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Editor's Choice

ORIGINAL ARTICLE

Predictive factors and prognostic effect of telomere shortening in pulmonary fibrosis

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ABSTRACT

Background and objective: **The abnormal shortening of telomeres is a mechanism linking ageing to idiopathic pulmonary fibrosis (IPF) that could be useful in the clinical setting. The objective of this study was to identify the IPF patients with higher risk for telomere shortening and to investigate the outcome implications.**
Methods: **Consecutive Spanish patients were included at diagnosis and followed up for 3 years. DNA blood samples from a Mexican cohort were used to validate the results found in Spanish sporadic IPF. Prior to treatment, telomere length was measured through quantitative polymerase chain reaction (qPCR) and Southern blot. Outcome was assessed according to mortality or need for lung transplantation. A multivariate regression logistic model was used for statistical analysis.**
Results: **Family aggregation, age of <60 years and the presence of non-specific immunological or haematological abnormalities were associated with a higher probability of telomere shortening. Overall, 66.6% of patients younger than 60 years with telomere shortening died or required lung transplantation, independent of functional impairment at diagnosis. By contrast, in patients older than 60 years with telomere shortening, the negative impact of telomere shortening in outcome was not significant.**
Conclusion: **Our data indicate that young sporadic IPF patients (<60 years) with some non-specific immunological or haematological abnormalities had higher risk of telomere shortening, and furthermore, they presented a poorer prognosis.**

SUMMARY AT A GLANCE

The study establishes predictive factors for telomere shortening in idiopathic pulmonary fibrosis (IPF) and demonstrates clinical implications in pulmonary fibrosis. Sporadic IPF patients younger than 60 years and/or presenting non-specific immunological or haematological abnormalities were at higher risk of telomere shortening. A poor prognosis is more frequently associated with a young disease onset.

Key words: familial pulmonary fibrosis, genetics, idiopathic pulmonary fibrosis, telomere disorders, telomere shortening.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most devastating interstitial lung disease (ILD). Although the pathogenesis remains unclear, there is evidence that IPF is an age-related disease. The mechanisms linking IPF to ageing, including abnormal shortening of telomeres,

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are currently under study.[1–11] The frequency of telomerase mutations accounts for 8–15% of cases of familial pulmonary fibrosis (FPF) and 1–3% for sporadic cases, with an autosomal-dominant inheritance and age-dependent penetrance.[1,6] Importantly, however, telomere attrition has been identified even in the absence of telomerase mutations.[2,9] There is also evidence that IPF may be more likely to develop in subjects with the shortest telomeres.[2,7] Thus, telomere shortening is a risk factor in developing the disease. Short telomeres are detected in lymphocytes, granulocytes and alveolar epithelial cells.[2] Moreover, pulmonary fibrosis may occur in the setting of a complex syndrome in which telomere dysfunction may associate extra-pulmonary manifestations.[1] This systemic effect could explain the post-transplant lung complications and drug-related toxicities.[12,13] Furthermore, poor patient outcome has been recently associated with short telomeres.[14,15]

In this context, the identification of a telomere-mediated disorder in lung fibrosis is relevant for prognostic implications, diagnostic approach and treatment of patients facing lung transplant. Therefore, the evaluation of telomere length (TL) would be helpful for personalizing the management of IPF patients. However, two facts must be considered: (i) telomere shortening has been described in a minority of IPF patients[14,16] and (ii) genetic studies are expensive and complex, and their availability depends on the skilled technicians and economic resources. Thus, optimizing the clinical suspicion of telomere involvement in IPF would be relevant to improve the diagnostic yield. Hence, we evaluated clinical features that help to identify IPF patients with higher risk of telomere shortening.

METHODS

Study cohorts

This observational prospective study was approved by the Ethics Committee of University Hospital of Bellvitge (approval number PR082/13), and all patients signed the written informed consent before inclusion in the study. The derivation cohort consisted of 106 consecutive IPF patients referred to the ILD unit from June 2013, who were evaluated at diagnosis and followed up for 3 years. Lung transplantation and mortality were reported. IPF diagnosis was established in accordance with guidelines.[17,18] FPF was considered when two or more relatives from the same family were affected.

