various sources, such as residual lung resected from tissue next to a tumour. Also, MMqPCR was used to assess telomere length in the lung. Although telomere restriction fragment (TRF) length analysis is often used for tissue analysis, literature showed that a strong correlation exists between TRF, MMqPCR and FISH [29]. Furthermore, we included a relatively young IPF case of 35 years old. However, since the WES

analysis for IPF-related genes was negative and no family history of disease was known, the subject was classified as sporadic IPF.

In conclusion, this study demonstrates that there is no difference in average lung telomere length in time and location, which make it plausible that lung telomere shortening occurs prior to disease diagnosis. This opens up possibilities for early detection of patients at risk of developing IPF if easily accessible markers would be available that associate with this process. Furthermore, a subgroup of patients with IPF shows similarities with subjects harbouring a *TERT* mutation. 20% of this IPF group have telomere related gene mutations. These patients have extremely short AT2 telomeres in fibrotic lesions and may have telomere driven pathology. On the other hand, IPF patients with lung telomere length above the range of TERT patients have normal lung telomere length, and mildly short AT2 cells. In these patients disease is unlikely to be telomere driven but might associate with other processes that affect AT2 cell turnover. Future measurement of lung telomere length in IPF may aid discrimination between telomere-related and telomere-unrelated pulmonary fibrosis.

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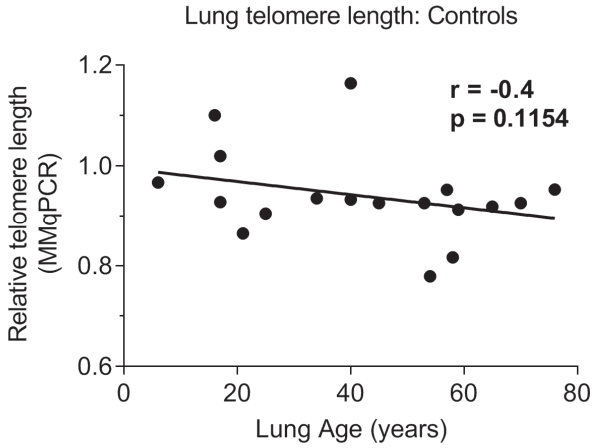
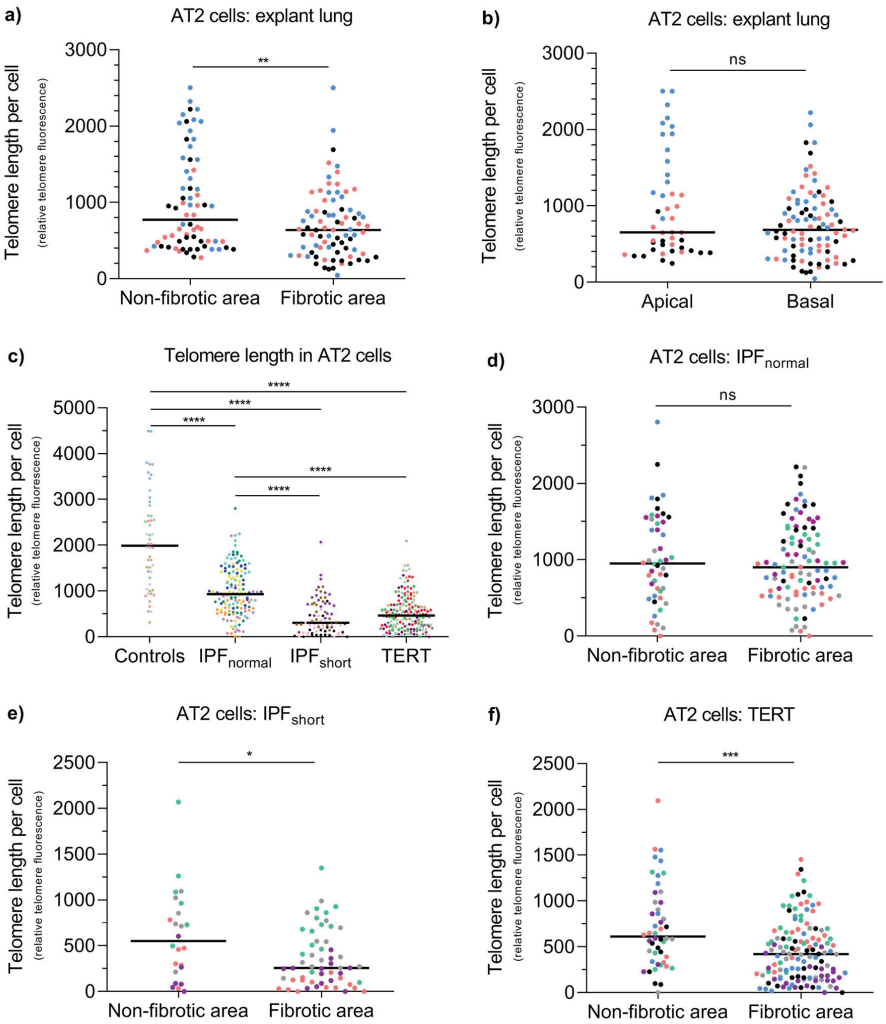
## Supporting information




**S2 Fig. Telomere length in control lung**. A trend towards a significant Spearman correlation was found between lung telomere shortening measured by MMqPCR and increasing age (n = 18, r = -0.4, p = 0.115).



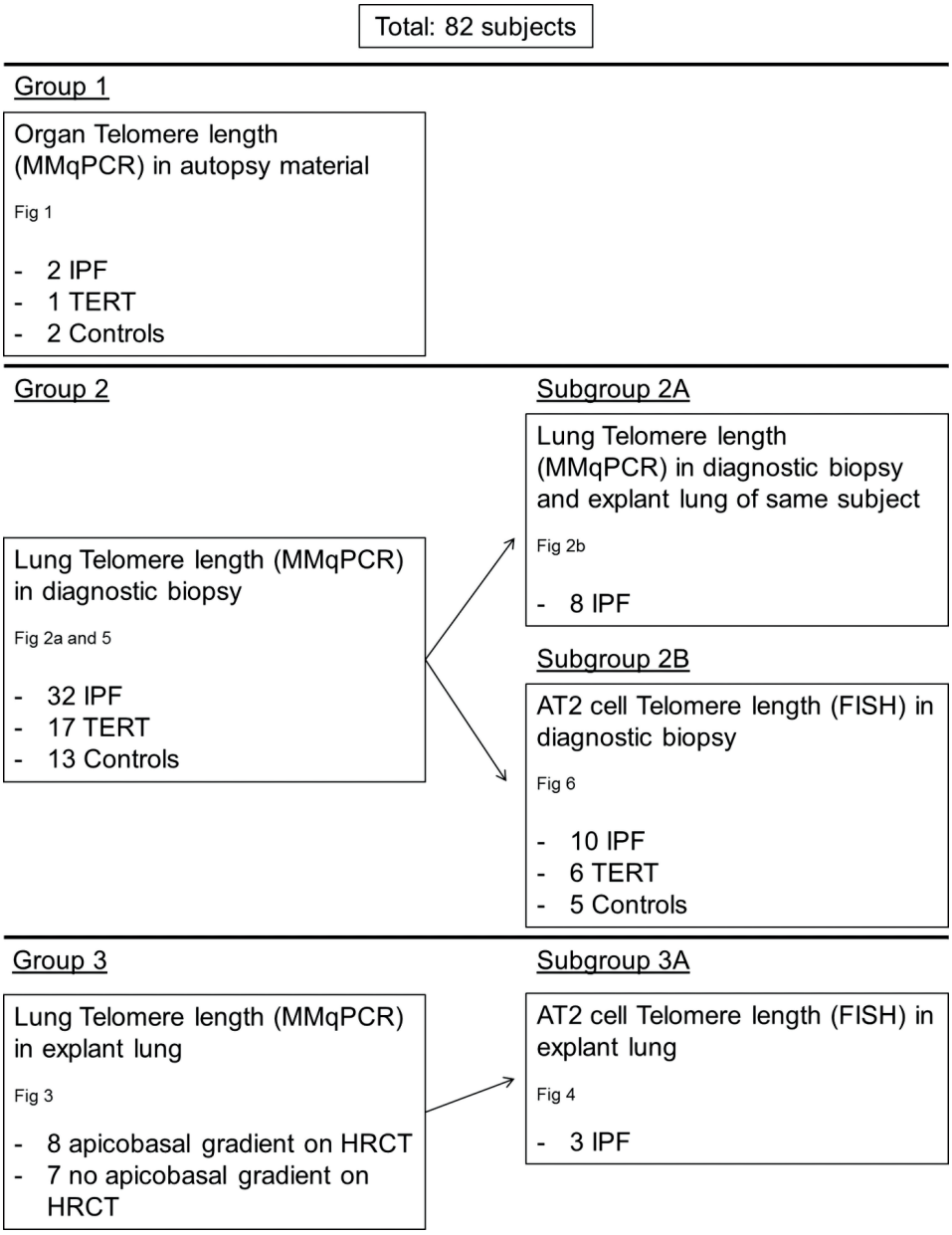

**S3 Fig. Raw data of the AT2 cell telomere length in explant lungs and diagnostic biopsies.** (**a, b**) Telomere length of AT2 cells in explant lungs measured by FISH. (**a**) Within explant lung specimens, telomere length in AT2 cells was significantly longer in non-fibrotic areas than in fibrotic areas. (n = 3; Mann-Whitney test: ** < 0.01). (**b**) No difference in AT2 cell telomere length was observed between apical and basal lung tissue. (**c, d, e, f**) Telomere length of AT2 cells in diagnostic biopsies measured by FISH. (**c**) All patient groups showed significant shorter AT2 cell telomere length than in controls ($p < 0.0001$).Telomere length of $IPF_{short}$ was significantly shorter than in controls ($p < 0.0001$) and in $IPF_{normal}$ ($p < 0.0001$). Telomere length in TERT was also significantly shorter than in controls ($p < 0.0001$) and in $IPF_{normal}$ ($p < 0.0001$). No difference in AT2 telomere length was present between $IPF_{short}$ and TERT. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests. (**b, c, d**) AT2 cell telomere length in non-fibrotic and fibrotic lung areas in (**b**) $IPF_{normal}$, (**c**) $IPF_{short}$ and (**d**) TERT lungs. In $IPF_{normal}$ no difference was found between areas. AT2 cell telomere length in non-fibrotic areas was significantly longer than fibrotic areas in $IPF_{short}$ ($p = 0.0237$) and TERT ($p = 0.0001$) lungs. Asterisks indicate significant differences calculated by Mann-Whitney analyses (ns = not significant, * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$). Every dot represents an individual AT2 cell and each subject is indicated by a different color.

## S1 File. Supplemental methods and results.

## Supplemental methods

**Patient selection and clinical data collection**

Patient inclusion for this study is presented in S1 Fig. Subject characteristics were included in Table 1 and S1 Table. 20 cases were included in our previous publication [1]. However, in the current study all FISH and MMqPCR experiments were original for every subject. In the TERT group, 15 out of 17 cases were found in the context of familial pulmonary fibrosis. Familial disease was determined when 2 or more first-degree family members also presented with pulmonary fibrosis. The remaining two *TERT* patients were discovered because of the relatively young age at which they were diagnosed with IPF. Patient characteristics were retrieved from medical reports between 6 months before and after date of biopsy. Moreover, in order to measure telomere length in apical and basal areas of the lung 8 IPF explant lungs with an apicobasal gradient and 7 explant lungs without an apicobasal gradient on High-resolution computed tomography (HRCT). HRCT scans within 6 months before transplantation were included. The mean fibrosis rate in these apical and basal specimens was measured by the amount of white fibrotic tissue as a percentage of the total tissue. As controls, lung tissue with no pathology, obtained during post-mortem examination (n = 8), from residual donor lobes (n = 3) and relatively normal lung resected from tissue next to a tumour (n = 7) were used. The age ranged from 6 to 76 years old. For comparison of control lung telomere length with that of subjects with pulmonary fibrosis, controls were age matched (n = 13). To assess telomere length of multiple organs of control and IPF patients, residual lung, kidney, thyroid, liver and bladder tissues were included from autopsy section material. One of the three subjects (IPF 1 in Fig 1) in this study died because of an acute exacerbation. Control autopsies were age-matched to subjects with pulmonary fibrosis and did not have lung related pathology, but died of heart failure (n = 2).




**S1 Fig. Schematic overview of the three subject groups included in this study.** In total 82 individual cases were included. No overlapping cases were present between the groups. Patients were distributed over three groups based on origin of the tissue (autopsies in group 1, diagnostic biopsies in group 2 and explant lung in group 3). MMqPCR = Monochrome multiplex quantitative polymerase chain reaction; FISH = Fluorescence *in situ* hybridization; HRCT = High-resolution computed tomography.

**S1 Table. Characteristics of controls, explant lungs and autopsies.**

| | Controls | Explant lungs | Autopsies |
|---|---|---|---|
| N | 15 | 15 | 3 |
| Male/Female | 11/4 | 8/7 | 3/0 |
| Biopsy Age (SD) | 50.3 (16.2) | 57.5 (9.3) | 69 (11.5) |
| Mean FVC%pred (SD) | N/A | 44.2 (17.9) | 78.5 (10) |
| Mean DLCO%pred (SD) | N/A | 30.1 (12.3) | 36.3 (12.4) |
| Smoke status (CS:FS:NS:U) | N/A | 0:13:2:0 | 0:1:2:0 |
| Pack years | N/A | 23 (21.2) | 5 (8.7) |

FVC = Forced Vital Capacity; DLCO = Diffusing Capacity of the Lungs for Carbon Monoxide. Pred = Predicted; CS = Current Smoker; FS = Former Smoker; NS = Never Smoker; U = Unknown; N/A = Not Applicable



**Telomere length measurements by MMqPCR in FFPE tissue and peripheral blood leukocytes**

Genomic DNA was extracted from peripheral leukocytes using a magnetic beads-based method (chemagic DNA blood 10k kit; Perkin Elmer Inc. Waltham, MA, USA). In the case of FFPE tissue, DNA was isolated 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) and sample purity was determined using the absorbance ratio of 260 and 280nm. Samples within a ratio of 1.8 - 2.0 were included. To measure whole lung biopsy and leukocyte telomere length, monochrome multiplex qPCR (MMqPCR) was performed as described previously [4,5]. 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 telomere length 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 [4]. Quadruplicate reactions were performed on a MyiQ™ Single-Color Real-Time PCRDetection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Samples were analysed in triplicate in at least two runs, of which only coefficients of variation below 10%, with an overall mean of 2.5, were included.

**AT2 cell telomere length measurements with fluorescence *in situ* hybridization (FISH)**

In short, FFPE slides were pre-treated with a trisaminomethane-ethylenediaminetetraacetic acid solution (Tris-EDTA; 40-mM Tris, 1-mM EDTA, pH 9) for 20 min. Slides were incubated overnight at 37 $^{0}$C with a telomere-Cy3 peptide nucleotide acid (PNA) probe (2.70 µg/ml, F1002; Panagene, Daejeon, South Korea) which was diluted in a 2x saline-sodium citrate buffer (SSC) (0.3-M NaCl, 0.03-M sodium citrate), 5% dextran sulphate, 50% deionized formamide, and 0.5% Tween-20 hybridization mixture. Excess probe was cleared with a PNA wash solution (1% 1-M Tris, 29% Aquadest, and 70% Formamide). Next, to identify AT2 cells, a rabbit anti-

human proSP-C antibody (AB3786; Merck Millipore) was applied 1:100 in 1% BSA/PBS and incubated 1 hr at room temperature. Slides were incubated with a secondary Goat anti-rabbit Alexa Fluor 488 (1:80 in 1% BSA/PBS, A11008, Thermo Fisher Scientific) antibody for 30 min at room temperature. Subsequently,
4',6-diamidino-2-phenylindole (DAPI; 25 µg/ml) was used to stain nuclei. Pictures were taken with a LSM700 laser scanning confocal microscope (Zeiss, Jena, Germany) and images were analysed using the image analysis Telometer plugin (available at http://demarzolab.pathology.jhmi.edu/telometer/index.html) of ImageJ (http://rsb.info.nih.gov/ij/).

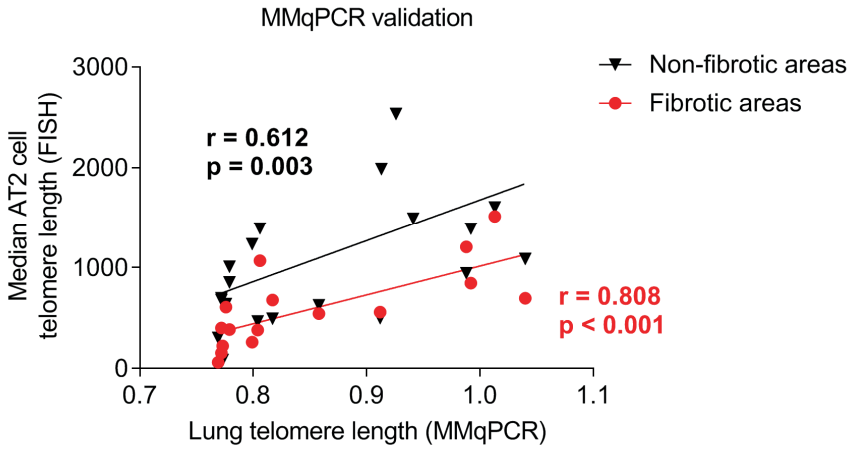
**Whole exome sequencing bioinformatics pipeline**

Whole exome sequence data was analysed with Ingenuity Variant Analysis (version 5.4.20181019, Qiagen, Hilden, Germany) using the following filters: variants were required to 1) have a call and genotype quality of at least 30, 2) have a read depth of at least 15, 3) be outside top 5% most exonically variable 100base windows in healthy public genomes, 4) be excluded when an allele frequency of at least 1% was observed in the 1000 genomes project, ExAC, gnomAD and NHLBI ESP exomes populations unless an established pathogenic common variant was known and 5) have no more than 20 bases in an intron that i) are experimentally observed to be associated with a pathogenic, likely pathogenic or an uncertain significance phenotype according to computed ACMG Guidelines classification [6] (or are listed in HGMD®) or ii) are associated with gain of function of a gene established in the literature or is inferred activating mutation by Ingenuity or iii) are associated with loss of function by frameshift, start/stop codon change, missense, splice site loss up to 2 bases into intron (or as predicted by MaxEntScan), copy number or promotor loss. Finally, variants were only selected when located within the telomere-related genes *TERT, RTEL1, TINF2, PARN, DKC1, TERC* or *NAF1*. Combined Annotation Dependent Depletion (CADD) scores were interpret using the Gene-Aware Variant INterpretation (GAVIN) method [7].

## Supplemental results

**Telomere length measured by MMqPCR validation by FISH**

We compared MMqPCR with AT2 cell FISH in the same FFPE biopsies of 10 IPF, 6 TERT and 5 control cases. Spearman correlation showed a good correlation between the two methods for fibrotic ($r = 0.808$, $p < 0.001$, S4 Fig) and non-fibrotic areas ($r = 0.612$, $p = 0.003$, S4 Fig).




**S4 Fig. Comparison of MMqPCR and AT2 cell FISH results.** Telomere length measurements in lungs of 10 IPF, 6 TERT and 5 control subjects by MMqPCR and FISH. A significant spearman correlation was found between both techniques for AT2 cell telomere length in fibrotic (red symbols; r = 0.808, p < 0.001) and in non-fibrotic areas (black symbols: r = 0.612, p = 0.003). Note that controls do not contain fibrotic areas.



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Chapter 4

# Cell type-specific quantification of telomere length and DNA double-strand breaks in individual lung cells by fluorescence *in situ* hybridization and fluorescent immunohistochemistry

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## Abstract

Telomeres are small repetitive DNA sequences at the ends of chromosomes which act as a buffer in age-dependent DNA shortening. Insufficient telomere repeats will be recognized as double-strand breaks. Presently it is becoming more evident that telomere attrition, whether or not caused by mutations in telomere maintenance genes, plays an important role in many inflammatory and age-associated diseases. In this report, a method to (semi-)quantitatively assess telomere length and DNA double-strand breaks in FFPE fixed tissue is described. Therefore, a novel combination of quantitative fluorescence in situ hybridization, tissue elution and immunofluorescence staining techniques was developed. Caveolin-1 (Type 1 pneumocytes), pro-Surfactant protein C (Type 2 pneumocytes), Club cell-10 (Club cells) and alpha smooth muscle actin (smooth muscle cells) markers were used to identify cell types. To visualize all the different probes, re-staining the tissue by heat mediated slide elution is essential. Fluorescent signals of telomeres and DNA double-strand breaks were quantified using the Telometer plugin of ImageJ. As example, we analyzed lung tissue from a familial pulmonary fibrosis patient with a mutation in the telomere associated gene Poly(A)-specific ribonuclease (*PARN*). The protocol displays a novel opportunity to directly quantitatively link DNA double-strand breaks to telomere length in specific FFPE cells.

## Introduction

Telomeres are DNA-protein complexes at the ends of chromosomes, which are involved in preservation of genetic information by acting as a buffer in chromosome end shortening [1-3]. In humans, telomeres consists of double-stranded TTAGGG repeats with a combined total length between four and fifteen kilobases [4]. In healthy subjects mean telomere length declines approximately 20 – 30 base pairs per year as a result of incomplete replication during DNA synthesis. Initial telomere shortening has no consequences [5,6]. However, when the remaining telomere length is insufficient, the termini of linear chromosomes are recognized as double-strand breaks, resulting in abnormal DNA repair or degradation and eventually in cell senescence [7-10].

Telomere shortening is associated with telomeropathy-related diseases, such as dyskeratosis congenita, liver cirrhosis and pulmonary fibrosis [11]. Mutations in genes associated with telomere maintenance are found to be causal to congenital forms of these diseases. For instance, Poly(A)-specific ribonuclease, encoded by the gene *PARN* [12-14], positively affects mRNA stability through deadenylation activity of the poly(A) tail. Deficiencies in the deadenylation process cause reduced production of proteins involved in telomere maintenance and induce telomere shortening [14]. Although many telomeropathy-associated mutations have been discovered, the type of cells affected by telomere shortening and how these are related to disease pathogenesis must still be elucidated. Interestingly, *PARN* mutations initiate an p53-regulated early DNA damage response [14]. To assess both telomere

length and DNA double-strand breaks in specific cells of formalin-fixed paraffin-embedded (FFPE) lung tissue, DNA and protein staining techniques need to be combined in one assay.

Quantitative fluorescence *in situ* hybridization (Q-FISH) is widely used to visualize and measure relative DNA or RNA with fluorescently-labeled probes containing sequences complementary to target DNA [15,16]. For the analysis of relative telomere length, fluorescent signals per individual immunofluorescence (IF) marked cell can be obtained by Q-FISH as previously described by Meeker and coworkers [17]. To visualize proteins, IF is a standard staining technique, using antibodies labeled with fluorescent tags [18]. Moreover, IF is suitable for quantification [19,20]. DNA double-strand breaks initially result in the phosphorylation of histon protein H2AX (gamma-H2AX) [21,22]. Therefore, gamma-H2AX staining is generally used in DNA damage assays [19,23]. In case of telomeres and DNA damage; FISH and IF are mostly used for co-localization studies [24,25]. However, no studies have quantified both telomere length and gamma-H2AX signals per cell type specifically. The telomere Q-FISH probe, gamma-H2AX and specific cell markers must all be identified separately in one tissue specimen. Spectral overlap will occur when all stainings occur simultaneously. To circumvent fluorophore spectral overlap, heat mediated antibody and FISH probe slide elution have been proposed to allow reuse of the same tissue for a different staining [26-28].

In FFPE material, cell-specific antibody stains are essential in identifying different cell subsets in lung material. Lung cells are subdivided in three main compartments; alveolar cells, bronchial and bronchiolar epithelium cells and pulmonary vascular cells [29]. To account for these three groups, we selected alveolar type I- (AT1, CAV-1+) and type II (AT2, pro-Spc+) pneumocytes, club- (CC10+) cells and smooth muscle (aSMA+) cells as proof of principle in the assessment of telomere length and gamma-H2AX.

A delicate way to study tissue biomolecules is laser scanning confocal microscopy (LSCM). Advantages include optical sectioning within a single cell, three-dimensional imaging and high signal-to-background ratios [30,31], which makes the system ideal for quantification of fluorescent labeled cell structures in fixed tissue [32,33]. In this study, the main challenge is to quantify FISH and IF signals simultaneously in multiple individually-stained cell types in FFPE tissue. Because LSCM can be used to image multiple fluorescent targets at once [34], this is the method of choice.

Here, we describe a novel, accessible method combining Q-FISH and IF staining techniques to quantitatively analyze the relationship between telomere length and DNA double-strand breaks in different cell types of FFPE lung tissue. To our knowledge, the procedures used in this assay were never combined into one protocol before. Lung FFPE material obtained from a pulmonary fibrosis patient with a *PARN* mutation was included as proof of principle.

