



**BRAZILIAN JOURNAL**  
OF MEDICAL AND BIOLOGICAL RESEARCH

www.bjournal.com.br

ISSN 0100-879X  
Volume 45 (7) 565-680 July 2012

CLINICAL INVESTIGATION

Braz J Med Biol Res, July 2012, Volume 45(7) 665-675

doi: 10.1590/S0100-879X2012007500066

## Vascular dysfunction by myofibroblast activation in patients with idiopathic pulmonary fibrosis and prognostic significance

E.R. Parra, R. Falzoni and V.L. Capelozzi

The Brazilian Journal of Medical and Biological Research is partially financed by



Ministério da Ciência e Tecnologia



Ministério da Educação



*Institutional Sponsors*



Explore High - Performance MS Orbitrap Technology In Proteomics & Metabolomics



All the contents of this journal, except where otherwise noted, is licensed under a [Creative Commons Attribution License](http://creativecommons.org/licenses/by-nc/4.0/)

# Vascular dysfunction by myofibroblast activation in patients with idiopathic pulmonary fibrosis and prognostic significance

E.R. Parra, R. Falzoni and V.L. Capelozzi

Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

## Abstract

In this study, we demonstrated the importance of telomerase protein expression and determined the relationships among telomerase, endothelin-1 (ET-1) and myofibroblasts during early and late remodeling of parenchymal and vascular areas in usual interstitial pneumonia (UIP) using 27 surgical lung biopsies from patients with idiopathic pulmonary fibrosis (IPF). Telomerase+, myofibroblasts  $\alpha$ -SMA+, smooth muscle cells caldesmon+, endothelium ET-1+ cellularity, and fibrosis severity were evaluated in 30 fields covering normal lung parenchyma, minimal fibrosis (fibroblastic foci), severe (mural) fibrosis, and vascular areas of UIP by the point-counting technique and a semiquantitative score. The impact of these markers was determined in pulmonary functional tests and follow-up until death from IPF. Telomerase and ET-1 expression was significantly increased in normal and vascular areas compared to areas of fibroblast foci. Telomerase and ET-1 expression was inversely correlated with minimal fibrosis in areas of fibroblast foci and directly associated with severe fibrosis in vascular areas. Telomerase activity in minimal fibrosis areas was directly associated with diffusing capacity of the lung for oxygen/alveolar volume and ET-1 expression and indirectly associated with diffusing capacity of the lungs for carbon monoxide and severe fibrosis in vascular areas. Cox proportional hazards regression revealed a low risk of death for females with minimal fibrosis displaying high telomerase and ET-1 expression in normal areas. Vascular dysfunction by telomerase/ET-1 expression was found earlier than vascular remodeling by myofibroblast activation in UIP with impact on IPF evolution, suggesting that strategies aimed at preventing the effect of these mediators may have a greater impact on patient outcome.

Key words: Idiopathic pulmonary fibrosis; Vascular activity; Immunohistochemistry; Survival

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating chronic fibrosing interstitial pneumonia of unknown etiology that typically increases in prevalence with advanced age, characterized by excessive collagen deposition and irreversible remodeling of the lung parenchyma (1,2). The histologic pattern associated with IPF is the usual interstitial pneumonia (UIP), characterized by patchy and temporally heterogeneous fibrosis with excessive extracellular matrix (ECM) and honeycomb change, interspersed with normal and collapsed areas (1). Although the etiology of IPF/UIP is not understood, many investigators hypothesize that dysregulated communication between injured epithelial cells and activated mesenchymal cells represents an important factor in its pathogenesis (3). Vascular changes

are another event frequently observed in IPF (4,5). In previous studies, we found a direct relationship between progressive vascular occlusion by collagen/elastic fiber deposition and tissue remodeling in surgical lung biopsies from patients with IPF (4,5). These findings probably are the consequence of persistent myofibroblast activation by mesenchymal cells, resulting in a heterogeneous fibroblast phenotype, intermediate between fibroblasts and smooth muscle cells (6). Although heterogeneity in fibroblast phenotype is evident, a subset of differentiated activated fibroblasts is characterized by the expression of alpha-smooth muscle actin ( $\alpha$ -SMA) (7). Depending on the precise stimulatory milieu, fibroblasts either transform to myofibroblasts or proliferate, resulting in vascular oc-

Correspondence: E.R. Parra, Departamento de Patologia, FM, USP, Av. Dr. Arnaldo, 455, 01246-903 São Paulo, SP, Brasil.  
Fax: +55-11-3064-2744. E-mail: erparra20003@yahoo.com.br

Received November 14, 2011. Accepted April 9, 2012. Available online April 27, 2012. Published July 2, 2012.

clusion or areas of fibroblastic foci, which are thought to be the sites of active ECM, collagen and elastic synthesis, and are considered to be the leading edge of fibrosis and vascular remodeling (8). In this regard, endothelin-1 (ET-1) is one of the more potent inducers of collagen deposition, with its fibrogenic effects being central in wound healing and tissue repair. A major role of ET-1 involves transition of quiescent fibroblasts to an "activated" phenotype, whereby matrix production and wound contraction are important consequences (9). Telomerase is a specialized polymerase that adds telomere repeats to the ends of chromosomes by using its intrinsic RNA component as a template, thereby compensating for the telomere loss that normally occurs with each cell division (10). Telomerase has been shown to be essential for unlimited cell proliferation and has been linked to immortality (11). Bleomycin-induced lung injury and fibrosis is also known to induce telomerase activity in the affected lung tissue and isolated lung fibroblasts (12). The fibroblasts isolated from such lungs undergoing fibrosis show increased intrinsic proliferative capacity and are able to differentiate into  $\alpha$ -SMA expressing myofibroblasts, which are also a key source of cytokines with inflammatory and fibrogenic properties (13,14). An injured lung fibroblast population may contain telomerase expressing cells with an extended life span, which could contribute to the observed increased numbers of lung fibroblasts (12).

In view of these considerations suggesting an interaction among telomerase, endothelium and myofibroblast activation, the aim of the present study was to evaluate 1) whether telomerase, ET-1 and myofibroblast  $\alpha$ -SMA are expressed in vascular and parenchymal areas of UIP; 2) to verify if the measures of mediator expression correlate with some measure of fibrosis, and 3) to determine the impact of the resultant parenchymal/vascular remodeling on patient survival. It was hypothesized that in UIP, a devastating chronic fibrosing interstitial pneumonia, the mediators under study exert their effect by influencing parenchyma/vascular remodeling/fibrosis with impact on patient outcome.