Epidemiological and clinical data were collected. All patients were tested using the same haematological and immunological panel (Tables S1, S2, Supplementary Information). The criteria for considering non-specific haematological abnormality was the presence of a decrease in the haemoglobin (Hb, <12 g/L for females and <13 g/L for males), platelet (<150 000/L) and/or leucocyte values (<3900/L), following the World Health Organization (WHO) definitions, in at least two different determinations, with no identified cause and no clinical relevance. The criteria for considering non-specific immunological abnormality was the increase of autoantibodies, in two separate blood analyses, but without the titre required to be considered an immunological domain (Table S2, Supplementary Information). An expert rheumatologist evaluated every patient with some of these immunological abnormalities. Patients with connective tissue disease (CTD) or interstitial pneumonia with autoimmune features (IPAF), asbestosis or any other type of fibrotic ILD were not included.[19] All patients signed a written informed consent for genetic testing. The telomere study was performed at the moment of diagnosis.

The independent validation cohort (Mexican cohort) consisted of 102 IPF patients, blindly selected, with no history of family aggregation and diagnosed at the 'Instituto Nacional de Enfermedades Respiratorias, Ismael Cosío Villegas', Mexico City, following published criteria.[17] The normal values for the Mexican cohort were the same except in the evaluation of anaemia; due to the difference in altitude, the WHO definitions were adjusted by altitude (2250 m) and anaemia was considered when Hb was <13.2 g/L in females and <14.8 g/L in males.[20] All patients consented to be included for genetic testing and the protocol was approved by the Bioethical Committee.

The control population utilized to obtain the telomere z-score consisted of 243 healthy subjects, with no cancer, no respiratory disease, nor any degenerative disorders such as diabetes, haematological, liver or kidney disease. Seventy-one percent were Caucasian and 29% Hispanic. DNA was obtained by oral swab and peripheral blood. More details are in Figures S1 and S2 (Supplementary Information). The Bioethical Committee approved the protocol and all subjects consented to inclusion.

Sample collection

TL analysis was performed using DNA samples isolated from mouth epithelial cells and peripheral blood mononuclear cells (PBMC). Oral swabs (Isohelix, SK-2S, Cell Projects Ltd), previously validated in normal subjects and other telomeropathies, were used for collection of cheek epithelial cells and DNA was extracted using a commercial kit (Isohelix, Cell Projects Ltd).[21–23] DNA from PBMC was obtained as previously described.[24]

TL analysis

The relative TL was assessed by quantitative polymerase chain reaction (qPCR), as previously described,[24] and then was confirmed by Southern blot. The qPCR determines the ratio of telomere (T) repeat copy number to single-copy (S) gene (*36B4*) copy number (T/S ratio) in experimental samples, as compared with a reference DNA sample. This methodology was also validated for the TL measurement in buccal cells. As TL changes with age, a z-score value was obtained to allow the comparisons between individuals of different ages. The z-score compared the T/S value in each individual with the age-matched mean and SD of the values obtained in the controls (individual's value − population mean/population SD, age-matched population of within 9 years on average). The z-score below the 25th percentile of a normal distribution was considered telomere shortening. Severe TL reduction was identified when z-score was below the 10th and 1st percentiles.

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Telomere shortening was also measured from blood DNA of each patient by Southern blot analysis of telomere restriction fragment (TRF) (TeloTAGGG Telomere Length Assay, Roche), which was considered the gold standard to determine TL.[24,25] Correlation of TL measurements by using both methods are detailed in Figure S3 (Supplementary Information). Analysis from both cohorts was done in the same laboratory.

Statistical analysis

Descriptive statistics were expressed as mean (SD) or median (interquartile range) and valid percentage for continuous and categorical data, respectively. The relationship between the length of telomeres and clinical variables was assessed using the chi-square test (exact Fisher test with observed frequencies <5) for categorical variables, whereas continuous variables were tested using t-test (Mann–Whitney U-test when not normally distribution).

Clinical variables that showed association with the presence of telomere shortening on univariate analysis (P–value < 0.1) were used to construct the corresponding multivariate logistic regression backward stepwise model. Receiver operating characteristic (ROC) curves were constructed to predict the presence of telomere shortening. The Hosmer–Lemeshow goodness-of-fit test was performed to assess the overall fit of the model.[26] Finally, predictors from this model were used to test the probability of telomere shortening, which was calculated by following this formula: Exp (b)/(1 + Exp(b)), where b = −1.088 + 1.069 (in the case of presence of immunological abnormalities) + 1.422 (in the case of presence of haematological abnormalities) + 0.987 (if age < 60 years).

The 3-year survival data was calculated through multivariate Cox regression model. Survival graphics were done using the Kaplan–Meier method, and differences were assessed with the log-rank statistic. Hazard ratios (HR) and 95% CI were calculated. Data for mortality analysis were censored at the date of death or lung transplant. Patients lost to follow-up were censored at the last visit date.