## Materials and Methods

**Tissue inclusion and study approval**

Residual tissue was obtained from formalin fixed paraffin embedded (FFPE) lung tissue from patients with pulmonary fibrosis. An experienced lung pathologist reviewed all tissues to select the biopsies showing all features of a distinct pathological usual interstitial pneumonia (UIP) pattern. Lung control tissue was collected from residual donor organ. The patient has written biobank informed consent and the study was approved by the Medical research Ethics Committees United (MEC-U) of the St Antonius Hospital (approval number R05-08A)

**Tissue preparation and fluorescence *in situ* hybridization**

Two serial sections of 4 µm were cut, air-dried for 10 minutes and heated at 56°C for 30 minutes. Slides were then placed at 4°C until staining. Material was incubated at 56°C for four hours. The sequential sections were deparaffinized and hydrated using a xylene and ethanol series respectively (20 seconds per step), after which they were rinsed in tab water (2 times 1 minute). Next, the two slides were washed in Phosphate-buffered saline (PBS) and boiled in a Trisaminomethane-Ethylenediaminetetraacetic Acid solution (Tris-EDTA; 40 mM Tris, 1mM EDTA, pH 9) for 20 minutes. The boiling pan containing the Tris-EDTA solution and the slides was cooled down to room temperature by placing the boiling pan in cold water. Samples were washed once with PBS and thereafter with demineralized water. Next, slides were air-dried and dry slides were incubated, using cover glasses, with a telomere-Cy3 PNA probe (2,70 µg/ml, F1002, Panagene, Daejeon, South-Korea) which was diluted in a 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), 5% dextran sulphate, 50% deionized formamide and 0.5% Tween-20 hybridization mixture. The PNA probe consists of the sequence CCCTAACCCTAACCCTAA, which is complementary to the human telomere repeat sequence. Slides with the probe were heated for 5 minutes on a stretching table at 84°C and subsequently placed on an ice-cooled metal plate (3-5 minutes). The slides were hybridized overnight at 37°C in a moistened environment to prevent evaporation. To remove the excess probe, PNA wash solution (1% 1M Tris, 29% Aquadest and 70% Formamide) was used for 15 minutes while shaking. This step was repeated once. Subsequently, slides were washed 2 times 5 minutes in PBS and PBS-0.05% Tween (PBST), respectively, before starting the immunofluorescent staining protocol. The flow diagram of methodological steps is presented in figure 1.

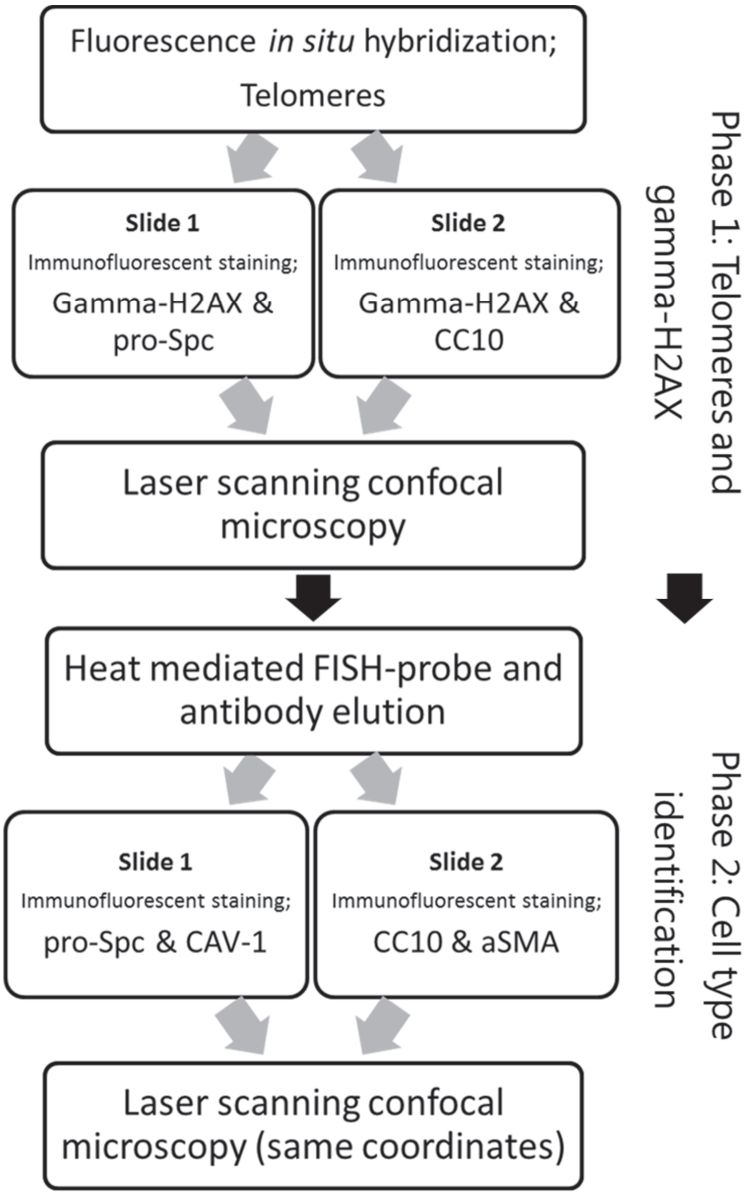


**Figure 1. Flow chart of methodological steps.** The methods are subdivided in a telomere and gamma-H2AX staining phase and a second, post elution phase, which include the IF staining of specific cell markers. Integration of data from phase 1 and 2 was done by scanning coordinates.

**Gamma-H2AX Immunofluorescent staining**

Both sequential slides were incubated with 1% Bovine Serum Albumin (BSA) in PBS for 20 minutes. On slide 1, a pre-dilution of Mouse anti-human gamma-H2AX antibody (05-636-I,

Merck Millipore, Darmstadt, Germany) and Rabbit anti-human pro-Spc antibody (AB3786, Merck Millipore, Darmstadt, Germany) was applied. The second slide was incubated with a pre-diluted combination of Mouse anti-human gamma-H2AX antibody and Rabbit anti-human CC10 antibody (sc-25554, Santa Cruz Biotechnology, Dallas, Texas, US). All primary antibodies were diluted 1:100 in 1% BSA/PBS and incubated 1 hour at room temperature. Slides were washed three times 5 minutes in PBST after each antibody cycle. Subsequent secondary antibodies Goat anti-mouse Alexa Fluor 647 (1:100 in 1% BSA/PBS, A21235, Thermo Fisher Scientific, Waltham, MA, US) and Goat anti-rabbit Alexa Fluor 488 (1:80 in 1% BSA/PBS, A11008, Thermo Fisher Scientific, Waltham, MA, US) were applied sequentially on both slides for 30 minutes at room temperature. Subsequently, tissue was washed in PBS for 5 minutes. Next, samples were incubated with 4',6-diamidino-2-phenylindole (DAPI, 25 µg/mL), washed in PBS for 5 minutes and rinsed in DEMI water. Samples were air-dried, Vectashield antifade mounting medium (Vector laboratories, Burlingame, CA, US) was applied and slides and were stored at 4°C until analysis with LSCM (see section Laser Scanning Confocal Microscopy).

**Tissue slide elution and Immunofluorescent staining to identify cell types**

Pro-Spc and CC10 positive cells were identified during the assessment of telomeres and gamma-H2AX and tissue coordinates of the locations were saved. However, to avoid spectral overlap due to the use of more than 4 colors, we introduced heat mediated FISH-probe and antibody elution. After the first phase of LSCM analysis, cover glasses were removed, slides were washed in PBS and subsequently boiled in Tris-EDTA (40 mM Tris, 1mM EDTA, pH 9) for 20 minutes to elute previously used FISH probe and antibodies. The proceeding steps were as described above using the following premixed primary antibodies for slide 1 and 2. Slide 1; Mouse anti-human Caveolin-1 (1:50, 610407, BD biosciences, Franklin Lane, New Jersey, US) and Rabbit anti-human pro-Spc (1:100, AB3786, Merck Millipore, Darmstadt, Germany). Slide 2; Mouse anti-human αSMA (1:50, Sigma-Aldrich, Darmstadt, Germany) and Rabbit anti-human CC10 antibody (1:100 sc-25554, Santa Cruz Biotechnology, Dallas, Texas, US).
Secondary antibodies were for both slides the same, respectively: Goat anti-mouse Alexa Fluor 546 (1:50 in 1% BSA/PBS, A11030, Thermo Fisher Scientific, Waltham, MA, US) and Goat anti-rabbit Alexa Fluor 488 (1:80 in 1% BSA/PBS, A11008, Thermo Fisher Scientific, Waltham, MA, US). Afterwards, LSCM pictures were taken of the same slide location, using previously saved coordinates.

**Laser scanning confocal microscopy**

Telomere and gamma-H2AX pictures were taken at the end of phase 1 for quantification (Fig. 1). 1024x1024 digital images were obtained on a LSM700 laser scanning confocal microscope (Zeiss, Jena, Germany) with a 63x/1,4 oil immersion objective (DIC, M27, Plan Apochromat). For AT1, at least 6 cells, but on average 15 cells were obtained from five images scattered over the biopsy and analyzed for each patient. The average of AT2 cells was 20. The minimum amount of images required for the analysis was determined by analyzing 10 images of a control lung using the method of cumulative means/medians [35]. Fluorescent probes were

excited at 405nm (DAPI), 488nm (AF488), 555nm (Cy3 and AF546) and 639nm (AF647), using solid-state diode lasers. Additionally, emission was detected between 406-479nm (DAPI), 489-533nm (AF488), 556-604nm (Cy3 and AF546) and 640-700nm (AF647), using corresponding emission filters. Pinholes were set equally between fluorescence channels. Z-stacking of 9 focal planes with 0.5µm intervals was used to maximize the coverage of cell nuclei. Tissue X- and Y-coordinates were saved. Next, z-stack images were captured by a photo multiplier tube (PMT) detector and per laser channel merged into one image by LSM2011 software for further analysis. Microscope and software settings were for all experiments the same, in which fluorescent signal saturation was avoided to maintain detection linearity. After slide elution and IF re-staining (see section slide elution, Fig. 1), LSCM was used for identification of cell markers. Previously imaged sites were located using the saved coordinates. To save time capturing the images in phase 2 for identifying cell types using specific fluorescent markers, a maximum z-stack of 4 focal planes (1.5µm) was used.

**Image analysis**

To quantify fluorescent signals of telomeres and gamma-H2AX, images were analyzed using the image analysis Telometer plugin (available at http://demarzolab.pathology.jhmi.edu/telometer/index.html) of ImageJ (http://rsb.info.nih.gov/ij/). First, images were converted into 16bit output. Rolling ball radius and threshold settings were set to 3 and 30 for telomeres and 3 and 15 for gamma-H2AX measurements respectively. To account for total fluorescence, maximum pixels was configured at 1000000. Next, pictures were normalized for background. Additionally, DAPI nuclei were cross checked with images showing cell specific markers, encircled and manually saved as regions of interest (ROIs) (Fig. 2 J and K). The advantage of using ROIs is that cell selection is concordant between separate telomere and gamma-H2AX images. To avoid background fluorescence in a ROI, cells overlapping unidentified (auto)fluorescent structures were not selected for analysis. Telometer draw mode in the software was not utilized. Next, ImageJ calculated total fluorescence per cluster of positive pixels. Multiple clusters were manually summed up to calculate total telomere or gamma-H2AX fluorescence within a cell.

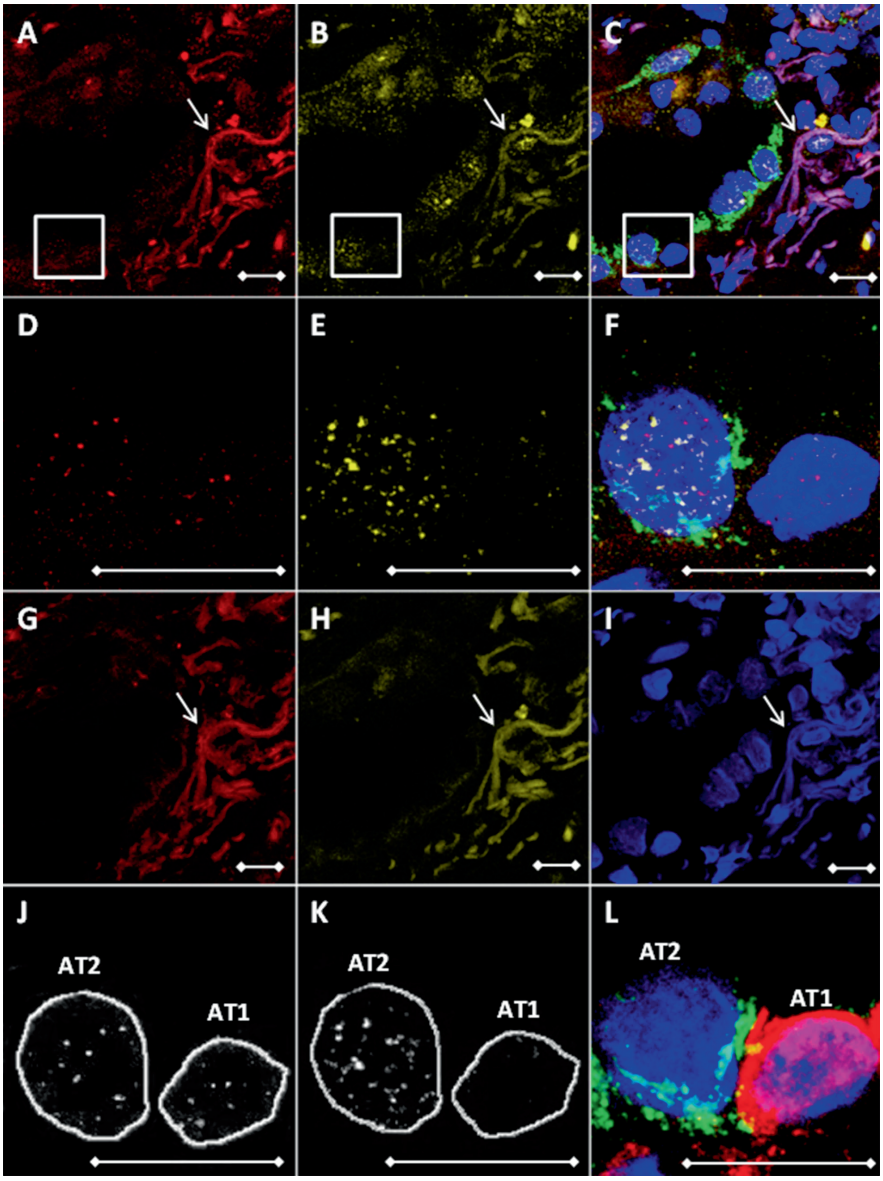


**Figure 2. Antibody and FISH probe elution procedures are essential for the use of multiple fluorophores.** Representative images of a patient with pulmonary fibrosis. Z-stacked LSCM pictures of (A) telomeres (red), (B) gamma-H2AX (yellow) and (C) overlay stains containing the AT2 cell-specific proSPC marker (green). (D-F) Magnifications of boxed areas in images A-C, respectively. (G and H) Telomere and gamma-H2AX channels after FISH probe and antibody elution, showing complete loss of cell nucleus specific signal and persistent presence of autofluorescent collagen and elastin fibers. (I) DAPI DNA staining (blue) and slide coordinates were used to determine the location. (J and K) ImageJ telometer images showing manually encircled cells of telomere and gamma-H2AX analyzed pictures. Both images were mirrored to (L), indicating proSPC (AT2) and Caveolin-1 (AT1) specific cell markers, to identify cell types. AT2 = Type 2 pneumocyte, AT1 = Type 1 pneumocyte. White arrows indicate autofluorescent collagen and elastin fibers. Scale bars represent 10 µm.

## Results

**Antibody and FISH probe elution procedures allows for the use of multiple fluorophores**

For the analysis of multiple fluorescent probes in one FFPE tissue slide we used antibody elution to avoid spectral overlap of different fluorophores. In phase 1, we obtained multiple fluorescent pictures from telomere (red) and gamma-H2AX (yellow) stained tissue (Fig. 2 A, B, D and E). For localization analysis, tissue was co-stained with a type 2 cell-specific pro-Spc antibody (green, Fig. 2 C and F). To remove fluorescent signals, a Tris-EDTA heat-mediated elution procedure was used, resulting in a complete loss of telomere FISH probe and gamma-H2AX signals (Fig. 2 G and H). This was confirmed by incubating tissue solely with the AF647-conjugated secondary antibody (data not shown). After obtaining telomere and gamma-H2AX pictures, antibodies against Caveolin-1 (red) and pro-Spc (green) were applied for identification of respectively AT1 and AT2 cells at the same coordinates (Fig. 2 L).

**Telomere length and gamma-H2AX quantification by the Telometer plugin of ImageJ**

To locate and select cells of interest, telomere and gamma-H2AX pictures were mirrored to pictures showing cell-specific markers. All antibodies were checked for cell specificity by experienced lung pathologists using consecutive HE stained slides for comparison. The DAPI-stained nuclei of selected cells were encircled as regions of interest (ROIs) and used in both telomere and gamma-H2AX channels to achieve an exact overlap of cell location between the channels. Image analysis was conducted using the ImageJ Telometer plugin, which was originally designed to quantify telomere fluorescence. In this study, visualization and detection of telomere fluorescence was verified (Fig. 2 J), similarly to the work of Meeker and coworkers [17]. To investigate whether gamma-H2AX signals could be reliably assessed, staining was performed on adenocarcinomic human alveolar basal epithelial (A549) cell cytospins which were treated with a concentration series of hydrogen peroxide ($H_2O_2$: 0 – 100 – 250 – 1000uM) for 1 hour at 37 °C to induce DNA double-strand breaks. Recovery of cells was done in Roswell Park Memorial Institute 1640 medium (52400-041, Thermo Fisher Scientific, Waltham, MA, US) + 10% Fetal Calf Serum (758093, Greiner Bio-one, Alphen a/d Rijn, the Netherlands) for 30 minutes at 37 °C, after which cytospins were produced. These data show that in treated cells, elevated concentrations of $H_2O_2$ resulted in significantly higher gamma-H2AX intensity (Fig. 3). Results were dose-dependent. In FFPE lung, gamma-H2AX signal clusters were predominantly found in the AT2 cell. In contrast, the AT1 cell contained no gamma-H2AX signal (Fig. 2 K). Furthermore, to assess a variety of lung cell types, pictures were taken in bronchus and artery areas to assess telomere length and gamma-H2AX in club cells (CC10 positive, Fig. 4 C) and smooth muscle cells (aSMA positive, Fig. 4 F), respectively.

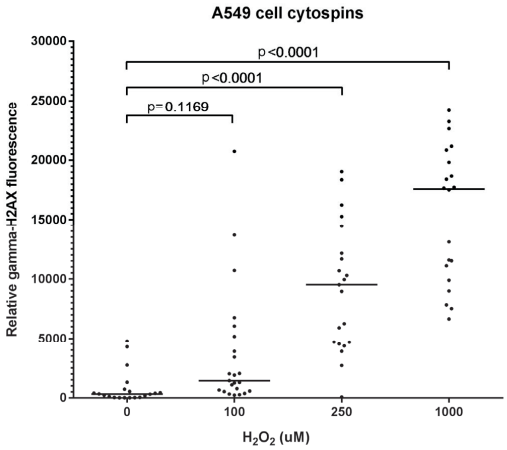


**Figure 3. Elevated concentrations of $H_2O_2$ result in dose-dependent increase in gamma-H2AX intensity.** Adenocarcinomic human alveolar basal epithelial (A549) cells were treated in four batches with 0 – 100 – 250 – 1000uM hydrogen peroxide ($H_2O_2$) respectively. Cells for cytospins were spun down on glass slides and stained using a gamma-H2AX antibody. Quantification of fluorescence showed that cells treated with 250uM and 1000uM $H_2O_2$ had significant stronger gamma-H2AX signals than null-treated cells. Each dot represents a cell. Medians are indicated with horizontal bars. P-values below 0.05 indicate significant differences (Kruskal-Wallis multiple comparison test).

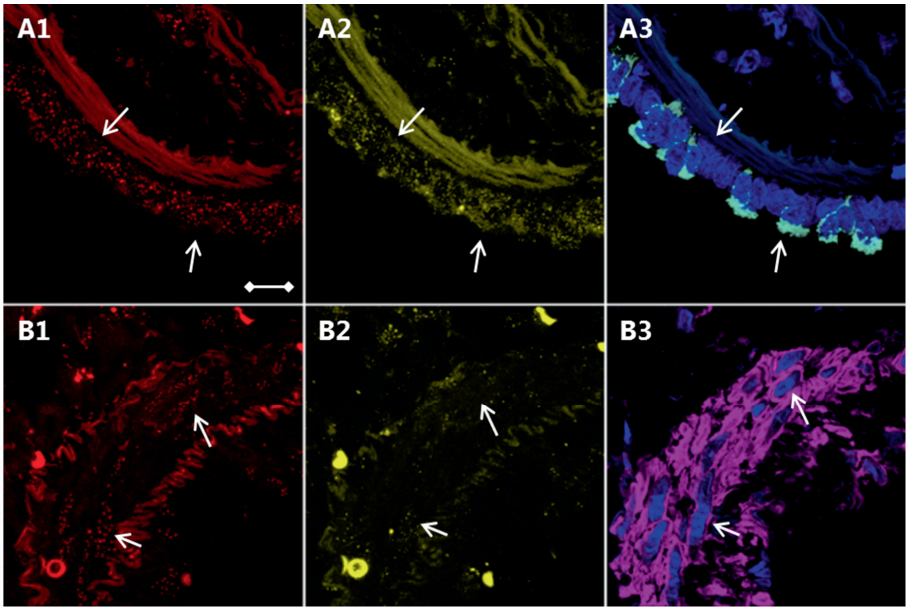


**Figure 4. Bronchial club and arterial smooth muscle cells identification using CC10 and aSMA selective immunofluorescence stains.** White arrows point to the (A1-A3) row of bronchus epithelial cells and to (B1-B3) cells of the arterial wall. Z-stacked LSCM pictures of (A1 and B1) telomere (red dots) and (A2 and B2) gamma-H2AX (yellow dots). DAPI DNA staining (blue) and cell-type identification of (A3) CC10 positive club cells (white) and (B3) aSMA positive smooth muscle cells (purple). Scale bar is valid for all images and represents 10 µm.

**Adjusting for the amount of DNA using nuclear surface area is preferable over DAPI intensity**
Telomere and gamma-H2AX signal in the nucleus is quantified, but requires adjustment for the amount of nuclear DNA captured in the LSCM images. To investigate whether either DAPI intensity or nucleus surface area is optimal for the adjustment of the telomere and gamma-H2AX signal, they were compared between two consecutive slides of the same tissue block. The consecutive slides were stained on different time points, using a 30 minutes incubation step with the same DAPI solution. Correlations between DAPI intensity and nucleus surface area in healthy controls showed a positive correlation ($r = 0.8521$, $p < 0.0001$, Fig. 5 A). In patients we show that AT2 DAPI intensity is significantly higher in one slide compared to the other slide ($p < 0.0001$), while nucleus surface area was constant between the two slides (Fig. 5 B and C). Similar results were found for AT1, club and smooth muscle cells (data not shown). Furthermore, telomere fluorescence was quantified and did not differ between slides (Fig. 5 D).

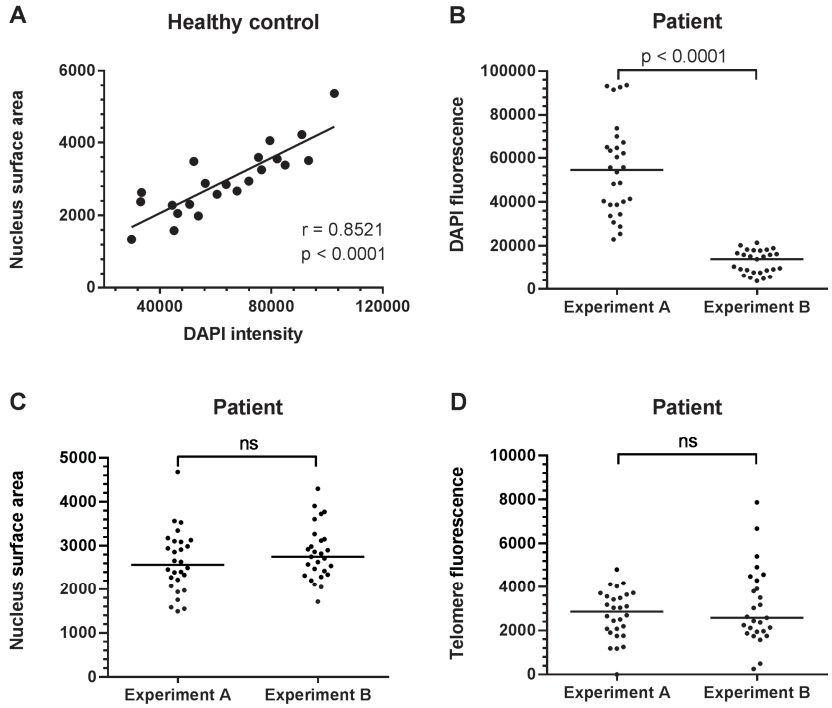


**Figure 5. Nucleus surface area, but not DAPI intensity is stable between experiments.** (A) Significant spearman correlation in AT2 cells of healthy controls between DAPI intensity and nucleus surface area ($r = 0.8521$, $p < 0.0001$). (B) Examples of one patient where AT2 DAPI intensity significantly differs between two independent experiments on two consecutive tissue sections. (C) Nucleus surface area and (D) unadjusted telomere fluorescence of the same AT2 cells indicate no significant differences between experiments. DAPI intensity, nucleus surface area and telomere intensity data were obtained from the telemeter plugin of ImageJ. Each dot represents one cell. Medians are indicated with horizontal bars. P-values indicate significant differences (Mann-Whitney tests; ns = non-significant).