## Material and Methods

This research was approved by the Institutional Ethics and Scientific Committees (protocol No. 0960/08) and all patients gave written informed consent to participate in the study.

### Open lung biopsies

Pulmonary specimens were obtained by surgical lung biopsy from 27 patients with IPF/UIP (14 males and 13 females; mean age  $64 \pm 1.4$  years), according to criteria outlined in the American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of idiopathic interstitial pneumonias (1). Only specimens from patients who fulfilled these consensus criteria were included. Three lung specimens obtained by

open surgical biopsy from each patient were collected according to the normal, intermediate and more affected areas in different parts of the lung determined by high-resolution computed tomography. The histologic UIP pattern of IPF was characterized by the patchy subpleural and paraseptal distribution of parenchymal injury. Temporal heterogeneity was seen at low magnification, with alternating areas of normal lung parenchyma with septal fibro-myxoid tissue (fibroblastic foci), honeycomb and vascular thickening.

### Baseline characteristics

The pulmonary function tests included vital capacity, forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), FEV1/FVC ratio 100X, total lung capacity (TLC), residual volume (RV), and carbon monoxide transfer factor (diffusing capacity of the lung for carbon monoxide, DLCO). The percentages of TLC, RV, and RV/TLC were measured by the helium dilution method using a Master Screen apparatus (Erich Jaeger GmbH, Germany). The DLCO and DLCO/alveolar volume (VA) were assessed by the single breath holding helium dilution method (15). Lung function results (Table 1) are reported as the percentage of patient lung function compared to predicted values for a standardized healthy population. In all patients, the arterial partial pressure of carbon dioxide (PaCO<sub>2</sub>) and partial pressure of oxygen (PaO<sub>2</sub>) were also measured at rest.

### Immunohistochemistry

A standard peroxidase technique was used, with Harris's hematoxylin as the counterstain, to identify telomerase+ epithelial cells, ET-1+ endothelium and  $\alpha$ -SMA+

**Table 1.** Clinical data of the patients.

Variables	Idiopathic pulmonary fibrosis
Age at biopsy (years)	64.5 $\pm$ 7.4
Gender (males/females)	13/14
Spirometry	
FEV1 (% predicted)	78 $\pm$ 4.4
FVC (% predicted)	69.5 $\pm$ 3.5
FEV1/FVC	20 $\pm$ 8.8
TLC (% predicted)	79 $\pm$ 5
RV (% predicted)	102 $\pm$ 15.9
TLC/RV (%predicted)	44.2 $\pm$ 3.4
DLCO (% predicted)	59.8 $\pm$ 8
DLCO/VA (% predicted)	59 $\pm$ 11.7
PaCO <sub>2</sub>	60.2 $\pm$ 3.7
PaO <sub>2</sub>	38.1 $\pm$ 1.3

Data are reported as means  $\pm$  SEM. FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; TLC = total lung capacity; RV = residual volume; DLCO = diffusing capacity of the lung for carbon monoxide; VA = alveolar volume; PaCO<sub>2</sub> = partial pressure of carbon dioxide; PaO<sub>2</sub> = partial pressure of oxygen.

myofibroblasts and caldesmon+ smooth muscle cells in normal tissue, fibroblastic foci and vascular areas of UIP. Since the  $\alpha$ -SMA antibody stains myofibroblasts as well as smooth muscle cells, which are a regular component of arteries, the caldesmon (C21) antibody sc-58700 was specifically used to characterize these cells. All antibodies used were biotinylated rabbit polyclonal antibodies. Anti-telomerase, anti-ET-1, anti- $\alpha$ SMA and anti-caldesmon polyclonal antibodies (Santa Cruz Biotechnology, Inc., USA) were incubated with tissue sections at 1:100 dilution. The Max Polymer Novolink amplification kit (Leica, Newcastle Inc., UK) was used for signal amplification and 3,3'-diaminobenzidine tetrachloride (0.25 mg dissolved in 1 mL 0.02% hydrogen peroxide) was used as a precipitating substrate for signal detection. The specificity of primary antibodies was confirmed by appropriate reagent controls (omitting the primary antibody or substituting non-immune serum for the primary antibody in the staining protocol), which revealed no staining.

### Histomorphometry

Regional differences between samples from the same patient in the three lung specimens obtained by open surgical biopsy in each patient were evaluated for telomerase+ epithelium, ET-1+ endothelium,  $\alpha$ -SMA+ myofibroblasts, caldesmon+ smooth muscle cells, and fibrosis severity in 30 fields by the point-counting technique (16) and a semi-quantitative score (4). As previously described, the histologic pattern of UIP was characterized by alternating areas of normal lung parenchyma, minimal fibrosis (fibroblastic foci), severe (mural) fibrosis, honeycombing, and vascular thickening. At 400X magnification, 30 fields were chosen from across multiple sites accounting/adjusting for cellularity or as number of positively staining cells per mesenchymal tissue. In other words, normal lung parenchyma, minimal fibrosis (fibroblastic foci), severe (mural) fibrosis, and vascular areas present in UIP in each field were determined according to the number of points hitting mesenchymal tissue, as a proportion of the total grid area. The number of positive cells within the normal lung parenchyma, minimal fibrosis (fibroblastic foci), severe (mural) fibrosis, and vascular areas were then counted. The immunostaining cellularity was determined as the number of positive cells in each field divided by the normal lung parenchyma, minimal fibrosis (fibroblastic foci), severe (mural) fibrosis, and vascular areas. The final results are reported as means  $\pm$  SEM of 3 lung specimens from each patient in 30 random, non-coincident microscopic fields.

The severity of fibrosis was assessed by semiquantitative analysis in UIP areas of 1) mild interstitial thickening by fibroblastic foci (minimal fibrosis, Figure 1A and B), and 2) severe mural-organizing fibrosis with honeycombing and foci of actively proliferating fibroblasts and myofibroblasts (severe fibrosis, Figure 1C-F).

Interobserver comparisons were performed for 20% of the

slides by 2 observers (E.R.P. and V.L.C.). The coefficient of variation for the interobserver error of cell count was <5%.

### Statistical analysis

Data are reported as means  $\pm$  SEM with 95% confidence intervals. ANOVA with the Bonferroni test were used to analyze the relationship between continuous variables and the residuals were examined to ensure that their distribution was approximately normal. The relationship between the immunostaining cellularity was evaluated by Pearson's correlation. Actuarial survival was estimated using the Kaplan-Meier method and compared using the log-rank test. Cox proportional hazards regression was used to ascertain the individual contribution of factors associated with survival and to compare adjusted survival between groups. The statistical program used was SPSS 18.0 (SPSS Inc., USA). The level of significance was set at 5% in all tests.