All tests were two-tailed, and significance was set at 5%. Statistical software package for Windows (SPSS version 20.0, IBM, Chicago, IL,USA) was used.

RESULTS

Patient characteristics

A total of 106 patients from derivation cohort, 68 IPF and 38 FPF (from 28 different pedigrees) were included. As shown in Table 1, FPF patients were younger than the IPF population (P = 0.021), while the male/female ratio was higher in the IPF group (P = 0.046). ANA < 1:320 titres were the most common non-specific immunological abnormality. Mild platelet count reductions and mild anaemia were the most prevalent haematological abnormalities (Table 1).

Table 1 Patient characteristics from derivation and Mexican cohorts

	Derivation cohort (*n* = 106)			Mexican cohort (*n* = 102)	
	n (%)			*n* (%)	
	FPF patients (*n* = 38)	IPF patients (*n* = 68)	*P*-value†	IPF patients (*n* = 102)	*P*-value‡
Age (years, mean (SD))	60.8 (11.9)	66.3 (10.9)	0.021	65.0 (8.3)	0.157
Males	21 (56.8)	52 (76.5)	0.046	85 (83.3)	0.268
Smoking history			0.324		0.521
Non-smoker	17 (45.6)	22 (32.4)		27 (31.8)	
Current smoker	2 (5.4)	2 (2.9)		6 (7.1)	
Former smoker	18 (48.6)	44 (64.7)		52 (61.2)	
Non-specific haematological abnormalities	5 (13.5)	10 (14.7)	0.881	49 (48)	**<0.001**
Platelet decrease	0 (0.0)	5 (7.4)		10 (9.8)	
Anaemia	4 (10.5)	1 (1.5)		38 (37.2)	
Non-specific immunological abnormalities	11 (29)	11 (16.2)	0.209	34 (33.3)	**0.013**
ANA 1 < 320 homogenic pattern	3 (7.9)	3 (4.4)		29 (28.4)	
Other	8 (21.1)	8 (11.8)		5 (4.9)	
Signs of telomere syndrome§	2 (5.4)	0 (0.0)	0.674	0 (0.0)	NA
Relative telomere length, mean (SD)	1.0 (0.3)	1.3 (0.3)	**<0.001**	1.65 (0.6)	**<0.001**
z-Score, mean (SD)	−1.5 (1.0)	−0.6 (0.99)	<0.001	−0.337 (1.5)	0.453
Telomere shortening	31 (81.6)	26 (38.2)	<0.001	42 (41.2)	0.048
<25th Percentile	7 (22.6)	10 (38.4)		20 (47.6)	
<10th Percentile 10	13 (41.9)	8 (30.8)		10 (23.8)	
<1st Percentile	11 (35.5)	8 (30.8)		12 (28.6)	

†Mann–Whitney U-test or chi-square test comparing IPF and FPF patients from derivation cohort.
‡Mann–Whitney U-test or chi-square test comparing IPF patients from derivation and Mexican cohort.
§Signs of telomere syndrome in these two patients included: premature hair greying, menopause and cryptogenic liver cirrhosis.
ANA, Anti-Nuclear Antibody; FPF, familial pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis; NA, not applicable.

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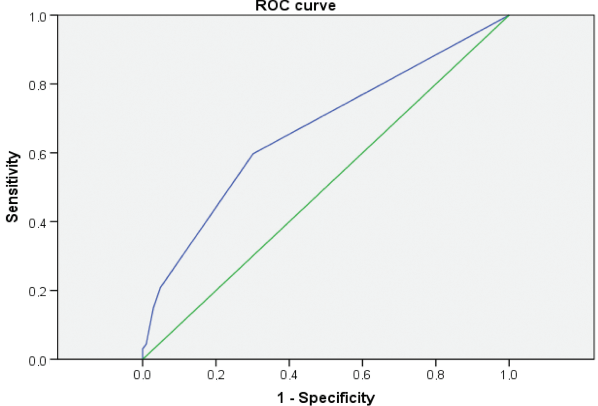

Figure 1 Probability of telomere shortening in idiopathic pulmonary fibrosis patients. Receiver operating characteristic analysis of significant variables derived from the logistic regression model in its capacity to predict presence of telomere shortness (area under the curve: 0.670, SE: 0.044, $P < 0.001$, 95% CI: 0.580–0.751).