**Specific association of telomere shortening with DNA double-strand breaks in AT1 and AT2 cells of a familial pulmonary fibrosis patient with a Poly(A)-specific ribonuclease (*PARN*) mutation**

In order to put the combined staining procedures into practice we assessed telomere length and double-strand breaks of 25 AT1 and 27 AT2 cells in a case of familial pulmonary fibrosis with a *PARN* mutation (NM 001134477:c.98C>T; NP 001127949:p.P33L). This mutation segregated in a family in which two first degree family members were diagnosed with pulmonary fibrosis. The biopsy contained extensive subpleural fibrosis, consistent with the usual interstitial pneumonia pattern. Multiple fibroblasts foci were present. In this subject, AT1 cells show significant higher telomere fluorescence (Fig. 6 A) and lower gamma-H2AX (Fig. 6 B) signals compared to AT2 cells. The correlation analysis between telomere length and DNA double-strand breaks on alveolar epithelial cells resulted in a significant negative correlation ($r = -0.5202$, $p < 0.0001$, Fig 5 C), implying increase in DNA double-strand breaks when telomeres shorten.

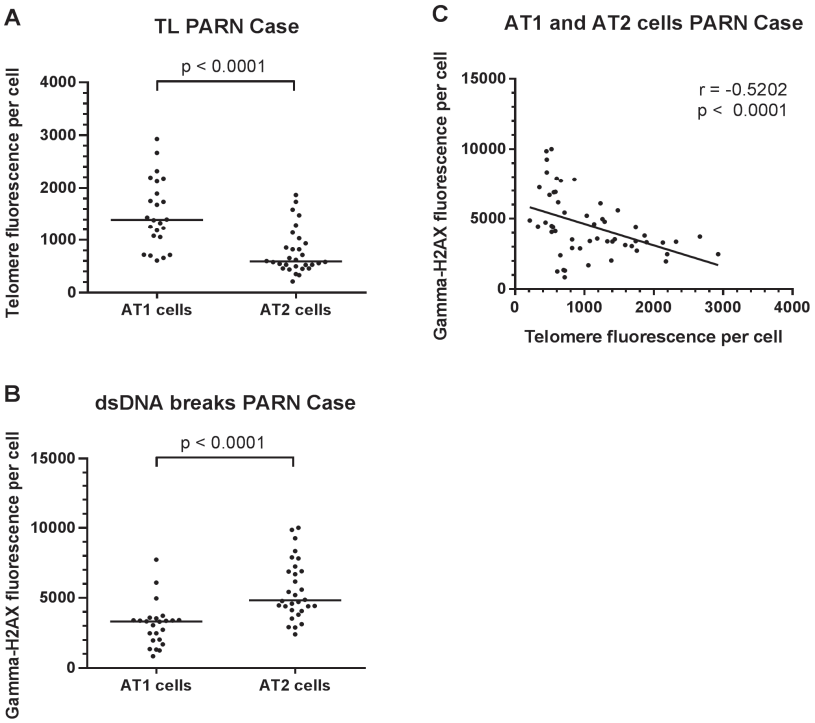


**Figure 6. Telomere and gamma-H2AX fluorescence measurements in AT1 and AT2 lung cells of a patient with a *PARN* mutation.** (A) FISH-stained telomere signals are significantly higher in AT1 than in AT2 cells ($p < 0.0001$). (B) Immunofluorescent staining of gamma-H2AX showed significantly lower signals in AT1 cells compared to AT2 cells ($p < 0.0001$). Medians are indicated with horizontal bars. Mann-Whitney tests were used for comparisons. (C) Significant negative spearman correlation in AT1 and AT2 cells between telomere and gamma-H2AX signals ($r = -0.5202$, $p < 0.0001$). Each dot represents one cell.

## Discussion

In this article, we describe a novel method to semi-quantitatively assess telomere length and gamma-H2AX associated DNA damage in specific cell types in FFPE lung tissue using serial staining, elution and re-staining of slides with probes and antibodies. In our hands, gamma-H2AX was a suitable detector of DNA double strand breaks. To obtain pictures a standard 4 color detection confocal microscope was utilized. Furthermore, we showed that DAPI-positive nuclear cross sectional surface area is preferable over DAPI intensity for the adjustment of DNA amount.

Previous approaches only quantified telomere length in one cell type using a cell identifying marker, or assessed cells only on histology features [17,36-38]. However, most organs consist of many different cell types and in some diseased tissues (e.g. fibrosis) histology alone is not sufficient to identify these different cells. Thus, to compare both telomere length and DNA double-strand breaks between multiple cell types, a more comprehensive approach is needed. Here, we identified multiple lung cells e.g. AT1 and AT2 pneumocytes, club cells and smooth muscle cells, using appropriate antibodies. The locations of AT2 and Club cells were selected by means of pro-Spc and CC10 staining, respectively. AT1 cells were identified juxtaposed AT2 cell coordinates, using a secondary staining after the elution procedure. Presence of artery smooth muscle cells was based on DAPI staining and checked afterwards using aSMA positivity (Fig. 1). Obtained LSCM pictures often contained fluorescent structures, which were visible in all channels (Fig. 2, white arrows). These structures could include extracellular matrix components, of which elastin fibers and collagen possess autofluorescent features [39,40]. Moreover, elastin is abundantly present in the lung [41]. Obviously, cells overlaying these structures were not included in the analysis in order to avoid interfering fluorescence signals during analysis.

The percentage of a cell nucleus captured in a picture is dependent on the cutting procedure of the paraffin block and the Z-stack selection. Both telomere and gamma-H2AX are located in the nucleus. Therefore, measurements had to be adjusted for the amount of cell nucleus captured in a picture. In literature, DAPI intensity and nuclear surface area have been used to adjust telomere and gamma-H2AX measurements [17,36,42]. However, we are not aware of any direct comparison of these procedures. Theoretically we expect that the amount of DNA (DAPI) for each cell type is comparable. First, we have shown that in healthy controls DAPI intensity correlated significantly with nuclear surface area ($r = 0.8521$, Fig 4 A), implying that in normal conditions factors are exchangeable. However, significant differences were found in between patient DAPI intensity in AT2 cells of two separately stained consecutive slides of the same biopsy (Fig. 5 B), while nucleus surface area was constant (Fig. 5 C). Moreover, no significant differences were found in telomere signal between the consecutive slides, which implies that DNA is evenly preserved (Fig. 5 D). This supports the idea that only DAPI intensity is fluctuating. Because nucleus surface area remained stable, we determined that this is a suitable factor to adjust for the amount of nuclear DNA present in an image.

Based on this methodological work, we analyzed telomere length and DNA double-strand breaks in AT1 and AT2 cells of a familial pulmonary fibrosis case with a rare c.98C>T; p.P33L *PARN* mutation. *PARN* is involved in the production of proteins related to telomere biology [14]. Therefore, defects in *PARN* leads to telomere shortening. Previously, we showed that telomeres of surrounding cells of patients with a mutation-induced telomere maintenance problem were significantly longer than telomeres of AT2 cells. We concluded that short AT2 cell telomere length might play a role in the pathogenesis of IPF, because AT2 cells are the progenitor cells of lung alveoli, thereby preserving the distal lung structure. This requires active telomere maintenance. However, in fibrotic lungs AT2 cell telomere conservation supposedly cannot be maintained [38]. To study different types of surrounding cells, we therefore constructed a method to identify subsets of surrounding cells to investigate whether telomere length differs among these different types of surrounding cells. In a case of *PARN*-mutation associated pulmonary fibrosis, we showed that in AT1 cell telomeres were significantly longer than AT2 cell telomeres. Whether this is a general feature of pulmonary fibrosis has to be investigated.

Telomere shortening plays a postulated role in pulmonary fibrosis according to the model of Chilosi and coworkers [10]. Additionally, in accordance with literature [12-14], we found that the mutation in *PARN* segregates with disease in a Dutch family of pulmonary fibrosis patients, implying the causative role of telomere shortening. However, the role of DNA double-strand breaks has not been elucidated in fibrogenesis. *PARN* mutations initiate an p53-regulated early DNA damage response [14]. We found that in this one case short telomeres correlated with elevated DNA double-strand breaks, specifically in AT2 cells. Crucial high numbers of DNA double-strand breaks results in degradation or abnormal DNA repair and eventually in cell senescence [7-10]. A limitation of this study is that only one case with an inherited telomere shortening syndrome is evaluated. However, these data cautiously indicate that DNA double-strand breaks might bridge the gap between telomere shortening and AT2 cell dysfunction in pulmonary fibrosis and deserve further investigation.

In conclusion, this study presents an accessible way to investigate cell type specific quantitative association of telomere attrition with DNA double-strand breaks in FFPE lung tissue.

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Chapter 5

# Telomere shortening and DNA damage in culprit cells of different types of progressive fibrosing interstitial lung disease

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## Abstract

Pulmonary fibrosis is strongly associated with telomere shortening and increased DNA damage. Key cells in the pathogenesis involve alveolar type 2 (AT2) cells, club cells and myofibroblasts, however to which extend these cells are affected by telomere shortening and DNA damage is yet unknown. We sought to determine the degree of, and correlation between telomere shortening and DNA damage in different cell types involved in the pathogenesis of progressive fibrosing interstitial lung disease. Telomere length and DNA damage were quantified, using combined fluorescence in situ hybridization and immunofluorescence staining techniques, in AT2 cells, club cells and myofibroblasts of controls and patients with pulmonary fibrosis and a telomerase reverse transcriptase mutation (TERT-PF), idiopathic pulmonary fibrosis (IPF) and fibrotic hypersensitivity pneumonitis (fHP). In IPF and TERT-PF lungs, AT2 cells contained shorter telomeres and expressed higher DNA damage signals than club cells and myofibroblasts. In fHP lungs, club cells contained highly elevated levels of DNA damage, while telomeres were not evidently short. In vitro, we found significantly shorter telomeres and higher DNA damage levels only in AT2 surrogate cell lines treated with telomerase inhibitor BIBR1532. Our study demonstrated that in IPF and TERT-PF lungs, telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting the importance of AT2 cells in these diseases, while in fHP the particularly high telomere-independent DNA damage signals in club cells, underscores it's bronchiolocentric pathogenesis. These findings suggest that cell type-specific telomere shortening and DNA damage may aid to discriminate between different drivers of fibrogenesis.

**Take home message:** In patients with IPF telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, while in fHP the particularly high telomere-independent DNA damage signals in club cells, underscores it's bronchiolocentric pathogenesis.

## Introduction

Progressive fibrosing interstitial lung disease (ILD) is a group of devastating disorders characterized by scarring of the epithelium and reduced survival (1, 2). Although the pathogenesis is incompletely understood, evidence is growing that processes associated with accelerated aging, such as telomere shortening and genetic instability, play a causative role in the destruction of the lung epithelium and subsequent fibrosis (3, 4).

Telomeres are DNA-protein complexes at the end of chromosomes which act as a buffer in cell-cycle dependent DNA shortening, thereby protecting the genetic information of the genome (5-7). Shortening of telomeres is associated with several forms of progressive fibrosing ILD, such as idiopathic pulmonary fibrosis (IPF) and fibrotic hypersensitivity pneumonitis (fHP) (8-11). In a subset of these patients, disease is caused by genetic mutations

in telomere-related genes such as telomerase reverse transcriptase (*TERT*), the catalytic subunit of telomerase involved in telomere elongation and maintenance (12-16). Furthermore, we have previously shown that telomere length in lung tissue of sporadic IPF patients is significantly reduced and associated with poorer survival (8, 17).

Critical telomere shortening is recognized as DNA damage similar to a double-strand DNA break. This results in the phosphorylation of H2A histone family member X (γH2AX) initiating the DNA damage response. In healthy circumstances, double-strand DNA breaks take approximately 72 hours to resolve (18). However, if not fixed properly in time, the DNA damage response becomes persistent and eventually leads to cellular senescence (19-21), a process associated with fibrogenesis in IPF lungs and fibrotic mouse models (22, 23). Previous studies using mice models with a telomere-repeat factor-1 (Trf1) knockout in alveolar or bronchiolar epithelial cells demonstrated telomere shortening and increased DNA damage foci in both cell types (24, 25). Similar results were found in TRF2-inactivated human cell lines showing an elevated amount of damage foci at uncapped telomeres (26). Furthermore, we previously found a significant inverse correlation between γH2AX signals and telomere length in alveolar epithelial cells of a patient with pulmonary fibrosis harbouring a *PARN* mutation (27). In contrast, other studies reported that, even though DNA damage signals were increased, no such correlation between average whole lung telomere length and DNA damage signals was found in IPF lungs (28). However, extensive cell-type specific measurements are missing in pulmonary fibrosis.

Several cell types have been associated with the pathogenesis of tissue remodelling underlying pulmonary fibrosis. Alveolar type 2 (AT2) cells, progenitor cells responsible for maintenance and renewal of the alveolar compartment, are generally considered to play a fundamental role in the onset of fibrogenesis in the lung (24, 29, 30). Loss of functional AT2 cells result in an impaired renewal capacity of alveolar cells and the production of pro-fibrotic factors, subsequently leading to activation of myofibroblasts and extracellular matrix deposition (31). However, there has been emerging attention for a role of club cells in pulmonary fibrosis. Similar to AT2 cells in alveoli, club cells are progenitor cells responsible for maintenance and renewal of bronchiolar epithelium. In pulmonary fibrosis, club cells drive bronchiolization, a process in which bronchiolar epithelial cells migrate to and repopulate the alveoli (32, 33). A third cell type, i.e. the myofibroblast is the main source of collagen deposition, thereby driving fibrogenesis. Clusters of myofibroblasts forming fibroblast foci are a histological hallmark of IPF (1, 34). We previously found that in IPF lungs, telomeres in AT2 cells were shorter than in other, as yet unclassified cells surrounding these AT2 cells (8). However, telomere length in the two other cell types involved in fibrogenesis has never been studied specifically in IPF or other types of pulmonary fibrosis such as fHP. Moreover, a possible quantitative and cell-type specific relation between telomere shortening and persistent activation of the DNA damage response remains to be elucidated.

To mimic lung-specific telomere shortening in an experimental setup, lung cell lines can be treated with BIBR1532, a telomerase inhibitor which binds noncompetitively to the active site of the telomerase protein (35). Previously, BIBR1532-dependent telomere shortening in a

A549 carcinoma cell line, which is derived from the alveolar epithelium and closely resemble AT2 cells, showed that after 140 population doublings telomeres were shorter in these cells (36). However, to date telomere shortening in surrogate cell lines of club cells and myofibroblasts with BIBR1532 treatment and its correlation with DNA damage was not assessed.

## Material and Methods

**Patient selection**

Lung material of three patient groups with progressive fibrosing ILD were included in the analysis, consisting of 32 patients with IPF, 17 patients with pulmonary fibrosis and a *TERT* mutation (TERT-PF), and 9 patients with fHP (Table S1). The study was approved by Medical research Ethics Committees United of the St Antonius Hospital (approval number W14.056 and R05-08A) and all patients provided written informed consent.

**Cell culture**

To investigate the relation between telomere shortening and DNA-damage, non-small-cell lung cancer cell lines A549 (cultured in Dulbecco's Modified Eagles Medium (DMEM)) and NCI-H460 (cultured in Roswell Park Memorial Institute (RPMI) 1640 medium), bronchial epithelial cell line 16HBE (cultured in Minimum Essential Medium (MEM)) and lung fibroblast cell line MRC5 (cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)) were treated with 0, 10 or 25 μM telomerase inhibitor 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid (BIBR1532; Selleckchem, Munich, Germany). Additional detail on cell culture experiments is provided in an online data supplement.

**Whole biopsy telomere length measurements in lung tissue**

Whole biopsy telomere length in DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue was measured by monochrome multiplex quantitative polymerase chain reaction (MMqPCR) as described previously (8, 17, 37). 13 controls, 17 TERT-PF, 32 IPF and 9 fHP lung biopsy samples were included. Additional detail on telomere length measurements by MMqPCR is provided in an online data supplement.

**Cell type-specific telomere and DNA damage staining**

Subsequent FFPE tissue slides of 4 μm were prepared and stained for telomere length and DNA damage analysis in specifically labelled AT2 cells, club cells and myofibroblasts as described previously (27). To investigate cell type-specific telomere length, we performed a fluorescence *in situ* hybridization (FISH) analysis in a random sub selection of control (n = 8), TERT-PF (n = 6), IPF (n = 10) and fHP (n = 5) lungs. Additional detail on telomere length and γH2AX DNA damage measurements by FISH and immunofluorescence (IF) is provided in an online data supplement.

**Statistical analysis**

Statistical significances were computed using non-parametric tests in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Telomere length and DNA damage signal differences were determined by Mann-Whitney tests and combined Kruskall-Wallis and Dunn's multiple comparisons tests. First, we used the data to compare differences in the signal between the different groups of patients (controls, TERT-PF, IPF and fHP), then we used the data to compare differences between the different types of cells (AT2, Club cells and myofibroblasts). Spearman's rank coefficient was used to calculate correlations per disease group between telomere length and γH2AX signal in all cell-types together. Next, we investigated if the correlation that was present in TERT-PF lungs would fit observed values in the specific cell-types AT2, Club cells or myofibroblasts in IPF or fHP lungs. The cell-type specific observed telomere length was used in the equation representing the correlation in TERT-PF lungs to calculate the cell-type specific expected DNA damage value. Statistical differences between observed and expected values for each cell-type were computed with the Mann-Whitney tests.

## Results

**No difference in whole biopsy telomere length between IPF and fHP lungs**

Analysis of whole biopsy telomere length measured by MMqPCR showed that telomeres in the control group were significantly longer than in the patient groups ($p < 0.05$). Furthermore, comparison between patient groups showed that telomere length in TERT-PF lungs was significantly shorter than in IPF and fHP groups ($p < 0.05$). No difference was found between whole biopsy telomere length of IPF and fHP groups (Table 1).



**AT2 cells of TERT-PF and IPF lungs have the shortest telomeres**

To determine cell type-specific telomere length, we stained telomeres by FISH together with cell type markers by IF (Fig1). Comparison between cell types showed that in controls there was no difference between telomere length in AT2 cells and club cells ($p = 0.961$, Fig 2a). In TERT-PF and IPF lungs, telomere length in AT2 cells was significantly shorter than in club cells ($p < 0.0001$, Fig 2b and c) and in myofibroblasts ($p < 0.0001$, Fig 2b and c), while in fHP lungs telomere length of AT2 cells was not different from that of club cells ($p = 0.168$, Fig 2d), and slightly shorter than that of myofibroblasts ($p = 0.0002$, Fig 2d). In all three patient groups, no difference in telomere length was found between club cells and myofibroblasts. Comparison between groups showed that telomere length in AT2 cells and club cells of control lungs were significantly longer than those of TERT-PF, IPF and fHP lungs (Table 1). In contrast, telomere length in AT2 cells, club cells and myofibroblasts of TERT-PF lungs were significantly shorter than those of control, IPF and fHP lungs (Table 1). Interestingly, telomeres in AT2 cells of IPF lungs were significantly shorter than in AT2 cells of fHP lungs ($p < 0.0001$), while telomere length in club cell and myofibroblasts did not differ between IPF and fHP (Table 1).

**Table 1. Group comparison of telomere length and DNA damage in lung tissue**

| | Controls | TERT-PF | IPF | fHP |
|---|---|---|---|---|
| *Average TL (T/S ratio) of whole biopsy by MMqPCR (IQR)* | 0.932[a] (0.909-0.947) | 0.772[b] (0.734-0.803) | 0.862 (0.810-0.959) | 0.849 (0.778-0.884) |
| *AT2 cell* | | | | |
| *γH2AX signal (IQR)* | 1005 (246-3354) | 4253[c] (3019-6390) | 3923 (2005-5364) | 3661 (2028-4749) |
| *FISH TL (IQR)* | 1764[a] (999-2484) | 464[b] (226-703) | 729[d] (342-1101) | 1049 (679-1605) |
| *Club cell* | | | | |
| *γH2AX signal (IQR)* | 1303 (248-3782) | 1879 (902-4624) | 1745 (487-3407) | 6769[c] (4025-8827) |
| *FISH TL (IQR)* | 1650[a] (769-2600) | 813[b] (467-1147) | 1405 (821-1949) | 1155 (847-1864) |
| *Myofibroblast* | | | | |
| *γH2AX signal (IQR)* | NA | 752 (0-1637) | 388 (9.2-2691) | 2080 (681-7381) |
| *FISH TL (IQR)* | NA | 924[b] (646-1345) | 1518 (1139-2105) | 1513 (1055-2069) |

TERT-PF = Patients with a TERT mutation and pulmonary fibrosis; IPF = Idiopathic Pulmonary Fibrosis; fHP = fibrotic Hypersensitivity Pneumonitis; AT2 = Alveolar Type 2; TL = Telomere Length; IQR = Inter Quartile Range; NA = Not Applicable. Numbers describe median telomere length or DNA damage signal.

[a] = Telomeres were significantly longer than in the other study groups ($p < 0.05$).

[b] = Telomeres were significantly shorter than in the other study groups ($p < 0.05$).

[c] = DNA damage signal was significantly higher than in the other study groups ($p < 0.05$).

[d] = Telomeres in AT2 cells of IPF lungs were significantly shorter than in AT2 cells of fHP lungs ($p < 0.0001$).

**Increased DNA damage in club cells of fHP lungs**

To determine cell type-specific DNA damage, we used immunofluorescent staining of γH2AX together with cell-type specific markers. Comparison between cell types showed that in TERT-PF and IPF lungs, γH2AX signal in AT2 cells was significantly higher than in club cells ($p < 0.0001$, Fig 2f and 2g) and in myofibroblasts ($p < 0.0001$, Fig 2f and 2g). In contrast, in fHP lungs γH2AX signal in club cells was higher than in AT2 cells ($p < 0.0001$, Fig 2h) and myofibroblasts ($p < 0.0001$, Fig 2h). Moreover, comparison between study groups showed that club cells in fHP lungs contained a significantly higher γH2AX signal than club cells in control ($p < 0.0001$), TERT-PF ($p < 0.0001$) and IPF lungs ($p < 0.0001$, Table 1). In AT2 cells of TERT-PF lungs, γH2AX signal was higher than in the other study groups (controls: $p < 0.0001$, IPF: $p = 0.038$ and fHP: $p = 0.005$, Table 1) while no difference in γH2AX signal of AT2 cells between fHP and IPF lungs was detected ($p = 0.248$, Table 1).

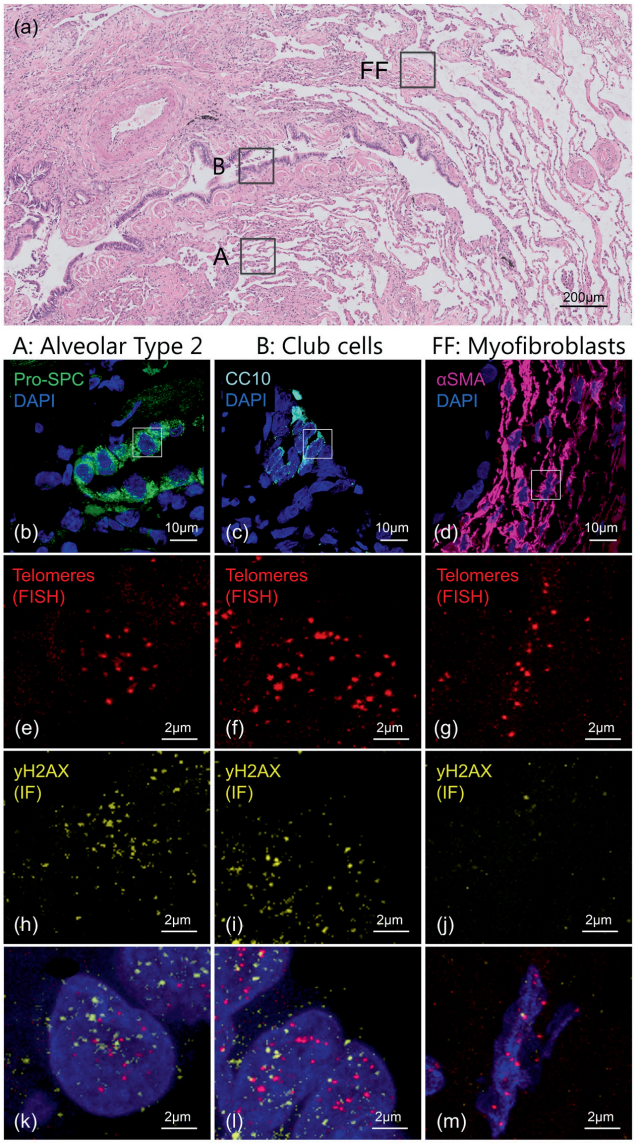


**Figure 1. Telomere and DNA damage in fibrotic lung tissue measured by combined FISH and immunofluorescence staining techniques.** (a) Hematoxylin and Eosin (H&E) staining representing a typical IPF lung biopsy. A = Alveolus, B = Bronchiolus and FF = Fibroblast Focus. (b-d) Representative fluorescent stained examples of boxed areas in image a, containing pro-Spc-positive AT2 cells (green), CC10-positive club cells (light blue) and αSMA-positive myofibroblasts (purple), respectively. (e-g) FISH-stained telomere signals (red dots) and (h-j) IF-stained γH2AX signals (yellow dots) in magnified boxed areas of images b-d, respectively. (k-m) Overlay pictures of telomere, γH2AX and DAPI stainings. All fluorescent pictures were captured and Z-stacked using a LSM700 laser scanning confocal microscope. Abbreviations: FISH, fluorescence in situ hybridization; IF, Immunofluorescence; AT2, alveolar type 2 pneumocyte; Spc, surfactant protein C; CC10, club cell protein 10; αSMA, alfa smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; γH2AX, phosphorylated histone protein from the H2A family.