## Results

### Telomerase activation

Immunohistochemical staining of epithelium telomerase+ in normal (Figure 2B), minimal fibrosis (Figure 2C), severe fibrosis (Figure 2D), and vascular (Figure 2F) areas of UIP, appears as brownish cells. Epithelium telomerase+ immunostaining was more exuberant in normal and vascular areas than in fibrosing areas of the histologic pattern of UIP than in control areas (Figure 2A and E).

Quantitative analysis confirmed the morphologic distribution of telomerase+ epithelium expression (Table 2). Telomerase+ epithelium was significantly overexpressed in normal and vascular areas than in UIP fibrosing and control areas.

### Myofibroblasts and smooth muscle cell replication

$\alpha$ -SMA+ myofibroblasts in normal (Figure 3A), fibrosing (Figure 3C) and vascular (Figure 3E) areas of UIP and control areas (Figure 3G), when stained by immunohistochemistry, appear as brownish cells.  $\alpha$ -SMA+ myofibroblasts were more prominent than smooth muscle cells caldesmon+ in vascular (Figure 3F) than fibrosing (Figure 3D) and control (Figure 3H) areas. Normal areas did not present  $\alpha$ -SMA+ myofibroblasts or caldesmon+ smooth muscle cells (Figure 3B).

Quantitative analysis confirmed the morphologic distribution of  $\alpha$ -SMA+ myofibroblasts and caldesmon+ smooth muscle cells (Table 2). Interestingly,  $\alpha$ -SMA+ myofibroblasts were significantly more increased than caldesmon+ smooth muscle cells in vascular areas of UIP ( $P < 0.001$ ).

### Endothelium activation

ET-1+ endothelium in normal (Figure 4B), minimal (Figure 4C) and severe (Figure 4D) fibrosing, as well as vascular (Figure 4F) areas of UIP, when stained by immunohistochemistry, appears as brownish cells. Numerous



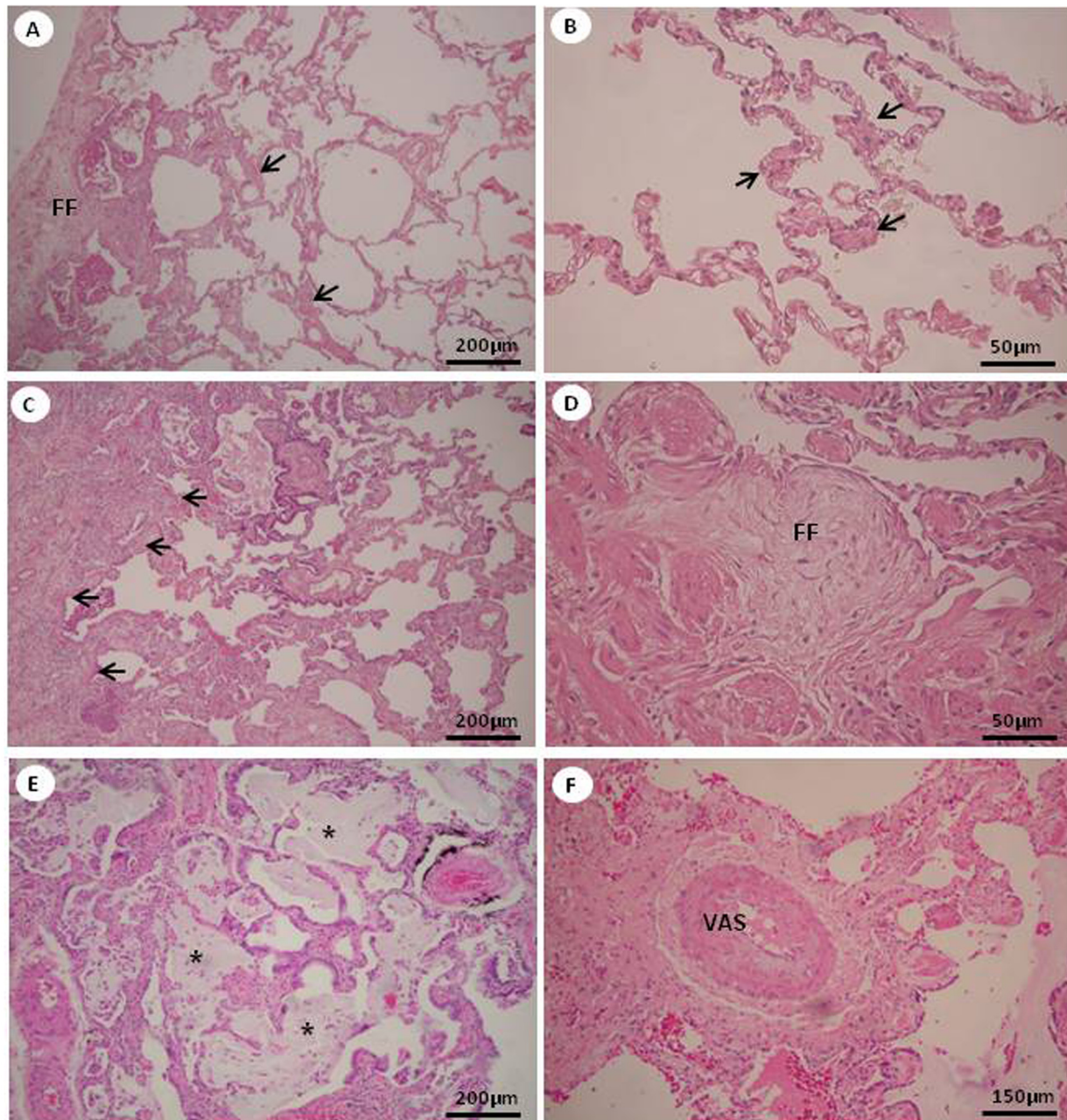
cells in normal and vascular areas expressed ET-1 when compared to fibrosing areas of the UIP histologic pattern and control areas (Figure 4A and E).

Quantitative analysis confirmed the morphologic distribution of ET-1 expression that was significantly increased

in normal and vascular areas of UIP when compared to fibrosing areas (Table 2).