Reduced TL was first identified by using oral swab (Supplementary Information, Fig. 1). Peripheral blood telomere shortening was found in 57 of 106 (53.8%) Spanish patients (Table 1). FPF patients presented higher prevalence of telomere shortening (81.6%) with a shorter TL compared with sporadic IPF (38.2%) ($P < 0.001$) (Table 1). A positive correlation was observed between the two sets of TL measurements performed by both the qPCR–Southern blot of blood DNA and by the qPCR on DNA extracted from buccal cells (R = 0.690) (Fig. S3, Supplementary Information). One hundred and two sporadic IPF patients from the Mexican cohort were evaluated to validate the predictive factors for telomere shortening in the derivation cohort by using blood DNA (Fig. S2, Supplementary Information). Table 1 summarizes the Mexican IPF patient characteristics.

Clinical features for predicting telomere shortening

To evaluate whether telomere attrition could be associated with some potential predictive factors, we analysed the differences between patients with and without reduced TL (Table 2). Patients with telomere shortening were younger ($P = 0.001$), without difference in smoking status (Table 2).

The main predictive factor for telomere shortening was family aggregation. To determine whether some clinical variables could be related to telomere shortening in patients with no family aggregation, we performed a multivariate logistic regression analysis. Independent variables were selected from the univariate analysis according to *P*-value <0.1: age transformed as a binary variable (<60 years), non-specific haematological and immunological abnormalities. Telomere shortening was considered a dependent variable. As shown in Figure 1, IPF patients younger than 60 years and those with non-specific immunological or haematological abnormalities had a higher risk of reduced TL. Pre-test probability modelling including these three independent clinical variables associated with telomere shortening was used. The probability of telomere shortening increased progressively with the number of putative predictors. Therefore, the probability for telomere shortening in an IPF patient without any of these characteristics would be 25.2%, rising to 91.6% for those having all three variables (Table 3). This final model was well calibrated, with *P*-values of 0.889, by using the Hosmer–Lemeshow test. The capacity of the significant variables derived from the logistic regression model to

Table 2 Patient characteristics according to telomere shortening

	IPF patients from derivation and Mexican cohort *n* = 170			FPF patients from derivation cohort *n* = 38		
	No telomere shortening *n* = 103	Telomere shortening *n* = 67	*P*-value	No telomere shortening *n* = 6	Telomere shortening *n* = 32	*P*-value
Age (years, mean (SD))	67.5 (8.6)	62.5 (9.9)	**0.001**	63 (10.6)	60.0 (12.4)	0.568
Males	81 (78.6)	56 (83.6)	0.426	4 (66.7)	18 (56.5)	0.635
Smoking history			0.354			**0.038**
Non-smoker	28 (30.8)	21 (33.9)		0 (0)	17 (53.1)	
Current smoker	3 (3.3)	5 (8.1)		1 (16.7)	1 (3.1)	
Former smoker	60 (65.9)	36 (58.1)		5 (83.3)	14 (43.8)	
Non-specific haematological abnormalities	32 (31.1)	25 (37.3)	0.339	1 (16.7)	5 (15.7)	0.947
Non-specific immunological abnormalities	19 (18.4)	26 (38.8)	**0.003**	2 (33.3)	8 (25)	0.671
Bone marrow aplasia, n = 68	0	1 (4)	0.186	0	1 (3.1)	0.661
Signs of telomeric syndrome (early greying hair)	0	0	0.186	0	2 (6.5)	0.661
Relative telomere length, mean (SD)	1.725 (0.53)	1.141 (0.28)	**<0.001**	1.405 (0.09)	0.979 (0.24)	**<0.001**
z-Score, mean (SD)	−0.283 (1.06)	−1.539 (0.74)	**<0.001**	−0.161 (0.23)	−1.794 (0.89)	**<0.001**

FPF, familial pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis.

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Table 3 Probability of telomere shortening in IPF patients with no family aggregation

Presence of immunological abnormalities	Presence of haematological abnormalities	Age < 60 years	Probability of presence of telomere shortness (%)	
–	–	–	25.2	
+	–	–	49.5	
–	+	–	58.3	
–	–	+	47.5	
+	+	–	80.3	
+	–	+	72.5	
–	+	+	78.9	
+	+	+	91.6	
Multivariate logistic regression model for telomere shortening				
Independent variables		OR	95% CI	*P*-value
Non-specific immunological disorders		2.913	1.409–6.024	0.004
Non-specific haematological disorders		4.145	1.010–17.655	0.049
Age < 60 years		2.684	1.297–5.553	0.008

IPF, idiopathic pulmonary fibrosis.

predict telomere shortening was evaluated through ROC curve with an area under the curve (AUC) of 0.670 (95%CI: 0.580–0.751, $P < 0.001$) (Fig. 1).