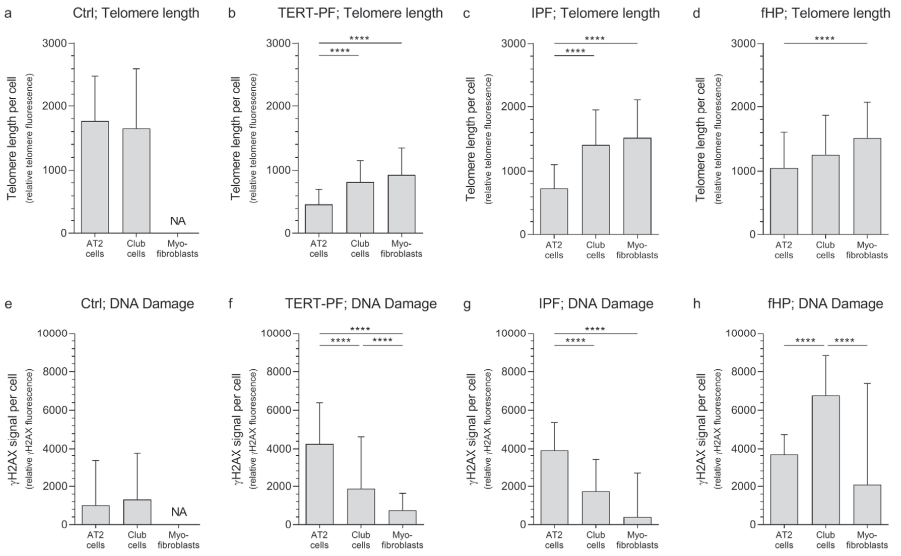


**Figure 2. Cell-specific quantification of telomere and DNA damage signals in control and fibrotic lungs.** Bar charts of telomere length (measured by FISH) and DNA damage signals (measured by IF) in AT2 cells, club cells and myofibroblasts in lungs of (a, e) 8 controls, (b, f) 6 patients with a *TERT* mutation, (c, g) 10 IPF and (d, h) 5 fHP patients. Controls contained long telomeres and low DNA damage signals in AT2 cells and club cells. In TERT-PF and IPF lungs, telomere length in AT2 cells was shorter than in club cells and myofibroblasts ($p < 0.0001$) and DNA damage signals in AT2 cells were significantly higher than in club cells and myofibroblasts ($p < 0.0001$). In fHP lungs, DNA damage signals in club cells were highly elevated compared to AT2 cells and myofibroblasts ($p < 0.0001$), while telomere length in club cells was comparable with AT2 cells and myofibroblasts. Boxes represent medians and whiskers extend up to values within the 3rd quartile. Abbreviations: FISH, Fluorescence in situ hybridization; IF, Immunofluorescence; γH2AX, phosphorylated histone protein from the H2A family; Ctrl, Controls; TERT-PF, Patients with a *TERT* mutation and pulmonary fibrosis; IPF, Idiopathic pulmonary fibrosis; fHP, fibrotic hypersensitivity pneumonitis; NA, Not applicable. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests (**** = $p < 0.0001$).

## DNA damage in club cells and myofibroblasts of fibrotic lungs is higher than expected from telomere shortening alone

Next, using data from all cell-types, we analysed the association between FISH telomere length and γH2AX signal and found in TERT-PF lungs a moderately strong correlation ($r = -0.689$, $p = 0.003$, Fig 3a). The correlation between telomere length and DNA damage in TERT-PF lungs, in which a causative mutation in the *TERT* gene is underlying telomere shortening and disease, shows that telomere shortening is likely the cause of the observed increase in DNA damage. However, using data from all cell-types, such a correlation was not found in the IPF or fHP groups. Next, we investigated whether a correlation exists for specific cell-types in IPF and fHP, rather than in cell types combined. Therefore, we used the equation representing the correlation between telomere length and DNA damage found in TERT-PF lungs to calculate

the expected DNA damage for each cell-type based on the observed telomere length for each cell-type in IPF and fHP. The expected and observed DNA damage signals per cell type are presented in Fig 3b – 3g. In AT2 cells of IPF and fHP no difference between expected and observed DNA damage signals was present (Fig 3b and 3e). However, in club cells and myofibroblasts of IPF and fHP lungs, observed DNA damage signals were significantly higher than the expected values ($p < 0.05$, Fig 3c, 3d, 3f and 3g).

**Induced telomere shortening and increased DNA damage in AT2 surrogate cell lines**

In order to experimentally study if telomere shortening causes an increase in DNA damage, we added BIBR1532, a highly specific telomerase inhibitor to cultures of surrogate lung cell lines A549 and NCI-H460 for AT2 cells, 16HBE for club cells and MRC5 for myofibroblasts. Inhibition of telomerase showed that only in AT2 surrogate cell lines A549 and NCI-H460 telomeres shortened significantly with 25μM BIBR1532 ($p < 0.05$, Fig 4a and 4b) and that the level of DNA damage increased significantly compared with no BIBR1532 ($p = 0.0001$, Fig 4a, 4b, 4e and 4f). In 16HBE and MRC5 cells treated with 25μM BIBR1532, however, telomeres did not shorten (Fig 4c, 4d), while DNA damage increased to very high levels when compared with no BIBR1532 ($p = 0.0001$, Fig 4g and 4h).

## Discussion



In this study, telomere length and DNA damage were investigated for the first time in different cell types involved in the pathogenesis of progressive fibrosing ILD. In AT2 cells of patients with IPF and patients with a *TERT* mutation we detected the shortest telomeres and highest DNA damage signals when compared to club cells and myofibroblasts. However, telomere length in AT2 cells of fHP lungs was not evidently short, while very high DNA damage signals were present in club cells. The observed increase of DNA damage in AT2 cells may be caused by telomere shortening. This was experimentally replicated in two AT2 surrogate cell lines, which showed BIBR1532-induced telomere shortening together with increased DNA damage. However, the level of DNA damage in club cells and myofibroblasts of IPF and fHP lungs could not be explained by telomere shortening alone.

Cell-type specific analysis is most important to better understand processes in fibrotic lungs. Our results demonstrate that although no differences in average whole lung biopsy telomere length was present between IPF and fHP, significant differences between cell-types exist. In both TERT-PF and IPF lungs, telomere length in AT2 cells was most affected, confirming the important role of AT2 cell telomere shortening in IPF pathogenesis (8, 10, 24, 30). Moreover, γH2AX signals were significantly elevated in AT2 cells of these groups. However, only in TERT-PF lungs, in which a causative mutation in the *TERT* gene is underlying telomere shortening and disease, a correlation between telomeres and γH2AX-related DNA damage exists, suggesting that telomere shortening causes DNA damage in these patients. This is consistent with a previous report showing that in IPF lungs no correlation was found between average

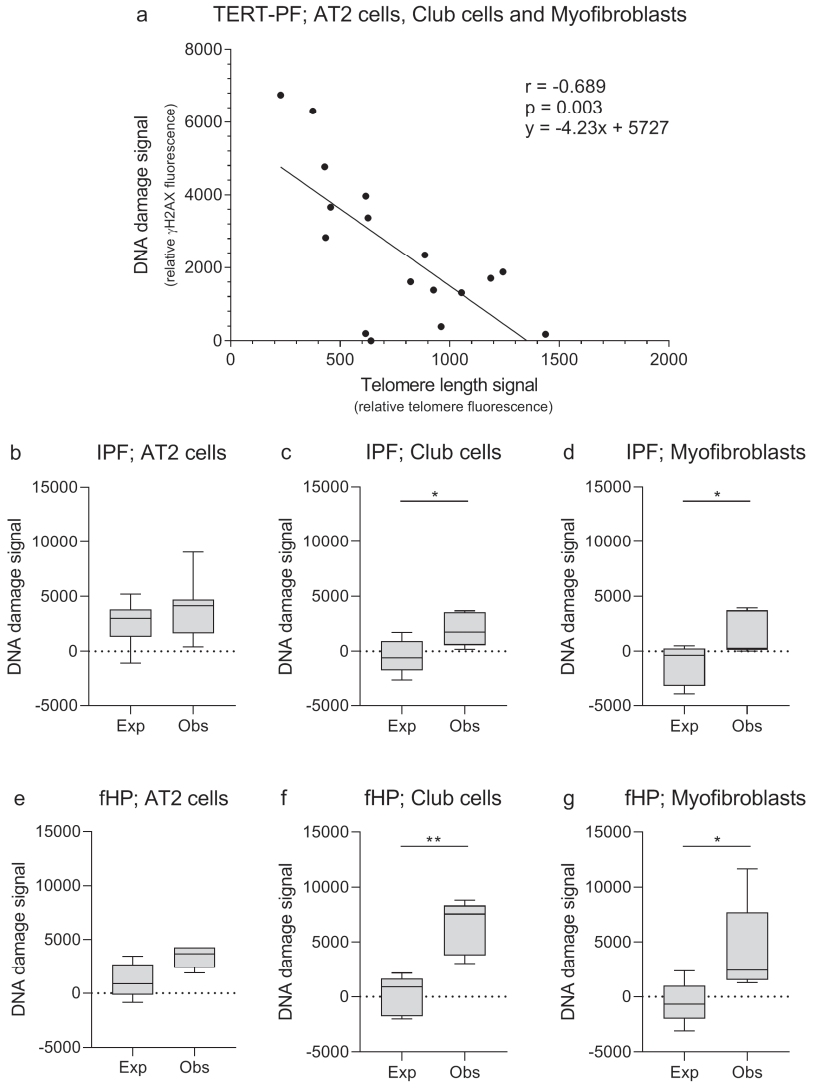


**Figure 3. Observed and expected DNA damage signals in AT2 cells, club cells and myofibroblasts of IPF and fHP lungs.** (a) Inverse spearman correlation (r = -0.689, p = 0.003) of median telomere length and median γH2AX signals in AT2 cells, club cells and myofibroblasts per biopsy specimen in 6 TERT-PF lungs resulting in a linear regression line with equation y = -4,23x + 5727. This equation was used to calculate expected DNA damage values from observed telomere length signals in (b) IPF AT2 cells (c) IPF club cells, (d) IPF myofibroblasts, (e) fHP AT2 cells (f) and fHP club cells, (g) fHP myofibroblasts. In IPF and fHP, club cells and myofibroblasts showed lower expected than observed DNA damage signals, while in AT2 cells no significant difference was found between expected and observed DNA damage signals. Statistical differences were computed by Mann-Whitney tests (* = $p < 0.05$, ** = $p < 0.01$). Abbreviations: TERT-PF, Patients with a *TERT* mutation and pulmonary fibrosis; IPF, Idiopathic pulmonary fibrosis; fHP, fibrotic hypersensitivity pneumonitis AT2, Alveolar type 2 pneumocytes; Exp, Expected; Obs, Observed.

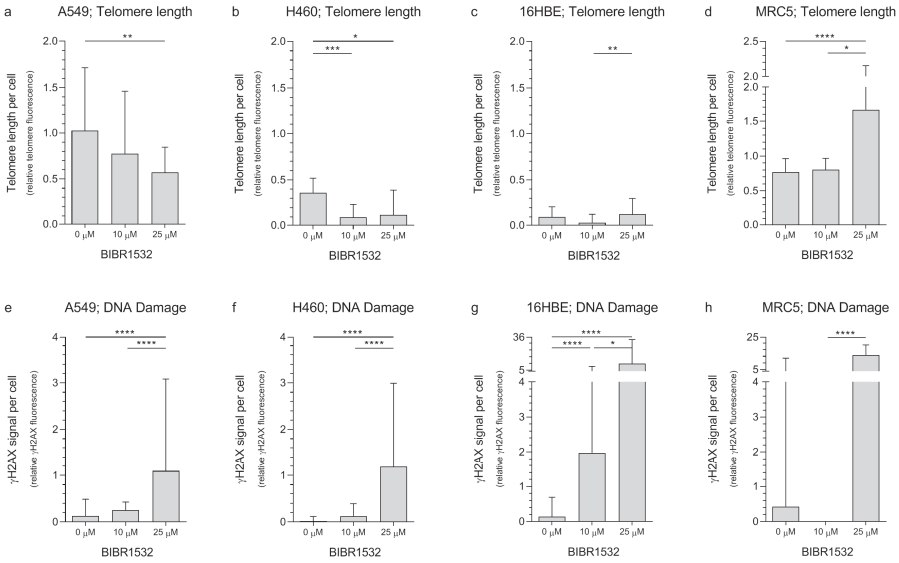


**Figure 4. Experimental induction of telomere shortening and DNA damage signals by inhibition of telomerase by BIBR1532**. Bar charts of telomere length (measured by FISH) and DNA damage signals (measured by IF) in cytospins of (a, e) A549, (b, f) H460, (c, g) 16HBE and (d, h) MRC5 cell lines. Induced telomere shortening with 25μM BIBR1532 was only observed in A549 (p = 0.004) and H460 cells (p = 0.013) compared to untreated cells, while in all cell lines increased DNA damage was found. Telomeres in MRC5 cells treated with 25μM BIBR1532 were significantly longer than untreated cells (p = 0.0001). Boxes represent medians and whiskers extend up to values within the 3rd quartile. Abbreviations: FISH, Fluorescence in situ hybridization; IF, Immunofluorescence; γH2AX, phosphorylated histone protein from the H2A family; BIBR1532, 2-[(E)-3-naphtalen-2-yl-but-2-enoylamino]-benzoic acid. Asterisks indicate significant differences calculated by Kruskal-Wallis multiple comparison tests (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).



whole lung telomere length and DNA damage signals (28) and supports the notion that DNA damage in IPF may be caused by other factors than telomere shortening. To investigate if a decrease in telomere length induces an increase in DNA damage for each cell-type in IPF and fHP lungs, we tested if observed DNA damage signals deviated from expected DNA damage signals based on the observed telomere length. In AT2 cells, observed and expected DNA damage signals were similar, while in club cells and myofibroblasts the amount of DNA damage was significantly higher than expected. These data imply that in AT2 cells of IPF and fHP lungs telomere shortening may be the primary cause of DNA damage, while in club cells and myofibroblasts other processes may be involved.

Progressive telomere shortening and accumulation of DNA damage are prominent features of aging and may eventually lead to cellular senescence or apoptosis (19-21). Our data demonstrates that telomere shortening in TERT-PF lungs and in AT2 cells of IPF and fHP lungs is associated with elevated DNA damage signals. This suggests that AT2 cells in fibrotic lungs are prone to become senescent or apoptotic. Senescent and apoptotic AT2 cells have been

observed in IPF lung (38-40) but not in fHP. Accumulation of senescent cells has been associated with progressive pulmonary fibrosis in IPF lungs and fibrotic mouse models (22, 23, 41, 42) and was postulated to drive pulmonary dysfunction in IPF. Whether the high level of DNA-damage observed in club cells of fHP lungs associates with senescence or apoptosis, remains to be investigated. This is of special interest because, treatment with senolytic dasatinib in combination with quercetin was proven to be effective in eliminating cultured senescent human lung cells (43) and recent clinical trials with these drugs demonstrated physical alleviation in patients with IPF (44).

In fHP, club cells most prominently contained highly elevated DNA damage signals, but showed no excessive telomere shortening. The causal trigger of fHP is a sustained allergic reaction against an extrinsic antigen, but how this allergic reaction leads to telomere-unrelated DNA damage in club cells is unclear. A possible cause may be the inflammation-induced accumulation of reactive oxygen species (ROS), a group of highly reactive, DNA damage-inducing molecules that are also associated with other allergic diseases, such as asthma (45, 46). Because fHP is characterized by inhaled antigens that, due to size, strand in the bronchioles (47), it is possible that the accumulation of DNA damage in club cells of fHP lungs at this location is caused by ROS, and not by telomere shortening. This is in line with a previous study that showed that bronchoalveolar lavage of fHP patients contained significantly higher carbonylated protein levels, a marker of ROS, compared to IPF and controls (48). However, another study showed that in mice with a club cell-specific knock out of telomere repeat-binding factor-1 (Trf1), rapid aging of club cells by telomere dysfunction alone was sufficient to induce DNA damage and subsequent bronchiolocentric fibrosis (25). In addition, it was reported that in 25% of the cases with an initial diagnosis of IPF, bronchiolocentric fibrosis is indicative for a revised diagnosis of fHP (49). The excess DNA damage in fHP club cells might therefore, regardless of the cause, be suggestive of an important role of these cells in disease development and is in congruence with the localization of fibrosis in fHP.

Next, we showed for the first time that experimental inhibition of telomerase resulted in telomere shortening in AT2 cell surrogate cell lines A549 and NCI-H460, suggesting that telomeres in these cells, similar to AT2 cells in diagnostic biopsies of pulmonary fibrosis, are most sensitive to telomerase dysfunction. This is in congruence with previous experiments where telomere shortening in BIBR1532-treated NCI-H460 cells was observed (36). Moreover, in both *in situ* and *in vitro* experiments a decrease in telomere length in AT2 cells is associated with an increase in DNA damage, underlining the telomere-dependent accumulation of DNA damage in AT2 cells. Furthermore, in surrogate club cell and myofibroblasts cell lines 16HBE and MRC5 treated with BIBR1532 no evident telomere shortening was found, while these cells showed high levels of DNA damage. This corresponds with the finding that club cells and myofibroblasts in IPF and fHP tissue accumulate DNA damage independent of telomere shortening. However, it is unclear why BIBR1532-treated 16HBE and MRC5 cells present with high telomere-independent levels of DNA damage.

Strengths of this study comprise of the detailed assessment of cell type-specific telomere length and DNA damage in a broad spectrum of progressive fibrosing ILD patients, including those harbouring a *TERT* mutation (TERT-PF), IPF and fHP. However, some limitations are worth noting. The data presented here are based on associations; no causative links could be concluded from telomere length and DNA damage analysis in human tissue samples. Also, control tissue was obtained from various sources, such as residual lung resected from tissue next to a tumour. Furthermore, in contrast to the other cell lines, MRC5 cells are mortal foetal cells. Even though the MRC5 cells used in this study were still actively replicating, no definitive conclusions can be drawn on telomere length or DNA damage signals compared to other cell lines. Finally, use of primary cells instead of surrogate cell lines for AT2 and club cells would have been optimal to investigate a relation between telomere shortening and DNA damage.

In conclusion, this is the first study addressing in detail telomere status and DNA damage signals in AT2 cells, club cells and myofibroblasts in different types of progressive fibrosing ILD. In IPF and TERT-PF lungs, telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting their central role in fibrogenesis of these groups, while the remarkably high DNA damage in club cells of fHP lungs underscores the more bronchiolocentric fibrogenesis and a prominent role for club cells and DNA damage in fHP (Fig 5). To further elucidate the link between club cells and the pathogenesis of fHP, future studies should focus on cellular aging due to a sustained allergic reaction and DNA damage in these cells.



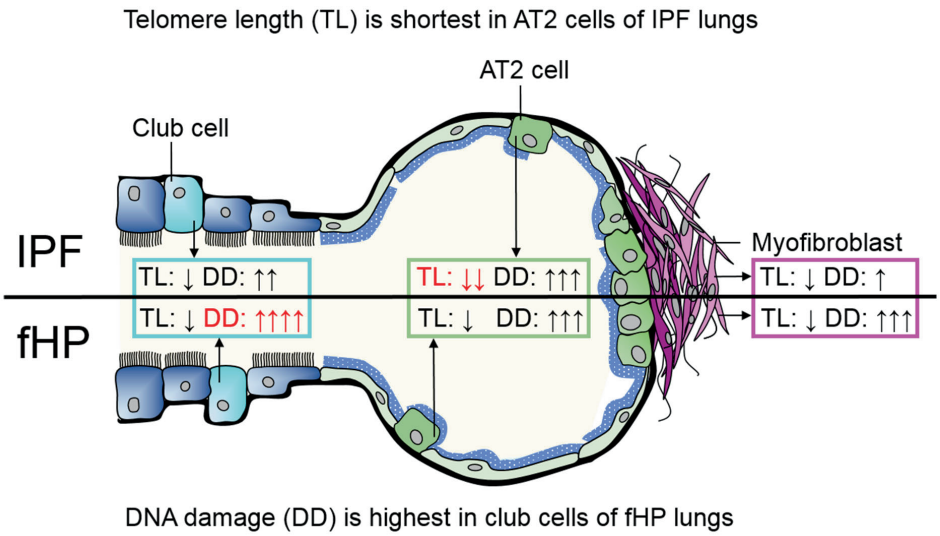


**Figure 5. Graphical abstract summarizing the main message of this study.** The extent to which key cells in progressive fibrosing lung disease are affected by telomere shortening and DNA damage is yet unknown. This study revealed that in patients with idiopathic pulmonary fibrosis (IPF) telomere shortening and accumulation of DNA damage is primarily affecting AT2 cells, further supporting the importance of AT2 cells in this disease, while in fibrotic hypersensitivity pneumonitis (fHP) the particularly high telomere-independent DNA damage signals in club cells, underscores it's bronchiolocentric pathogenesis.

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## Supplementary methods

### Tissue selection

Diagnostic surgical lung biopsies, taken between 1994 and 2015, were reviewed by an experienced pathologist (MFMvO) in accordance with the ATS/ERS/JRS/ALAT guidelines (1, 2). Furthermore, we cross-referenced data with 13 age matched control lung tissue specimens, which were obtained during post-mortem examination (n = 3), from residual donor lobes (n = 3) and from normal appearing lung resection tissue next to a tumour (n = 7).

### Cell culture

All media were supplemented with pen/strep and 10% FCS. Proliferative cultures were incubated at 37°C in a humidified 5% CO2 incubator. Cultures were split twice a week. To subculture the cell monolayers, they were washed with phosphate buffered saline (PBS) without calcium and magnesium before cells were detached from the culture glass using a Trypsin/EDTA solution at 37°C and seeded into fresh flasks. Trypsin was inactivated by the addition of growth medium. All media and supplements have been obtained from Thermofischer (Waltham, Massachusetts, USA). To achieve continuous exposure, the final concentration of 10 or 25 µM BIBR1532 was added after each passage. These concentrations were reported to be below levels of toxicity (3). Cells were cultured with the telomerase inhibitor BIBR1532 for 22 days before they were spun down on glass slides and stained for telomeres and DNA double strand breaks using FISH and a γH2AX antibody.



### Whole biopsy telomere length measurements by MMqPCR in FFPE tissue

In short, three sequential sections were cut from tissue samples and used for MMqPCR and FISH. DNA was isolated from the first section using an AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Whole biopsy telomere length assessment was performed using a monochrome multiplex qPCR (MMqPCR) on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) (4-6). The relative telomere length for each sample was calculated 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. Samples were analysed in triplicate in at least two runs, of which only coefficients of variation below 10% were included. The overall mean coefficient of variation was 2.5.

### Cell type-specific telomere and DNA damage staining

For a more detailed description of the methods on cell-specific telomere length and DNA damage measurements using combined FISH and immunofluorescence staining techniques, we refer to our methodological article (7). In short, slides were incubated with a telomere-Cy3 peptide nucleotide acid (PNA) probe (2.70 µg/ml, F1002; Panagene, Daejeon, South Korea). Excess probe was cleared with a PNA wash solution to subsequently stain for γH2AX (1:100, 05-636-I; Merck Millipore, Darmstadt, Germany) and specific cell markers with

immunofluorescence antibodies; AT2 cells (rabbit anti-human pro-Spc, 1:100, AB3786; Merck Millipore, Darmstadt, Germany), club cells (rabbit anti-human CC10, 1:100, sc-25554; Santa Cruz Biotechnology, Dallas, TX), Myofibroblasts (mouse anti-human αSMA, 1:50, A2547; Sigma-Aldrich, Darmstadt, Germany) and corresponding secondary antibodies. Lastly, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 25 µg/ml). Pictures were taken with a LSM700 laser scanning confocal microscope (Zeiss, Jena, Germany) and images were analysed using the image analysis Telometer plugin (available at http://demarzolab.pathology.jhmi.edu/telometer/index.html) of ImageJ (http://rsb.info.nih.gov/ij/). To this end the nuclear surface area was manually demarcated for the cell of interest. The telometer plugin provides the intensity of each fluorescent cluster within a demarcated area and the surface of this area. All clusters were manually summed up to calculate the total telomere or γH2AX fluorescence signal within a cell nucleus. To adjust the total signal intensity for the amount of DNA the cell's total telomere or γH2AX signal was divided by the nuclear surface area.