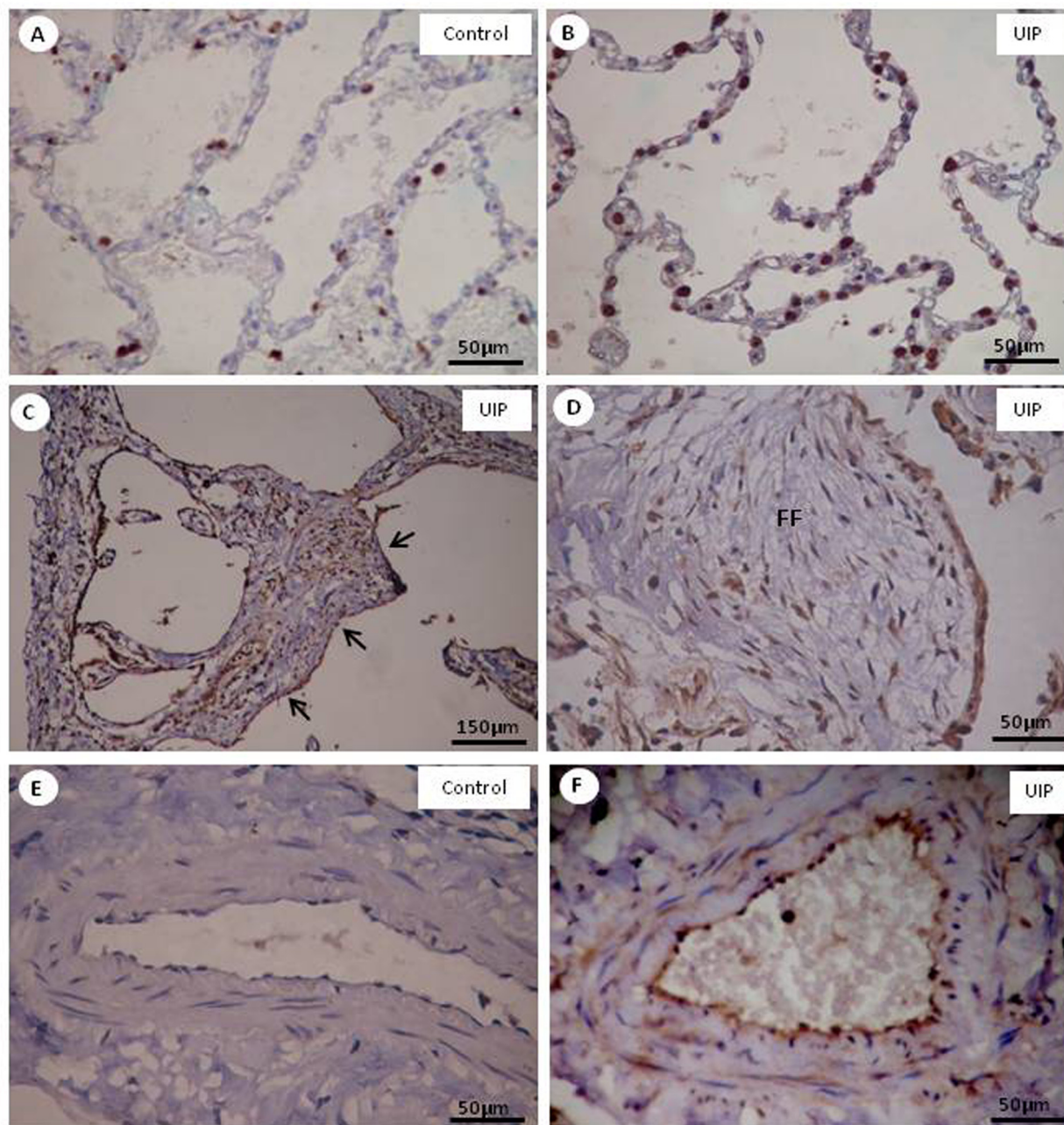
#### Association between mediators

A direct association was found between telomerase and



**Figure 1.** Degree of fibrosis in the pattern of usual interstitial pneumonia (UIP). UIP minimal fibrosis is characterized by interstitial thickening (arrows) and by fibroblastic foci (FF) (A and B) and UIP severe fibrosis is characterized by severe mural-organizing fibrosis (arrows) (C) with foci of actively proliferating fibroblasts and myofibroblasts (FF) (D) and honeycombing (stars) (E). Vascular (VAS) representation is observed in the middle of pulmonary fibrosis (F). Hematoxylin and eosin.





**Figure 2.** Telomerase expression in control areas (A and E) and in a normal area (B), in an area of minimal fibrosis (arrows) (C), of fibroblastic foci (FF) of severe fibrosis (D) and in vascular (F) areas of usual interstitial pneumonia (UIP), when stained by immunohistochemistry. Numerous cells are observed in the different areas of UIP pattern when compared with the control areas.

ET-1 expression in vascular areas of UIP ( $r = 0.45$ ;  $P = 0.03$ ). An inverse association was found between telomerase and ET-1 expression in normal and minimal fibrosis areas of UIP ( $r = -0.42$ ;  $P = 0.03$ ). Telomerase and ET-1 expression were positively associated with vascular areas ( $r = 0.54$ ;  $P = 0.004$ ) and negatively associated with minimal fibrosis

and normal areas ( $r = -0.44$ ;  $P = 0.02$ ) of UIP.

#### Association between mediators and fibrosis severity

Telomerase and ET-1 expression were inversely correlated with minimal fibrosis in normal and fibroblast foci areas ( $r = -0.38$ ;  $P = 0.04$  and  $r = -0.39$ ;  $P = 0.03$ , respec-

tively) and directly associated with severe fibrosis in vascular areas ( $r = 0.41$ ;  $P = 0.03$  and  $r = 0.54$ ;  $P = 0.004$ ) of UIP.

### Association between mediators and functional tests

A direct association was found between diffusing capacity of the lung for oxygen/alveolar volume (DLCO/VA) and telomerase activity ( $r = 0.49$ ;  $P = 0.01$ ) in minimal fibrosis areas. An indirect association was found between DLCO and severe fibrosis in vascular areas displaying high ET-1 expression ( $r = -0.74$ ;  $P = 0.02$ ).

### Survival analysis

The median follow-up was 42.70 months. Ten patients were still alive and 17 died from causes related to IPF. ROC curve analysis showed that the optimum cut-off point of expression was 14.55% for telomerase and 12.54% for ET-1. These cut-off points resulted in a significant difference in Kaplan-Meier curves. The 5-year survival rate of patients with minimal fibrosis in vascular areas displaying ET-1  $\leq 12.54\%$  was 57.49 vs 31.13% in the group with minimal fibrosis displaying higher ET-1 levels ( $P < 0.01$ ). The 5-year survival rate of patients with minimal fibrosis in vascular areas displaying telomerase  $\leq 14.55\%$  was 59.55 vs 28.88% in the group with minimal fibrosis and higher telomerase levels ( $P < 0.01$ ).