Disease outcome and telomere shortening

To evaluate the role of telomere shortening in prognosis, the 3-year survival (lung transplantation and mortality) was compared among patients with and without telomere attrition. Of the 106 patients, 16 (15.1%) returned to their referral hospital and the follow-up was lost. Among the 90 patients who were followed up, 29 patients received anti-fibrotic treatment (pirfenidone $n = 16$, nintedanib $n = 13$, 15 cases with telomere shortening and 14 cases without). 27.8% of IPF patients and 34.4% of FPF died or required lung transplant. Lung transplantation was performed in 12 (13.3%) cases, most of them (83.3%) with telomere shortening. Mortality was reported in 15 (16.7%) patients and 9 (60%) showed telomere shortening. The cause of death was related to IPF progression. No impact of anti-fibrotic treatment was observed on survival differences, although the number of treated cases was limited as both drugs were commercially approved after the recruitment began. Thus, reduced TL would have a negative impact on the 3-year survival rate (Table 4).

A multivariate logistic COX regression analysed the possible implication of clinical characteristics associated with telomere shortening (immunological or haematological minor abnormalities and age) and disease progression (baseline forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DL_{CO}) and gender). Age was the strongest related factor associated with poor outcome. Remarkably, the 3-year survival rate was significantly lower in younger patients (<60 years, 44% vs 81%, respectively, $P = 0.010$) (Table 4, Fig. S4 (Supplementary Information)), although all of them had a GAP index I, stage I, at diagnosis. No differences were found in FVC and DL_{CO} among patients ($P > 0.05$). IPF and FPF patients younger than 60 years with telomere shortening presented higher probability for lung transplant or death (HR = 3.579, 95% CI: 1.074–10.900) (Fig. 2).

Data on extra pulmonary affectation and non-specific immunological and haematological abnormalities are included in Tables S1–S5 (Supplementary Information).

Table 4 Lung transplant and mortality in patients according to age and telomere shortening

	Age < 60 years (*n* = 27)				Age > 60 years (*n* = 63)			
	IPF *n* = 15		FPF *n* = 12		IPF *n* = 46		FPF *n* = 17	
Final number of patients in follow-up	Telomere shortening *n* = 9	No telomere shortening *n* = 6	Telomere shortening *n* = 12	No telomere shortening *n* = 0	Telomere shortening *n* = 15	No telomere shortenings *n* = 31	Telomere shortening *n* = 10	No telomere shortening *n* = 7
% Death or lung	**56%** (*n* = 14)				**19%** (*n* = 12)			
transplant	**66.6%**	16.6%	**66.6%**	0	26.6%	19.4%	10%	14.3%
Transplant (*n*)	5	0	4	0	0	2	1	0
Deaths (*n*)	1	1	4	0	4	4	0	1

FPF, familial pulmonary fibrosis; IPF, idiopathic pulmonary fibrosis.

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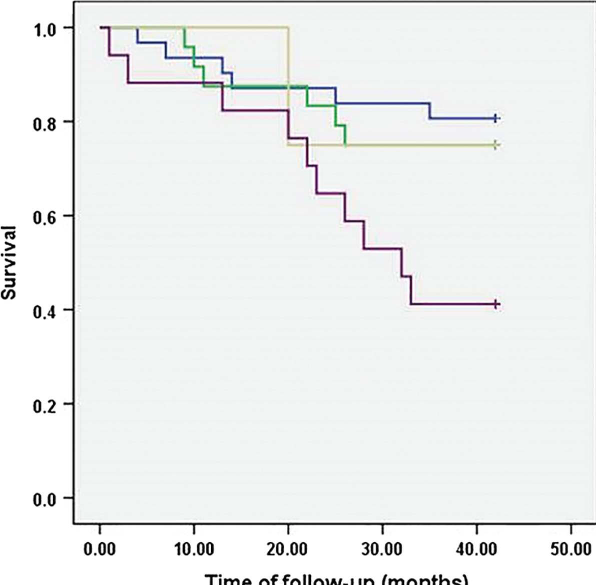