## Supplementary tables

**Table S1. Baseline characteristics of study groups**

| | Controls | TERT-PF | IPF | fHP |
|---|---|---|---|---|
| *N* | 13 | 17 | 32 | 9 |
| *Male/Female* | 10/3 | 14/3 | 29/3 | 6/3 |
| *Mean Age at time of biopsy (SD)* | 50.3 (16.2) | 58.7 (9.8) | 60.1 (9.5) | 60.1 (10.8) |
| *Mean FVC%predicted (SD)* | NA | 81 (15.6) | 70.5 (21.6) | 75.3 (27.5) |
| *Mean DLCO%predicted (SD)* | NA | 43.6 (6.2) | 46.5 (16.4) | 46.5 (19.3) |
| *Smoking status (CS:FS:NS:U)* | NA | 0:14:3:0 | 3:20:6:3 | 0:7:2:0 |
| *Pack years (SD)* | NA | 18 (15.5) | 20.2 (19.4) | 8.7 (12.4) |

TERT-PF = patients with a *TERT* mutation and pulmonary fibrosis; IPF = Idiopathic Pulmonary Fibrosis; fHP = fibrotic Hypersensitiviy Pneumonitis; FVC = Forced Vital Capacity; DLCO = Diffusing Capacity of the Lungs for Carbon Monoxide. CS = Current Smoker; FS = Former Smoker; NS = Never Smoker; U = Unknown; NA = Not Available. No significant differences in baseline characteristics were observed between the study groups (Kruskal-Wallis multiple comparison tests).

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Chapter 6

# The extent of inflammatory cell infiltrate and fibrosis in lungs of telomere- and surfactant-related familial pulmonary fibrosis

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## Abstract

Familial pulmonary fibrosis (FPF) is a monogenic disease most commonly involving telomere- (*TERT*) or surfactant- (*SFTP*) related mutations. These mutations have been shown to alter lymphocytic inflammatory responses and FPF biopsies with histological lymphocytic infiltrates have been reported. Recently, a model of a surfactant mutation in mice showed that disease initially started with an inflammatory response followed by fibrogenesis. Since inflammation and fibrogenesis are targeted by different drugs, we investigated whether the degree of these two features co-localize or occur independently in different entities of FPF, and whether they influence survival.

We quantified the number of lymphocyte aggregates per surface area, the extent of diffuse lymphocyte cell infiltrate, the number of fibroblast foci per surface area and the percentage of fibrotic lung surface area in digitally scanned H&E-sections of diagnostic surgical biopsies of patients with *TERT*-related FPF (TERT-PF; n = 17), *SFTP*-related FPF (SFTP-PF; n = 7), sporadic IPF (sIPF; n = 10). For comparison we included biopsies of patients with cellular non-specific interstitial pneumonia (cNSIP; n = 10), an inflammatory interstitial lung disease with high lymphocyte influx and usually responsive to immunosuppressive therapy.

The degree of inflammatory cell infiltrate and fibrosis in TERT-PF and SFTP-PF was not significantly different from that in sIPF. In comparison with cNSIP, the extent of lymphocyte infiltrates was significantly lower in sIPF and TERT-PF, but not in SFTP-PF. However, in contrast with cNSIP, in sIPF, TERT-PF, and SFTP-PF, diffuse lymphocyte cell infiltrates were predominantly and lymphocyte aggregates were only present in fibrotic areas ($p < 0.0001$). Furthermore, fibroblast foci and percentage of fibrotic lung surface associated with survival ($p = 0.022$ and $p = 0.018$, respectively), while this association was not observed for lymphocyte aggregates or diffuse lymphocytic infiltration.

Inflammatory cells in diagnostic lung biopsies of TERT-PF, SFTP-PF and sIPF are largely confined to fibrotic areas. However, based on inflammation and fibrosis, no differences were found between FPF and sIPF, substantiating the histological similarities between monogenic familial and sporadic disease. Furthermore, the degree of fibrosis, rather than inflammation correlates with survival, supporting that fibrogenesis is the key feature of FPF for therapeutic targeting.

## Introduction

Familial pulmonary fibrosis (FPF) is a devastating interstitial lung disease (ILD) characterized by progressive scarring of lung parenchyma, most commonly manifesting as idiopathic pulmonary fibrosis (IPF) (1).

Current international guidelines state that, although FPF develops at an earlier age (2) and the HRCT might differ (3), FPF and sIPF are clinically and histologically indistinguishable (1, 4). However, the studies upon which this statement is based compared sIPF with familial disease of unknown genetic etiology. Since then, it was discovered that nearly half of the FPF patients

harboured disease causing mutations (5). The mutated genes discovered in FPF can be subdivided in genes involved in telomere maintenance, most commonly in the telomerase reverse transcriptase (*TERT*) gene, and in genes involved in surfactant protein C, A1 and A2 (*SFTPC, SFTPA1* and *SFTPA2*) (6–12). While telomere dysfunction is a systemic disease that can cause immunodeficiency (13, 14), for example in common variable immunodeficiency disease (15, 16), genetic variations in surfactant genes were also associated with changes in immune cell activation and severity of infection (17). Moreover, lungs of patients with *SFTP* mutations have been frequently described to contain lymphocytic infiltrates and were sometimes classified as desquamative interstitial pneumonia, organizing pneumonia or cellular non-specific interstitial pneumonia (cNSIP) (12, 18–20). cNSIP is an inflammatory interstitial lung disease characterized by a high lymphocyte influx in the lungs and a diffuse fibrotic component (21). Furthermore, a genetic surfactant protein C deficiency was reported to be associated with pulmonary infections in childhood and immunosuppressive treatment is often beneficial in these patients (22), as well as in adults with cNSIP (21). Studies in *SFTP*-knockout mice and alveolar epithelial cell models showed that surfactant protein is important in inflammatory activation and regulation (23, 24). Interestingly, a recent study in a mouse model with a heterozygous knockin of the known human Sftpc I73T mutation showed that disease initially started with an inflammatory response (marked by increased numbers of macrophages, neutrophils, eosinophils and lymphocytes in BAL fluid) followed by fibrogenesis, suggesting that immunosuppressive treatment in early disease may be beneficial (25). Furthermore, inflammation was shown to be associated with disease progression (26–28). Altogether, these findings suggest an important inflammatory component in monogenic inflammatory disease that may be responsive to immunosuppressive drugs. However, immunosuppressive therapies were described to be harmful in sIPF patients (29, 30), while anti-fibrotic therapy was proven to beneficially reduce the decline of FVC in sIPF and other progressive fibrotic ILD (31–34). Therefore, caution is advised and more knowledge on the subject is urgently needed.
A characteristic of IPF lungs is the presence of temporal and spatial heterogeneous fibrosis with fibroblast foci (FF). FF are defined as clusters of matrix-depositing myofibroblasts, located at discrete sites of lung injury. They are considered a marker for the level of fibrosis in IPF lungs (35), and may relate also to the activity of the fibrotic process, since the presence of FF has been associated with the prognosis of IPF (26, 27, 36, 37), although not consistently so (38). Furthermore, in IPF lungs, by definition lymphocytes are predominantly located in dense fibrotic areas of the lung, rather than in non-fibrotic areas, and are considered to be reactive to the fibrosis.
In this study we quantified the extent and localization of diffuse lymphocyte cell infiltrate and lymphocyte aggregates as well as the number of fibroblast foci and percentage of fibrotic lung surface in diagnostic biopsies of adult monogenic *TERT*- and *SFTP*-associated FPF patients. These data were compared with lungs of patients with sIPF and cNSIP and related with survival.

## Material and Methods

**Patient and tissue selection**

Diagnostic lung biopsies of 17 FPF patients with a *TERT* mutation (TERT-PF), 7 FPF patients with a *SFTP* (4 *SFTPC* and 3 *SFTPA2)* mutation (SFTP-PF), 10 sIPF and 10 cNSIP patients were included in this study. FPF was determined when 2 or more first-degree family members also presented with pulmonary fibrosis. Furthermore, in the sIPF group, familial subjects were excluded and patients were screened negative for mutations in *TERT, TERC*, surfactant protein C (*SFTPC*), surfactant protein A2 (*SFTPA2*) exon 6 and TRF1-Interacting Nuclear Factor 2 (*TINF2*) exon 6. Upper and lower lobe specimens were both included in the comparison analysis. For the survival analysis, upper and lower lobe data was averaged. Specimens were reviewed by an experienced lung pathologist (MFMvO) and diagnoses were in accordance with the ATS/ERS/JRS/ALAT guidelines (1).

**Digital quantification of inflammatory cell infiltrate and fibrosis in diagnostic lung biopsies**

In this study hematoxylin and eosin (H&E) stained tissue sections were used (Fig 1). Sections were scanned for digital images at 20x magnification using an ultra fast scanner 1.8 (Philips, The Netherlands) and analysed at 5x magnification using image management system (IMS) web application (version 3.2, Philips, The Netherlands) on a calibrated Barco display. All parameters were assessed by two independent pathologists (SNK and MFMvO). In the sections, fibroblast foci (FF) were defined as a group of spindle-shaped myofibroblasts in a matrix of collagen, mostly present in the transition zone of fibrotic and non-fibrotic areas (35). Lymphocyte aggregates (LA) were defined as sharply edged dense groups of at least 50 lymphocytes. The size of these LAs was irrelevant. Both FFs and LAs were counted and adjusted for the total surface area of the tissue specimen. The amount of diffuse inflammatory cell infiltrate of the whole biopsy was scored using a histological grade from 1 to 4, while in subanalysis of non-fibrotic and fibrotic areas an percentage was estimated. The histological grades refer to a very little amount (1), a little amount (2), a moderate amount (3) and a severe amount (4) diffuse inflammatory cell infiltrate. Also the percentage of fibrotic lung surface was scored.

The digital scoring of the inflammatory cell infiltrate and fibrosis was executed by an experienced (MFMvO) and resident (SNK) lung pathologist. In order to master the method and to align scoring between pathologists, a study set of 14 biopsies was used. The final scoring for this study was performed on the analysis set. Subanalyses of fibrotic versus non-fibrotic areas were performed in a randomly selected subset of five sIPF, TERT and SFTP samples each.

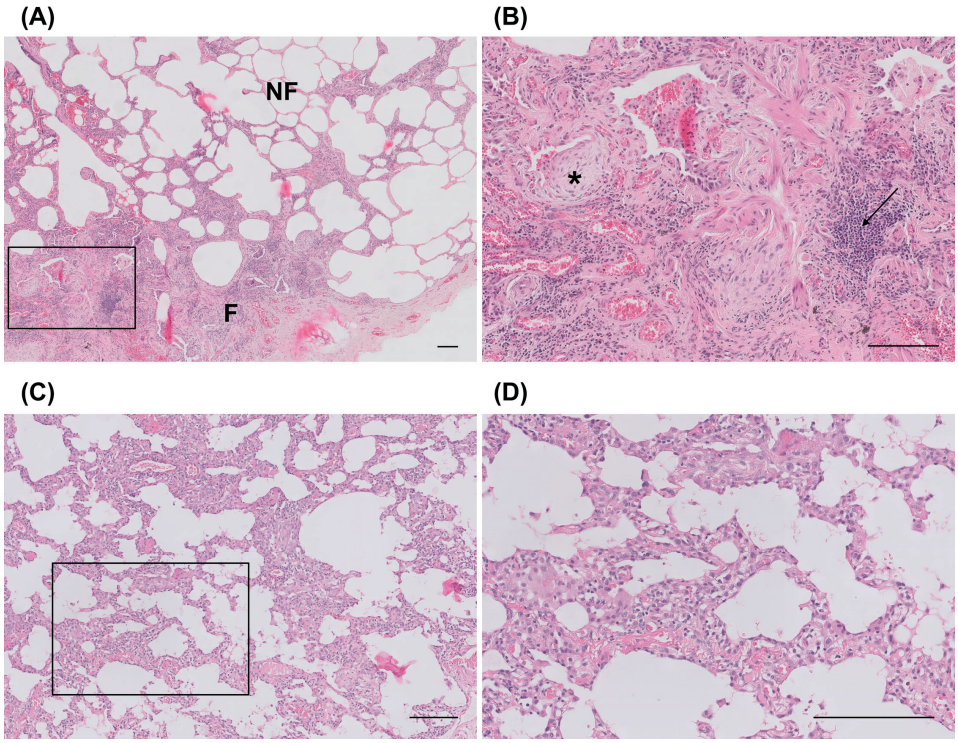


**Fig 1. Representative Hematoxylin and Eosin (H&E) stained diagnostic biopsies of a FPF/IPF and cNSIP lung.** Images of typical (**A**) FPF/IPF and (**C**) cNSIP lung biopsies. (**B, D**) Magnifications of boxed areas in images A and C, respectively. Fibroblast foci (asterisk) and lymphocyte aggregates (arrow) were digitally quantified in the whole biopsy and the amount of diffuse inflammatory cell infiltrate (blue-purple nuclei) of the whole biopsy was scored using a histological grade from 1 to 4. Horizontal bars represent a size of 200μm. Abbreviations: FPF, Familial pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis; cNSIP, cellular non-specific interstitial pneumonia; NF, non-fibrotic area; F, fibrotic area.



### Statistical analysis

Statistical significances were computed using non-parametric tests in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). In all patient groups, no significant differences were found between upper and lower lobe specimens for all parameters. Therefore, we combined upper and lower lobe data per patient group. Differences between inflammatory cell infiltrate and fibrosis were determined by Mann-Whitney tests and combined Kruskall-Wallis and Dunn's multiple comparisons tests. Spearman's rank coefficient was used to calculate correlations between the number of fibroblast foci and the percentage of fibrotic lung surface. Survival was computed by logrank (Mantel-Cox) testing on the total population of patients with pulmonary fibrosis (TERT-PF, SFTP-PF and sIPF combined) and divided the group at the median.

## Results

### Patient inclusion

Baseline characteristics of included patients are presented in Table 1. According to the ACMG classification of genetic variants (39), 6 SFTP variants were labelled as pathogenic and 1 as a variant of uncertain significance. Furthermore, we included 17 patients with a *TERT* mutation consisting of 6 pathogenic variants, 2 likely pathogenic variants and 9 variants of uncertain significance.

**Table 1. Baseline characteristics of study groups**

| | TERT-PF | SFTP-PF | sIPF | cNSIP |
|---|---|---|---|---|
| N | 17 | 7 | 10 | 10 |
| Male/Female | 14/3 | 3/4 | 9/1 | 4/6 |
| Mean age at time of biopsy (SD) | 58.7 (9.8) | 33.6 (14.1)* | 60.1 (5,1) | 57.8 (10.1) |
| Mean FVC%predicted (SD) | 81 (15.6) | 54.8 (14.1) | 73.6 (25) | 78.2 (25.5) |
| Mean DLCO%predicted (SD) | 43.6 (6,2) | 35.4 (14.1) | 47.1 (25) | 48.5 (12.8) |
| Smoking status (CS:FS:NS:U) | 0:14:3:0 | 0:2:5:0 | 1:6:2:1 | 1:8:1:0 |
| Pack years (SD) | 18 (15.5) | 3.7 (8) | 20.2 (25.1) | 9 (9.5) |

TERT-PF = Patients with pulmonary fibrosis and a *TERT* mutation; SFTP-PF = Patients with pulmonary fibrosis and a SFTP mutation; sIPF = sporadic Idiopathic Pulmonary Fibrosis; cNSIP = Cellular non-specific interstitial pneumonia; FVC = Forced Vital Capacity; DLCO = Diffusing Capacity of the Lungs for Carbon Monoxide; CS = Current Smoker; FS = Former Smoker; NS = Never Smoker; U = Unknown.

* = Mean age at time of biopsy in the SFTP-PF group was significantly lower than in the other groups (Kruskall-Wallis multiple comparison test: $p < 0.01$)

### Inflammatory cell infiltrate in familial and sporadic IPF

To study the degree of inflammatory cell infiltrate, we quantified the number of lymphocyte aggregates and the extent of diffuse lymphocyte cell infiltrate in TERT-PF, SFTP-PF, sIPF and cNSIP lungs. Analysing whole biopsies, no significant differences in lymphocyte and fibrotic parameters were found between TERT-PF, SFTP-PF and sIPF study groups (Fig 2a and 2b). Comparison with cNSIP showed that in TERT-PF and sIPF significantly less diffuse lymphocyte infiltrates were present. There was no correlation between lymphocyte aggregates and diffuse infiltrates. Detailed subanalysis of inflammatory cell infiltrate in fibrotic versus non-fibrotic areas showed that lymphocyte aggregates were completely and diffuse lymphocyte cell infiltrate were strongly associated with fibrotic areas in TERT-PF, SFTP-PF and sIPF (Mann-Whitney tests between non-fibrotic and fibrotic areas; $p < 0.0001$, Fig 2c and 2d). The number and extent of lymphocyte aberrations in cNSIP biopsies was comparable with that observed in the fibrotic areas of the TERT-PF, SFTP-PF and sIPF study groups (Fig 2c and 2d).

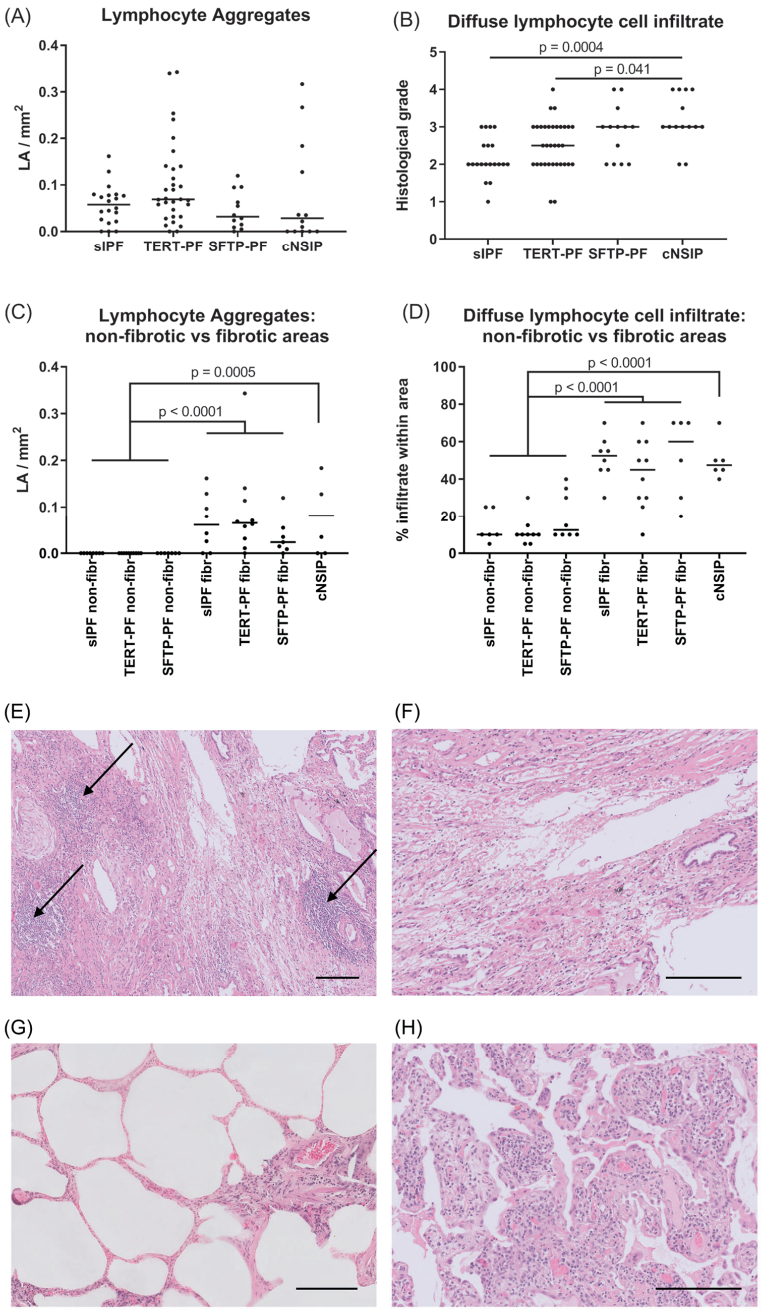


**Fig 2. Quantification of lymphocyte aggregates and diffuse lymphocyte cell infiltrate.** Scatter plots of lymphocyte aggregates (arrows in figure 1B and figure 2E) and diffuse lymphocyte cell infiltrate in diagnostic biopsies of 17 TERT-PF, 7 SFTP-PF, 10 sIPF and 10 cNSIP lungs analyzed (**A, B**) throughout the biopsy and in (**C, D**)

**-Continuation caption Figure 2-**
5 randomly selected biopsies per group to specifically assess non-fibrotic and fibrotic areas. In whole lung specimens, no significant differences were found between TERT-PF and SFTP-PF groups. In cNSIP lungs, the degree of diffuse lymphocyte cell infiltrate was significantly higher than in sIPF ($p = 0.0004$) and TERT-PF lungs ($p = 0.04$, Kruskall-Wallis test). The inflammatory cell infiltrate in non-fibrotic versus fibrotic areas showed that lymphocyte aggregates and the diffuse lymphocyte cell infiltrate were associated with fibrotic areas in TERT-PF, SFTP-PF and sIPF lungs (Mann-Whitney tests; $p < 0,0001$). Also the extent of lymphocyte aggregates ($p = 0,0005$) and the diffuse lymphocyte cell infiltrate ($p < 0,0001$) in cNSIP lungs was significantly higher than in non-fibrotic areas in PF lungs. No significant differences were found between fibrotic areas in PF and cNSIP lungs. Horizontal bars represent medians. (**E-H**) Examples of lymphocyte aggregates and diffuse lymphocyte cell infiltrate in Hematoxylin and Eosin (H&E) stained diagnostic biopsies; (**E**) fibrotic area in a TERT-PF case with 3 lymphocyte aggregates (arrows) and (**F**) grade 2 diffuse lymphocyte cell infiltrate; (**G**) non-fibrotic area in a sIPF case with no lymphocyte aggregates and grade 1 diffuse lymphocyte cell infiltrate; (**H**) cNSIP case with grade 4 diffuse lymphocyte cell infiltrate. Horizontal bars represent a size of 200µm. Abbreviations: TERT-PF, Patients with lung fibrosis and a TERT mutation; SFTP-PF, Patients with lung fibrosis and a surfactant mutation; sIPF, Sporadic idiopathic pulmonary fibrosis; cNSIP, Cellular non-specific interstitial pneumonia; LA, Lymphocyte aggregates.

### Fibrosis in familial and sporadic IPF

Next, we investigated the degree of fibrosis by assessing the number of fibroblast foci and the percentage of fibrotic lung surface in all four patient groups. Similar to the inflammatory cell infiltrate, no differences were found for these fibrotic parameters between TERT-PF, SFTP-PF and sIPF lungs (Fig 3a and 3b). Fibroblast foci in cNSIP lungs were almost absent and significantly lower than in sIPF lungs ($p = 0.001$, Fig 3a) and TERT-PF lungs ($p = 0.0009$, Fig 3a). Also, the percentage of (diffusely orientated) fibrotic lung surface in cNSIP lungs was significantly lower than in sIPF lungs ($p = 0.007$, Fig 3b) and SFTP-PF lungs ($p = 0.039$, Fig 3b). Furthermore, we found a significant moderate correlation between the number of fibroblast foci and the percentage of fibrotic surface in the fibrotic lungs ($r = 0.465$, $p = 0.005$, Fig 3c).

### Elevated numbers of fibroblast foci and a high percentage of fibrotic lung surface associate with worse survival

We investigated whether the number of fibroblast foci, percentage of fibrotic lung surface, number of lymphocyte aggregates and extent of lymphocyte cell infiltrate associated with survival. Because no differences were observed between TERT-PF, SFTP-PF and sIPF groups, we combined the data. Median values of each parameter were used as cut-off. Kaplan-Meier curves showed that both elevated numbers of fibroblast foci (cut-off value: 0.075 FF/mm$^2$, median survival: 85 vs 20 months; $p = 0.022$, Fig 4c) and high percentage of fibrosis (cut-off value: 50%, median survival: 82 vs 17 months; $p = 0.018$, Fig 4d) were significantly associated with survival. The number of lymphocyte aggregates (cut-off value: 0.062 LA/mm$^2$, $p = 0.91$, Fig 4a) and the extent of diffuse lymphocyte cell infiltrate (cut-off value: 2.5, $p = 0.99$, Fig 4b) did not associate with survival.