Several combinations of clinical and morphological variables were analyzed to generate a mathematical model with an impact on IPF patient survival. The results of the best combination, determined by Cox model analysis, appear in Table 3. The following variables showed a significant impact on patient survival: gender, age, fibrosis severity, telomerase in UIP normal lung parenchyma, telomerase in minimal (fibroblastic foci) fibrosis, ET-1 in normal lung parenchyma, and minimal (fibroblast foci) fibrosis. Multivariate analyses showed a low risk of death for females, UIP normal lung parenchyma or minimal (fibroblastic foci) fibrosis displaying high telomerase and ET-1 expression in UIP normal lung parenchyma areas.

## Discussion

We investigated the participation of telomerase and the factors responsible for its activation or suppression in the

**Table 2.** Histomorphometric markers and cellularity in non-fibrosing lung tissue (control) and different areas of fibrosing lung (usual interstitial pneumonia, UIP).

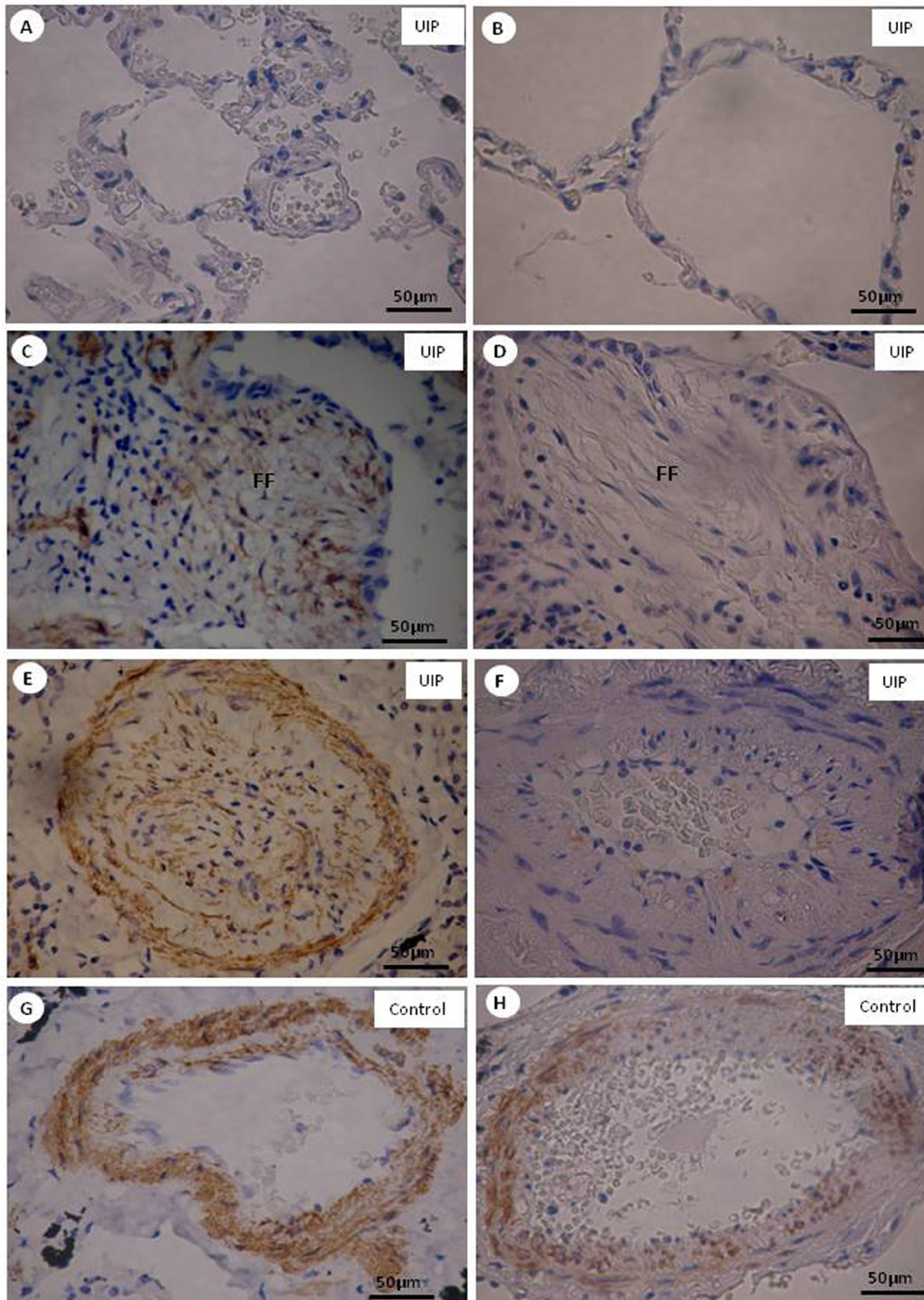
Lung tissue	Telomerase	$\alpha$ -SMA	Caldesmon	Endothelin-1
Non-fibrosing lung (control)				
Septal areas	0.78 $\pm$ 0.42	0	0	0.01 $\pm$ 0.02
Vascular areas	0.01 $\pm$ 0.02	9.57 $\pm$ 2.90	5.2 $\pm$ 1.15	0.0003 $\pm$ 0.0003
Fibrosing lung (UIP)				
Normal lung parenchyma	17.8 $\pm$ 6.7 <sup>+</sup>	0	0	17.2 $\pm$ 6.4 <sup>#</sup>
Minimal fibrosis	7.4 $\pm$ 2.1	8.2 $\pm$ 3.7	0.002 $\pm$ 0.0005 <sup>§</sup>	5.7 $\pm$ 2.2
Severe (mural) fibrosis	6.4 $\pm$ 2.5	12.0 $\pm$ 6.6	0.06 $\pm$ 0.001 <sup>§</sup>	7.2 $\pm$ 2.9
Vascular areas	18.2 $\pm$ 7.2 <sup>+</sup>	51.7 $\pm$ 10.4 <sup>*</sup>	0.45 $\pm$ 0.02 <sup>§</sup>	18.0 $\pm$ 4.3 <sup>#</sup>