Figure 2 Transplant-free survival (TFS) time according to the presence or absence of telomere shortening (TS) and age lower or higher than 60 years. TFS period was considered the time from study baseline to the date of death or lung transplantation. A combined outcome variable (death or lung transplantation) was estimated. Patients who survived or did not have a transplant throughout follow-up were censored at the date of the end of the study. A total of 26 patients had a transplant or died during follow-up study period. Patients younger than 60 years with TS had statistically lower mean follow-up time ($P = 0.034$). ——, Absence of TS and age ≥60 years; ——, presence of TS and age ≥60 years; ——, absence of TS and age <60 years; ——, presence of TS and age <60 years

DISCUSSION

The study reveals for the first time that sporadic IPF patients younger than 60 years display a high pre-test probability for reduced TL that increases even more when non-specific haematological and/or immunological abnormalities are present. Furthermore, telomere shortening would be a valuable marker of outcome, especially in those cases younger than 60 years of age. Therefore, while age has been described as a prognostic factor in the global IPF population (worse prognosis in older patients), our results show the poorest prognosis in young IPF patients with telomere shortening. Hence, telomere (biological) age would be a better prognostic factor than chronological age.

Our most important finding was the different ratio of shortened telomeres depending on the clinical phenotype. Thus, although the overall probability of telomere shortening was 25%, in those patients younger than 60 years of age who presented some non-specific immunological and haematological abnormalities, the probability was almost 100%. In addition, even in IPF patients older than 60 years, the age group at which most of them are diagnosed,[17] the presence of the identified blood or immunological abnormalities may increase suspicion of an impact of telomere shortening on outcome. Family aggregation is the most robust independent predictive factor for telomere shortening.[27,28] However, another strong predictive factor evidenced by our results is age, in accordance with the recent data from Newton *et al.*[29] and Borie *et al.*[30] for telomere mutations in FPF.

A reduction of TL has been recently associated with worse survival in retrospective cohorts.[12,29–34] Newton *et al.* reported a 2–3-year survival rate in the analysis of telomere-related gene mutations from 64 families. However, the mean age for those mutation carriers was 58 ± 10 years.[29] The results from our prospective cohort highlight that the prognosis associated with telomere shortening depends in part on the age of disease onset, suggesting that the sooner the lung fibrotic process develops the faster the lung ageing and disease progression occur. Several hypotheses could explain this observation, including genetic anticipation and higher cell turnover in younger patients with the same gene defect.

Another important novelty of this study is the use of an oral swab as an easy non-invasive method for TL assessment that presents a good correlation with the blood DNA analysis. This test could be performed at the patient's home, thereby facilitating the screening of gene mutation carriers.

The prevalence of telomere attrition in our FPF patients is higher than described, probably as most previous studies considered telomere shortening under the 10th percentile.[16,35,36] However, other telomere diseases may develop with a telomere reduction under the 25th percentile.[37] Furthermore, we also found a negative impact on disease outcome with this degree of telomere reduction.

The main limitations of the present study are the small sample size of the subgroup of patients younger than 60 years (which reduces the statistical power) and the lack of other genetic analysis such as MUC5B or SP gene variants.[28] Furthermore, the possible effect of environmental exposures on TL could only be analysed for tobacco.

In conclusion, identification of telomere shortening in pulmonary fibrosis is important in predicting patient outcome. Our study determines a probability pre-test model for telomere shortening that detects IPF patients with higher risk and, therefore, that most benefit from genetic study.

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Abbreviations: ANA, Anti-Nuclear Antibody; DL_{CO}, diffusing capacity for carbon monoxide; DNA, deoxyribonucleic acid; FPF, familial pulmonary fibrosis; FVC, forced vital capacity; HR, hazard ratio; ILD, interstitial lung disease; PBMC, peripheral

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blood mononuclear cell; qPCR, quantitative PCR; ROC, receiver operating characteristic; T/S ratio, ratio of telomere (T) repeat copy number to single-copy (S) gene (*36B4*) copy number; TFS, transplant-free survival; TL, telomere length; TS, telomere shortening; WHO, World Health Organization.

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Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Figure S1. Telomere length analysis of derivation cohort.
Figure S2. Telomere length analysis of Mexican cohort.
Figure S3. Correlation between the two telomere length assays and the different cell types.
Figure S4. Differences in the 3-year survival time in IPF cases older versus younger than 60 years.
Table S1. Immunological data in IPF/FPF patients of derivation cohort.
Table S2. Haematological data reported in IPF/FPF patients of derivation cohort.
Table S3. Age distribution of the control population.
Table S4. Non-specific immunological/haematological disorders in patients aged <60 years.
Table S5. Non-specific immunological/haematological disorders in patients aged >60 years.

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