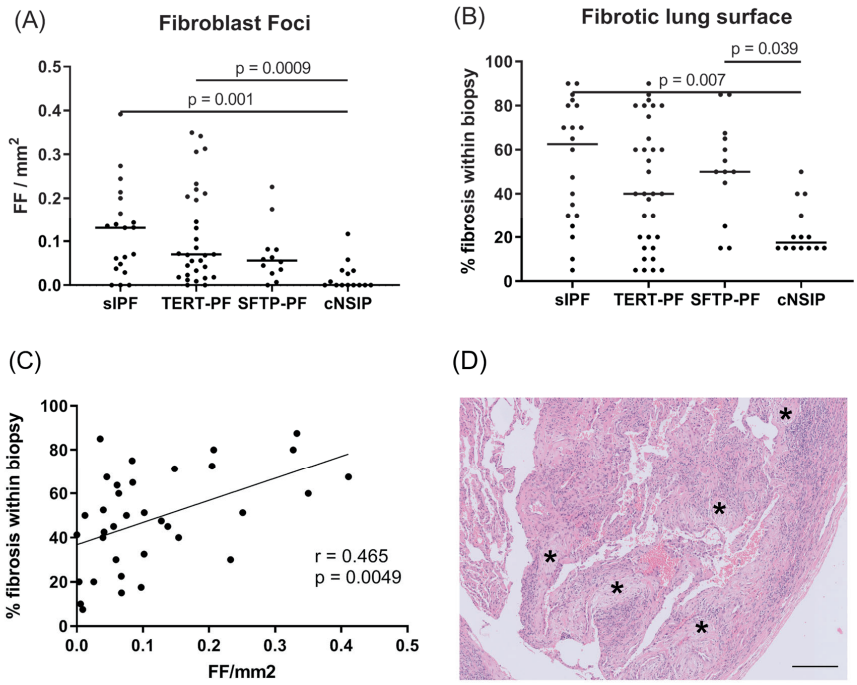


**Fig 3. Quantification of fibrotic foci and fibrotic lung surface.** Scatter plots of (**A**) the number of fibroblast foci (asterisk in figure 1B) and (**B**) the percentage of fibrotic lung surface in diagnostic biopsies of 17 TERT-PF, 7 SFTP-PF, 10 sIPF and 10 cNSIP lungs. For both parameters, no significant differences were found between TERT-PF, SFTP-PF and sIPF lungs. The amount of fibroblast foci per $mm^2$ in cNSIP lungs was significantly lower than in sIPF ($p = 0.001$) and TERT-PF lungs ($p = 0.0009$), while the percentage of fibrotic lung surface in cNSIP lungs was significantly lower than in sIPF ($p = 0.007$) and SFTP-PF lungs ($p = 0.04$). P-values were calculated using Kruskall-Wallis tests. Bars represent medians. (**C**) Positive spearman correlation between the number of fibroblast foci and percentage fibrotic lung surface of TERT-PF, SFTP-PF and sIPF lungs ($r = 0.465$, $p = 0.005$). (**D**) Example of a Hematoxylin and Eosin (H&E) stained fibrotic area in a SFTP-PF lung containing 5 fibroblast foci (asterisks). Horizontal bar represents a size of 200μm. Abbreviations: TERT-PF, Patients with lung fibrosis and a TERT mutation; SFTP-PF, Patients with lung fibrosis and a surfactant mutation; sIPF, Sporadic idiopathic pulmonary fibrosis; cNSIP, Cellular non-specific interstitial pneumonia; FF, Fibroblast foci.

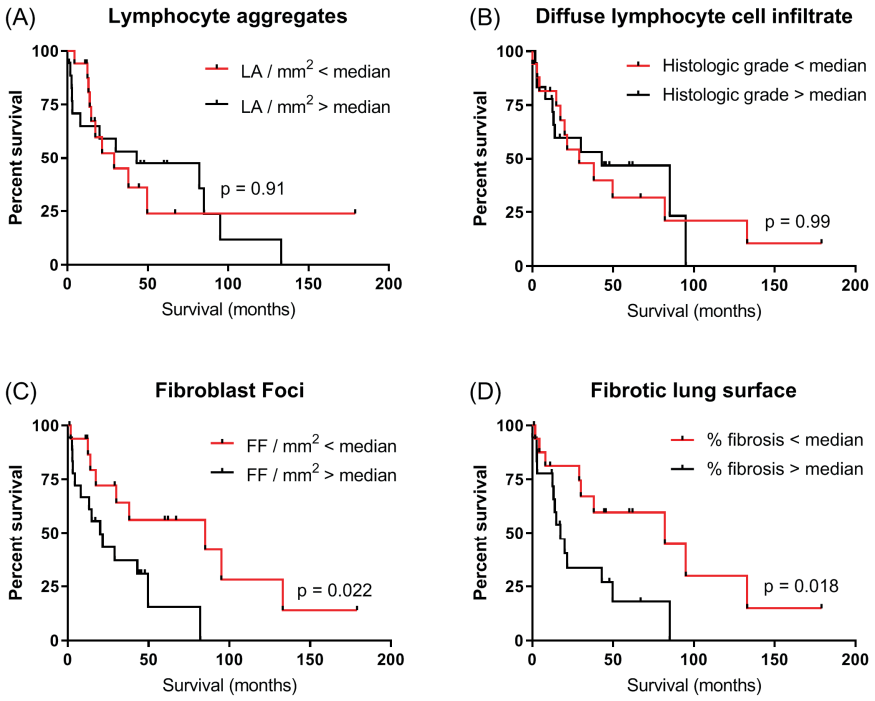


**Fig 4. Survival of joined TERT-PF, SFTP-PF and sIPF patients.** Kaplan-Meier curves showing the association between survival in months and the degree of (**A**) lymphocyte aggregates, (**B**) diffuse lymphocyte cell infiltrate, (**C**) fibroblast foci and (**D**) fibrotic lung surface in 34 patients with pulmonary fibrosis. Median values of each parameter were used as cut-off. For the degree of lymphocyte aggregates and diffuse lymphocyte cell infiltrate, no significant differences were found between survival above (black line) and below median (red line). For the degree of fibroblast foci and fibrotic lung surface, the significant difference in median survival was respectively 65 months (85 versus 20 months, p = 0.02) and 64.6 months (82 versus 17.4 months, p = 0.02). Significances were calculated using Log-rank (Mantel-Cox) tests. Abbreviations: LA, Lymphocyte aggregates; FF, Fibroblast foci.

## Discussion

In this study, we quantified the levels of inflammatory cell infiltrate and fibrosis in FPF with a *TERT* or *SFTP* mutation, sIPF and cNSIP lungs. The degree of inflammatory cell infiltrate and fibrosis were not different between TERT-PF, SFTP-PF and sIPF. Importantly, inflammatory cell abnormalities were almost exclusively located in the fibrotic areas of the lung, with similar levels to what we observed throughout biopsies of cNSIP lungs, while non-fibrotic locations were histologically healthy. Furthermore, survival in patients with FPF and sIPF combined associates with the number of fibroblast foci and percentage of fibrotic lung surface, but not with inflammatory cell infiltration and aggregation.

Previous reports on *SFTP*-related FPF lung biopsies frequently described lymphocyte cell infiltrate and used a classification such as desquamative interstitial pneumonia, organizing pneumonia or cNSIP (12, 18–20). Furthermore, it was reported that in mice induction of *SFTP*- or *TERT*-related dysfunctional alveolar epithelial cells was sufficient to cause inflammatory cell infiltrate and fibrosis (25, 40, 41). Since surfactant mutations have been associated with immunological changes (17, 24) we expected that inflammatory status was higher in SFTP-PF lungs than in sIPF. However, we found no differences in inflammatory cell infiltrate between monogenetic fibrotic lung disease and sIPF, further supporting the histological similarities between pulmonary fibrosis of known and unknown cause.

Interestingly, comparison of the grade of diffuse lymphocyte cell infiltrate showed no difference between SFTP-PF and cNSIP biopsies. However, while lymphocyte aggregates and diffuse lymphocyte cell infiltrate are present throughout the biopsy of cNSIP lung, they were respectively absent and nearly absent (approximately 10% of the tissue surface) in non-fibrotic areas of SFTP-PF, TERT-PF and sIPF lungs. Previously it was shown that mice with a heterozygous knockin of the known human Sftpc I73T mutation, developed early inflammation and no fibrosis, while in homozygous mice development of fibrosis occurred (25). This study suggested that in mice an early inflammatory phase precedes the development of fibrosis, and that disease evolution is influenced by the number of *SFTP* mutant alleles. In human disease it is unclear whether inflammation is a cause or a consequence of the fibrosis. We demonstrated that an inflammatory component was absent in the non-fibrotic areas, suggesting that inflammation does not precede fibrogenesis and may even be reactive to fibrogenesis. Furthermore, in contrast to cNSIP lungs (42, 43) we found no association between lymphocytes and survival in FPF lungs, further supporting that the degree of lymphocyte aberrations in FPF lungs may not contribute significantly to disease pathogenesis (26, 44). In contrast, it has been reported that elevated numbers of lymphocytes are associated with less progressive fibrosis in patients with sIPF (45, 46) and cause resistance to bleomycin in mouse models of pulmonary fibrosis (47).

The role of different lymphocyte subtypes in IPF lungs is currently open to discussion. Previously it has been shown that T-lymphocytes were diffusely present and lymphocyte aggregates were organised in tertiary lymphoid structures with a core of B-lymphocytes surrounded with T-lymphocytes (48, 49). Furthermore, even though we showed that the amount of diffuse lymphocyte infiltrate in cNSIP was significant higher than in IPF, it was previously demonstrated that the amount of different lymphocyte subtypes in IPF lungs did not significantly differ from NSIP lungs (50). However, this does not indicate that subtyping is unimportant. It has been reported that lymphocyte aggregates in IPF consist of active non-proliferating CD40L-positive lymphocytes and mature dendritic cells (49). The presence of these particular cells in IPF lungs might explain the ineffectiveness of anti-inflammatory drugs, because they are less sensitive to these agents (51). Therefore, other drugs targeting the CD40L pathway could potentially be an anti-inflammatory target in IPF. However, although CD40L immunotherapy is used in cancer, future studies should focus on the effectiveness in

IPF. Continued efforts to understand the precise role of inflammation and possible anti-inflammatory treatment remain warranted in fibrotic lungs.

Importantly, the degree of fibrosis in FPF biopsies was significantly higher than cNSIP but not than sIPF. Combining PF data we found that the number of fibroblast foci and the percentage of fibrotic lung surface in PF lung were significantly associated with survival. This corresponds to previous findings in sIPF (26, 27, 36, 46) and underlines the critical role of fibrotic remodelling in the outcome of patients with FPF.

Little is known about drug effect in FPF. We recently showed in a review that in patients and cell or mouse models with a surfactant-related mutation outcome of drugs was highly variable and most likely mutation specific (52). Furthermore, immunosuppressive therapies require careful consideration because of the harm observed in patients with sIPF (29, 30), further emphasizing the non-causative role of inflammatory cell infiltrate in these fibrotic lungs. We showed that the absence of lymphocytic abnormalities in non-fibrotic areas of FPF lung, the similarities with sIPF and the association between increased fibrosis and decreased survival support fibrogenesis and not inflammation as the primary target for therapy. Since familial disease resembles sIPF, the first choices of drugs are anti-fibrotics pirfenidone and nintedanib (31, 32). However, antifibrotics in SFTP-PF patients were not studied yet and a previous small retrospective study including 33 TERT-PF patients treated with pirfenidone demonstrated no beneficial effect post treatment initiation (53). These data emphasize the need for prospective FPF gene or mutation-specific therapies and registration of treatment effect of current antifibrotic drugs. However, a recent retrospective European studies on the effect of antifibrotics in 89 IPF patients carrying a telomere related mutations showed that pirfenidone and nintedanib were safe an reduce decline of forced vital capacity (54).

Strengths of this study comprise the detailed analyses of inflammatory cell infiltrate and fibrosis, consisting of the number of lymphocyte aggregates, extent of diffusely-orientated lymphocytes, number of fibroblast foci and percentage of fibrotic lung surface in well characterized groups of pulmonary fibrosis. Furthermore, this is the first study to show the association between survival and the degree of fibrosis as well as number of fibroblast foci in FPF. However, two limitations are worth noting. Lymphocyte aggregates were assessed in diagnostic biopsies. However, we did not routinely differentiate between different types of lymphocytes in these aggregates and we cannot exclude the possibility that we might have accidently included some monocytes within the infiltrate. Second, while for TERT-PF and sIPF patients tissue was sufficiently available, lung biopsies of adult SFTP-PF patients are extremely rare and only 7 lungs were included.

The international guidelines state that generally FPF and sIPF are histologically and clinically indistinguishable (1). However, to date no study compared histologic inflammatory and fibrotic features of FPF patients with specifically a *TERT* or *SFTP* gene mutation and sIPF. This is the first study investigating in detail the degree of inflammatory cell infiltrate and fibrosis in diagnostic biopsies of TERT-PF, SFTP-PF, sIPF and cNSIP. We found no differences between FPF and sIPF, further supporting the histological similarities between monogenetic familial pulmonary fibrosis and sIPF. Furthermore, this study showed that FPF and sIPF patient survival

depends on the number of fibroblast foci and percentage of fibrotic lung surface, but no clinically relevant correlation with inflammatory cell infiltrate was found. This corresponds with the general failure of trials with anti-inflammatory drugs and the more promising results of therapies targeting fibrosis.

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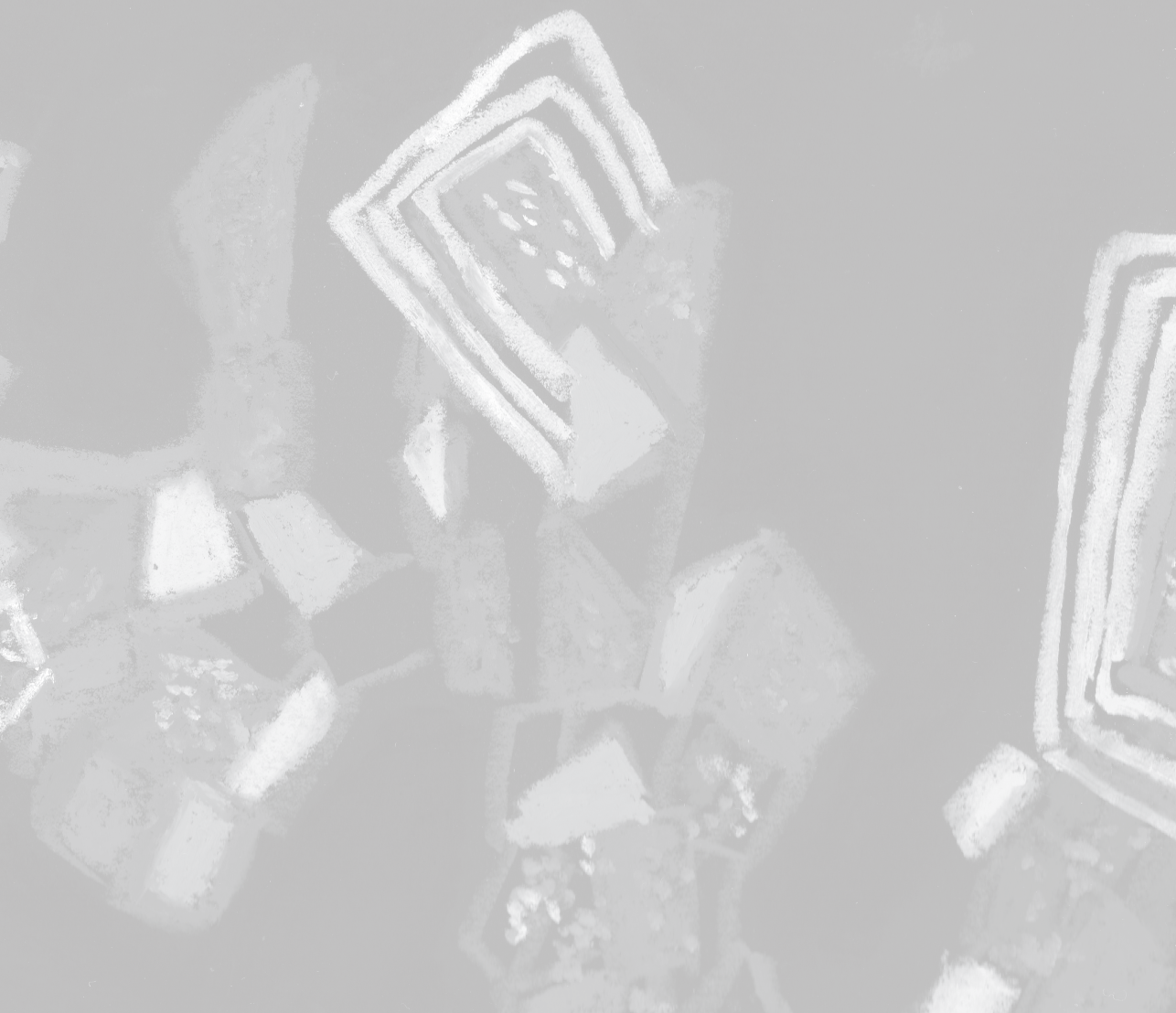
Chapter 7

# Summary and general discussion



## Summary of key findings

This summarizing discussion further addresses the question how telomere shortening is associated with IPF and other PF-ILDs at multiple levels, and the consequences of these findings for our understanding of pulmonary complications in short telomere syndromes. We have shown that at the organ level, average telomere length was most strongly declined in the lungs of patients with pulmonary fibrosis, compared to other organs. Within the lung, average telomere length did not differ between upper and lower lobes, or between diagnostic biopsies and explant lungs over time. At the cellular level, however, AT2 cells had significantly shorter telomeres than surrounding cells, such as club cells and myofibroblasts. Furthermore, AT2 cells in fibrotic areas had significantly shorter telomeres than AT2 cells in non-fibrotic areas. Next, at the patient level, approximately half of IPF patients showed excessive lung telomere shortening similar to that in pulmonary fibrosis driven by known telomerase mutations. In this "IPF short group" we found three cases with a genetic mutation in a telomere-related gene, which explains the disease. In these cases the "I" was removed from IPF.

In addition to the above findings regarding short telomeres, we also found elevated levels of DNA damage in AT2 cells of IPF and TERT-PF lungs. Since critically short telomeres are recognized as DNA double-strand breaks and initiate pro-fibrotic DNA damage responses, the AT2 cell-specific DNA damage signals further substantiates the importance of this cell type in IPF/TERT-PF lungs. Interestingly, we did not find excessively short telomeres in fHP, another PF-ILD. Remarkably, however, also in these cases DNA damage levels in club cells were extremely high, corresponding to the bronchiolocentric localization of this disease. Lastly, in histological slides of diagnostic lung biopsies we found no significant difference in fibrotic and inflammatory features between sporadic IPF and familial IPF due to a *SFTP*- or *TERT* mutations. It was the degree of fibrosis, rather than inflammation, which correlated with survival, supporting the view that fibrogenesis is the most essential mechanism to be targeted in FPF. The following paragraphs further discuss our results in the context of the available literature, leading to the concept that malfunction of AT2 cells due to telomere shortening is the main driver of disease in PF-ILD. Furthermore, we postulate that also in 50 percent of IPF cases telomere shortening is the initial defect, causing maladaptive wound healing and repair response to injury with extensive deposition of extracellular matrix (ECM) proteins and fibrosis.

## General discussion

### Telomere length at the organ level

In general, germline mutations in telomere-related genes cause telomere shortening in all cells of an individual. Several studies have, however, demonstrated that differences in telomere length exist between various organs. A telomere study in macaques demonstrated

that there is age-dependent telomere attrition in some organs, such as lungs, more than others (1–5). This is in agreement with a recent study demonstrating that in 556 human lung tissue-samples telomere length was among the shortest compared to other organs (6). However, it is unknown if all organs in patients with pulmonary fibrosis contain short telomeres. We showed that, among samples from five organs, in one TERT-PF and two sIPF subjects the lungs had the shortest telomeres while in two control subjects telomeres were not shorter in the lung samples than in those from other organs. Moreover, in the TERT-PF case, telomeres in the lung were shorter than in other organs, consistent with the fact that telomere-related mutations lead to accelerated fibrosis. This may be indicative of accelerated aging of fibrotic lungs and may be the reason why TERT-PF only suffer from a diseased lung. It might therefore also be interesting to know whether in other telomere-related disease telomere shortening occurs in the specific organ affected, in order to investigate the role of organ-specific telomere. However, we should note that, if the HRCT is conclusive for IPF, it would not be justified to perform a lung biopsy in view of the negative balance of benefit and risk of this invasive diagnostic test (7).

**Telomere length within the fibrotic lung**

Based on the hypothesis that telomere shortening in lungs might be causally related to progressive fibrosis, we further investigated the spatial connection between lung telomere length and fibrosis in IPF lungs.

During healthy ageing, telomeres shorten over time (8, 9). By comparing diagnostic biopsy samples with samples from explant lungs, we were able to follow the evolution of telomere length during the fibrosis. To our surprise, no difference in the average telomere length of the total biopsy was observed over the course of the disease. Furthermore, when we assessed telomere length in IPF explant lungs with and without a fibrotic apicobasal gradient, no difference was found between apical and basal lung telomere length, irrespective of the presence of the gradient. This suggests that genetic and/or environmental factors might already have led to critically short telomeres prior to the time of diagnosis. It is possible that there is a long-term preclinical phase of disease in which the observed shortening occurs. Since there appear to be no differences in average lung telomere length in IPF, in time or location, it can be hypothesized that the duration of the presence of short telomeres determines the amount of connective tissue accumulation. Short telomeres can induce cellular senescence, which through the senescence-associated secretory phenotype (SASP) leads to fibrosis (10–12). Therefore, as long as cellular senescence is not resolved, a pro-fibrotic microenvironment may lead to progressive fibrosis in the lungs over time. To study when telomeres reach their critical length, it may be worthwhile to assess preclinical telomere shortening during the lifetime of subjects who can be expected to develop progressive fibrosing lung disease, for example in families presenting with familial pulmonary fibrosis.

Another explanation for the observed lack of differences in telomere length might be that chromosomal instability and cellular senescence can be induced by a single critically short telomere on only one chromosome (13, 14). Because tissue sections not only contain

complete, but also partially included nuclei, the FISH procedure we used could only measure total telomere signal within a cell and could not detect shortening of telomeres of individual chromosomes. In metaphase spreads of cells cultured in vitro, chromosome-specific telomere alterations could be detected (15). In this way, it was shown that in CD34-positive cells derived from bone marrow of healthy humans telomeres of the 17p arm were the shortest (16). However, the authors did not take into account that telomere length correlates with the size of the chromosome arm (17). Therefore, the length of a telomere should be adjusted for the size of the chromosome arm. Since every individual chromosome have different size q- and p-arms, it might be possible that the adjusted telomere length does not differ between chromosomes. Future studies should focus on the use of this approach to study patients and their as yet healthy relatives, especially those with the familial forms of pulmonary fibrosis with or without a proven telomere-related mutation.

**Telomere shortening in AT2 cells**

The pivotal role of AT2 cells in IPF pathogenesis was first identified by the discovery of disease causing mutations in *SFTPC*. Only AT2 cells produce Surfactant Protein C (18–20) and the mutations cause intracellular toxicity. Hence, aberrations in this cell type may in some cases be sufficient to cause fibrogenesis. Since this observation, several studies in human tissues and mice have focussed on the role of AT2 cell in fibrosis.

In general, mouse studies are widely used in the development of new therapeutic strategies to treat IPF and to yield new insights into the pathogenesis of the disease (21). Gene knockout studies in mice have contributed significantly to the understanding of disease pathogenesis. For example, in homozygous *TERT* knockout mice treated with bleomycin as well as in mice with AT2 cell-specific knockout of telomere repeat binding factor 1 (TRF1) (leading to dysfunctional telomeres due to uncapping, independent of shortening) it was demonstrated that for development of pulmonary fibrosis excessive telomere shortening or severe telomere dysfunction was critical (22, 23). Furthermore, mice with a dysfunctional telomere maintenance machinery specifically in AT2 cells showed impaired response to injury (22–25) and mice with critically shortened telomeres in AT2 cells had elevated levels of pro-fibrotic TGF- β1 release (23), leading to the lung fibrosis (26). However, even though results in rodents have sometimes been promising, efforts to translate these results to humans have generally not been very successful. Therefore new models are needed (27).

In general, critically short telomeres eventually lead to cell senescence or apoptosis, limiting the regenerative capacity of tissue (28, 29). We showed that telomeres in AT2 cells of IPF and TERT-PF lungs were significantly shorter than those in AT2 cells of controls, confirming the results of Alder and coworkers (25). We also found that AT2 cell telomeres in fibrotic areas were significantly shorter than in non-fibrotic areas, while this difference was not observed in the surrounding cells. Importantly, assessment of control lung tissue found no difference between AT2 and surrounding cells. The association of telomere shortening in AT2 cells specifically with the fibrotic lesions of TERT-PF lungs underscores the relevance of findings in *TRF-1* knockout mouse models for the human situation and suggests that defective telomere

length maintenance in stem cells of alveoli is critically involved in the pathogenesis of TERT-PF. Of note, IPF lungs generally display an increase in apoptosis, and senescence signalling has been reported in fibrotic areas, which further emphasizes that the critical link between telomere shortening or dysfunction and IPF onset (30–33) deserves further exploration.

**Telomere shortening in sporadic IPF**

After we showed that telomeres in AT2 cells of both TERT-PF and sIPF lungs were significantly shorter than in surrounding cells such as Club cells and Myofibroblasts, we wondered if the short telomeres could be caused by genetic variants in telomere-related genes. In our sIPF cohort, average lung telomere length was highly variable and we decided to divide patients in two groups: one group with sIPF patients with short lung telomere length in the range of that found in TERT-PF, and the other group with lung telomere length above that of TERT-PF. The sIPF group with TERT-like telomere length had very short AT2 cell telomere length, particularly in fibrotic lesions, similar to what was found in the lungs of TERT-PF patients. The IPF group with normal telomere length did not show particular short telomeres in AT2 cells of fibrotic lesions. These findings convinced us that sIPF patients with TERT-like short lung telomeres might have a telomere related pathogenesis which may be caused by a genetic mutation in a telomere-related gene.