Data are reported as means  $\pm$  SEM percent of markers for 3 lung specimens obtained by open surgical biopsy from each patient (10 random, non-coincident microscopic fields were analyzed in each specimen). All lung specimens were analyzed for epithelial, myofibroblast, endothelial and smooth muscle cells from normal lung parenchyma, and minimal fibrosis (fibroblastic foci), severe (mural) fibrosis and vascular areas of UIP for percentage of telomerase,  $\alpha$ -SMA, caldesmon, and endothelin-1 fractional areas. <sup>+</sup>Telomerase expression values in UIP normal lung parenchyma and vascular areas differed significantly from minimal (fibroblastic foci) and severe (mural) fibrosis ( $P < 0.01$ ). <sup>\*</sup> $\alpha$ -SMA expression values in UIP vascular areas were significantly different from normal lung parenchyma and fibrosis areas ( $P < 0.01$ ). <sup>§</sup>Caldesmon expression values in UIP fibrosis and vascular areas differed significantly from normal lung parenchyma ( $P < 0.001$ ). Endothelin-1 expression values in normal lung parenchyma and vascular areas differed significantly from fibrotic and control areas ( $P < 0.01$ ). All markers in UIP were significantly increased compared to non-fibrosing control lungs ( $P < 0.0001$ ). ANOVA with the Bonferroni test were used for statistical analysis.

fibrotic process found in pulmonary specimens obtained by surgical lung biopsy from patients with IPF. Specifically, we investigated telomerase and ET-1 expression in alveolar epithelium, endothelium, and myofibroblast cells present in normal lung parenchyma, minimal (fibroblast foci) fibrosis and vascular areas of UIP to verify the impact of these markers on the remodeling/fibrosis process. In the present study, telomerase and ET-1 were significantly increased in normal lung parenchyma and vascular areas compared to fibroblast foci areas of UIP. Unexpectedly, myofibroblast  $\alpha$ -SMA<sup>+</sup> expression was more prominent than smooth muscle caldesmon<sup>+</sup> cells in vessels. Interestingly, we found a positive association between telomerase and ET-1 in UIP vascular areas, whereas a negative association was found between telomerase and ET-1 in normal lung parenchyma. A positive association was found between telomerase and myofibroblast  $\alpha$ -SMA in UIP vascular areas. In addition, the expression of these mediators correlated with the measurement of fibrosis degree, suggesting that they exert their effect by influencing remodeling/fibrosis in UIP.

The current study revealed that telomerase increases myofibroblast activation in vascular areas of UIP. In fact, telomerase can be expressed by different types of cells and its presence indicates not only a persistent activity to maintain a supply of activated cells (e.g., fibroblasts), but is also important in the regulation of cell proliferation (17,18), apoptosis (19,20), and cell differentiation (21,22). Although



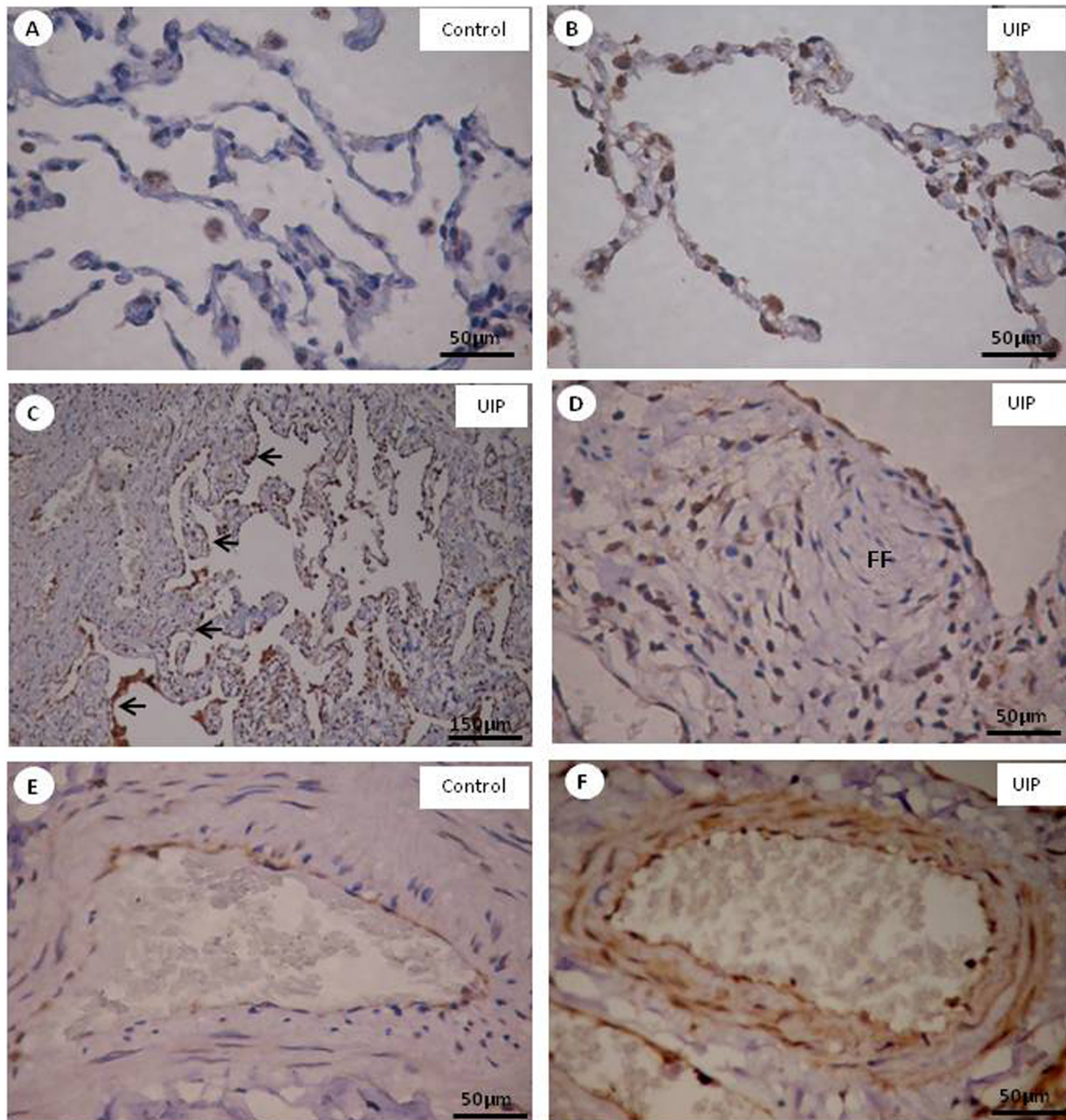


**Figure 3.** Alpha-smooth muscle actin ( $\alpha$ -SMA) (A, C, E, G) and caldesmon (B, D, F, H) expression in normal (A, B), fibroblastic foci (FF) of fibrosing (C, D) and vascular (E, F) areas of usual interstitial pneumonia (UIP) pattern, and vascular control areas (G, H) stained by immunohistochemistry.  $\alpha$ -SMA+/myofibroblasts are more prominent than caldesmon+ smooth muscle cells in vascular areas than in fibrosing and control areas. Normal areas do not show  $\alpha$ -SMA+ myofibroblasts or caldesmon+ smooth muscle cells.



telomerase activity is a nonspecific signal of cell proliferation involving different cell types besides fibroblasts, this last assumption does not disagree with our results, which show an influence of telomerase expression on vessels and prognosis. Our findings agree with the study of Nozaki et al.