Whole exome sequencing yielded three patients with telomere related gene mutations in the sIPF group with short lung telomere length, providing evidence for a telomere related cause of their disease. One patient carried a *RTEL1* c.3028C>T sequence variant that was previously associated with IPF, dyskeratosis congenita and Hoyeraal-Hreidarsson Syndrome (34, 35). Another patient carried the *RTEL* c.2258G>A variant, which resulted in an amino acid substitution in the same codon as a recently described variant (*RTEL1* c.2257C>T) in a patient with pulmonary fibrosis (35). Moreover, *in silico* prediction tools CADD scores, SIFT and Polyphen-2, and the low frequency of the variants in the ExAc population database supported the pathogenicity. The relatively high percentage of patients with telomere related gene variants, 9.4%, in our cohort was comparable to that observed in another sporadic IPF cohort of which 11.3% carried a genetic variant in *TERT*, *RTEL1* or *PARN* (35). Moreover, in the subjects of the sIPF group with TERT-like short telomeres that did not carry a telomere related gene mutation, pathogenesis could still be driven by critically short telomeres in AT2 cells, as the number of genes involved in telomeres maintenance is still growing. Future studies should focus on the discovery of new telomere-related genes and whether variations in these genes are associated with disease. Furthermore, it was shown that critical telomere shortening of a single chromosome is sufficient to induce cellular senescence (13, 14), but current measurements of total telomere length in cells would not be able to identify cells with a single short telomere. Pathogenesis in our group of sIPF patients with normal lung telomere length may therefore still be driven by excessive short telomeres.

**Multiple hit hypothesis**

We did not discover telomere-related mutations in the group with sIPF with normal average lung telomere length and slightly shorter AT2-specific telomere length than controls. This supports the view that IPF is, generally speaking, a heterogeneous disease which can be the result of an interaction between genetic and environmental factors. Disease may be the accumulative result of multiple events in individuals prone to IPF. According to this 'multiple hit' hypothesis, damage for example by cigarette smoke, irradiation, endogenous damage by protein misfolding or endoplasmic reticulum stress, could induce tissue remodelling in genetically susceptible hosts (36). Apart from telomere-related gene mutations, also other genetic predisposing variants, such as common polymorphisms and sex may affect disease development (37). In Trf1 knockout mice it was shown that telomere dysfunction in AT2 cells alone was sufficient for development of spontaneous lung fibrosis (23). However, *TRF1* gene mutations in humans have not yet been discovered. In contrast, mice harbouring a knockout of the *Tert* gene, which is associated with human disease, only developed pulmonary fibrosis upon a second hit, namely exposure to low dose bleomycin (22), which supports the hypothesis that multiple hits are needed. We found no correlation between telomere shortening and DNA damage in AT2 cells of sporadic IPF patients, suggesting that all patients have elevated DNA damage levels irrespective of telomere length. These elevated DNA damage levels may be induced by second hits such as smoking and subsequent oxidative stress as demonstrated in various cancers (38), ultimately leading to AT2 cell dysfunction followed by fibrogenesis. Furthermore, in mice with bleomycin-induced fibrosis elevated levels of DNA damage were found in the lung parenchyma, suggesting that various stressors can induce DNA damage and fibrogenesis and may act as a second hit (39). Therefore, future studies should focus on other causes of DNA damage in sIPF patients with relatively normal telomere length.

**DNA damage in AT2 cells of fibrotic lungs**

Critically short telomeres are recognized by the DNA repair mechanism as DNA double strand breaks. These breaks result in the phosphorylation of H2A histone family member X (γH2AX), initiating the DNA damage response. If breaks are not repaired in time, damage persists and results in cellular senescence (40–43) or apoptosis (44). Moreover, in healthy conditions, the DNA damage repair mechanism is inhibited at telomeres in order to prevent telomere fusions. Therefore, in contrast to DNA damage in other areas of the chromosome, telomeric DNA damage is usually irreparable and persists over time (45). The presence of a high number of DNA double-strand breaks result in degradation of DNA or abnormal DNA repair and eventually leads to cell senescence (42, 43, 46, 47). Indeed, a study by Minagawa *et al*. showed increased levels of senescence in epithelial cells lining fibroblast foci (31). AT2 cells in *Trf1* knockout mice also contained elevated levels of γH2AX DNA damage signal, compared to AT2 cells of wild-type mice (23). We showed that γH2AX signals were significantly elevated in AT2 cells of IPF and TERT-PF groups. However, of these groups, it was only in the lungs of TERT-PF cases as well as in one case with a *PARN* mutation, - in which causative mutations underly telomere shortening and disease - that a correlation existed between telomeres length and

degree of γH2AX-related DNA damage, suggesting that telomere shortening causes DNA damage in these patients. Furthermore, we showed for the first time that experimental inhibition of telomerase resulted in telomere shortening in AT2 cell surrogate cell lines A549 and NCI-H460, suggesting that telomeres in these cells, similar to those in AT2 cells from diagnostic biopsies of pulmonary fibrosis, are most sensitive to telomerase dysfunction. This is in agreement with previous experiments, in which telomere shortening in BIBR1532-treated NCI-H460 cells was observed (48). Moreover, both telomerase-defective in situ and in vitro experiments have found a decrease in telomere length in AT2 cells to be associated with an increase in DNA damage, underlining the telomere-length dependent accumulation of DNA damage in AT2 cells in TERT-PF. These findings show that in pulmonary fibrosis, anomalous telomere shortening and elevated DNA damage primarily affect AT2 cells, promoting the critical role of these cells in fibrogenesis within the lung. Moreover, based on the results from TERT-PF lungs and cell cultures treated with BIBR1532, we conclude that DNA damage is secondary to telomere shortening in AT2 cells. Previously, it was found in mice that inhibition of telomere-specific DNA damage alone is sufficient to reduce the severity of complications in another premature aging disease called Hutchinson–Gilford progeria syndrome (49), suggesting that DNA damage might be not the initial trigger, but a central downstream player in the pathogenesis of pulmonary fibrosis. Other triggers causing DNA damage in AT2 cells may therefore also be causative in pulmonary fibrosis. That raises the question whether other downstream targets of telomere shortening and DNA damage, such as factors in senescence and apoptosis pathways can also be telomere-specific and could possibly be targeted therapeutically.

**DNA damage in Club cells of fibrotic lungs**

In clinical practice, the differential diagnosis of IPF often includes the possibility of fibrosing hypersensitivity pneumonitis (HP) (50, 51). HP is caused by an ongoing delayed allergic reaction against an extrinsic antigen. One of the distinctive histopathological differences between IPF and fibrosing HP (fHP) is the location of fibrosis in the lung. Whereas in IPF, fibrosis is usually located in the subpleural and basal areas of the lung, fibrosis in fHP is more concentrated around small airways, typically causing mosaic attenuation and air trapping on HRCT. It was previously demonstrated that in 25% of the cases with an initial diagnosis of IPF, bronchiolocentric fibrosis led to a revised diagnosis of fHP (52). However, fHP can also present with UIP, the characteristic pattern on HRCT and in diagnostic biopsies of patients with IPF. This raises the question whether the multiple hit pathophysiology hypothesis in IPF can also play a role in a subgroup of fHP patients.

Interestingly, unlike AT2 cells in sIPF and TERT-PF lungs, Club cells (which are mainly located in terminal bronchioles) in fHP lungs prominently contained highly elevated DNA damage signals, but showed no excessive telomere shortening. If and how the ongoing delayed allergic reaction in HP leads to telomere-unrelated DNA damage in club cells is unclear. A possible explanation could be the inflammation-induced accumulation of reactive oxygen species (ROS), a group of highly reactive, DNA damage-inducing molecules that are also associated

with other allergic diseases, such as asthma (53, 54). Since fHP is characterized by inhaled antigens that, due to their size, are left stranded in the bronchioles (51), it is possible that the accumulation of DNA damage in club cells of fHP lungs at this location is caused by ROS, and not by telomere shortening. This is in line with a previous study, which showed that bronchoalveolar lavage of fHP patients contained significantly higher carbonylated protein levels, a marker of ROS, compared to IPF and controls (55). However, another study showed that in mice with a Club cell-specific knock out of telomere repeat-binding factor-1 (*Trf1*), rapid aging of club cells by telomere dysfunction alone was sufficient to induce DNA damage and subsequent bronchiolocentric fibrosis (56). The excess DNA damage in fHP Club cells might therefore, regardless of the cause, be suggestive of an important role of these cells in fibrogenesis, and is in agreement with the localization of fibrosis in fHP. To further elucidate the link between Club cells and the pathogenesis of fHP, future studies should focus on cellular aging due to a sustained allergic reaction and DNA damage in these cells.

**Proposed pathophysiological concept based on telomere shortening and DNA damage**

Since the fibrosis in lungs of patients with IPF is completely replacing the healthy air-containing alveoli, we hypothesize that it is the injury to AT2 cells that ultimately lead to destruction of alveoli. It has been conceptualized that excessive repetitive injury might lead to telomere shortening and DNA damage, which in turn induce senescence and apoptosis of AT2 cells and eventually cause failure of normal regenerative capacity of AT2 cells and triggering pulmonary fibrogenesis (57). AT2 cells produce and regulate surfactant in the alveoli and are the progenitor cells of the gas diffusing AT1 cells (58). This regenerative function of AT2 cells requires active telomere maintenance by the telomerase complex (59). Indeed, telomerase has been shown to be active and upregulated in subpopulations of rat AT2 cells after hypoxic injury (59–61). In normal circumstances, epithelial cell damage is dealt with an effective wound healing response, consisting of (temporary) fibroblast recruitment and conservation of basement membrane integrity (62, 63). Once the epithelial barrier is restored through AT2 cell proliferation and differentiation, fibroblasts disappear and alveolar functions are restored. However, in the case of sustained AT2 injury, fibroblasts persist and differentiate into myofibroblasts. The characteristic fibroblast foci close to areas with AT2 cell hyperplasia (64) in IPF lungs (65, 66) consist of both types of fibroblasts, actively regulating the deposit of extracellular matrix. Therefore, the fibrotic lesions in IPF are thought to be produced by these cells. Meanwhile, the destruction of alveolar cells and stiffness of subsequent fibrosis causes mechanical stress in the yet compliant neighbouring alveoli, leading to the collapse of these alveoli and expansion of the fibrosis (67).

It was previously demonstrated in pulmonary fibrosis that, in the case of AT2 cell failure in alveolar wound healing, also bronchiolar epithelial cells such as Club cells have the possibility to grow into areas where in normal circumstances AT2 cells are expected in a process called bronchiolization (68, 69). Furthermore, it has been reported in a bleomycin mouse model that a selection of cells located in the bronchoalveolar duct junction expressed markers of both AT2 and Club cells (70), suggesting that during fibrogenesis, Club cells might differentiate into

AT2 cells to take over their cellular functions (71, 72). However, the progressive nature of lung fibrosis in IPF suggest that these last resort mechanisms are insufficient to stop disease progression, let alone heal lesions. We hypothesize that damage to alveolar cells and fibrogenesis extends over time to adjacent endothelial cells at the other side of the alveolar-capillary basement membrane. In normal circumstances, if the damage is restricted to epithelial and endothelial cells but the basement membrane is still intact, repair of lung tissue by recellularization of epithelial and endothelial cells might still possible. However, even in the hypothetical instance that the cause of pulmonary fibrosis can be removed, loss of the basement membrane dictates the collapse of alveoli and the progression of fibrosis in the lung and therefore is considered the point of no return in tissue repair (73).

**Other causes of telomere shortening**

Telomere shortening is caused by several other factors than telomere-related gene mutations, which therefore potentially can influence fibrogenesis in the lung. First, in healthy aging, telomeres shorten due to incomplete cell-cycle dependent duplication (44, 74, 75). This way, leukocyte telomeres shorten 20-30 base pairs per year in normal circumstances (76) and elevated proliferative activity causes telomeres to shorten more quickly. Another important factor in telomere attrition is stress induced by life style factors, such as diet and (the lack of) physical activity, causing the production of oxidative stressors. In contrast to non-telomeric DNA, telomeres are more prone to oxidative stress induction because oxaditive factors react predominantly with triple guanine repeats, which are abundantly present in telomeric DNA. These reactions generate oxidative modified bases, possible subsequent base excision repair and eventually telomere shortening (77).

**Inflammatory and fibrotic tissue remodelling in pulmonary fibrosis**

To further study the effect of telomere shortening on lung histology, we compared sporadic IPF with monogenetic SFTP- and TERT-related disease. Previous reports based on SFTP-related FPF lung biopsies frequently described lymphocyte cell infiltrate and used classifications such as desquamative interstitial pneumonia, organizing pneumonia or cNSIP (78–81). Furthermore, it was reported that induction of SFTP- or TERT-related dysfunctional alveolar epithelial cells in mice was sufficient to cause inflammatory cell infiltrate and fibrosis (22, 23, 82). Since surfactant mutations have been associated with immunological changes (83, 84) we expected that the inflammatory status would be higher in SFTP-PF lungs than in sIPF. However, results showed no differences in inflammatory cell infiltrate between the main types of monogenetic lung fibrosis and sIPF, supporting the concept of histological similarity between pulmonary fibrosis of known and unknown cause.

Interestingly, a comparison of the levels of diffuse lymphocyte cell infiltrate showed no difference between SFTP-PF and cNSIP biopsies. However, whereas lymphocyte aggregates and diffuse lymphocyte cell infiltrate are present throughout the biopsy of cNSIP lungs, they were respectively absent and nearly absent (approximately 10% of the tissue surface) in non-fibrotic areas of SFTP-PF, TERT-PF and sIPF lungs. It was previously shown that mice with a

heterozygous knockin of the known human Sftpc I73T mutation developed early inflammation and no fibrosis, while in homozygous mice developed fibrosis (82). This study suggested that in mice, an early inflammatory phase precedes the development of fibrosis, and that disease evolution is influenced by the number of SFTP mutant alleles. In human disease, it is unclear whether inflammation is a cause or a consequence of the fibrosis. We demonstrated that an inflammatory component was absent from the non-fibrotic areas, suggesting that inflammation does not precede fibrogenesis and may even be reactive to fibrogenesis. Furthermore, in contrast to cNSIP lungs (85, 86), we found no association between lymphocytes and survival in FPF lungs, further supporting the view that the degree of lymphocyte aberrations in FPF lungs may not contribute significantly to disease pathogenesis (64, 87). In fact, it has been reported that elevated numbers of lymphocytes are associated with less progressive fibrosis in patients with sIPF (88, 89) and cause resistance to bleomycin in mouse models of pulmonary fibrosis (90).

Importantly, the degree of fibrosis in FPF biopsies was significantly higher than in cNSIP, but not higher than in sIPF. Combining PF data we found that the number of fibroblast foci and the percentage of fibrotic lung surface in PF lungs were significantly associated with survival. This corresponds to previous findings in sIPF (87, 89, 91, 92) and underlines the critical role of fibrotic remodelling in the outcome of patients with FPF. Whether inflammatory cell infiltrate is a driver (or co-driver) of disease or whether it is merely an epiphenomenon in IPF needs to be further investigated, for example by comparing biopsies with fHP which is characterized by an allergic inflammatory response. Emerging evidence suggests that the etiology of acute exacerbations of IPF is different from that of less progressive IPF and that this could therefore be an interesting subpopulation of IPF to study inflammatory cell infiltrate in fibrotic lung disease (93–95).

**Future prospects**

The progress of our understanding of IPF has greatly accelerated in recent years, in part due to the incorporation of concepts of aging biology in disease pathogenesis. Critical gaps in our understanding of the pathogenesis include how the pathophysiological role of telomere shortening leads to fibrosis, and undiscovered potential genetic variants underlying this disease. Further insights into these mechanisms might lead to the development of novel therapeutics for IPF and other progressive fibrosing ILDs.

**Therapies targeting telomerase and short telomeres**

One of the potential targets in the development of drugs for pulmonary fibrosis is telomere shortening. Currently, a phase 2 trial is underway with the synthetic androgen danazol, which has demonstrated elongation of telomeres in patients with telomere diseases (96). Androgens are able to restore telomerase activity in subjects with TERT mutations through sex steroid-dependent responsiveness of the TERT promotor (97). Furthermore, a recently started phase 2 clinical trial aims to investigate the inhibition of telomere shortening with danazol in adults and children with pulmonary fibrosis (98).

It has been demonstrated that adeno-associated vector therapy with telomerase in mice with low-dose bleomycin-induced short telomeres results in significantly elongated telomeres, less DNA damage, less senescence and subsequent restoration of the regenerative capacity of AT2 and Club cells. In addition, improvement of lung function and a disappearance of collagen deposition was demonstrated in these mice after 1-3 weeks of therapy (99, 100).
Besides therapeutically targeting telomere shortening itself, it is also possible to inhibit its direct effector, the telomeric DNA damage response. A study using a mouse model of Hutchinson–Gilford progeria syndrome, which is a genetic disorder characterized by premature aging features, demonstrated that specific inhibition of the telomeric DNA damage response prevents cellular senescence and significantly enhances skin homeostasis and lifespan (49, 101). Future investigations should help determining whether these approaches are effective and tolerable in human fibrotic disease.

**Cell therapy**

In the last few years, replacement of pulmonary stem cells has emerged as a possible therapeutic option for IPF (102). Since defective AT2 cells are considered the most important cell type in IPF development, and since AT2 cells exhibit stem cell characteristics, possible therapies targeting these cells were explored. In two animal models with bleomycin-induced lung fibrosis, insertion of AT2 cells or a pooled mix of lung epithelial cells in the lungs inhibited fibrosis (103–105). A clinical trial including human subjects with mild to moderate fibrosing disease, demonstrated that donor-derived AT2 cell therapy was well tolerated with no significant adverse reactions, stable lung function outcomes and no change on HRCT scans, while the quality of life and 6 minute walking distance were improved (106). Despite these small positive effects, AT2 cell therapy needs further validation.

**Final remarks**

This thesis has explored the extent of telomere shortening in lungs of patients with IPF, and compared data of patients with and controls. From the work presented here and literature reviewed, it becomes clear that telomere shortening in AT2 cells is not only a fundamental problem in lung of patients carrying telomerase-related gene mutations but also in many sporadic IPF. It is possible that in the latter group telomere-related mutations underlying the disease yet have to be discovered. Given the data described in this thesis, the following priorities for future research are given. First, since no difference in telomere shortening during different stages of disease was found, the role of telomere shortening before the clinical diagnosis needs to be studied. Second, a single critical short telomere has been shown sufficient to induce cellular dysfunction. Whether this is the case in IPF remains unclear. Finally, as we found highly elevated DNA damage levels in Club cell in fHP lungs, the question raises whether this pathologic finding is critical for the pathophysiology of this type of PF-ILD. Although the pathogenesis of progressive pulmonary fibrosis remains complex, the findings and interpretations made in this thesis highlight the important role of telomeres in this devastating disease.

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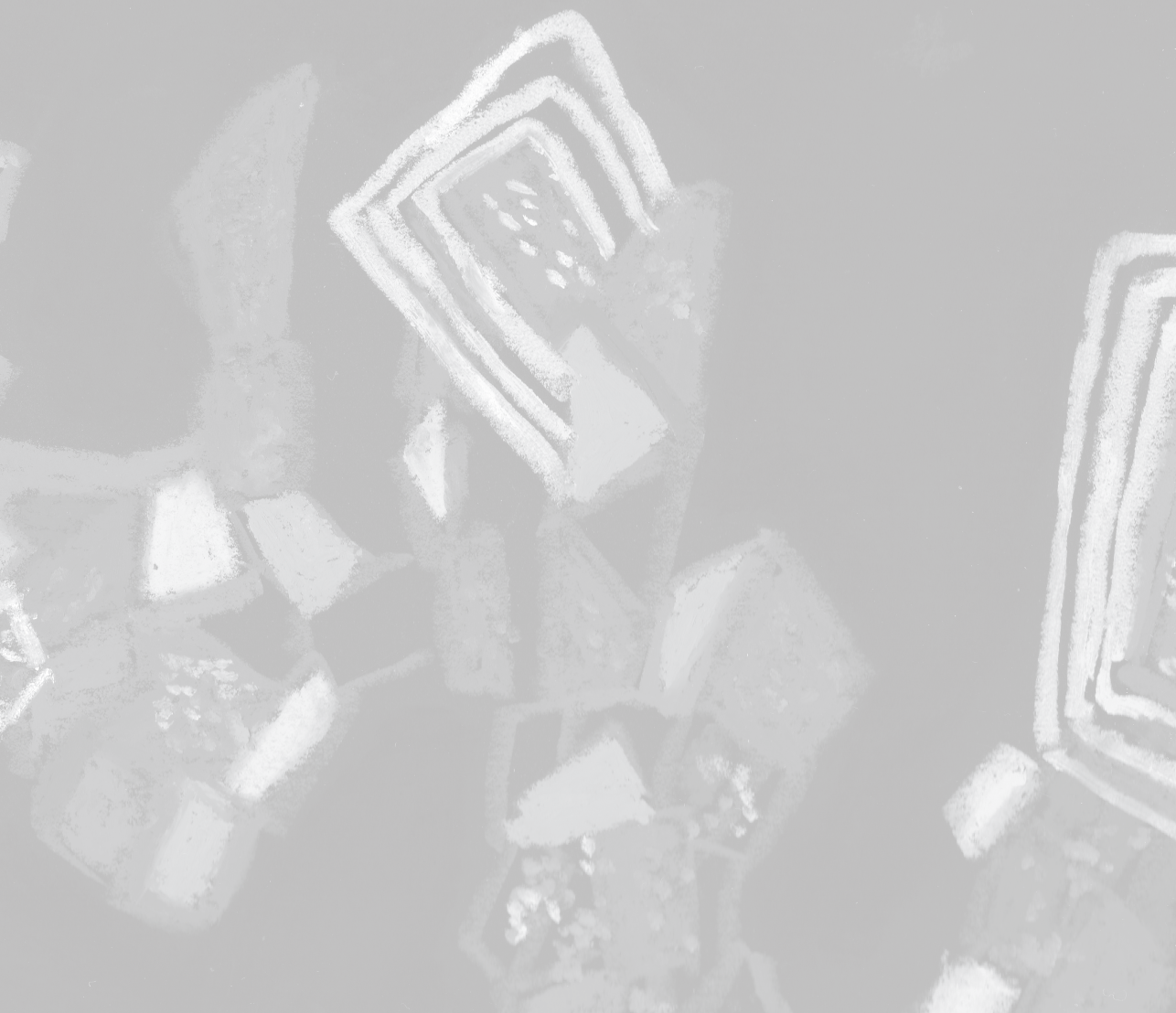
Appendix: Nederlandse samenvatting

# Telomeerverkorting en fibrotische verandering in progressief fibrotische interstitiële longaandoeningen



## Nederlandse samenvatting

**Hoofdstuk 1: Algemene inleiding en opzet van het proefschrift**

Interstitiële longziekten (ILD) zijn een groep van zeldzame ziekten die gekenmerkt worden door schade in het interstitium van het long weefsel. De meest ernstige vorm van ILD is progressief fibroserende ILD (pf-ILD), waarin het interstitium ernstig is verlittekend en dus gasuitwisseling tussen de lucht en het bloed onmogelijk wordt gemaakt. PF-ILD kan zich presenteren in een sporadische of een familiaire vorm. In de meeste patiënten met pf-ILD is de oorzaak onbekend. Het archetype van deze groep is idiopathische pulmonale fibrose (IPF), een ziekte met een zeer slechte prognose van gemiddeld 2 tot 5 jaar na het stellen van de diagnose. IPF wordt geclassificeerd middels radiologische en/of histologische kenmerken van het zogeheten 'usual interstitial pneumonia' (UIP) patroon met heterogene fibrose dat voornamelijk subpleuraal in de basale longvelden voorkomt. Hoewel de oorzaak van IPF onbekend is, worden er in de literatuur een combinatie van meerdere factoren geassocieerd met het ontstaan van de ziekte. Hierbij kan een combinatie van eventueel reeds aanwezige genetische vatbaarheden of omgevingsfactoren opgevolgd worden door een secundaire stressor zoals sigarettenrook. Dit kan leiden tot een ongecontroleerde respons en daaropvolgende fibrose. De vraag welke factoren een rol spelen in de ontwikkeling van IPF blijft echter onbeantwoord.

De behandeling van IPF bestaat uit de anti-fibrotische medicijnen Pirfenidon en Nintedanib. Deze therapie is niet genezend, maar heeft aangetoond dat de geforceerde vitale capaciteit (FVC) minder snel daalt. Echter, verbeteringen in FVC zijn bijna nooit gerapporteerd en de vele bijwerkingen zorgen voor een slechte verdraagzaamheid. Het laatste therapeutische redmiddel voor mensen met progressieve longfibrose is een longtransplantatie, maar is alleen haalbaar in een selecte groep patiënten. Mede daardoor blijft de noodzaak van een curatief medicijn bestaan.

In tegenstelling tot IPF, is de oorzaak van andere pf-ILD, zoals hypersensitiviteits pneumonitis (HP), wel bekend. HP is in de meeste gevallen een gevolg van een allergische reactie tegen extrinsieke factoren zoals bacteriën, schimmels of eiwitten van vogels. Bij langdurige blootstelling kan de schade die hierbij ontstaat resulteren in progressieve fibrose (fibrotische HP of fHP). Patiënten met fHP vertonen verschillende fibrotische patronen in de long, waaronder UIP, wat de differentiatie tussen fHP en IPF bemoeilijkt. HP wordt oorspronkelijk behandeld met immunosuppressiva maar recentelijk is aangetoond dat ook patiënten met progressief fibroserende HP baat kunnen hebben bij anti-fibrotische therapie. Hierom is het heel erg belangrijk om verschillende entiteiten op een vroegst mogelijk moment te herkennen.