(12) who demonstrated that bleomycin-induced lung injury and fibrosis are also known to induce telomerase activity in the affected lung tissue and isolated lung fibroblasts. The activity, however, is expressed only during the period of active fibrosis, and appears to be localized primarily in fibroblasts



**Figure 4.** Endothelial ET-1+ expression in control areas (A, E) and in normal (B), minimal (C, arrows) and severe (D) fibrosing, showing a fibroblastic foci (FF) as well as vascular (F) areas of usual interstitial pneumonia (UIP) pattern when stained by immunohistochemistry. Numerous cells in normal and vascular areas expressed endothelin-1 when compared to fibrosing areas of the UIP and control areas. Endothelin-1 immunohistochemistry.

**Table 3.** Cox proportional hazards regression to ascertain the individual contribution of clinical and morphological factors associated with survival and to compare adjusted survival between groups [-2 log likelihood = 28.90; chi-square = 38.26; P < 0.0001].

	$\beta$	SE	Wald test	P value	Exp ( $\beta$ )	95%CI for Exp ( $\beta$ )	
						Lower	Upper
Gender	-3.74	1.48	6.38	0.01	41.94	2.31	761.02
Age (years)	1.02	0.34	8.94	0.003	2.78	1.42	5.43
UIP fibrosis			9.26	0.01			
UIP severe (mural) fibrosis	-9.70	3.26	8.87	0.003	0.00	0.00	0.03
UIP minimal (fibroblastic foci) fibrosis	-12.04	3.98	9.16	0.002	0.00	0.00	0.01
Telomerase in UIP minimal fibrosis	-1.85	0.97	3.60	0.05	6.35	0.94	42.78
Telomerase in UIP normal lung parenchyma	6.05	2.35	6.62	0.01	0.002	0.00	0.23
Endothelin-1 in UIP normal lung parenchyma	-5.13	1.81	8.00	0.005	0.006	0.00	0.20
Endothelin-1 in UIP minimal (fibroblast foci) fibrosis	5.72	2.13	7.24	0.007	0.003	0.00	0.21

UIP = usual interstitial pneumonia;  $\beta$  = beta coefficient; SE = standard error; Exp ( $\beta$ ) = exponential beta; CI = confidence interval.

rather than myofibroblasts (23). The activity also declines with increased passaging *in vitro*, which is associated with increased differentiation into myofibroblasts. These studies suggest that the increased enzyme activity could be secondary to higher cell division rates and therefore the increase in telomerase activity would be secondary to the inflammatory and fibrotic process instead of its cause. The factors responsible for regulating telomerase activity in these tissues and cells are mostly unknown. More recent data from our group have shown that abnormal expression of telomerase/apoptosis limits type II alveolar epithelial cell replication in the early remodeling of UIP (24). In fact, we found that epithelial telomerase expression in normal lung parenchyma and fibroblastic foci areas was lower than in normal septal areas, probably reflecting the loss and shortening of telomeres activity. It is tempting to speculate about the existence of a specific checkpoint beyond which loss of telomerase activity due to injurious stimuli and/or inherited mutations may lead to loss of epithelial cells and promote genomic instability. Activation of telomerase after the onset of genomic instability, coupled with a repeatedly injurious microenvironment, may affect the physiological differentiation of normal alveolar epithelial progenitors towards a transformed mesenchymal phenotype, resulting in abnormal reepithelization in normal parenchymal areas and contributing to the fibrogenic process characterized by fibroblastic foci. Telomerase would inhibit the senescence of many normal cells, such as epithelial and mesenchymal cells, and most probably would increase the incidence of tumors and fibrosis in the patient. Thus, carcinogenesis and fibrogenesis may be similar processes respectively involving epithelial and mesenchymal (fibroblasts) neoplastic cells.

We also found that telomerase and ET-1 expression were positively associated with vascular tissue and

negatively associated with normal lung parenchyma and fibroblast focus areas of UIP. ET-1 can also be synthesized by endothelial and epithelial cells, vascular smooth muscle cells, airway epithelium, granulocytes, and macrophages (25). Moreover, increased concentrations and biosynthesis of ET-1 were demonstrated in the lungs of patients with pulmonary hypertension and systemic sclerosis (26). Furthermore, preproendothelin-1 messenger RNA is elevated in airway epithelial cells, proliferating type II pneumocytes, endothelial cells, and inflammatory cells present in IPF (27,28). The increased cell population expressing ET-1 and telomerase may indicate a continuous stimulation and transformation of these cells. In fact, we found a more evident expression in epithelial cells of normal alveolar areas, reflecting not only the stress to which these cells were submitted but also a probable attempt to maintain a large population to avoid alveolar collapse, as previously demonstrated by our group (24). We also found that many epithelial cells and myofibroblasts present in fibroblast focus areas expressed ET-1 and telomerase showing a high activity of these areas. Actually, ET-1 plays a role in several stages of wound healing: stimulating fibroblast differentiation and collagen metabolism, promoting the wound healing response by the production of collagens IV, V and VII, involvement in the reconstitution of the basement membrane and appropriate wound closure and other unknown effects on different types of cells (29,30).

Our study has clinical consequences. In order to establish the relevance of these findings to the evolution of the patients, the mediators under study were evaluated as a function of fibrosis severity and physiological testing. We found a direct association between DLCO/VA and minimal fibrosis areas displaying high telomerase activity and between DLCO and severe fibrosis areas of UIP displaying

high ET-1 expression. Although hypoxia is universal in IPF patients, decreases in DLCO caused by a defect in gas transfer certainly present in the pulmonary parenchyma of UIP, also contribute to hypoxemia. These functional changes observed are probably explained in part by the vascular involvement. The expression of these mediators probably represents another step in the wound healing process in IPF. We believe that high expression of these markers is crucial to maintain the activity of parenchymal injury by the regulation of compromised vascular tone. Many lines of evidence suggest that telomerase activation under chronic hypoxia may result in enhanced and sustained smooth muscle proliferation and vessel wall remodeling, findings characteristic of proliferative vascular disorders, such as pulmonary hypertension (31). On the other hand, the continuous ET-1 stimulus from the endothelium and smooth muscle of vascular cells activates the proliferation and the remodeling process of these vessels. Clearly, in our study poor patient survival was associated with high vascular expression of ET-1 and telomerase, suggesting

that a major damage to vessels leads to more parenchymal alterations during the development of pulmonary fibrosis caused by maintained hypoxia.