In 0.5 tot 20 procent van de IPF en fHP gevallen betreft het een familiaire vorm van pulmonale fibrose (familiaire pulmonale fibrose of FPF), waarin minimaal 1 eerstegraads familielid ook longfibrose heeft. Genetische testen hebben uitgewezen dat in bijna de helft van alle familiaire longfibrose er een bekende mutatie ten grondslag ligt aan de ziekte, voornamelijk in genen die coderen voor surfactant- en telomeer-gerelateerde eiwitten. Surfactant

(aanwezig als een dunne laag vloeistof aan de binnenkant van de longblaasjes) houdt de oppervlaktespanning van de longblaasjes in stand, zodat deze goed open blijven gedurende de ademhaling, en speelt tevens een rol in het immuun systeem. Telomeer-gerelateerde genen zijn betrokken bij het functioneel opereren van het telomerase eiwit-RNA complex. De belangrijkste functie van dit complex is het verlengen van de uiteinden van de chromosomen, de telomeren, in geslachts- en stamcellen.

Telomeren zijn repetitieve TTAGGG DNA sequenties die tezamen met de eiwitten telomere repeat factor 1 en 2 (TRF-1 en TRF-2), als buffer fungeren bij celcyclusafhankelijke verkorting van DNA. Daarnaast verzorgen zij de stabiliteit van de chromosoom structuur. Hierdoor wordt verlies van genetische informatie voorkomen. Door specifieke mutaties in de genen van het telomerase complex (het merendeel aanwezig in het telomerase reverse transcriptase (*TERT*) gen) verkorten telomeren sneller, wat gepaard kan gaan met een functioneel ontregelde cel. Als telomeren namelijk een kritiek korte lengte hebben, veranderen ze van structuur waardoor ze, door de DNA schade-machinerie, herkend worden als een dubbel-strengs DNA breuk. Als deze DNA breuk niet op de juiste manier gerepareerd kan worden zal de DNA schade respons persisterend aanwezig blijven waardoor de cel zal stoppen met delen en in een cellulair senescente staat terecht komt dan wel dood gaat door middel van apoptose. Beide processen zijn in de literatuur geassocieerd met IPF longen en fibrotische muis modellen. Indien een cel in senescence is, dan is de cel stabiel aanwezig maar werkt verder niet meer, met als doel het weefsel te beschermen tegen de vermeerdering van cellen met veel (DNA) schade. De aanwezigheid van teveel senescente cellen dan wel de afwezigheid van cellen die apoptose hebben ondergaan zal echter de functionaliteit van de long ook niet ten goede komen. Daarom is het interessant om te onderzoeken in hoeverre longcellen zijn aangedaan door telomeerverkorting en DNA schade.

Er zijn verschillende type cellen in de long die mogelijk een rol spelen in de ontwikkeling van pf-ILD. Ten eerste de alveolaire type 2 (AT2) cellen, die een essentiële rol spelen in het onderhoud en de regeneratieve processen in de longblaasjes. AT2 cellen produceren surfactant eiwitten, hebben een zeer lange zelf-vernieuwingstijd en kunnen daarnaast differentiëren in alveolaire type 1 (AT1) cellen, de cellen die gas uitwisselen tussen de lucht en het bloed. Aangezien AT2 cellen deze belangrijke functies vervullen in de distale luchtwegen, worden ze beschouwd als de belangrijkste cellen die kunnen worden aangetast door chronische (repetitieve) schade. Er zijn tevens studies gepubliceerd die aantonen dat mutaties in het surfactant eiwit C gen zijn geassocieerd met de ziekte. Aangezien dit eiwit uitsluitend tot expressie komt in AT2 cellen, is dit overtuigend bewijs dat AT2 cellen een pathogene rol spelen in de ontwikkeling van longfibrose. Senescence en apoptose signalen worden dan ook het meest vastgesteld in AT2 cellen van IPF longen en in fibrotische muis modellen. Ten tweede zijn club cellen geïmpliceerd in de ontwikkeling van longfibrose. Net als AT2 cellen in de longblaasjes, zijn club cellen de voorlopercellen die verantwoordelijk zijn voor het onderhoud en de vernieuwing van bronchiolair epitheel. Er is ook gesuggereerd dat ze bij kunnen dragen aan alveolaire epitheelvernieuwing door vervanging van AT2 cellen. Recente studies hebben aangetoond dat cellen die positief zijn voor zowel de specifieke AT2 cel marker

SFTPC als de club cel marker CC10 het potentieel hebben om bij te dragen aan herstel van de luchtwegen dan wel de longblaasjes. De lokalisatie van deze voorlopercellen in de bronchioalveolaire duct junction (BADJ), een tussenliggende zone die de bronchioli en de longblaasjes verbindt, lijkt hierbij een rol te spelen. In het geval van de ontwikkeling van longfibrose, zijn club cellen bovendien betrokken bij bronchiolisatie, een proces waarbij epitheelcellen uit de bronchioli migreren van de BADJ naar de kapotte longblaasjes om zich daar te vestigen als noodverband. Het is echter niet bekend in hoeverre telomeer verkorting plaatsvindt in club cellen van patiënten met longfibrose. Een derde celtype, de myofibroblast, reguleert de extracellulaire matrix tijdens wondgenezing en is, wanneer de schade aanhoudt, de belangrijkste bron van collageenafzetting, waardoor fibrose ontstaat. Hoewel clusters van myofibroblasten, die fibroblastfoci (FF) vormen, als reactie op fibrose worden beschouwd, zijn ze histologisch kenmerkend voor IPF. Bovendien is de aanwezigheid van FF een marker van de activiteit van het fibrotische proces, aangezien FF in verband zijn gebracht met een slechte prognose van IPF.

**Doel van dit proefschrift**

Bij de meeste ILD-patiënten is het onderliggende pathologische mechanisme nog steeds onduidelijk. Recente bevindingen van studies naar de genetica in familiaire longfibrose kunnen echter helpen om processen te ontrafelen die geassocieerd zijn met ziekteprogressie van fibroserende ILD. Het algemene doel van het onderzoek dat aan dit proefschrift ten grondslag ligt, is het ophelderen van de pathofysiologische rol van telomeerverkorting in progressieve fibroserende ILD. Dit hebben we gedaan door de telomeerlengte in AT2 cellen in histologische biopten van IPF longen in kaart te brengen. Zowel in de ruimte, door verschillende locaties in de long te vergelijken, als in de tijd, door op verschillende momenten tijdens de ziekte telomeerlengte te meten in longweefsel van patienten met IPF en patiënten met telomeer-gerelateerde longfibrose. Daarnaast hebben we een methode ontwikkeld om gelijktijdig telomeerlengte en DNA schade te meten in verschillende celtypes die essentieel zijn voor de pathogenese van longfibrose. Tevens hebben we de distributie van ontstekingscelinfiltraat en fibrose in longweefsel van patiënten met telomeer en niet-telomeer-gerelateerde fibroserende ILD onderzocht.

**Hoofdstuk 2: Korte telomeerlengte in IPF longen associeert met fibrotische gebieden en voorspelt overleving**

In dit hoofdstuk hebben we telomeerlengte vergeleken tussen de typische heterogene verdeling van niet-fibrotische en fibrotische gebieden in longen van patiënten met IPF en patiënten met familiaire longfibrose. Het doel hiervan was het mechanisme te onderzoeken hoe telomeerlengte verband houdt met fibrose in de longen. Hiervoor hebben we gebruik gemaakt van een fluorescentie in situ hybridisatie techniek, waarbij we specifiek in AT2 cellen, maar ook in omliggende cellen, de telomeerlengte hebben bepaald. Daarnaast hebben we de gemiddelde telomeerlengte van alle cellen in de longbiopten bepaald met behulp van een

monochroom multiplex kwantitatieve PCR (MMqPCR). In sporadische IPF patiënten waren de telomeren van AT2 cellen in niet-fibrotische gebieden 56% langer dan in die van AT2 cellen in fibrotische gebieden. Een dergelijk verschil werd niet waargenomen in de omringende longcellen. Bij longfibrose patiënten met een telomerase reverse transcriptase (*TERT*)-mutatie waren de telomeren in AT2 cellen significant korter dan in patiënten met sporadische IPF. Tussen sporadische IPF patiënten en patiënten met een *TERT* mutatie werd echter geen telomeerlengte verschil in de omringende cellen waargenomen. Ten slotte werd met behulp van MMqPCR metingen vastgesteld dat IPF patiënten met de kortste telomeerlengte in de long een significant slechtere overleving hadden dan patiënten met langere telomeren. Deze resultaten laten zien dat korte telomeren een zeer belangrijke invloed heeft op AT2 cellen in fibrotische gebieden, wat impliceert dat telomeerverkorting een oorzaak van het fibrotisch proces is. Bovendien is het hebben van korte telomeren in de longen geassocieerd met een slechtere overleving.

**Hoofdstuk 3: Van orgaan tot cel: Telomeerlengte bepalingen op meerdere niveaus in patiënten met idiopathische pulmonale fibrose**

Een subgroep van patiënten met IPF hebben korte telomeren in bloedcellen en/of hebben telomeer-gerelateerde mutaties. We hebben in hoofdstuk 2 aangetoond dat AT2 cellen korte telomeren hebben in fibrotische gebieden van de long. In hoofdstuk 3 hebben we getracht om te begrijpen hoe telomeerverkorting associeert met fibrose in de IPF long en tevens hebben we ons het doel gesteld om een subgroep van patiënten met telomeer-gerelateerde ziekte te identificeren. De gemiddelde telomeerlengte werd bepaald in meerdere organen, basale en apicale longen, en diagnostische en eindstadium fibrotische longbiopten. Daarnaast hebben we de telomeerlengte in AT2 cellen in verschillende gebieden van de IPF longen gemeten. Uit de resultaten blijkt dat in IPF patiënten, maar niet in controles, de telomeerlengte in de longen korter was dan in andere organen, wat een reden was om te focussen op de telomeerlengte in de longen. De telomeerlengte correleerde niet met de leeftijd in dit IPF cohort en er werd geen verschil in telomeerlengte gevonden in de tijd tussen een diagnostische longbiopt en een long na explantatie. Ook was er geen verschil in telomeerlengte tussen basale en apicale gebieden binnen een long, ongeacht de aanwezigheid van een radiologische apicobasale fibrose gradiënt. Verder hadden 15 van de 28 IPF patiënten longcellen met een gemiddelde telomeerlengte die in het bereik van patiënten met een telomerase (*TERT*)-mutatie lagen, de zogeheten '$IPF_{short}$' groep. Alleen in deze $IPF_{short}$- en TERT-groep waren de telomeren van de AT2 cellen extreem kort in de fibrotische gebieden. Verder werden er twee genetische variaties in *RTEL1* en één in *PARN*, twee andere telomeer-gerelateerde genen, aangetoond mbv whole exome sequencing analyse in 3 patiënten uit de $IPF_{short}$ groep. Hieruit hebben we geconcludeerd dat gemiddelde telomeerverkorting van longweefsel niet geassocieerd is met fibrotische patronen in longen van patiënten met IPF. Echter, ongeveer de helft van de IPF patiënten heeft extreme korte telomeren in de long en is vergelijkbaar met de telomeerlengte-verdeling in niet-fibrotische en fibrotische gebieden van patiënten

longfibrose veroorzaakt door een telomeer-gerelateerde mutatie. Deze resultaten suggereren dat er mogelijk telomeer-gerelateerde mutaties ten grondslag kunnen liggen aan de longfibrose in een deel van de patiënten met sporadische IPF.

**Hoofdstuk 4: Celtype-specifieke kwantificatie van telomeerlengte en DNA dubbelstrengs breuken in individuele longcellen met behulp van fluorescentie in situ hybridisatie en fluorescentie immunohistochemie**

Dit hoofdstuk beschrijft een methode om telomeerlengte en DNA dubbelstrengs breuken in formaline gefixeerd weefsel (semi-)kwantitatief te kunnen meten. Hiervoor hebben we een nieuwe combinatie van kwantitatieve fluorescentie in situ hybridisatie en immunofluorescentie kleuringen ontwikkeld. Caveolin-1 (AT1 cellen), pro-surfactant eiwit C (AT2 cellen), club cel-10 (Club cellen) en alfa-SMA (gladde spiercellen) markers zijn gebruikt om celtypes te identificeren. Om alle verschillende fluorescente signalen te visualiseren, is het essentieel om na de eerste kleuringsronde de fluorescente signalen te verwijderen door middel van warmte gemedieerde elutie om vervolgens het weefsel opnieuw te kleuren. Fluorescerende signalen van telomeren en DNA dubbelstrengs breuken zijn gekwantificeerd met behulp van de Telometer-plug-in van ImageJ. Als voorbeeld analyseerden we longweefsel van een familiaire longfibrosepatiënt met een mutatie in het telomeer-geassocieerde gen Poly(A)-specifieke ribonuclease (*PARN*). Dit protocol biedt een nieuwe mogelijkheid om DNA dubbelstrengs breuken direct kwantitatief te koppelen aan telomeerlengte in specifieke cellen.

**Hoofdstuk 5: Telomeerverkorting en DNA schade in sleutelcellen van verschillende soorten progressief fibroserende interstitiële longziekten**

In dit hoofdstuk laten we zien hoe we de methode van hoofdstuk 4 tot uitvoer hebben gebracht met als doel te onderzoeken in hoeverre AT2 cellen, club cellen en myofibroblasten worden beïnvloed door telomeerverkorting en DNA schade in de pathogenese van pf-ILD. Om de mate van en correlatie tussen telomeerverkorting en DNA schade te bepalen hebben we deze factoren gekwantificeerd in deze verschillende longcellen van controles, TERT-PF, IPF en fHP patiënten. De resultaten lieten zien dat in IPF en TERT-PF longen AT2 cellen significant kortere telomeren en hogere DNA schade signalen hadden dan club cellen en myofibroblasten. In fHP longen hadden club cellen sterk verhoogde niveaus van DNA schade terwijl telomeren niet duidelijk kort waren. Daarnaast is er in vitro gekeken wat het effect was van telomerase remmer BIBR1532 op telomeerlengte en DNA schade in surrogaat cellijnen voor de verschillende celtypes. Alleen in AT2 surrogaat cellijnen, en niet in club cel en fibroblast surrogaat cellijnen, vonden we significant kortere telomeren en hogere DNA schadeniveaus. De resultaten van deze studie toonden aan dat in IPF en TERT-PF longen telomeerverkorting en accumulatie van DNA schade voornamelijk AT2 cellen aantast. Dit benadrukt het belang van AT2 cellen bij deze ziekten, terwijl in fHP de bijzonder hoge telomeer-onafhankelijke DNA schade in club cellen de bronchiolocentrische pathogenese benadrukt. Deze bevindingen suggereren dat celtype-specifieke telomeerverkorting en DNA

schade kunnen helpen om onderscheid te maken tussen verschillende entiteiten van fibrosering.

**Hoofdstuk 6: De omvang van ontstekingsinfiltraat en fibrose in longen van telomeer- en surfactant-gerelateerd familiaire longfibrose**

FPF is een monogenetische ziekte die meestal gepaard gaat met telomere- (*TERT*) of surfactant- (*SFTP*)-gerelateerde mutaties. Van deze mutaties is aangetoond dat ze de lymfocytaire ontstekingsreactie kunnen beïnvloeden. Verder is er beschreven dat in longbiopten van patiënten met FPF lymfocytaire infiltraten verhoogd aanwezig zijn. Onlangs is aangetoond dat in een muismodel met een surfactant mutatie de ziekte aanvankelijk begon met een ontstekingsreactie, welke werd gevolgd door fibrosering van de long. Omdat ontsteking en fibrose behandeld worden met verschillende medicijnen, hebben we onderzocht of de mate van deze twee kenmerken co-lokaliseren of onafhankelijk voorkomen in verschillende entiteiten van FPF, en of ze de overleving beïnvloeden. Hiervoor hebben we het aantal lymfocytenaggregaten per oppervlaktegebied, de mate van diffuus lymfocytair infiltraat, het aantal fibroblasten foci per oppervlaktegebied en het percentage fibrotische longoppervlak in diagnostische longbiopten van patiënten met TERT-PF, SFTP-PF en sporadische (sIPF) bepaald. Deze hebben we vergeleken met dezelfde factoren in biopten van patiënten met cellulaire niet-specifieke interstitiële pneumonie (cNSIP), een inflammatoire interstitiële longziekte waarbij patiënten meestal goed reageren op immunosuppressieve therapie. De mate van ontstekingscelinfiltraat en fibrose in TERT-PF en SFTP-PF was niet significant verschillend ten opzichte van dat in sIPF. In vergelijking met cNSIP was de mate van lymfocytair infiltraat significant lager in sIPF en TERT-PF, maar niet in SFTP-PF. Echter, in tegenstelling tot cNSIP waren in sIPF, TERT-PF en SFTP-PF de diffuse lymfocytaire infiltraten voornamelijk, en lymfocytenaggregaten uitsluitend aanwezig in fibrotische gebieden. Verder hebben we aangetoond dat fibroblasten foci en het percentage fibrotisch longoppervlak geassocieerd zijn met overleving, terwijl deze associatie niet werd waargenomen voor lymfocytenaggregaten of het diffuse lymfocytaire cel infiltraat. Hieruit hebben we geconcludeerd dat ontstekingscellen in diagnostische longbiopten van TERT-PF, SFTP-PF en sIPF grotendeels zijn beperkt tot fibrotische gebieden. Op basis van ontsteking en fibrose werden echter geen verschillen gevonden tussen FPF en sIPF, wat de histologische overeenkomsten tussen monogenetische familiale en sporadische ziekte bevestigt. Bovendien correleert de mate van fibrose, in tegenstelling tot ontsteking, met overlevingsduur. Al deze bevindingen bij elkaar onderbouwen het algemene beeld dat in het kader van behandeling, de ontwikkeling van fibrose het belangrijkste kenmerk is in longen van patiënten met FPF.

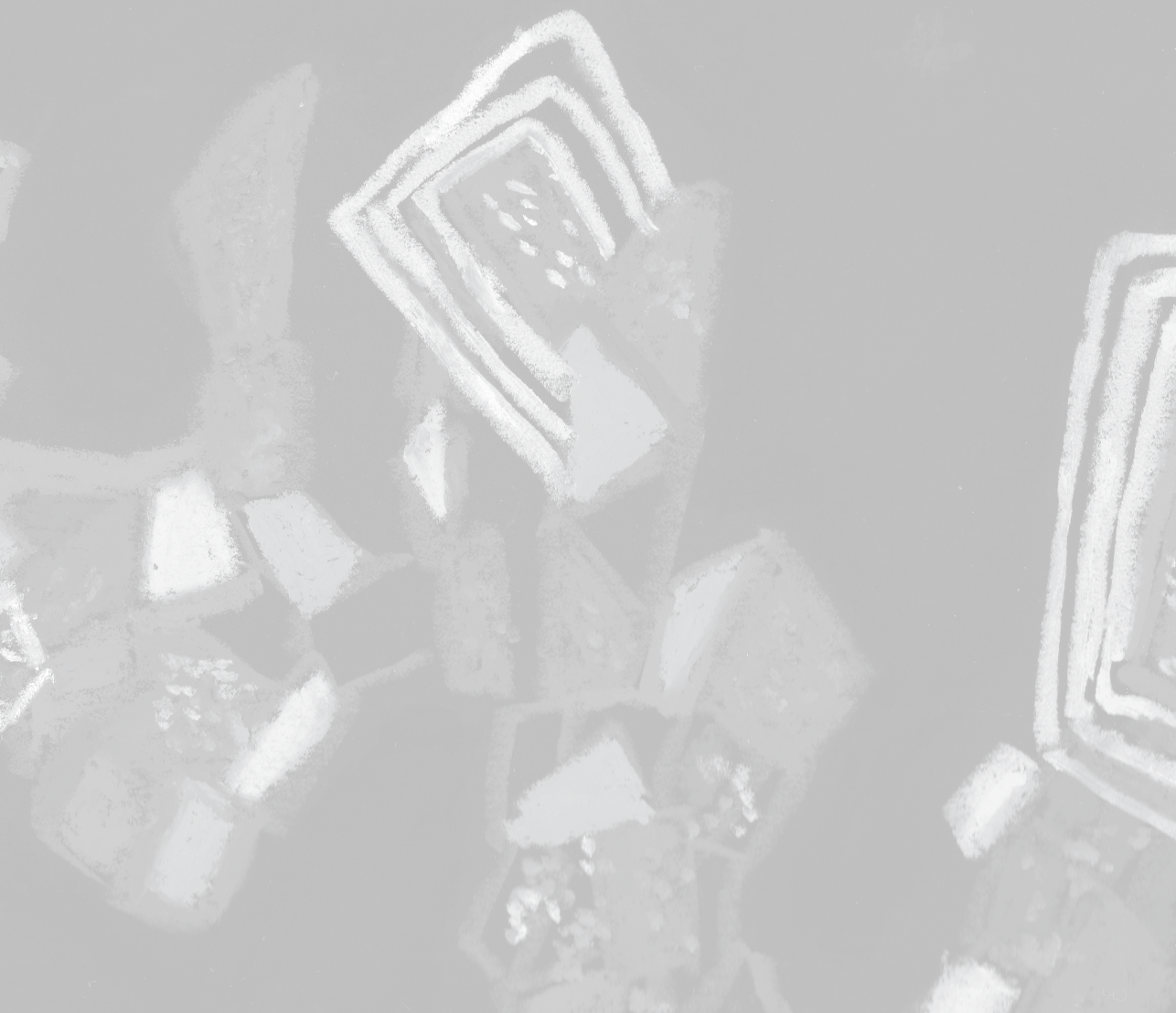
**Hoofdstuk 7: Algemene conclusie en suggesties voor toekomstig onderzoek**

Dit proefschrift beschrijft de mate van telomeerverkorting in de longen van patiënten met IPF en FPF en gaat tevens in op de vergelijking van deze data met controles. Uit het hier gepresenteerde werk en de gerelateerde literatuur wordt duidelijk dat telomeerverkorting in AT2 cellen niet alleen een fundamenteel probleem is bij longfibrose patiënten met telomeer-

gerelateerde mutaties, maar ook bij veel sporadische IPF patiënten. Het is mogelijk dat in de groep van sporadische IPF de telomeer-gerelateerde mutaties die ten grondslag liggen aan de ziekte nog ontdekt moeten worden. Op basis van de resultaten die in dit proefschrift worden beschreven, zouden we graag de volgende punten voor toekomstig onderzoek willen prioriteren. Ten eerste, aangezien er geen verschil in telomeerverkorting over de tijd in verschillende stadia van de ziekte werd gevonden, moet de rol van telomeerverkorting vóór de klinische diagnose worden bestudeerd. Bijvoorbeeld in familieleden van patiënten met FPF die mogelijk de ziekte nog gaan ontwikkelen. Ten tweede is in de literatuur aangetoond dat een enkele kritiek korte telomeer voldoende is om cellulaire disfunctie te induceren. Echter, of dit in patiënten met pf-ILD het geval is blijft onduidelijk. Ten slotte, aangezien we een zeer verhoogd DNA schadeniveau hebben aangetoond in club cellen van fHP longen, rijst de vraag of deze pathologische bevinding cruciaal is voor de pathofysiologie van dit type pf-ILD. Hoewel de pathogenese van progressieve longfibrose complex blijft, benadrukken de bevindingen en interpretaties in dit proefschrift de belangrijke rol van telomeren in deze ernstige ziekte.

Appendices

# Author affiliations
# List of publications
# Dankwoord
# Curriculum Vitae



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## Publications in this thesis

*The Extent of Inflammatory Cell Infiltrate and Fibrosis in Lungs of Telomere- and Surfactant-Related Familial Pulmonary Fibrosis.*
**van Batenburg AA**, van Oosterhout MFM, Knoppert SN, Kazemier KM, van der Vis JJ, Grutters JC, Goldschmeding R, van Moorsel CHM.
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## Curriculum Vitae

Aernoud Alexander van Batenburg was born in Sneek on July 17th 1988. After graduating with a culture and society profile from the Vrije School Groningen in 2006 and with a nature and health profile from the Friesland College department of adult education in 2007, he started studying Biomedical sciences at the University of Utrecht. After obtaining his bachelor degree in 2010, Aernoud went abroad for 6 months to work and to travel. In 2011, he continued his studies with the master specialization Biology of Disease at the University of Utrecht. During his master studies he did two internships: at the department of Medical Microbiology, UMC Utrecht (on the topic of inhibition of neutrophil granular proteases by Staphylococcus aureus immune-evasion proteins) and at the department of Cell Biology I, Netherlands Cancer Institute in Amsterdam (on the topic of Epac-based cAMP biosensors: Properties and affinity Improvements). In Amsterdam he developed his interest for microscopy and after he received his master's degree in 2013, he started as a PhD student in the group of Interstitial Lung Diseases Center of excellence at the St. Antonius Hospital in Nieuwegein. Here, Aernoud could further explore the use of microscopy techniques on the topic of telomere shortening and fibrotic remodeling in progressive fibrosing ILD, of which the results are presented in this thesis. During his PhD studies, he attended several courses and congress meetings. While presenting his work at the WASOG conference in Gdansk (Poland) in June 2016 as well as at the European congress of Pathology in Amsterdam in September 2017, he received at both congresses a best poster award. Furthermore, he won the NRS Young investigator award at Week van de Longen in Ermelo (The Netherlands).