In conclusion, telomerase activity increases endothelial and myofibroblast activation in early remodeling/fibrosis of IPF with impact on evolution, suggesting that strategies aimed at preventing the effect of these mediators may have a greater impact on patient outcome.

## Acknowledgments

We are grateful to the biologist Sandra de Morais Fernezlian from the Laboratory of Immunohistochemistry. We would like to thank Dr. Ronaldo Adib Kairalla and Dr. Carlos Roberto Ribeiro de Carvalho from the Division of Respiratory Diseases – Heart Institute (InCor), Faculdade de Medicina, Universidade de São Paulo, for providing many pulmonary function tests of these patients. Research supported by CNPq and FAPESP (#2008/53022-3).

## References

1. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* 2002; 165: 277-304.
2. Gribbin J, Hubbard RB, Le Jeune I, Smith CJ, West J, Tata LJ. Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. *Thorax* 2006; 61: 980-985.
3. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001; 134: 136-151.
4. Parra ER, David YR, da Costa LR, Ab'Saber A, Sousa R, Kairalla RA, et al. Heterogeneous remodeling of lung vessels in idiopathic pulmonary fibrosis. *Lung* 2005; 183: 291-300.
5. Parra ER, Kairalla RA, de Carvalho CR, Capelozzi VL. Abnormal deposition of collagen/elastic vascular fibres and prognostic significance in idiopathic interstitial pneumonias. *Thorax* 2007; 62: 428-437.
6. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002; 3: 349-363.
7. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 1999; 250: 273-283.
8. Elias JA, Zitnik RJ, Ray P. Fibroblast immune-effector function. In: Phipps RP (Editor), *Pulmonary fibroblast heterogeneity*. Boca Raton: CRC Press; 1992. p 295-322.
9. Shichiri M, Adachi S, Sedivy JM, Marumo F, Hirata Y. Biphasic regulation of the preproendothelin-1 gene by c-myc. *Endocrinology* 1997; 138: 4584-4590.
10. Greider CW. Telomere length regulation. *Annu Rev Biochem* 1996; 65: 337-365.
11. Counter CM, Hirte HW, Bacchetti S, Harley CB. Telomerase activity in human ovarian carcinoma. *Proc Natl Acad Sci U S A* 1994; 91: 2900-2904.
12. Nozaki Y, Liu T, Hatano K, Gharaee-Kermani M, Phan SH. Induction of telomerase activity in fibroblasts from bleomycin-injured lungs. *Am J Respir Cell Mol Biol* 2000; 23: 460-465.
13. Phan SH, Varani J, Smith D. Rat lung fibroblast collagen metabolism in bleomycin-induced pulmonary fibrosis. *J Clin Invest* 1985; 76: 241-247.
14. Zhang HY, Gharaee-Kermani M, Zhang K, Karmiol S, Phan SH. Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1996; 148: 527-537.
15. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993; 16: 5-40.
16. Hsia CC, Hyde DM, Ochs M, Weibel ER. An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. *Am J Respir Crit Care Med* 2010; 181: 394-418.
17. Greider CW. Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci U S A* 1998; 95: 90-92.
18. Sarin KY, Cheung P, Gilson D, Lee E, Tennen RI, Wang E, et al. Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature* 2005; 436: 1048-1052.
19. Haendeler J, Hoffmann J, Rahman S, Zeiher AM, Dimmeler



- S. Regulation of telomerase activity and anti-apoptotic function by protein-protein interaction and phosphorylation. *FEBS Lett* 2003; 536: 180-186.
20. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev* 1999; 13: 2388-2399.
  21. Rosenberger S, Thorey IS, Werner S, Boukamp P. A novel regulator of telomerase. S100A8 mediates differentiation-dependent and calcium-induced inhibition of telomerase activity in the human epidermal keratinocyte line HaCaT. *J Biol Chem* 2007; 282: 6126-6135.
  22. Savovsky E, Yoshida K, Ohtomo T, Yamaguchi Y, Akamatsu K, Yamazaki T, et al. Down-regulation of telomerase activity is an early event in the differentiation of HL60 cells. *Biochem Biophys Res Commun* 1996; 226: 329-334.
  23. Liu T, Nozaki Y, Phan SH. Regulation of telomerase activity in rat lung fibroblasts. *Am J Respir Cell Mol Biol* 2002; 26: 534-540.
  24. Waisberg DR, Barbas-Filho JV, Parra ER, Fernezlian S, de Carvalho CR, Kairalla RA, et al. Abnormal expression of telomerase/apoptosis limits type II alveolar epithelial cell replication in the early remodeling of usual interstitial pneumonia/idiopathic pulmonary fibrosis. *Hum Pathol* 2010; 41: 385-391.
  25. Battistini B, Dussault P. Biosynthesis, distribution and metabolism of endothelins in the pulmonary system. *Pulm Pharmacol Ther* 1998; 11: 79-88.
  26. Abraham DJ, Vancheeswaran R, Dashwood MR, Rajkumar VS, Pantelides P, Xu SW, et al. Increased levels of endothelin-1 and differential endothelin type A and B receptor expression in scleroderma-associated fibrotic lung disease. *Am J Pathol* 1997; 151: 831-841.
  27. Giaid A, Polak JM, Gaitonde V, Hamid QA, Moscoso G, Legon S, et al. Distribution of endothelin-like immunoreactivity and mRNA in the developing and adult human lung. *Am J Respir Cell Mol Biol* 1991; 4: 50-58.
  28. Saleh D, Furukawa K, Tsao MS, Maghazachi A, Corrin B, Yanagisawa M, et al. Elevated expression of endothelin-1 and endothelin-converting enzyme-1 in idiopathic pulmonary fibrosis: possible involvement of proinflammatory cytokines. *Am J Respir Cell Mol Biol* 1997; 16: 187-193.
  29. Guidry C, Hook M. Endothelins produced by endothelial cells promote collagen gel contraction by fibroblasts. *J Cell Biol* 1991; 115: 873-880.
  30. Desmouliere A. Factors influencing myofibroblast differentiation during wound healing and fibrosis. *Cell Biol Int* 1995; 19: 471-476.
  31. Minamino T, Mitsialis SA, Kourembanas S. Hypoxia extends the life span of vascular smooth muscle cells through telomerase activation. *Mol Cell Biol* 2001; 21: 3336-3342.